A Study of the Molecular Pathology of Ductal Carcinoma In Situ and Invasive Ductal Carcinoma of the Breast

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ABSTRACT:

The biological validity of the histopathological classification of ductal carcinoma in situ (DCIS) of the breast was evaluated in this study by correlating the three histopathological grades of DCIS to immunohistochemical expression of Ki67, p53, cerbB-2, markers of poor prognosis in invasive ductal carcinoma (IDC) and also to bcl2 and ER, markers of good prognosis in invasive breast cancer. DCIS grades correlated positively to Ki67, p53, cerbB-2 and negatively to bcl2 and ER, suggesting validity of the classification.

The incidence of bax protein expression was determined immunohistochemically in DCIS and IDC. It did not correlate to histopathological grades of DCIS or IDC. The relationships of bax protein to the above mentioned biological markers were also determined in DCIS and IDC.

Furthermore, the expression of bax, bcl2, Ki67, ER, p53 and cerbB-2 within DCIS grades was compared with the expression of these markers within IDC grades. The DCIS grades were determined subjectively as well as objectively by means of computer assisted image analysis with significant correlation found between subjective and objective measures. Image analysis was also used to determine percentage of positive cells per case for the nuclear stains (Ki67, ER, p53).

Immunohistochemically positive p53 cases were analysed for p53 mutation by polymerase chain reaction (PCR) and subsequent DNA sequencing to compare the incidence of p53 mutation in DCIS to that of IDC.

Biochemical changes within tissue may either initiate disease or occur as the result of the disease process and these changes can be studied by both fourier transform infrared (FTIR) and FT-Raman spectroscopic techniques. FTIR and FT-Raman were employed to distinguish the DCIS and IDC grades. It has the potential to distinguish between DCIS grades, between IDC grades and also between DCIS and IDC as whole groups.

The implications of the obtained data for the understanding of the molecular biology of DCIS of the breast and IDC are discussed and future investigations to further elucidate the molecular and cellular mechanisms involved are proposed.

DEDICATION:

To my beloved husband; Ihtesham ur Rehman and lovely children; Hamza, Juwairiya and Sumaiya, without their continuous love, support and patience I could not have done this work.

> *"Oh our Lord, grant us spouses and offspring who will be comfort to our eyes, and make us leaders for the pious."*
>
> **[Al – Quran (Sura Al – Furqan, number 74)]**

To the quest for knowledge,

> *"O my Lord, increase my knowledge".*
>
> **[Al-Quran (Sura Taha, number 114)]**

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ABBREVIATIONS:

ABC	avidin-biotin complex
ACTH	adrenocorticotrophic hormone
ADH	atypical ductal hyperplasia
AFP	alpha-fetoprotein
AI	apoptotic index
AIB1	amplified in breast 1 gene
AJCC	American Joint Committee on Cancer
AMP	adenosine monophosphate
ANC	axillary node clearance
ANN	artificial neural network
ANS	axillary node sampling
APAAP	alkaline phosphatase-anti-alkaline phosphatase
APC	adenomatous polyposis coli gene
APES	3-aminopropyl-triethoxysilane
AR	androgen receptor
AT	ataxia-telangiectasia
ATR	attenuated total reflectance
BC	breast cancer
BCL2	B-cell leukaemia/lymphoma 2 gene
BMT	bone marrow transplant
bp	base pairs
BRCA1&2	breast cancer susceptibility genes 1 and 2
BRDU	bromodeoxyuridine
BRISH	bright field in situ hybridization
BSE	breast self examination
CAS	cell analysis system
CAS	cellular apoptosis susceptibility gene
Ca^{2+}	calcium
CCD	charged couple device
CDC2	cell division cycle gene 2, the protein product of which is called p34
CDK	cyclin dependent kinase
cDNA	complementary deoxyribonucleic acid
CEA	carcinoembryonic antigen
CGH	comparative genomic hybridisation
Chr	chromosome
cm	centimetre
CMF	cyclophosphamide, methotraxate, 5-fluorouracil
CNS	central nervous system
CV	coefficient of variation
DAB	3,3diaminobenzidine
DCC	dextran-coated charcoal
DCIS	ductal carcinoma in situ
DFS	disease-free survival
DGGE	denaturing gradient gel electrophoresis
DDGE	double-strand denaturing gel electrophoresis
DNA	deoxy-ribonucleic acid
ddNTP	2', 3'-dideoxynucleotide
dNTP	deoxynucleotide tri-phosphate

DOB	date of birth
EBCTC	Early Breast Cancer Triallists Collaborative (Group)
EBV	Epstein-Barr virus
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EIA	enzyme immuno-assay
EIC	extensive in situ component
ELISA	enzyme-linked immunoabsorbent assay
EORTC	European Organisation for Research and Treatment of Cancer
EPWG	European Pathologists' Working Group
EQA	external quality assurance
ER	oestrogen receptor
ERE	oestrogen response element
FBC	familial breast cancer
FDA	Food and Drug Agency
FEC	5-fluorouracil, epirubicin, cyclophosphamide
FGF	fibroblast growth factor
FISH	fluorescence in situ hybridization
fmol	femtomoles
FNAC	fine needle aspiration cytology
FTIR	fourier transform infrared microscopy
G_1	first gap phase of cell cycle
G I	grade I of IDC
G_2	second gap phase of cell cycle
G II	grade II of IDC
G III	grade III of IDC
GMP	guanosine monophosphate
H&E	haematoxylin and eosin
HCG	human chorionic gonadotrophin
HER	human epidermal growth factor receptor
HGF	hepatocyte growth factor
HIV	human immunedeficiency virus
HNG	high nuclear grade (of DCIS)
HNPCC	hereditary non-polyposis colon cancer
Hpf	high power field
HPV	human papilloma virus
HRP	horseradish peroxidase
HRT	hormone replacement therapy
Hsp	heat shock protein
HTLV-1	human T lymphotropic virus-1
IA	image analysis
ICC	immunocytochemistry
ICE	interleukin-1 β-converting enzyme
IDC	invasive/infiltrating ductal carcinoma
IGF-1	insulin like growth factor-1
IGSS	immunogold staining with silver precipitation
IHC	immunohistochemistry
ING	intermediate nuclear grade (of DCIS)
IR	infrared

IRS	immuno-reactive score
JNK	JUN N-terminal kinase
kb	kilobase pair
LABC	locally advanced breast cancer
LCIS	lobular carcinoma in situ
LHRH	luteinizing hormone releasing hormone
LI	labelling index (of Ki67)
LN	lymph node
LNG	low nuclear grade (of DCIS)
LOH	loss of heterozygosity
M	mitotic phase of cell cycle
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase
MBC	male breast carcinoma
MDM2	murine double minute 2 gene
Mg^{2+}	magnesium
MI	mitotic index
MIC	micro-invasive carcinoma
ml	millilitre
µl	microlitre
mm	millimetre
µm	micrometer
MMM	mitozantrone, methotraxate, mitomycin
MMP	matrix metalloproteinase
MMPI	matrix metalloproteinase inhibitor
MMTV	mouse mammary tumour virus
MNA	mean nuclear area
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NCAM	neural cell-adhesion molecule
NCGBSP	National Coordinating Group for Breast Screening Pathology
NEQAS	National External Quality Assessment Scheme
NF	neurofibromatosis
NGS	normal goat serum
NHL	non-Hodgkin's lymphoma
NICE	National Institute for Clinical Excellence
nm	nanometers
NME	non-metastatic cells, expressed gene
NM23	non-metastatic 23 gene
NMR	nuclear magnetic resonance
NOS	not otherwise specified
NPI	Nottingham prognostic index
NSABP	National Surgical Adjuvant Breast and Bowel Project
NSCLC	non-small-cell lung cancer
NST	no special type
OCP	oral contraceptive pill
OS	overall survival
PAP	peroxidase-antiperoxidase
PAS	periodic acid Schiff

PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PFGE	pulsed field gel electrophoresis
PI	proliferative index
PLL	Poly-L-Lysine
PR	progesterone receptor
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
S	synthesis phase of cell cycle
SAPK	stress-activated protein kinase (another name for JNK)
SERM	selective oestrogen receptor modulator
SLNB	sentinel lymph node biopsy
SPF	S-phase fraction
SSCP	single-strand conformation polymorphism
STATs	signal transducers and activators of transcription
TAF-I	transcription activator factor I
TAF-II	transcription activator factor II
TBE	Tris borate EDTA buffer
TBP	TATA binding protein
TBS	Tris buffered saline
TDLU	terminal duct lobular unit
TGFα	transforming growth factor alpha
TGFβ	transforming growth factor beta
TGGE	teperature-gradient gel electrophoresis
TIMP	tissue inhibitor of matrix metalloproteinase
TNF	tumour necrosis factor
TNM	tumour, node, metastasis
tPA	tissue plasminogen activator
UDH	usual ductal hyperplasia
UICC	International Union Against Cancer Criteria (Union Internationale Contre le Cancer)
UKCCCR	United Kingdom Co-ordinating Committee on Cancer Research
uPA	urokinase-type plasminogen activator
UV	ultraviolet
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau disease
VNTR	variable number of tandem repeats
WHO	World Health Organisation
WLE	wide local excision

CHAPTER 1: THE BREAST – EMBRYOLOGY, ANATOMY AND PHYSIOLOGY:

EMBRYOLOGICAL DEVELOPMENT:

The breasts are often described as modified sweat glands. The phylogenetic relationship of the mammary glands to sweat glands is fully established (1). However, this is an oversimplified view, since the breasts undergo complex physiological changes during reproductive life, which are not seen in sweat glands.

The mammary line, also known as the primitive milk streak or galactic streak, is the initial stage in development of the breast tissue, which appears at about the 7 mm stage of embryonic growth (fifth to sixth week) and extends on each side of the body from the axilla to groin (1). The caudal two thirds of each line disappears by the 20 mm stage (seventh to eighth week) and eventually only the mid portion of the cephalic third remains. It is from this residuum of the mammary line that the mammary primordium develops. By the 60-90 mm stage (12-16 weeks) mesenchymal cells differentiate into the smooth muscle of the nipple and areola. Then the epithelial buds develop and branch to form 15-25 sprouts representing the future secretory apparatus and adipose tissue forms around these sprouts. This is followed by hair follicle, sebaceous gland and sweat gland differentiation. It appears that the developments until this stage are independent of hormonal influences.

From about 20^{th}-32^{nd} week of pregnancy, placental sex hormones enter the fetal circulation and induce canalisation of the epithelial sprouts to form a branching system composed of 15-25 major ducts. Distinction into the two layers of epithelium, secretory and myoepithelial, can be recognized ultrastructurally at about 20 weeks (2), but the biochemical phenotype is not yet established (3). Between 32 and 40 weeks, secretory activity begins, with the production of colostrum. The nipple-areolar complex develops further and becomes pigmented.

Apart from minor growth and branching of the duct system, no significant changes occur in the mammary tissue from then onwards until the onset of puberty. The changes at puberty are described on page 6.

ANATOMY:

Surface and Macroscopic Anatomy:

The adult female breasts lie on the upper chest wall, the upper edges at the level of the second or third rib and the lower edge at the level of the sixth rib. Medially they extend to the edge of the sternum and laterally to the anterior axillary line, although the tail may extend further into the axilla. The superficial pectoral fascia covers the breast from the anterior aspect and deep pectoral fascia from the posterior aspect. Fibrous bands called Cooper's suspensory ligaments connect these two layers providing support to the breast. The breasts vary greatly in size and contour from individual to individual.

Microanatomy:

The anatomy and microanatomy of the breast has been discussed in great detail by some authors (4;5). Briefly, the breasts consist of three major components - skin, the subcutaneous adipose tissue and the functional glandular tissue, which comprises both parenchyma and stroma. The nipple-areolar complex is centrally placed and contains abundant sensory nerves and sebaceous and apocrine glands. Morgagni's tubercles are elevations located in the areola, formed by the openings of the ducts of Montgomery's glands, which are sebaceous glands. At the tip of the nipple are the openings of collecting ducts. Immediately beneath the nipple the collecting ducts dilate to form the lactiferous sinuses. Deep to this the breast is divided into 15-25 lobes, each of which is based on a branching duct system and consists of 20-40 lobules which drain through ductules into subsegmental ducts, then segmental ducts and finally into the collecting ducts via the lactiferous sinuses. A lobule itself consists of 10-100 acini bound together in a fine vascular connective tissue. A lobule, with its draining ductule, is termed a terminal duct-lobular unit (TDLU), which is the functional site of milk production (figure 1.1)(6). The main bulk of each lobe is made up of adipose tissue and fibrous stroma called interlobular connective tissue. A low power view of normal breast lobules is shown in figure 1.2a.

The nipple is covered by stratified squamous epithelium, which extends for a short distance into the collecting ducts. Then there is an abrupt change to the glandular epithelium, which is present throughout the duct and the lobular system. This epithelium is composed of two distinct types of cell, the secretory or luminal cell and the myoepithelial cell. In the collecting ducts the luminal cells are generally columnar

whereas in the lobular acini they are cuboidal. Detailed microanatomical studies have shown that there are two types of luminal secretory cell (5). Basal cells have relatively clear cytoplasm and form microvilli where they are in contact with the lumen; the nucleus is oval and lacks a nucleolus. Superficial cells are darker with basophilic cytoplasm rich in ribosomes. The myoepithelial cells form an often discontinuous layer between the luminal secretory cells and the basement membrane (figures 1.1 and 1.2c). The myoepithelial cells are small and flat with dark nuclei and clear cytoplasm. Their cytoplasm contains myofibrils 50-80 nm in diameter which are inserted by hemidesmosomes into the basement membrane. These myoepithelial cells may be readily identified by immunostaining with anti smooth muscle actin (3;7).

Figure 1.1; Ductal system of human mammary gland

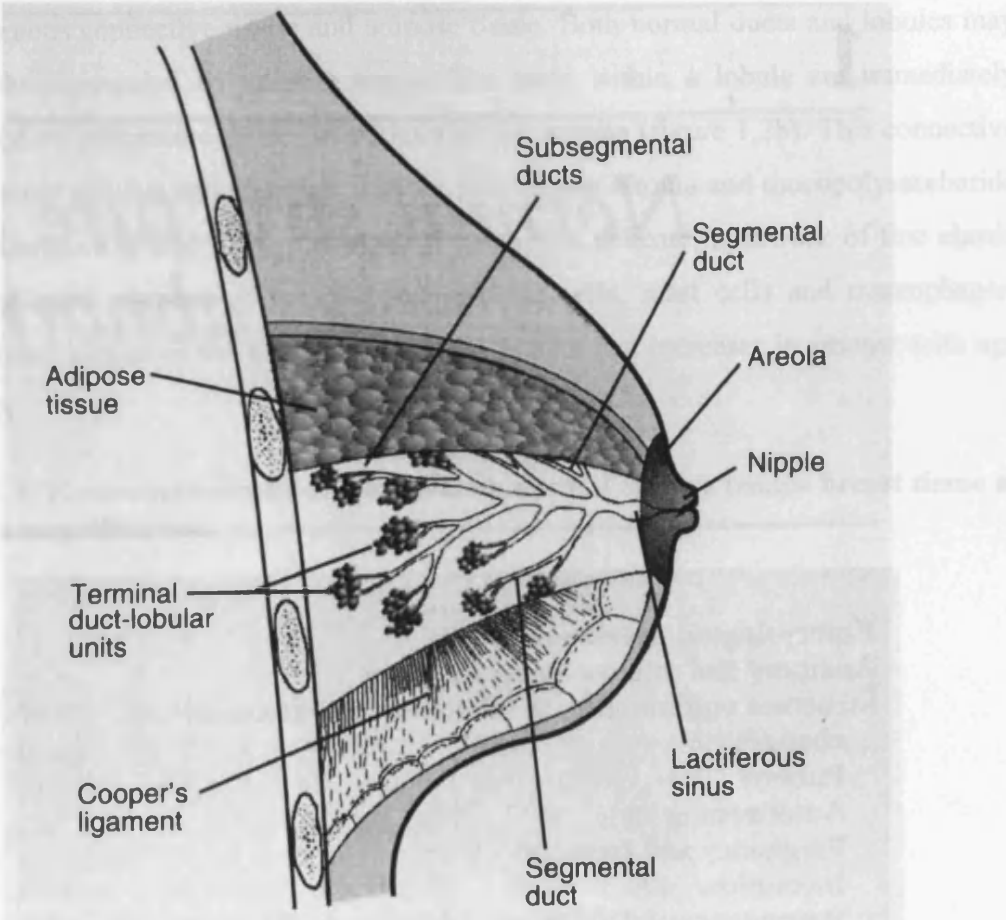


Figure 1.1a; Schematic diagram of the ductal system of human mammary gland

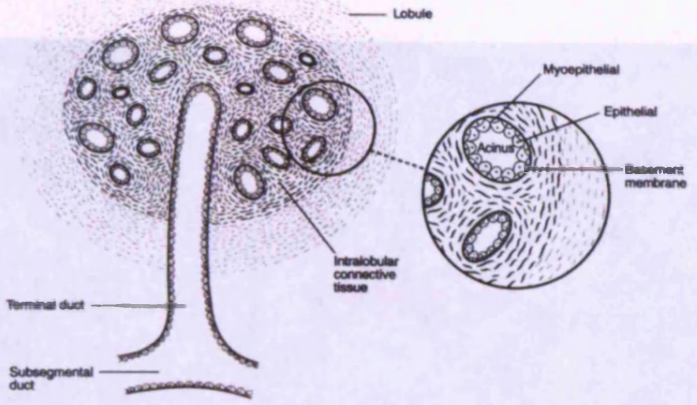


Figure 1.1b; Diagram of a single terminal duct – lobular unit showing the inter-relationship between the epithelial and stromal components

The TDLUs are set within interlobular stroma, which is composed of variable proportions of collagenous connective tissue and adipose tissue. Both normal ducts and lobules may be entirely surrounded by adipose tissue. The acini within a lobule are immediately surrounded by connective tissue called intralobular stroma (figure 1.2b). This connective tissue is more cellular and vascular than the interlobular stroma and mucopolysaccharide ground substance is also more abundant. It contains a delicate meshwork of fine elastic fibres and small number of lymphocytes, plasma cells, mast cells and macrophages. Elastic tissue surrounds the extralobular ductal system and increases in amount with age and parity.

Figure 1.2; Photomicrographs of lobules from normal mature female breast tissue at different magnifications

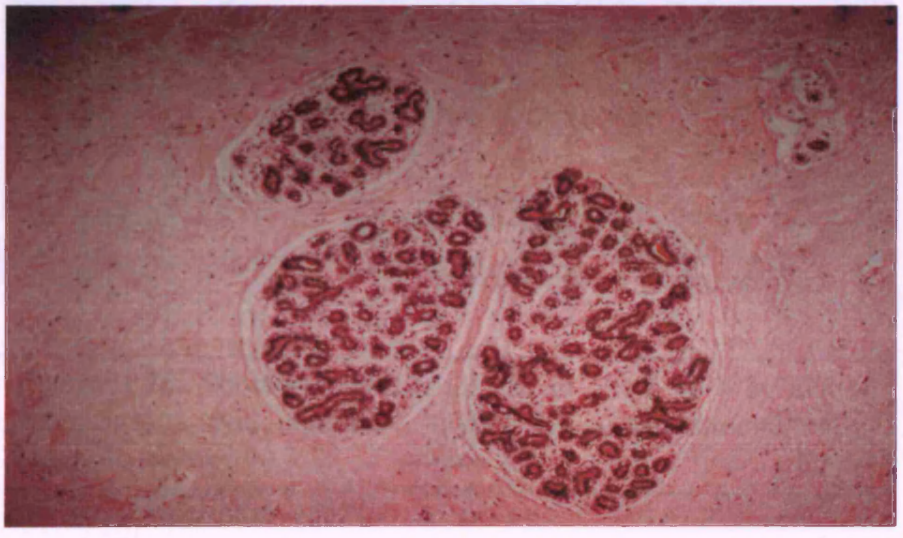


Figure 1.2a; Low power view of lobules from normal mature female breast tissue. Magnification x 50.

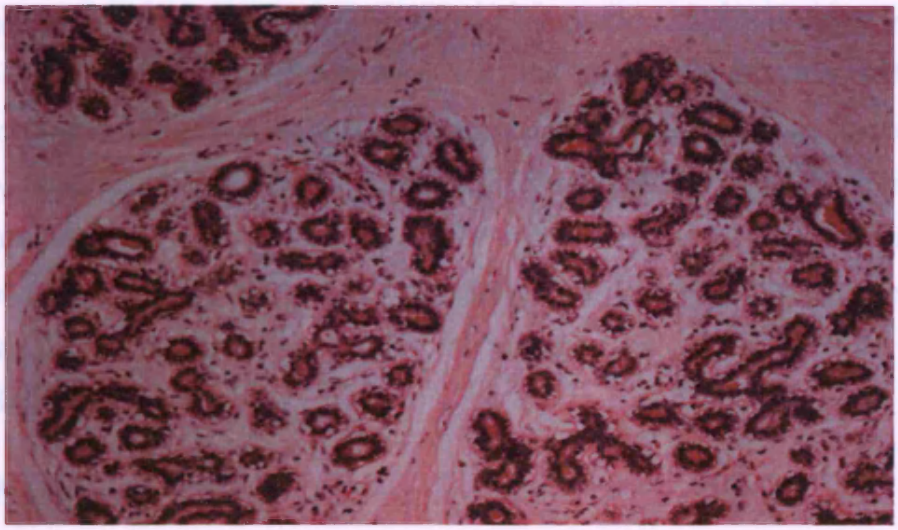


Figure 1.2b; Same lobules as in previous photomicrograph. The interlobular and intralobular connective tissues can be seen. Magnification x 100.

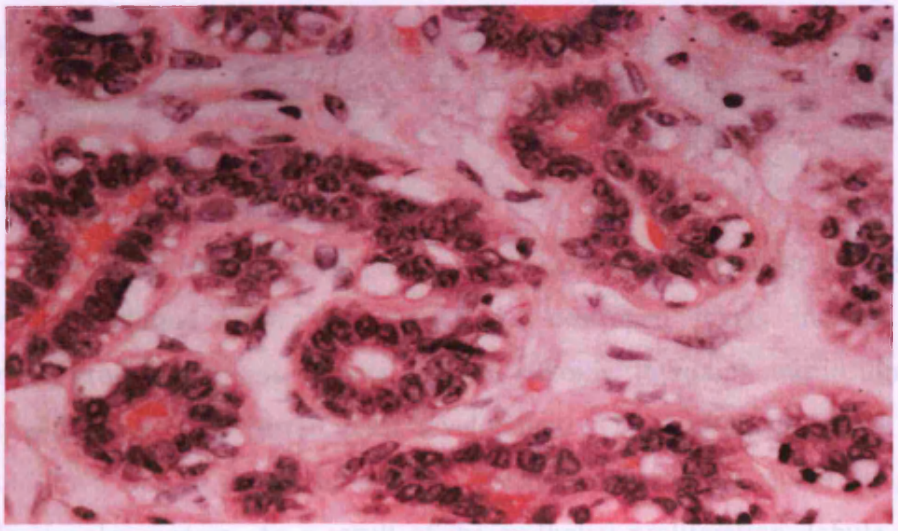


Figure 1.2c; Showing same lobule as in 1.2 a & b at higher magnification with epithelial, myoepithelial and stromal cells. Magnification x 400.

Lymphatic Drainage:

The breasts have a rich and complex network of lymphatic channels and the drainage to the loco-regional lymph nodes has been well documented (5;8). Over 95% of the lymphatic flow is towards the axillary lymph nodes, although drainage to the internal mammary chain can occur from any quadrant of the breast (9). Small intramammary lymph nodes may be present within the interlobular connective tissue in any quadrant of the breast (10). Drainage also occurs to subclavicular and supraclavicular nodes and periareolar plexus. The first node draining the primary tumour (sentinel node) is briefly discussed on page 25.

PHYSIOLOGY:

During childhood, both male and female breasts are relatively insignificant structures composed only of a small nipple-areolar complex and underlying rudimentary duct system. At puberty, the female breast develops its characteristic adult structure described above and can then be regarded as being in its 'resting' state. Minor variations occur during the menstrual cycle but major physiological changes are seen during pregnancy and lactation. Thereafter the appearances alternate between these two states until the menopause when there is a major regression and atrophy associated with aging. These physiological changes can result in quite different histological appearances, which still fall within the range of normality.

Changes at Puberty:

At puberty, elongation and branching of the ducts occurs, mainly under the influence of oestrogenic stimulation from the ovaries, as oestrogen levels increase 20-fold. Progesterone, growth hormone, adrenal cortical steroids, thyroxine and insulin are also thought to play a role in pubertal breast development (11). Oestrogens mainly cause the growth and branching of the ductal system, whereas progesterone causes the growth of the lobulo-alveolar system. As a result of the epithelial proliferation, lobule formation occurs and these bud out from terminal ducts to form the normal adult resting breast structure. There is also an increase in connective tissue stroma, interstitial matrix and adipose tissue and an increase in pigmentation and size of the nipple.

Changes During Menstrual Cycle:

The majority of women experience cyclical changes in the breasts during the menstrual cycle including increase in size and nodularity during the latter part of the cycle with increased sensitivity. There is a feeling of engorgement 3-4 days before menstruation and then the breasts return to the resting state at the end of menstruation. These changes are related to the cyclical variation in the levels of ovarian hormonal secretion and can manifest as morphologically recognizable parenchymal changes (12). During the follicular phase of the menstrual cycle, proliferation of the breast epithelium occurs with an increase in mitotic index (13;14). In the luteal phase, breast ducts and acini dilate and secretory changes occur in the acinar cells, with lipid material in the lumina. The premenstrual engorgement has been attributed to increased intralobular oedema and the epithelial proliferation. At the onset of menstruation secretory activity ceases and the breast epithelium returns to the resting state. Apoptosis occurs approximately 3 days after the peak of mitotic activity in order to restore the balance between cell proliferation and cell death. Changes in the stromal cells during the menstrual cycle are not well understood.

Changes During Pregnancy and Lactation:

Well defined changes occur in the breasts during pregnancy and lactation under the influence of oestrogen, progesterone, placental lactogen and prolactin. These hormones stimulate the proliferation of ductular and lobular epithelium to form new lobular units. Microscopically, by the 5^{th}-6^{th} month, a great increase in the amount of lobular tissue has taken place. Though oestrogen and progesterone are essential for the physical development of the breasts during pregnancy, they inhibit the actual secretion of milk, which is promoted by prolactin. This hormone is secreted by the female pituitary gland and its concentration in the blood rises steadily from the 5^{th} week of pregnancy till the baby's birth, at which time it is 10 times the non-pregnant level. Secretory activity becomes prominent within the luminal secretory cells and lipid droplets appear in acinar lumina. Myoepithelial cells become flattened and relatively less prominent as does the intralobular stroma. Interlobular connective tissue and adipose tissue also appear relatively insignificant. Further changes occur at parturition when the uninhibited action of prolactin and the stimulus of suckling by the infant quickly lead to the establishment of lactation. There is sudden loss of both oestrogen and progesterone. Other hormones,

which play an important role at this stage are growth hormone, cortisol and parathyroid hormone. These are necessary to provide the amino acids, fatty acids, glucose and calcium for milk formation. Once lactation starts, the acini become dilated. The cells may be tall or columnar and the cytoplasm granular or vacuolated.

Secretory activity is cyclical within the breast. Milk is expressed at the nipple following sensory nerve stimulation by the infant suckling, which results in release of oxytocin from the posterior pituitary gland. The impulses travel from sensory nerves of the nipple to spinal cord and then to the hypothalamus and finally along the neurosecretory fibres of the hypothalamoneurohypophyseal tract. Oxytocin causes the myoepithelial cells of the breast acini to contract and eject milk into the duct system and then the lactiferous sinuses.

Involution:

When lactation ceases, there is a gradual return to the 'resting' state, which takes three months on average (15). The reduction in prolactin level is a major contributory factor to this. Involution of the epithelial tissue occurs, with regression towards the resting ratio of connective tissue to lobules. However, the breasts never return completely to their former state and there is a variation in the degree of involution from one area to another.

Postmenopausal Atrophy:

At the end of reproductive years, there is marked reduction in ovarian hormonal secretions, accompanied by progressive atrophy of the epithelial components of the breast (16;17). There is an increase in the amount of adipose tissue, with hyalinization of connective tissue stroma. Most of the main duct system is preserved but there is considerable loss of lobular units. Remaining lobules show a marked reduction in size due to loss of acini. Hyalinization of intralobular stroma is also seen. Some ducts appear ectatic and focal microcyst formation is common in residual lobules. With advancing age any residual lobules become very small and indistinct and ductal structures predominate within a dense hypocellular stroma. Sometimes, an apparent myoepithelial hyperplasia is seen in the atrophic lobules (18;19). Occasionally, some apparently normal, resting type lobules, may be found in post menopausal breast tissue; and some of these may exhibit secretory features (lactational lobules). These have also been observed in nulliparous women (20).

CHAPTER 2: BREAST CANCER:

INCIDENCE:

Breast cancer (BC) is increasing worldwide with the highest rates reported in affluent western societies, (1 in 8 to 1 in 12). In the UK alone, it accounts for 15,000 deaths per year. The lifetime risk in the UK is 9% and average incidence 65 per 100,000 persons per year (1). Breast cancer remains the leading cause of cancer death of adult women under 54 years of age and the second most common cause after age 54 (1). Among women of all ages, breast cancer is second only to lung cancer as the leading cause of cancer deaths. Less than 1% of all breast cancer cases occur in men; in whom the course of disease and its clinical management are very similar to that in women.

RISK FACTORS:

Women at a higher risk for developing breast cancer are those with a strong family history of breast cancer (2), a personal history of breast cancer (2-11% of women diagnosed with BC will develop contralateral BC in their lifetime) (3), past history of benign breast biopsy especially if this shows atypical hyperplasia (4), early menarche or late menopause or a first full term pregnancy after age 30 (5), age at the last full term pregnancy may be important as well (6). The risk of developing breast cancer also increases with age, being most common after age 50. Long term oestrogen therapy [oral contraceptive pill (OCP) (7), hormone replacement therapy (HRT)(8;9)], a high fat diet, obesity (10) and alcohol use (11) have all been reported as risk factors. Ionizing radiation has also been associated with increased risk (12;13). It has been suggested that breast cancer may be associated with exposure to oestrogens and other hormones in utero. Twin pregnancies have higher levels of pregnancy-associated hormones than singleton pregnancies, and these levels may be higher in dizygotic than in monozygotic twin pregnancies. Twins seem to have a higher risk than the general population, especially dizygotic twins and females with twin brothers (14). However, the risk is less in mothers of twins (15), due to proposed inhibitory effect of human chorionic gonadotrophin (HCG) and alpha-feto protein (AFP) (16).

CLINICAL PRESENTATION:

Most of the patients (85%) with breast cancer have a lump in the breast. Less commonly, nipple discharge (5%), skin deformity or ulceration (1%), an axillary lump (1%) or Paget's disease of the nipple may be presenting features. Breast pain (5%) can also be a symptom of early breast cancer (17). With the advent of mammographic screening, asymptomatic cancers are being increasingly diagnosed. These are more likely to be node negative (65%), small (50% < 15mm) and more likely to be of good prognostic type, well differentiated or in situ cancers (18;19).

Patients may present with metastatic disease and its complications e.g., bone pain, hypercalcaemia, pleural effusion, liver disease etc.

SCREENING:

With modern technology, breast cancer can be detected at a very early stage of development when the chance for cure is highest (20). The key to cure is early detection and prompt treatment. Physical examination, mammography and breast self examination comprise an early detection approach.

Population screening of women over 50 with mammography has been shown to reduce breast cancer mortality at 10 years follow up by 30% (21). National screening programmes examine women aged 50-64 years with a single oblique mammogram every 3 years, there is increasing evidence that screening during the fifth decade (40-49 years age group) may also be effective (22;23).

DIAGNOSIS AND INVESTIGATIONS:

1. Mammography: Mammography can detect the majority of breast cancers, although 10-14% of clinically diagnosable cancers will not be detected, therefore, a normal mammogram does not exclude breast cancer. Radiological signs of malignancy include spiculate masses, microcalcification, stellate lesions, parenchymal distortions and skin tethering.

2. Ultrasound: Ultrasound is not useful as a screening tool. Cancers are typically seen as solid, irregular and vascular. Ultrasound is not as sensitive or specific as mammography but it is particularly useful in younger women. Ultrasound can

categorize palpable masses, which are indeterminate mammographically. Ultrasound is sometimes used to improve the accuracy of fine needle aspiration.

3. MRI: The role of magnetic resonance imaging (MRI) as a modality to detect, diagnose and stage breast cancer is as yet unclear. It is costly but very accurate to detect breast implant failure (24). It is sensitive for the visualization of both DCIS and IDC and can detect these when they are clinically and mammographically occult, offering the future potential for more accurate breast cancer staging and optimized treatment planning.

4. Fine Needle Aspiration Cytology (FNAC): This is a simple technique with 58% specificity. It can differentiate between cysts and solid tumours, however, it cannot distinguish between in situ and invasive carcinoma.

5. Core Biopsy: A core biopsy performed under local anaesthetic, can accurately confirm the diagnosis preoperatively. It provides a large core of tissue for histological study and allows further investigations (e.g., hormone receptor staining to be performed). It is a highly sensitive and specific technique for the management of patients with mammographic abnormalities.

6. Localization Biopsy: This form of biopsy is done for impalpable mammographically suspicious lesions.

7. Tumour Markers: The major role of tumour markers (CA 15.3, CEA) is in the diagnosis and monitoring of metastatic BC.

STAGING:

Staging is a method of grouping patients by the extent of disease to determine the choice of treatment and predict prognosis. The more advanced the stage of the disease, the poorer the prognosis. Staging investigations include: full blood count, serum biochemistry, chest X-ray in all patients and liver ultrasound and bone scan for patients with stage II, III and IV disease.

The staging system (TNM) recommended by the American Joint Committee on Cancer (AJCC) in the 5th edition is shown in table 2.1:

Table 2.1; The staging system (TNM) recommended by the American Joint Committee on Cancer (AJCC)

Stage	Tumour (T)	Nodes (N)	Metastasis (M)
0	Tis	N0	M0
I	T1	N0	M0
IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T3	N1, N2	M0
IIIB	T4	Any N	M0
	Any T	N3	M0
IV	Any T	Any N	M1

Explanation:

(1) Primary tumour (T):

Tx - Primary tumour cannot be assessed

T0 - No evidence of primary tumour

Tis - Carcinoma in situ (DCIS, LCIS, Paget's disease of nipple)

T1 - Tumour size < 2cm

T1a - <0.5 cm

T1b - 0.5 – 1 cm

T1c - 1 – 2 cm

T2 - Tumour size 2-5 cm

T3 - Tumour size >5 cm

T4 - Tumour of any size with direct extension to chest wall or skin

T4a - Chest wall extensions

T4b - Oedema or ulceration of skin or satellite skin nodules

T4c - Both T4a and T4b

T4d - Inflammatory carcinoma

(2) Regional Lymph Nodes (N):

Nx - Regional lymph nodes cannot be assessed

N0 - No nodal metastasis

N1 - Metastasis to mobile ipsilateral axillary lymph node/s

N2 - Metastasis to fixed ipsilateral axillary lymph node node/s

N3 - Metastasis to ipsilateral internal mammary, supraclavicular or infraclavicular nodes.

(3) Distant Metastasis (M):

Mx - Presence of distant metastasis cannot be assessed.

M0 - No distant metastasis

M1 - Distant metastasis

An alternative staging system used quite often in clinical situations is shown in table 2.2.

Table 2.2; Staging of breast cancer by defining risk groups of patients

Stage	Group	Five year Survival	Example	Treatment
0	Minimal risk	>90%	Screen detected <1cm DCIS	Local
I	Low risk	70 – 90%	Node – negative histological Grade I and II	Loco – regional
II	High risk	30 – 70%	Node – positive or Grade III	Loco – regional and systemic
III	Locally advanced	10 – 30%	Large tumour or skin fixation/ulceration	Primary systemic
IV	Metastatic	<10%	Liver and lung metastases	Primary systemic

CLASSIFICATION & PATHOLOGY:

Classification of breast malignancy is based on the morphological features described by the World Health Organisation (WHO) (25-29) and is as follows:

Epithelial:

1- Ductal carcinoma in situ (high grade, intermediate grade, low grade and mixed).

2- Micro-invasive carcinoma (MIC).

3- Invasive carcinoma (the usual types are ductal carcinoma of no special type 45-70%, lobular 5-10%, medullary 2-10%, tubular 1-7%, invasive cribriform 0.8-3.5%, tubular mixed 14%, mucinous 1-3%, invasive papillary <2%, metaplastic , mixed, and the rare types are squamous cell, mucoepidermoid, low grade adenosquamous, adenocystic, malignant adenomyoepithelioma, malignant myoepithelioma, apocrine, primary oat cell, clear cell and secretory) (30).

Mesenchymal:

These include angiosarcoma, lymphangiosarcoma, fibrosarcoma, malignant fibrohistiocytoma, liposarcoma, osteogenic sarcoma, leiomyosarcoma, rhabdomyosarcoma, chondrosarcoma. Primary malignant mesenchymal tumours are exceedingly uncommon in the breast.

Miscellaneous:

These are malignant lymphoma (Non-Hodgkin's and Hodgkin's), plasmacytoma, leukaemia and metastatic tumour from cancer at other sites.

It has been shown that majority of breast carcinomas arise from the terminal duct lobular unit (TDLU)(31).

The commonest histological type (70%) of breast cancer is invasive or infiltrating ductal carcinoma (IDC)(32). Fisher et al have added the term "not otherwise specified (NOS)" (33) and Page et al have used the term "no special type (NST)" (34). IDC is subdivided into well differentiated (Grade I), intermediate differentiated (Grade II) and poorly differentiated (Grade III) on the basis of histological features. Elston and Ellis (1991)

reported 16 years survival in grade I tumours to be 90%, 55% in Grade II and 48% in Grade III (35). The Elston and Ellis grading system has been modified from the one described by Bloom and Richardson (36). The following protocol is based on that described by Elston and Ellis. The method involves the assessment of three components of tumour morphology: tubule formation, nuclear pleomorphism and frequency of mitoses. Each is scored from 1-3. Adding the scores gives the overall histological grade as shown below.

Tubule Formation:

Score:

1. majority of tumour (greater than 75%)
2. moderate amount (10 - 75%)
3. little or none (less than 10%)

Nuclear Pleomorphism:

Score:

1. nuclei small, with little increase in size in comparison with normal breast epithelial cells, regular outlines, uniform nuclear chromatin, little variation in size.
2. cells larger than normal with open vesicular nuclei, visible nucleoli and moderate variability in both size and shape.
3. vesicular nuclei, often with prominent nucleoli, exhibiting marked variation in size and shape, occasionally with very large and bizarre forms.

The evaluation of nuclear grade in the grading of invasive breast cancer is one of the most difficult and least reproducible category compared with tubule formation and mitotic count (37).

Mitosis:

The score depends on the number of mitoses per 10 high power fields (hpf) assessed at the tumour periphery. For example, for a field diameter of 0.48,

Score:

1. 0 – 6 mitoses/hpf
2. 7 – 12 mitoses/hpf
3. >12 mitoses/hpf

A minimum of 10 fields is counted at the periphery of the tumour where the proliferative activity has been demonstrated to be the greatest (38;39).

Overall Grade:

The scores of tubule formation, nuclear pleomorphism and mitoses are then added together and assigned to grades as below:

Well differentiated	(Grade I)	=	score 3 - 5	(Figure 2.1 a-b)
Intermediately differentiated	(Grade II)	=	score 6 - 7	(Figure 2.2 a-b)
Poorly differentiated	(Grade III)	=	score 8 – 9	(Figure 2.3 a-b)

Histological grade is a powerful independent prognostic factor like lymph node stage (35) (40)and the consistency and reproducibility of histological grading has now been confirmed in several studies (41-43). Both grade and type should be assessed in all cases of invasive breast cancer (44), although both histological grading and typing are subject to interobserver variability (45;46).

Invasive lobular carcinoma has a greater tendency to be multifocal (10-15% of cases) and bilateral than ductal tumours and is often (60%) associated with in situ lobular carcinoma (LCIS) (47;48). Tubular, medullary and mucinous (colloid) constitute 10% of all breast cancers collectively and have a notably better prognosis (49). These histological types are found more frequently among mammographically detected cancers (50-52).

In situ carcinoma of the breast may be ductal, lobular or mixed. Ductal carcinoma in situ (DCIS) is discussed in detail in chapter 6. Lobular carcinoma in situ (LCIS) is usually an incidental finding as it is typically impalpable and undetectable mammographically. It is usually diagnosed in premenopausal women, multicentric foci may be present in up to 90% of cases and contralateral LCIS is found in 25% when a mirror image biopsy is performed (53). It is generally regarded as a risk factor rather than a premalignant condition (relative risk increased 10 fold) because invasive cancer, when it develops, can occur in either breast with equal frequency and can be of either ductal or lobular type. Approximately 25% of women with LCIS will develop invasive breast cancer within 20 years from diagnosis giving an estimated cumulative annual risk of 1% per year.

Figure 2.1; Photomicrographs of a case of invasive ductal carcinoma (histological grade I)

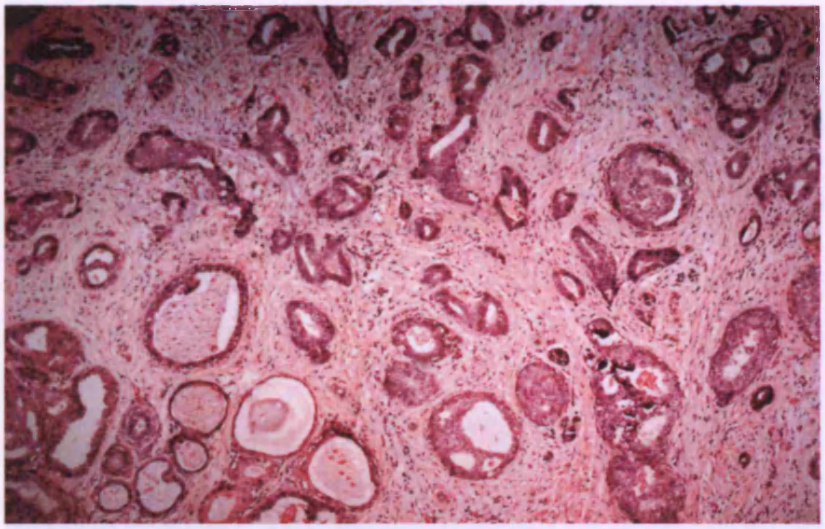


Figure 2.1a; Low power view of a grade I IDC showing marked tubule formation (score = 1). Magnification x 50.

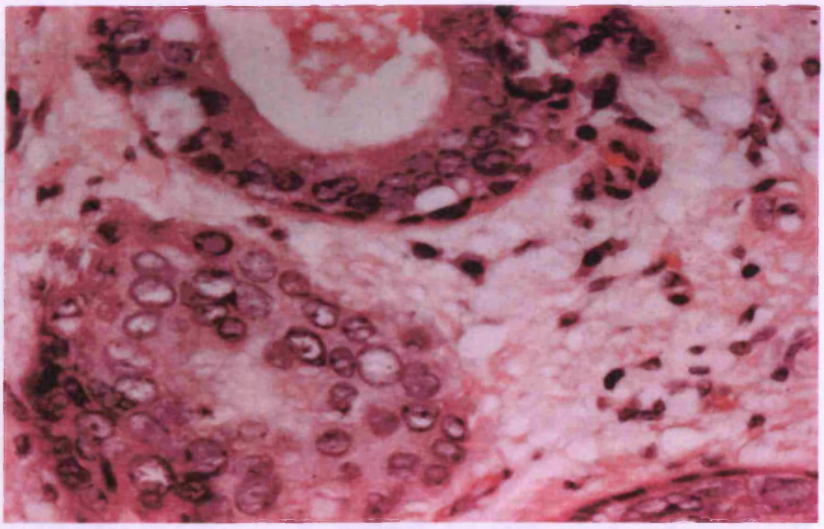


Figure 2.1b; Same case as in 2.1a, showing mild degree of nuclear pleomorphism and scanty mitosis. Magnification x 400.

Figure 2.2; Photomicrographs of a case of invasive ductal carcinoma (histological grade II)

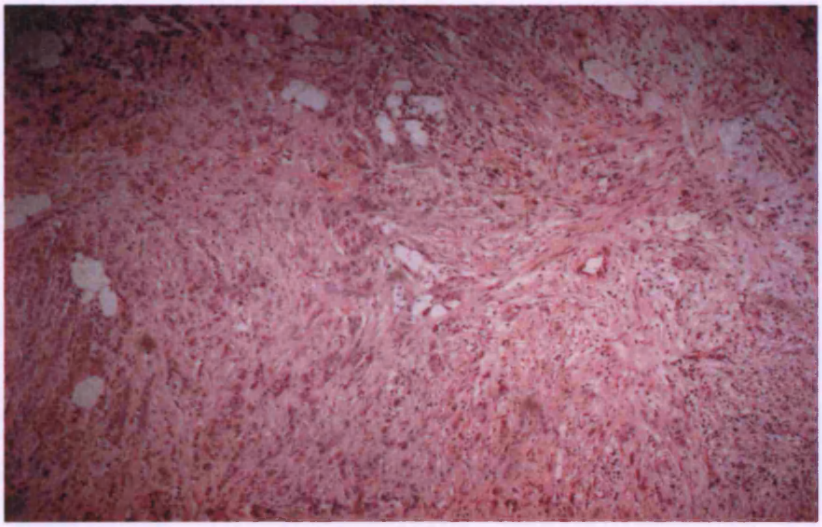


Figure 2.2a; Low power view of a grade II IDC showing some tubule formation (score = 2). Magnification x 50.

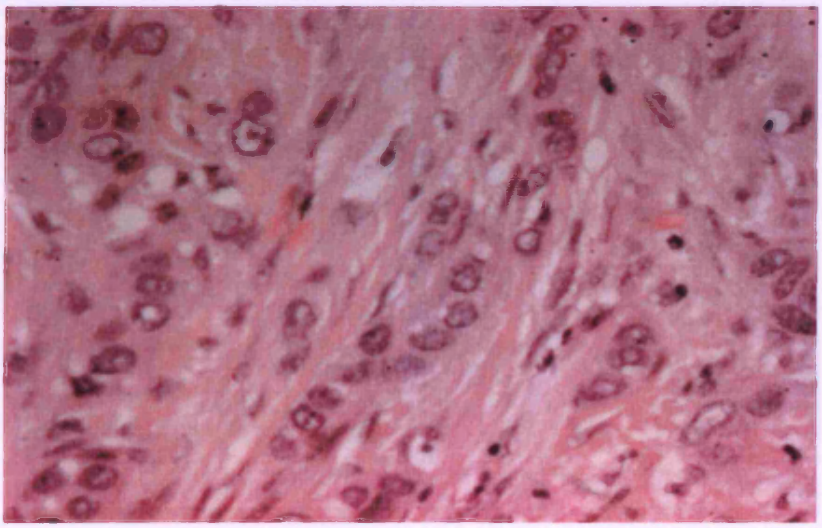


Figure 2.2b; Same case as in 2.2a, showing moderate degree of nuclear pleomorphism and mitosis. Magnification x 400.

Figure 2.3; Photomicrographs of a case of invasive ductal carcinoma (histological grade III)

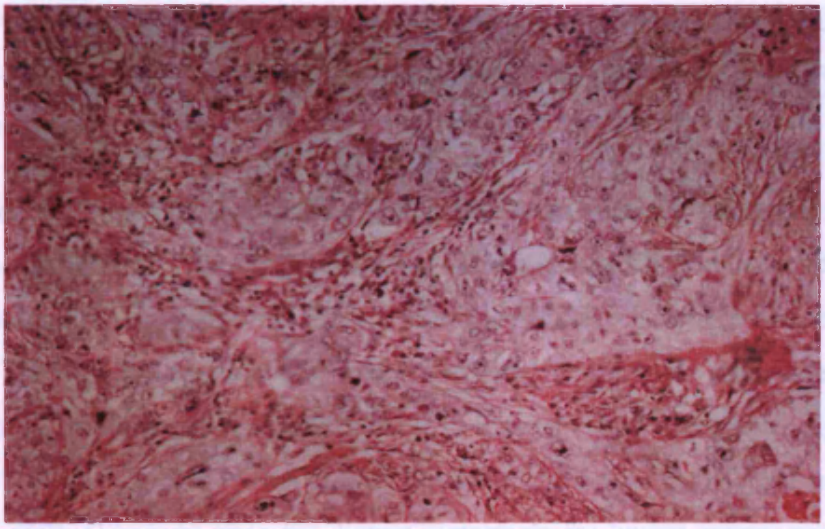


Figure 2.3a; Low power view of a grade III IDC showing scanty tubule formation (score = 3). Magnification x 50.

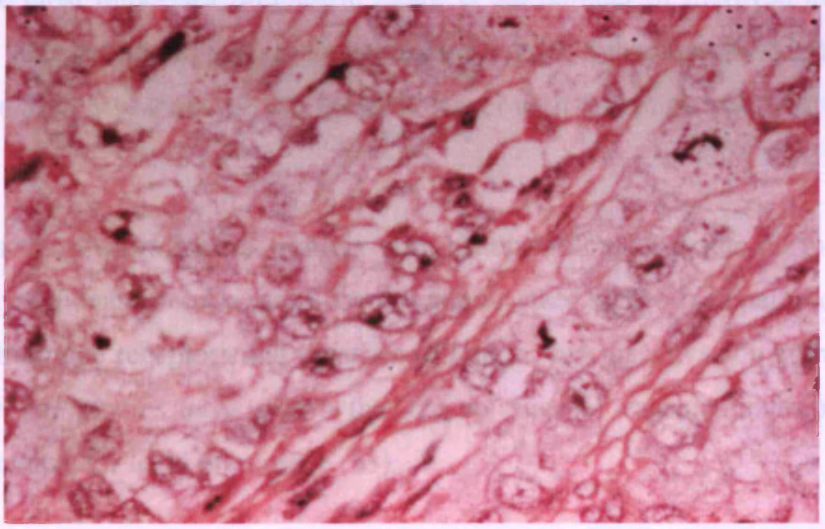


Figure 2.3b; Same case as in 2.3a, showing marked nuclear pleomorphism and abundant mitosis. Magnification x 400.

Extensive in Situ Component (EIC):

Extensive in situ component (EIC) is defined as the presence of DCIS within an invasive carcinoma, in which the DCIS comprises 25% or greater of the tumour mass and extends clearly beyond the infiltrating margins of the tumour into grossly normal adjacent breast tissue (54;55). The presence of EIC is a significant risk factor for local recurrence in patients treated with conservative surgery and radiotherapy (54-57).

Breast cancer commonly metastasizes to ipsilateral axillary nodes and nodal involvement is found in up to 50% of symptomatic cases at presentation (58). The more axillary nodes that are involved, the greater the risk of micrometastasis elsewhere and of relapse or recurrence. The reported incidence of 'occult' axillary lymph node metastases in patients with apparently 'node-negative' breast cancer is between 9-33% but their significance is debated (59). However, one recent study has demonstrated these to be of no prognostic significance (60). Common sites for distant metastasis for breast cancer are bone, liver, lung and brain. Lobular carcinoma may also spread to atypical sites such as the meninges or the serosal surfaces of pleura and peritoneum.

PROGNOSIS:

Overall survival (OS) for breast cancer at 5 years is 50%, at 10 years 35% and at 15 years is 30%. The most important prognostic factor is the number of involved axillary nodes with 78% 5 years survival for node-negative cancer, 63% 5 years survival if 1-3 nodes are involved and 32% 5 years survival for breast cancer with 4 or more involved lymph nodes (61;62).

Other prognostic factors include tumour size, histopathological grade, hormone receptor status and lymphovascular invasion. Large tumour size, higher histopathological grade, absence of hormone receptors and presence of lymphovascular invasion correlate to poor prognosis. Some studies have shown that the presence of vascular invasion in tumour specimen predicts for local recurrence (63).

Analysis of a range of prognostic factors, using the multiple regression model of Cox (64), showed that only tumour size, histological lymph node stage and histological grade give a significant correlation with overall survival (65). Based on these findings, the Nottingham prognostic index (NPI) has been formulated as follows:

NPI = 0.2 x tumour size (in centimetres) + lymph node stage (1 – 3) + histological grade (1 – 3)

[Lymph node stage 1 = 0 nodes involved, stage 2 = 1 – 3 nodes involved and stage 3 = >3 nodes involved.]

Prognosis worsens as the numerical value of NPI increases and by using cut-off points of 3.4 and 5.4, patients may be categorized into good, moderate and poor prognostic groups having an annual mortality of 3, 7 and 30% respectively (66). The predictive value of the NPI has been confirmed after long-term (15 year) follow-up (67) and also validated independently by the Yorkshire Breast Group (68).

Increased tumour proliferation as measured by S-phase fraction, thymidine labelling index, tumour ploidy and Ki67 immunostaining, has also been related to worse prognosis. Furthermore cerbB-2 oncoprotein and cathepsin D expression are also associated with a worse prognosis. However, these molecular markers are not yet widely used in clinical decision making.

Recently, under the auspices of the College of American Pathologists, a multidisciplinary group of clinicians, pathologists and statisticians considered prognostic and predictive factors in breast cancer and stratified them into categories reflecting the strength of published evidence. Category I included factors proven to be of prognostic importance and useful in clinical patient management and these were TNM staging, histological grade, histological type, mitotic figure counts and hormone receptor status. Category II factors were those which have been studied extensively biologically and clinically, but whose importance remains to be validated in statistically robust studies and these included cerbB-2, proliferation markers, p53 and lymphatic and vascular channel invasion. Category III factors were all other factors not sufficiently studied to demonstrate their prognostic value and included DNA ploidy analysis, microvessel density, epidermal growth factor receptor (EGFR), transforming growth factor-alpha (TGF-α), bcl2, pS2 and cathepsin D (69).

FAMILIAL BREAST CANCER:

Familial breast cancer (FBC) accounts for 10-15% of all breast cancers. The genes responsible include BRCA-1 on chromosome 17q21, BRCA2 on 13q12-13, p53 on

17p13, ataxia telangectasia (AT) on 11q23, HRAS minisatellite locus on 11p15 and the androgen receptor (AR) gene on the X chromosome. These genes do not account for all high risk breast cancer families, therefore probably there are other genes responsible that remain to be identified yet. The above mentioned genes differ markedly in the risks of breast cancer they confer and the proportion of breast cancer cases which they explain. BRCA-1 mutations account for 5% of all breast cancer cases, that is, a high proportion of familial BC cases and the relative risk of developing BC is 50% by the age of 50.This is discussed further in chapter 3. BRCA-2 has a strong association with male breast cancer. Germline p53 mutations are rare and cause less than 1% of breast cancer cases, although the risk of developing BC is high, over 50% by age 50 (70;71). Germline mutations of the androgen receptor (AR) gene are an even rarer cause of breast cancer, and have been shown to cause male breast cancer in only two families (72). Ataxia-telangiectasia (AT) gene is more common, probably responsible for 7% of BC cases, but confers a low risk, about 11% by age 50 (73). The HRAS minisatellite locus has alleles, which are mutated much more frequently, but confers a very low risk (about 3% by age 50). Thus only BRCA-1 explains a substantial proportion of 'familial breast cancer' cases. The other genes are either too rare (p53, AR) or confer too low a risk (AT, HRAS) to have a measurable familial effect.

Familial Genotypes:

Familial genotypes include: 1) site-specific breast cancer which is characterized by early age of onset and frequent bilaterality, 2) breast cancer displaying a familial association with ovarian and endometrial carcinoma, 3) breast cancer displaying a familial association with carcinomas of gastrointestinal tract, 4) breast cancer in Cowden's disease (multiple hamartoma syndrome), 5) breast cancer in Klinefelter's syndrome, 6) breast cancer showing a familial association with cutaneous malignant melanoma (74) and 7) Li Fraumeni syndrome which is characterized by bone/soft tissue sarcoma, breast cancer, brain tumour, adrenocortical carcinoma and leukaemia (75) and is associated with germline p53 mutation. The criteria for diagnosis include 1) a proband with bone or soft tissue sarcoma diagnosed under the age of 45 years, 2) one first degree relative with cancer under the age of 45 years and 3) one first or second degree relative in the same lineage with cancer under 45 years of age or sarcoma diagnosed at any age.

MALE BREAST CANCER:

Male breast carcinoma (MBC) is uncommon, with the reported incidence in the literature being 1% or less (76-78). MBC affects an older population, presents late and is frequently fatal (78). The mean age at presentation is 60-65, which is approximately 10 years later than in females (77). Over 80% of patients present with a breast lump and histologically the majority of the male breast carcinomas are ductal, NST (78). Other histological subtypes occur rarely. Pure DCIS has also been reported in males, but the reported frequency varies from 2.5-14% of total cancers in males (78;79). The potential risk factors include hyperoestrogenism, gynaecomastia, family history in either female or male relatives and prior radiation exposure. Klinefelter's syndrome is known to be a definite risk factor (80). The differential diagnosis is from gynaecomastia and secondaries from other sites. Cancers which frequently metastasize to male breast are prostate, lung, kidney and malignant lymphoma. MBC was thought to have a worse prognosis than BC in females, the overall 5- and 10-year survival rates being 38 and 17% respectively in one series (81). However, one new study has shown that there is no real difference in OS between males and females when known prognostic factors are taken into account (82). Biological factors such as ER, MIB1, cerbB-2 and p53 have been studied in some MBC patients but there are insufficient data available on the prognostic value of these in MBC. The management of MBC is similar to BC in females.

BREAST CANCER IN CHILDREN:

This is exceedingly rare. Less than 50 cases were reported by 1973. Isolated cases since then have been reported (83-85). The most common pattern is secretory carcinoma (86;87). It may develop local recurrence, and can rarely metastasize to regional lymph nodes (88) or to systemic sites. Usually breast cancer in children has an excellent prognosis (89). Isolated examples of rhabdomyosarcoma, angiosarcoma and non-Hodgkin's lymphoma (NHL) of the breast have been reported in patients in the second decade of life.

CLONALITY OF HUMAN BREAST CANCER:

Tumours are generally considered to be monoclonal (90;91), including breast cancer. Some hyperplasias are also monoclonal. However, Going et al (92) have recently demonstrated that at least some breast cancers are likely to be polyclonal by analysis of

X-chromosome inactivation in DNA extracted from microdissected in situ and invasive breast carcinoma by *Hpa* II restriction and polymerase chain reaction (PCR) of the androgen receptor (AR) exon I CAG polymorphism.

MANAGEMENT:

1. Carcinoma in situ: (1a) Ductal carcinoma in situ (DCIS) : Management of DCIS is dealt with in chapter 6. (1b) Lobular carcinoma in situ (LCIS): Wide local exision (WLE) is inappropriate as the condition is usually multifocal. As LCIS is a marker of increased risk of BC, patients should be observed clinically with regular mammography. Tamoxifen is currently being used in a randomized clinical trial by the European Organization for Research and Treatment (EORTC).

2. Invasive carcinoma of the breast: Management depends on the type and stage of disease at the time of diagnosis. Treatment is generally multidisciplinary with a combination of surgery, radiotherapy and chemotherapy (93). Stage I and II disease is potentially curable by surgery and is considered early breast cancer. Stage III is locally advanced breast cancer and Stage IV is metastatic breast cancer. Locally advanced and metastatic breast cancers are incurable but the natural history is very variable and ranges from months to many years.

(2a) Early breast cancer: This is generally treated by breast conservation surgery (which is usually wide local excision and axillary dissection to either level I, II or III) followed by radiotherapy to the whole breast or by modified radical mastectomy with or without breast reconstruction. Simple excision of the tumour in the absence of radiotherapy is associated with a high incidence of local recurrence (about 15-40%) (94). Studies have shown no survival difference between breast conservation surgery followed by radiotherapy and modified radical mastectomy provided tumours are small in size and there is no evidence of multifocal disease or extensive in situ carcinoma. Criteria for selecting patients for conservation include tumour size <3cm, histological grade I or II, no lymphatic or vascular invasion and no extensive in situ changes. The indications for mastectomy include tumour size >4cm, multifocal disease, recurrent disease, centrally located tumour, DCIS component >40mm and the patient's preference.

Axillary node status is the best single predictor of disease outcome in patients with early stage breast cancer (95). There are three main methods of determining axillary node

status- axillary node sampling (ANS), axillary node clearance (ANC) and sentinel lymph node biopsy (SLNB). Recent studies have demonstrated that the sentinel lymph node biopsy (SLNB) is a simple and reliable method for determining the status of the regional lymph nodes in patients with clinically node-negative breast cancer (96). In this technique, subdermal or peritumoral injection of vital blue dye or radiolabelled colloid, or both substances together can be used to identify the first lymph node (sentinel node) draining the primary tumour. The sentinel node can be examined intra-operatively by frozen section. Axillary node clearance can subsequently be performed if intra-operative examination of the node is positive for malignancy, thus avoiding the need for a second surgical procedure.

Surgery and radiotherapy are local treatments and will not affect undetectable micrometastasis at the time of diagnosis. Adjuvant therapy may be given to patients with early stage disease who are at higher risk of developing metastatic disease based on the number of involved axillary nodes, menopausal status and hormonal receptor status (97).

Adjuvant Therapy:

Adjuvant therapy is considered in three different groups of patients as follows:-

(I) Premenopausal node positive: These patients should receive polychemotherapy. The commonest regime used is FEC (5-fluorouracil, epirubicin and cyclophosphamide) for 6 months. The other common regime is CMF (cyclophosphamide, methotraxate, 5-fluorouracil).

(II) Premenopausal node negative: 30% of women with node negative cancers relapse. Consideration for chemotherapy in this group should be given particularly to those with large, poorly differentiated, oestrogen receptor negative tumours. These patients can also benefit from tamoxifen unless the tumours are ER (oestrogen receptor) negative.

Trials are still being conducted of chemotherapy in node negative patients.

(III) Post menopausal: Irrespective of nodal and ER status, postmenopausal patients benefit most from tamoxifen and increased benefit may occur with combined chemotherapy in node-positive patients. Tamoxifen reduces the risk of relapse and

mortality by 25% and 17% at 5 years respectively, the benefits of adjuvant tamoxifen are greater for node positive patients compared with node negative and also for women older than 50.

Neo-adjuvant Therapy:

Primary medical therapy for tumours >4cm may allow breast conservation without detriment to survival. Several different regimes are being evaluated. The most commonly used regime is AC (adriamycin 60mg/m^2 and cyclophosphamide 600mg/m^2). Follow-up is usually by three monthly reviews for first two years following treatment, six monthly reviews uptil five years after treatment and yearly afterwards with an annual mammogram.

(2b) Locally advanced breast cancer:

Patients with locally advanced (stage III) breast cancers have a poorer prognosis and are not candidates for curative surgery. However, good local control may be achieved with combination surgery, and radiotherapy, followed by chemotherapy, to improve disease-free (DFS) and overall survival (OS).

(2c) Metastatic breast cancer:

The main aim of therapy for patients with metastatic breast cancer is palliation of disease related symptoms and improved quality of life. Premenopausal patients are treated by combination chemotherapy and/or hormonal therapy such as LHRH agonists and tamoxifen. Chemotherapeutic regimes include CMF, FEC (5-fluorouracil, epirubicin and cyclophosphamide), MMM (mitozantrone, methotrexate and mitomycin) and taxol.

Post menopausal patients in the past were primarily treated by tamoxifen followed by aromatase inhibitors such as anastrozole or progestogens. At relapse, the overall objective response rate with tamoxifen based on 5353 patients in 86 clinical trials was 34% with disease stabilisation in a further 19%. The median duration of response varied between 2 and 24 months (98). Breast cancer patients more likely to respond to tamoxifen are those that are ER positive, progesterone receptor positive, with a long disease-free interval (>2 years) and predominantly soft-tissue and/or bone disease and a single site of disease. Ten percent of ER negative BC patients also respond to endocrine therapy (98). In the light of

recent randomised trial data, aromatase inhibitors are now 1st line treatment for hormone positive metastatic breast cancer.

Patients with metastatic disease rarely exhibit a lasting response to standard treatments, therefore, researchers are finding the use of high dose chemotherapy regimes followed by autologous bone marrow transplant (BMT) or peripheral blood stem cell rescue to be promising. In early phase II studies in patients with measurable disease, there were high response rates with a complete remission rate of up to 60% (99).

New chemotherapy drugs in the treatment of metastatic breast cancer include navelbine (100), taxol (101) and Herceptin (discussed further in chapter 4).

Other treatment modalities include management of specific problems, for example, radiotherapy for bone metastasis, central nervous system (CNS) metastasis and spinal cord compression, bisphosphonates for hypercalcaemia, surgical fixation of pathological fractures, pleurodesis for recurrent pleural effusions, Leveen shunt for recurrent ascites, pleuropericardial window for recurrent symptomatic pericardial effusion, adequate analgesia, psychological support and good palliative care.

PROPHYLACTIC THERAPY (CHEMO-PREVENTION):

Trials are in progress to assess the value of tamoxifen and raloxifene in the prevention of breast cancer in patients with a strong family history of BC. The use of tamoxifen in prevention is being tested in an international clinical trial (IBIS). Striking reductions in breast cancer risk were observed for raloxifene in a randomised, placebo-controlled trial that had been designed for the prevention of osteoporosis (102).

CHAPTER 3: MOLECULAR BIOLOGY OF BREAST CANCER:

In this chapter, an account of the molecular biology of cancers in general is given for basic understanding, followed by the description of specific genetic alterations in breast cancer. The recommendations of the International Standing Committee on human gene nomenclature have been followed. Thus human genes are written in italicised capitals and their gene products in non-italicized capitals (1). For genes of other species, the nomenclature recommended for murine genes has been followed, genes are italicised with an initial capital letter followed by lower case letters and the corresponding proteins are written in non-italicized capitals (2).

GENETIC EVENTS IN CANCER DEVELOPMENT:

Cancer develops through a succession of stages marked by the accumulation of genetic events within the cell (3). The progression from normal cell to cancer cell is a complex multistep process. Initially genetic alterations are thought to confer a growth advantage to individual cells by either decreasing tumour suppressor gene activity or increasing oncogene activity or both. Further genetic alterations result in the development of cell clones that have the ability to invade adjacent tissue, establish metastatic deposits and evade immune surveillance. At some point in the process, these malignant cell clones also lose the normal ability to respond to hormonal growth regulatory signals.

Tumours result from disruption of the processes that control the normal growth and the mortality of cells. This loss of normal control mechanisms arises from the acquisition of mutations in three broad categories of genes:

1. Proto-oncogenes, the normal products of which are components of signalling pathways that regulate proliferation and which in their mutated form, become dominant oncogenes (4).

2. Tumour suppressor genes, which generally exhibit recessive behaviour, the loss of function of which leads to deregulated control of cell cycle progression and inability to undergo apoptosis.

3. DNA repair genes, mutations in which promote genetic instability.

Cancers arise mainly from deregulated proliferation; however, modulation of the normal processes that leads to cell loss (programmed cell death, apoptosis) is also sufficient, although not necessary, for tumour development. A further consequence of the activation of oncogenes or the ablation of tumour suppressor genes may be that the growth arrest process associated with ageing (senescence) may be over-ridden. Senescence usually correlates with decrease in the length of telomeres which are short, tandem repeats of the hexanucleotide 5'-TTAGGG-3' at the ends of each chromosome. Telomeres are synthesized by telomerase, a ribonucleo-protein DNA polymerase that is active in many tumours but is not common in normal tissues (5). In tumour cell lines telomerase activity reaches a maximum in the S phase and a variety of inhibitors of cell cycle progression (e.g. transforming growth factor β1) inhibit telomerase activity (6).

ONCOGENES:

Oncogenes were first identified in viruses capable of inducing tumours in animals and of transforming cells in vitro. These viruses have RNA genomes and are called 'retroviruses'. They can replicate through a DNA intermediate in infected cells (e.g. mouse mammary tumour virus-MMTV). The oncogenes carried by these viruses are strongly homologous in sequence to normal cellular genes (proto-oncogenes) that are highly conserved in evolution. Despite their potent tumorigenic capacity in appropriate animal hosts, no retrovirus has yet been shown to be directly oncogenic in humans. However, it is quite likely that the latent development of cancers commonly associated with infection by human T-lymphotropic viruses (HTLV-I and II) and human immunodeficiency virus (HIV) occurs from subversion of normal cellular control mechanisms by the transcription factors encoded in the virus genomes (3). The DNA viruses of the adenovirus, herpesvirus, poxvirus and papovavirus families also possess oncogenic potential. Some DNA tumour viruses are oncogenic in humans (hepatitis B virus, Epstein-Barr virus) but most are only tumorigenic in other species. The oncogenes of DNA viruses differ from those of retroviruses and their transforming genes have not yet been shown to have proto-oncogene homologues within the normal human genome, except for a few including the presence of *BCL2* sequences in the Epstein-Barr virus (EBV) *BHRF1* gene.

There are nearly 200 known oncogenes among about 60,000 functional human genes (3). The main classes of oncoproteins (proteins encoded by oncogenes) include growth factors

(e.g.HSTF1, INT2, PDGFB/SIS, WNT1, WNT2, WNT3 etc.), tyrosine kinases (FMS, EGFR, KIT, MET, HER2/NEU, RET, TRK, SRC), serine-threonine kinases (AKT1, AKT2), membrane associated guanine nucleotide binding proteins (HRAS, KRAS, NRAS), cytoplasmic regulators (CRK) cell cycle regulators (INK4A, INK4B, CYCLIN D1, CDC25A, CDC25B), transcription factors (BCL3, E2F1, ERBA, ETS, FOS, JUN, MYB, MYC, REL, TAL1, SKI), intracellular membrane factor (BCL2), RNA binding proteins (EWS) and others; and these act at different points in signalling pathways. Oncogene activation mainly is the result of somatic events rather than hereditary genetic causes transmitted by mutation in the germline. In normal cells proto-oncogene activation may occur by mutation, DNA rearrangement or gene amplification. Point mutations may arise from the action of chemicals or radiation. Mutation of the coding sequence can result in formation of hyperactive protein in normal amounts. The mechanism of gene amplification can result in overproduction of a normal protein product. DNA rearrangement (chromosome translocation) can give rise to elevated cellular concentrations of the normal protein product or to the expression of new proteins created by in-frame fusion of coding sequences from separate genes.

GROWTH FACTORS AND PROLIFERATION CONTROL PATHWAYS:

The regulation of normal cell proliferation occurs through the activation of biochemical pathways by growth factors (mitogens) interacting with their receptors on the plasma membrane (figure 3.1). There are many different growth factors like epidermal growth factor (EGF), platelet derived growth factor (PDGF), insulin, bombesin etc. and a single cell often possesses a variety of different receptors. Normal and malignant breast epithelial cells co-express a number of epidermal growth factor (EGF) related peptides including EGF, transforming growth factor α (TGFα), amphiregulin and cripto-1 (7;8). TGFα is a single chain polypeptide which can stimulate growth by binding to and activating the EGF receptor (9;10). In normal breast epithelial cell lines and some breast cancer cell lines, the production of TGFα is controlled partially by oestrogen. It is secreted by all tumour cell lines including breast and studies have demonstrated that TGFα is an important modulator of malignant progression of mammary epithelial cells in breast cancer (8). TGFβ is a two-chain polypeptide which has a growth inhibitory effect on epithelial cells including mammary epithelium (11;12).

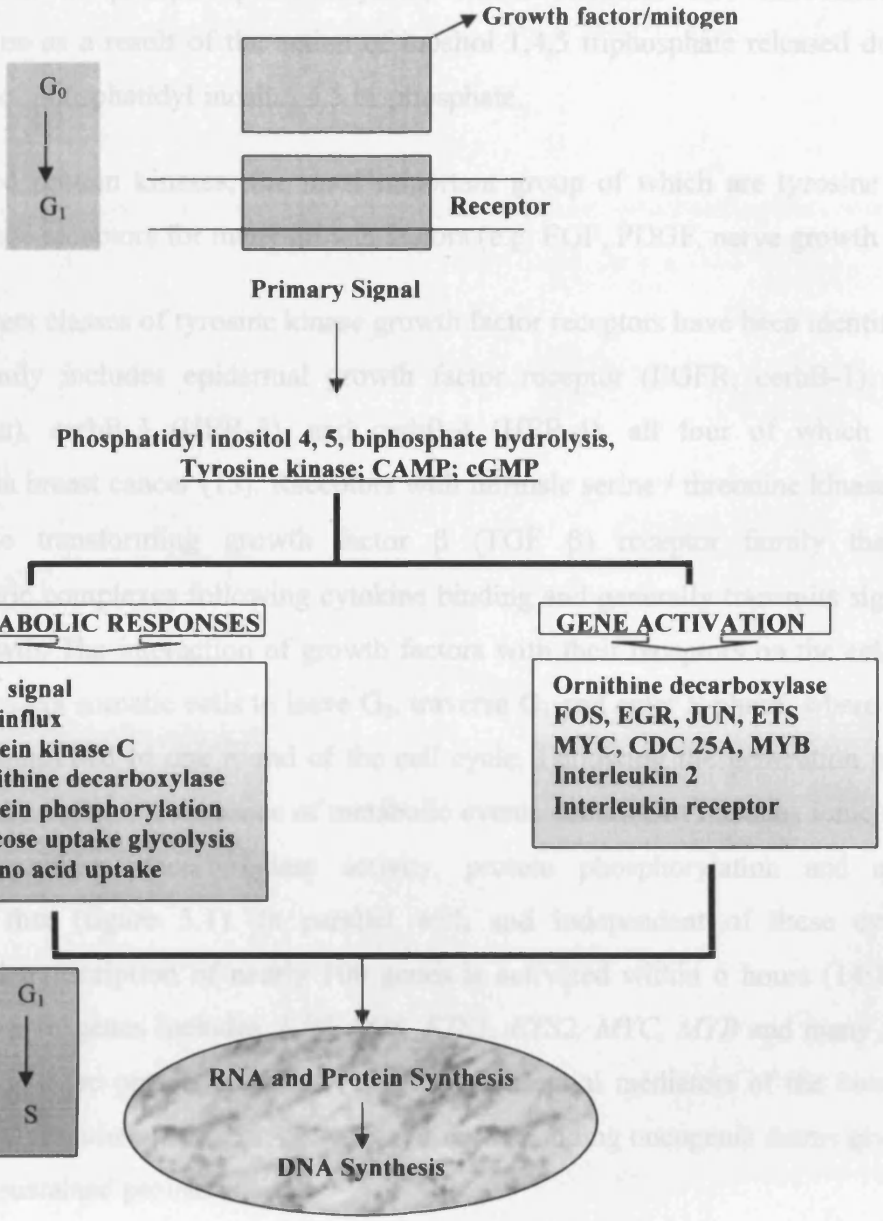


Figure 3.1; Biochemical events during proliferation in eukaryotic cells (3)

The four basic signalling mechanisms that can be activated by transmembrane receptors are:

1. Activation of adenylate cyclase, which generates cyclic AMP

2. Activation of guanylate cyclase to generate cyclic GMP.

3. Activation of phospholipase enzymes, which increases free intracellular Ca^{++} concentration as a result of the action of inositol 1,4,5 triphosphate released during the hydrolysis of phosphatidyl inositol 4,5 bisphosphate.

4. Activated protein kinases, the most important group of which are tyrosine kinases. These include receptors for many growth factors (e.g. EGF, PDGF, nerve growth factors).

Nine different classes of tyrosine kinase growth factor receptors have been identified. The type 1 family includes epidermal growth factor receptor (EGFR, cerbB-1), cerbB-2 (HER-2/neu), cerbB-3 (HER-3), and cerbB-4 (HER-4), all four of which may be expressed in breast cancer (13). Receptors with intrinsic serine / threonine kinase activity include the transforming growth factor β (TGF β) receptor family that forms heterodimeric complexes following cytokine binding and generally transmits signals that inhibit growth. The interaction of growth factors with their receptors on the cell surface causes quiescent somatic cells to leave G_0, traverse G_1 and enter S phase, where cells are normally committed to one round of the cell cycle. Following the generation of one or more primary signals, a sequence of metabolic events occurs that includes ionic changes, increased ornithine decarboxylase activity, protein phosphorylation and enhanced glycolytic flux (figure 3.1). In parallel with and independent of these events the coordinated transcription of nearly 100 genes is activated within 6 hours (14;15). This large number of genes includes *JUN, FOS, ETS1, ETS2, MYC, MYB* and many more. In normal cells, these proto-oncogenes function as essential mediators of the biochemical pathways that regulate proliferation and their corresponding oncogenic forms give rise to cancers by sustained proliferation.

Oncoproteins function at most of the known steps in signalling pathways associated with cell proliferation (figure 3.2). As mentioned above, they may arise as extracellular growth factors (SIS), ligand-independent transmembrane proteins (FMS, ERBB, RET), membrane associated proteins (SRC, YES, RAS, RAF1), cytosolic factors (ABL, MOS), nuclear transcription factors (ERBA, ETS, FOS, JUN, MYB, MYC) or DNA repair enzymes (MSH2). A major signal transduction pathway in which growth factors or oncoproteins cause the activation of mitogen-activated protein serine/threonine kinases (MAPKs or extracellular signal-regulated kinases ERKs) is shown in figure 3.2. In this pathway, growth factor-activated receptors or oncoproteins (SRC, BCR/ABL) interact with SH2 domain adaptor proteins (SHC, GRB2, SOS) to activate RAS to its GTP-bound

form. Activated RAS then causes the initiation of a cascade of protein phosphorylation by serine/threonine kinases including RAF, MAPKK (MEK MAP kinase) and MAPKs (16).

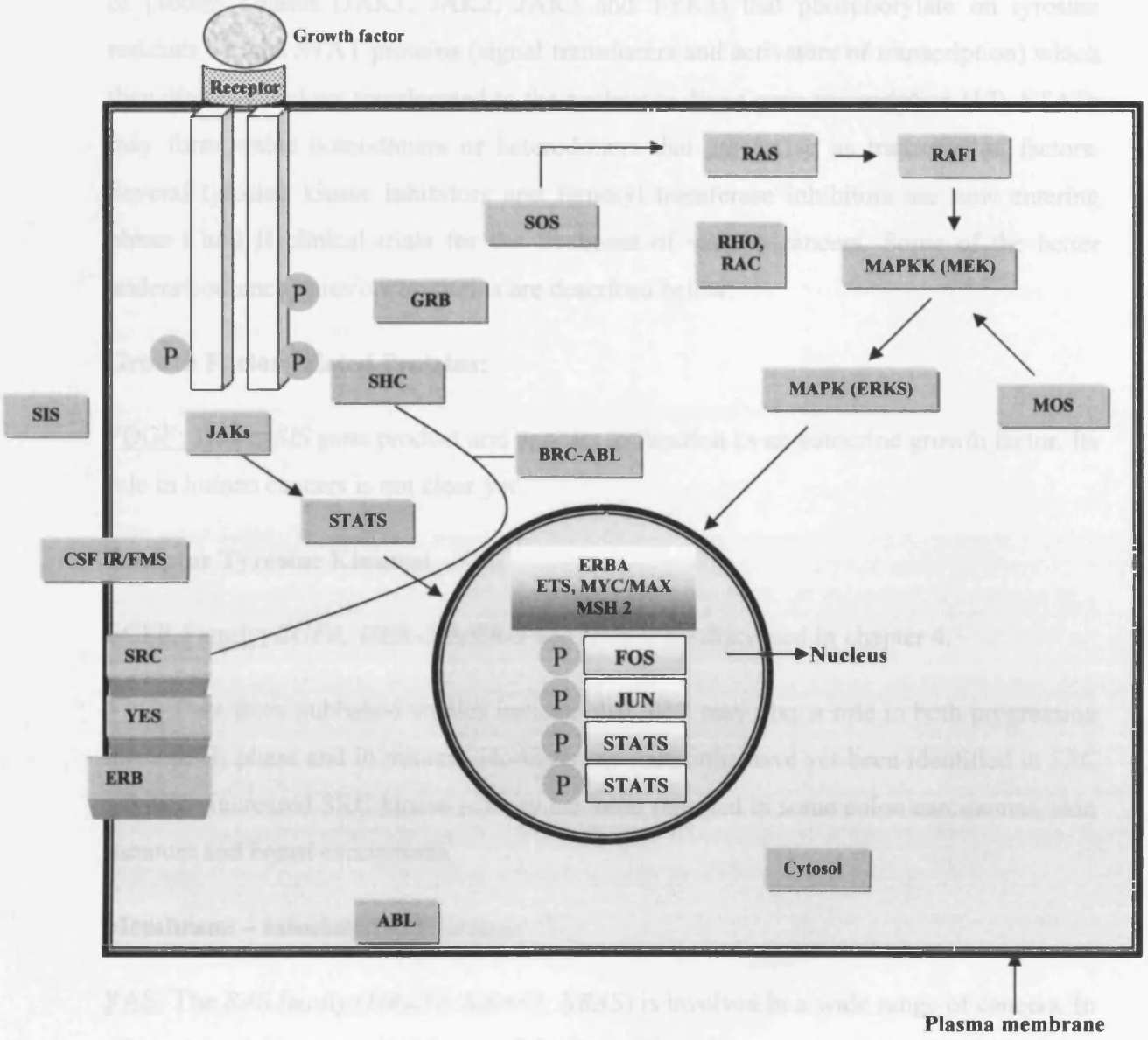


Figure 3.2: Schematic diagram showing cellular location of some oncoproteins

Thus RAS functions as a relay switch, the activity of which is dependent on the enzyme farnesyl transferase. Some cancers contain RAS mutations encoding a protein product that is permanently activated in the 'on' position resulting in enhanced cell proliferation. In germ cells, MOS activates MAPKK by serine phosphorylation. MAPKs target transcription factors like ETS, FOS, JUN. A variety of cytokines (including interleukins,

interferons, erythropoietin, growth hormone, prolactin and granulocyte colony-stimulating factor) and growth factors signal by activating members of the Janus family of protein kinases (JAK1, JAK2, JAK3 and TYK3) that phosphorylate on tyrosine residues various STAT proteins (signal transducers and activators of transcription) which then dimerize and are translocated to the nucleus to direct gene transcription (17). STATs may form stable homodimers or heterodimers that are active as transcription factors. Several tyrosine kinase inhibitors and farnesyl transferase inhibitors are now entering phase I and II clinical trials for the treatment of various cancers. Some of the better understood oncogenes/oncoproteins are described below:

Growth Factor-related Proteins:

PDGF: This is *SIS* gene product and appears to function as an autocrine growth factor. Its role in human cancers is not clear yet.

Receptor Tyrosine Kinases:

EGFR Family: *EGFR, HER-2, HER-3* and *HER-4* are discussed in chapter 4.

SRC: Data from published studies indicate that SRC may play a role in both progression through G_1 phase and in mitosis. However, no mutations have yet been identified in *SRC* although increased SRC kinase activity has been reported in some colon carcinomas, skin tumours and breast carcinomas.

Membrane – associated G Proteins:

RAS: The *RAS* family (*HRAS1, KRAS2, NRAS*) is involved in a wide range of cancers. In all cancers the average incidence of *RAS* mutations is approximately 15% (18) but in pancreatic carcinomas *KRAS2* mutations occur in 95% of tumours and in colorectal carcinoma, the reported range is from 20-50%. *NRAS* mutations occur in acute mylogenous leukaemia. Mutations in *RAS* reduce its GTP-ase activity, the oncogenic protein thus remaining in an active, GTP–bound state. This results in sustained activation of RAS–MAPK pathways. *RAS* mRNA is overexpressed in a variety of tumours including breast (19). The minisatellite region of *HRAS1* also appears to be mutated in some cancers. Minisatellites are tandem arrays of between 14 and 100 bp in length of a locus-specific consensus sequence. They are often polymorphic in the number of tandem

repeats and hence called "variable number of tandem repeats" (VNTRs). Minisatellites are dispersed throughout the genome and many VNTR loci display dozens of alleles. In the *HRAS1* minisatellite locus, there are 4 common and 25 rare alleles, the latter being more than twice as common in the genotypes of cancer patients than they are in normal individuals (20). Mutant alleles of the *HRAS1* minisatellite locus represent a major risk factor for 10% of breast, colorectal and bladder carcinomas (21). HRASp21 protein expression has been studied in human breast tumours by immunohistochemistry. There are conflicting results, some groups finding increased p21 expression in carcinomas and pre-malignant lesions (22-25) and others finding similar expression in benign and malignant lesions (26;27).

RAF: *RAF1* is amplified in some non-small cell lung cancers and there is over-expression in many small cell lung cancers.

Nuclear Oncoproteins:

ETS: ETS proteins may be involved in tumour-associated angiogenesis and invasiveness. However, most epithelial tumours do not express *ETS.*

FOS: There are few reports of involvement of *FOS* in human cancers, although in one study its overexpression was detected in 60% of osteosarcomas (28).

JUN: *JUN* overexpression has been detected in small cell and non-small cell lung cancers (29;30).

MYB: Amplification of *MYB* has been detected in a small proportion of leukaemias, colon carcinomas, melanomas and breast carcinomas. In breast cancer, *MYB* expression may correlate inversely with that of *HER-2* and constitutes a good prognostic factor (31). Abnormally high levels of *MYB* transcripts have been detected in some ovarian and cervical carcinomas (32;33).

MYC: MYC proteins (MYC, MYCN and MYCL) induce proliferation, inhibit terminal differentiation in adipocytes and myeloid cells and in the absence of appropriate growth factors, induce apoptosis. MYC has long been known to activate ornithine decarboxylase, which is necessary for cell proliferation. Also, it can trans-activate cyclin D1 and cyclin A, both of which control passage through the cell cycle. In addition, the MYC/MAX

complex trans-activates *CDC25A*, which itself has oncogenic capacity. Furthermore, *MYC* is repressed as a component of p53-mediated growth arrest.

Amplification and/or overexpression of *MYC* commonly occurs in a wide range of tumours. *MYCN* is amplified in neuroblastomas and also in retinoblastomas, astrocytomas, gliomas and small cell lung carcinomas. A high level of *MYCN* expression is associated with the advanced stages of cervical intraepithelial carcinoma. Amplification of *MYCL1* has been detected in small cell lung carcinoma (34). Deregulated *MYC* expression occurs also in breast carcinomas (35-37). This is further discussed later in the chapter. The most common genetic rearrangements in B cell lymphomas involve *MYC* (8q24) or *BCL2* (18q21.3).

REL: Amplification of *REL* occurs in some lymphomas and rearrangement of its chromosomal region (2p12-15) is associated with some non-Hodgkin's lymphomas.

THR/ERBA: This encodes thyroid hormone receptor α. Loss of heterozygosity at the *THRA1* locus has been detected in sporadic breast carcinomas and in a breast carcinoma cell line (38).

Other oncogenes include *BCL1*, which is also known as *CCND1* (cyclin D1 gene) or *PRAD1*, and is located on chromosome 11q13 along with the *INT2* and *HSTF1* genes. *BCL1*, *INT2* and *HSTF1* are co-amplified in 22% of breast carcinomas (36;39). Cyclin D1 together with cyclin D2&3 is a rate-limiting controller of G_1 phase progression in mammalian cells and is discussed in a later section on cell cycle control. The expression of cyclin D1 in breast cancer is considered later on in the chapter. *BCL2* is discussed in the section of apoptosis. *BCL3*, located on chromosome 19q13.1, encodes for a transcription factor. It is involved in chromosomal translocations in some B-cell leukaemias.

TUMOUR SUPPRESSOR GENES:

The existence of tumour suppressor genes was first suggested in studies of rare inherited cancer syndromes. In familial retinoblastoma, for example, a genetic change was inherited through the germline, but mutation or ablation of the other allele on the homologous chromosome was required for tumour development (Knudson's two hit hypothesis) (40;41). In sporadic cases, independent mutational events in both homologous

genes are required; as a result, sporadic retinoblastoma is less common than the familial form (42).

In normal organisms, cell proliferation is balanced by regulated cell loss through apoptosis. Many tumour suppressor genes are involved in cell proliferation, genetic stability and cell death, and they often show mutation in cancer. Such mutations in these genes generally ablate the ability to cause apoptosis and result in increased cell numbers.

There are approximately 50 known tumour suppressor genes, the normal function of which is to control cell proliferation. The two best understood tumour suppressor genes are retinoblastoma (*RB1*) gene and *TP53* (also called *P53*). Retinoblastoma provides the classical model for a recessive tumour suppressor gene in that both paternal and maternal copies of *RB1* must be inactivated for the tumour to develop. For *TP53* and some other tumour suppressor genes, mutation at one allele may be sufficient to give rise to the altered cell phenotype. Some of the better understood tumour suppressor genes are described below.

RB1: Retinoblastoma protein (pRb) is a signal transducer, connects the cell cycle clock with transcriptional control mechanisms and mediates progression through the first phase of the cell cycle (figure 3.3). It inhibits transcription factors that are required for the expression of genes involved in DNA replication. Its loss releases a brake at the G_1/S phase transition and drives proliferation and hence the accumulation of mutations. Retinoblastoma protein binds to and inhibits the E2F family of cellular proteins. The phosphorylation of pRb appears to be essential for progression through the cell cycle and inhibition of pRb phosphorylation causes cell cycle arrest. The *RB1* gene is defective in all retinoblastomas, whether familial or sporadic, and there is loss of both copies of the gene by inactivating mutations. Inactive *RB1* alleles also occur commonly in small cell lung carcinomas, non-small cell lung carcinomas, bladder, pancreatic and 20% of breast carcinomas (43-46).

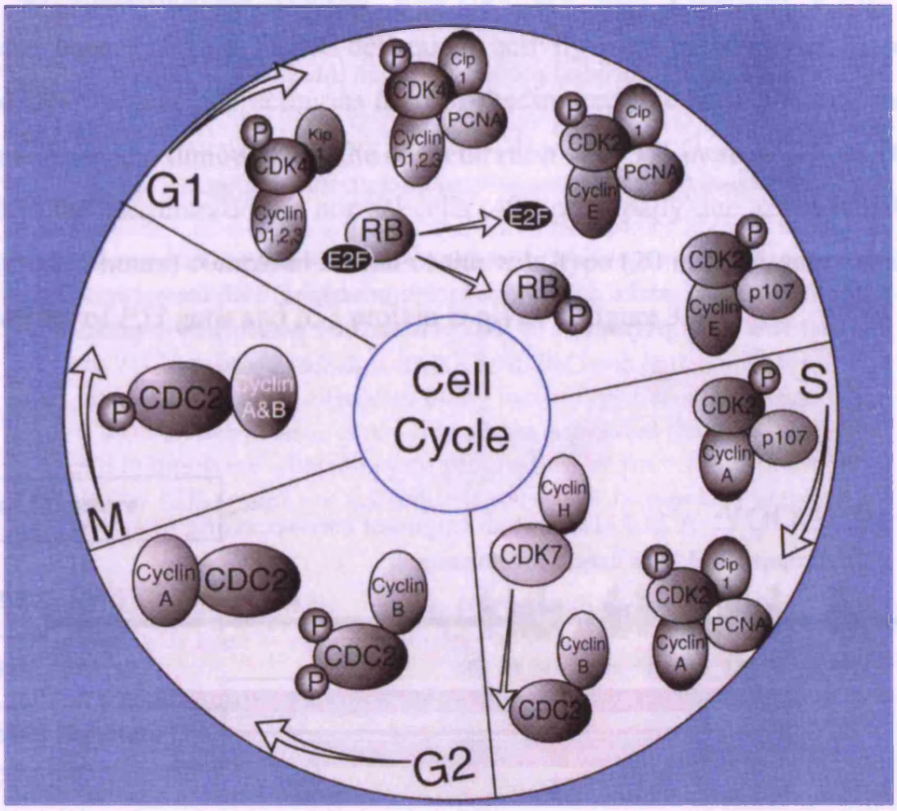


Figure 3.3; Cyclin dependent kinases in the control of the cell cycle

Cyclin dependent kinases (CDKs) regulate progression through the cell cycle. They are activated by specific association with cyclins and the rate-limiting controllers of G_1 phase are D and E cyclins (also called G_1 cyclins). D cyclins associate with CDK2, CDK4, CDK5 and CDK6, of which CDK4 is the most abundant. The activation of CDK4/cyclin D in the cell cycle precedes that of other CDKs in the order:

D/CDK4 → D/CDK6 → E/CDK2 → A/CDK2 → A/CDC2 → B/CDC2.

A critical substrate of D/CDK4 is pRb, the phosphorylation of which causes its inactivation and permits entry from G_1 to S-phase. CDKs are inhibited by WAF1 (also called cip1and p21), INK4 (also called p16), KIP1 and KIP2. See list of abbreviations for the abbreviations listed in the figure.

P53: The *P53* gene is located on chromosome 17p13.1 and consists of 11 exons. The protein product p53, is located in the nucleus and functions as a transcription factor that regulates normal cell growth cycle by activating transcription of genes which control progression through the cycle. It also controls other genes that cause arrest in G_1 when the genome is damaged and in some cell types, promotes apoptosis (47). Hypoxia, occurring in areas of tumours with poor blood supply, promotes p53 dependent apoptosis, while cells with mutated *P53* are resistant to killing by hypoxia (48). Genes activated by *P53* include *WAF1, MDM 2, GADD45, BAX* and cyclin G, whilst *P53* represses *MYC, BCL2,*

FOS, JUN, MYB, PCNA, RB1 and others. In addition to these activities, which are mediated by transcriptional control, wild-type p53 also possesses 3' to 5' DNA exonuclease capacity. This Mg++ dependent activity may be involved in replication associated DNA repair, p53 acting as a proof-reading enzyme for DNA polymerases. In most transformed and tumour cells, the concentration of p53 is increased 5- to 100-fold as compared to the concentration in normal cells (49), principally due to the half-life of the mutant forms (4 hours) compared to that of the wild-type (20 min). A schematic diagram of the structure of *P53* gene and p53 protein is given in figure 3.4.

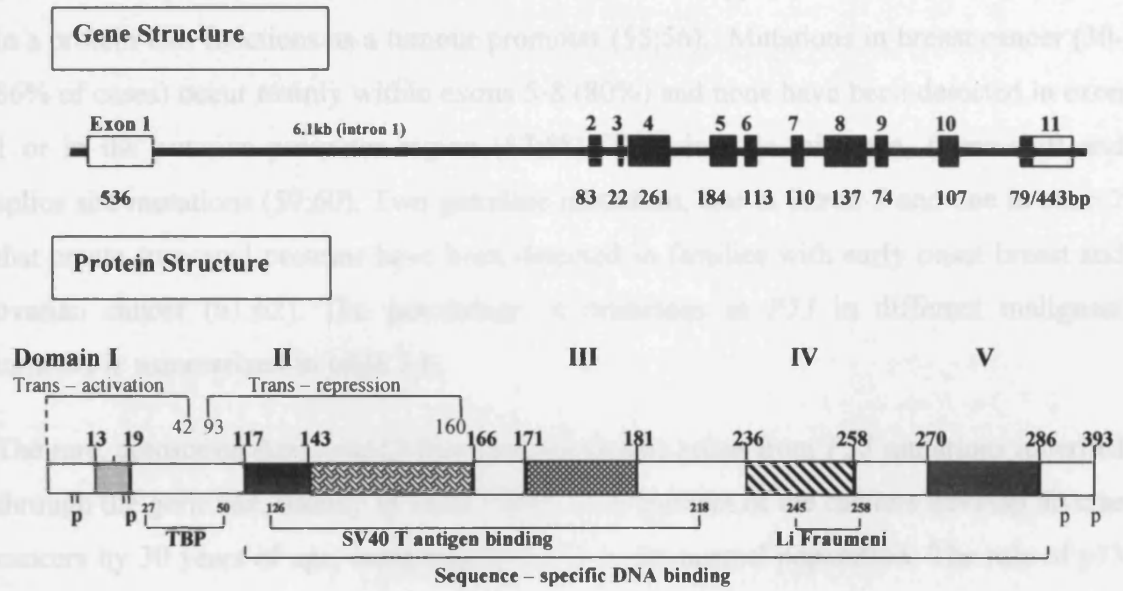


Figure: 3.4; Schematic diagram of the structure of *P53* gene and protein.
TBP=TATA binding protein

Normal function of p53 is regulated post-translationally and could be influenced by phosphorylation state, sub-cellular localization and interaction with many other cellular proteins. Wild type p53 is a negative regulator of cell growth, thought to act by forming homodimers around DNA, allowing DNA repair to occur before cell division, or if repair does not occur then inducing cell death through apoptosis. Mutations of *P53* result in more stable forms of protein, which form ineffective dimers around wild type p53 and lead to a failure of growth regulation. Other effects of mutation include nuclear exclusion of protein and interaction with MDM2 protein. Most documented mutations result from a

single amino acid substitution with 50% of mutations occurring between exons 5-8, which are highly conserved during evolution (50).

Mutations in the single copy *P53* gene are the most frequent genetic changes yet shown to be associated with human cancers (51;52), over 6000 of which have been detected (53). Point mutations, deletions or insertions in *P53* occur in approximately 70% of all tumours. The most frequently mutated residues lie in the regions that directly bind to DNA but other mutations modulate function by affecting the overall three-dimensional structure of the protein (54). Functionally, mutations in *P53* may (i) be of the dominant negative type when the protein overrides the action of the suppressor wild-type p53 (*trans*-dominant mutations), or (ii) result in the loss of suppressor function, or (iii) result in a protein that functions as a tumour promoter (55;56). Mutations in breast cancer (30-86% of cases) occur mainly within exons 5-8 (80%) and none have been detected in exon 1 or in the putative promoter region (57;58). They include missense, frame-shift and splice site mutations (59;60). Two germline mutations, one in intron 5 and one in exon 7 that create truncated proteins have been detected in families with early onset breast and ovarian cancer (61;62). The percentage of mutations in *P53* in different malignant tumours is summarized in table 3.1.

The rare, autosomal dominant Li-Fraumeni syndrome arises from *P53* mutations inherited through the germline, usually in exon 7 (63). Fifty percent of the carriers develop diverse cancers by 30 years of age, compared with 1% in the normal population. The role of p53 in breast cancer is considered later in this chapter.

Table 3.1; Percentages of *P53* mutations in different tumours.

TUMOUR TYPE	% CASES
Breast carcinomas	30 – 86
Ovarian carcinomas	44 – 61
Lung carcinomas	60
Colorectal carcinomas	30 – 60
Acute mylogenous leukaemias	6
Brain tumours	10
Malignant astrocytomas	30
Oesophageal adenocarcinomas	80
Gastric carcinomas	57
HBV-positive hepatomas	18
Head and neck cancers	69
Primary melanomas	97
Multiple myelomas	20
Osteosarcomas	41
Pancreatic carcinomas	40
Papillary thyroid carcinomas	50
Renal cell carcinomas	79
Rhabdomyosarcomas	45
Squamous cell laryngeal carcinomas	60
Wilm's tumours	73

BRCA1 and 2: *BRCA-1* (breast cancer susceptibility gene 1) was located on chromosome 17q21(17q21)$^{12\text{-}21}$ by genetic linkage analysis in 1990 by King et al (64). It encodes a 220 kDa protein expressed predominantly in the nucleus and involved in DNA repair. The protein has 1863 amino acids with an amino terminal domain that exhibits similarity to a zinc finger domain found in several regulatory and transcription factors (65). Germline mutations in *BRCA-1* confer a high risk of developing early onset breast cancer (66) and ovarian cancer (67), which is inherited in an autosomal dominant manner. Germline mutations in the *BRCA1* gene appear to be responsible for approximately 50% of the families that have a dominant predisposition to breast cancer and between 80 and 90% of those in which multiple cases of both breast and ovarian cancer occur. There is significant correlation between the location of mutations and the relative risk of breast or ovarian cancer within a family. Mutation in the N- terminal of the protein carries a significant predisposition to ovarian cancer whereas C- terminal mutations are strongly associated

with breast cancer. Several studies of sporadic breast tumours have suggested that *BRCA-1* mutations also play an important role in the development of non-inherited breast cancer. Studies by the International Breast Cancer Linkage Consortium have indicated that approximately half of all cases of inherited breast cancer are attributed to germline *BRCA-1* mutations (68). Prostate (69;70) and colon (71) cancer risks are also increased in individuals harbouring germline *BRCA-1* mutations. Carriers of *BRCA2* (breast cancer susceptibility gene 2 located on chromosome 13q12-13) mutations also have a high risk of developing breast cancer (72).

Other tumour suppressor genes not discussed in this thesis include: *APC* (adenomatous polyposis coli) gene which gives rise to familial adenomatosis polyposis, *NF1* (neurofibromatosis type 1) gene, *VHL* (Von-Hippel Lindau) gene, *WT1* (Wilm's tumour 1) gene and others.

CELL CYCLE CONTROL:

If the cell cycle is considered to be like an automobile in motion under normal conditions, proto-oncogenes are like the accelerator and tumour suppressor genes are like the brakes. The cell cycle comprises of 4 phases: G_1 (gap1), S (synthesis), G_2 (gap2) and M (mitosis). Progression through the cell cycle is governed by a family of cyclin-dependent kinases (CDKs) and their regulatory subunits, the cyclins (73). As cells enter the cell cycle from G_0, D- and E-cyclins are synthesized sequentially and both are rate limiting for S-phase entry (figure 3.3). CDKs inactivate pRB and its relatives p107 and p130 by phosphorylation, these proteins being negative regulators of progression, so that exit from G_1 occurs with entry into S-phase.

D-cyclins bind pRb directly and pRb is the critical substrate of CDK4 and CDK6 although cyclin E/CDK2 may also phosphorylate pRb. Phosphorylation of pRb relieves its inhibitory effect on the trans-activation function of both E2F family transcription factors and UBF (which regulates RNA polymerase I) that are required for S-phase. The activities of the CDK family are regulated by CDK inhibitors. These include WAF 1, the INK4 family, KIP 1 and KIP 2. WAF1 mediates *P53*-dependent G_1 arrest by inhibiting cyclin D/CDK4, cyclin E/CDK2 and cyclin A/CDK2, therefore preventing G_1/S transition. WAF1 exists as a quaternary complex with a cyclin/CDK and proliferating cell nuclear antigen (PCNA), a subunit of DNA polymerase delta involved in DNA

replication and repair. WAF1 inhibits PCNA and its capacity to activate DNA polymerase delta and also prevents interaction of PCNA with GADD45, which is involved in stimulating DNA excision repair. Thus the induction of WAF1 by p53 causes both cell cycle arrest and inhibition of DNA synthesis. WAF1 also inhibits the JUN N-terminal kinases (JNKs), also called stress-activated protein kinases (SAPKs), and MAP kinases that are activated by a variety of oncoproteins, cytokines, heat or ultraviolet (UV) radiation. Therefore WAF1 plays a central role in mediating the transition from a proliferative to a differentiated phenotype through its capacity to inhibit cyclin D1 activity and to prevent the G_1/S transition. Maintenance of the differentiated state requires the abrogation of signals that normally drive proliferation, as this, in combination with a block to cell cycle progression, often promotes apoptosis.

MYC expression causes activation of both cyclin D1 and cyclin E and hyperphosphosylation of pRb, thus increasing proliferation. This is a result of direct activation of CDC25 transcription. However, in the absence of growth factors or in combination with p53, the expression of MYC will result in apoptosis (figure 3.5).

Similar to components of growth factor signalling pathways, any cell cycle regulator is a potential oncogene or tumour suppressor gene. In addition to loss of *RB1* and the high frequency of mutations in *P53*, mutation and deletion of *INK4A* is also very common in primary tumours. Mutations in *WAF1* and *KIP1* are much less common.

Abnormal expression of cyclin D1 occurs in a considerable variety of cancers, amplification of cyclin D2 has been detected in colorectal carcinoma and abnormally high expression of both cyclins D1 and D3 occurs in some primary breast carcinomas. The cyclin-dependent kinase activator CDC25B is over-expressed in approximately 30% of primary human breast carcinomas and this correlates with a less favourable prognosis (74).

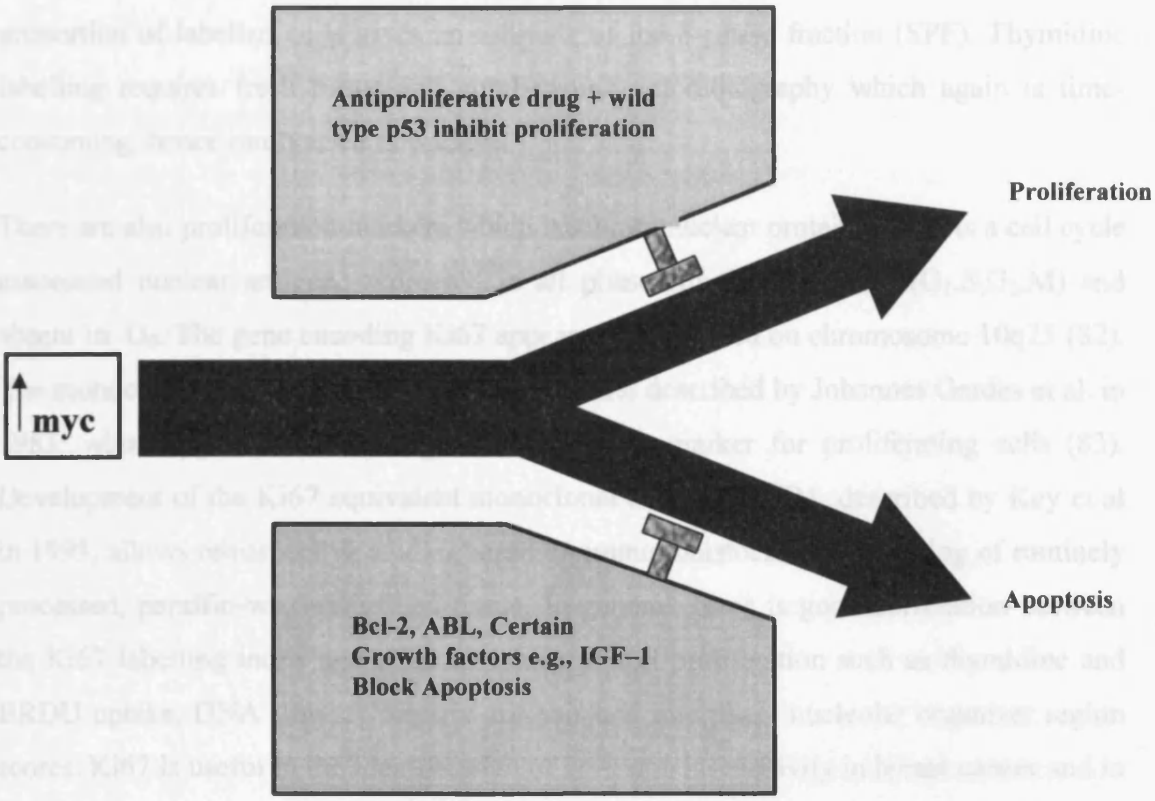


Figure 3.5; Myc up-regulation can lead to proliferation or apoptosis

PROLIFERATIVE ACTIVITY AND PROLIFERATION MARKERS:

It has been shown by many groups that estimation of proliferative activity of a tumour, no matter what method is employed, can give prognostic (75;76) and therapeutic (77;78) information. It is important to note that true assessment of the growth rate of a tumour can only be determined by combining measurements of the proliferative fraction and the length of the cell cycle (79). A single measurement in time of the growth fraction of a tumour is regarded as an index of proliferation only (79;80), which is a measure of proliferation *state*, not *rate*. The growth fraction can be assessed by counting the number of cells in mitosis called mitotic index (MI) (81), but this requires high quality tissue sections and is time-consuming. An alternative is to employ a technique by which cells in cycle can be labelled. Cells in the synthesis phase of the cell cycle (S-phase) take up thymidine which may be tritiated or its analogues such as 5-bromodeoxyuridine (BRDU) and incorporated molecules can be identified by prior radiolabelling, or in the case of BRDU, by immunocytochemistry or flow cytometry using anti-BRDU antibody. The

proportion of labelled cells gives an estimate of the S-phase fraction (SPF). Thymidine labelling requires fresh tissue and cumbersome autoradiography which again is time-consuming, hence rarely used nowadays.

There are also proliferation markers which label intranuclear proteins. Ki67 is a cell cycle associated nuclear antigen, expressed in all phases of the cell cycle (G_1,S,G_2,M) and absent in G_0. The gene encoding Ki67 appears to be located on chromosome 10q25 (82). The monoclonal antibody to Ki67 antigen was first described by Johannes Gerdes et al. in 1983, who suggested that it might be used as a marker for proliferating cells (83). Development of the Ki67 equivalent monoclonal antibody MIB1, described by Key et al in 1993, allows retrospective studies based on immunohistochemical staining of routinely processed, paraffin-wax embedded tissue. In general, there is good correlation between the Ki67 labelling index and other measures of cell proliferation such as thymidine and BRDU uptake, DNA flow cytometric analysis and interphase nucleolar organizer region scores. Ki67 is useful in the identification of hormone insensitivity in breast cancer and in the prediction of tumour growth rates and patient survival (84;85). Therefore it is used prognostically (86). It has been shown that elevated levels of this antigen are associated with earlier breast cancer recurrence (85), shorter survival time and shorter disease free interval (87), as well as a poorer response to therapy.

KiS1 antibody recognises another cell cycle associated antigen, which is expressed at increasing levels during DNA synthesis and reaches a peak in mitosis and has been shown to be prognostically useful.

Proliferating cell nuclear antigen (PCNA) has received a lot of attention, particularly as one of the first antibodies which could be used on paraffin-wax embedded material. However studies have shown that PCNA has a dual role in cell replication and DNA repair. These two functions contribute to the conflicting data about the value of measuring proliferation with PCNA antibodies. Whilst some authors have shown it to be useful in lymphoid tissue, others have found it unreliable in solid tumours.

Both the proteins recognised by KiS1 and PCNA have relatively long half lives. Expression of PCNA can also be induced by growth factors in normal and benign cells, which are in the vicinity of a malignant lesion. Hence more cells are labelled than are actually proliferating.

APOPTOSIS:

Apoptosis (programmed cell death) is an actively regulated cellular process that leads to the destruction of individual cells (88-91). It is a mechanism by which cells commit suicide and is thought to play a key role in the development and growth regulation of normal and neoplastic tissues. It is distinct from necrosis where a cell loses its homeostatic control and becomes distended with fluid, leading to lysis and release of intracellular contents with stimulation of inflammation. Apoptosis, by contrast, involves shrinkage and fragmentation of cells with intact membranes and their subsequent removal by phagocytosis before release of harmful intracellular contents can occur. It can be triggered by several stimuli, such as radiation, drugs and toxins or by deprivation of hormones or growth factors (92;93). As with proliferation, apoptosis appears to show cyclic variations in normal breast tissue with a peak at the end of luteal phase (94). The apoptotic process is controlled by several genes, including inducers (*P53, BCLX$_S$, BAX, BAD, BAK, BIK*) and repressors (*BCL2, BCLX$_L$, MCL-1, BRAG-1, BFL-1*). The balance between expression of these genes regulates the cell cycle and apoptosis. The balance between these proteins is also regulated by other stimuli such as p53 protein or oestrogen receptors in breast carcinomas.

Apoptosis is characterised morphologically by chromatin condensation, internucleosomal fragmentation of DNA by a Mg^{2+} dependent endonuclease, blebbing of the cell membrane and vesicularisation of the cell contents. Apoptosis plays an important role in normal development and as a defence against viral infection. Inhibition of apoptosis can lead to cancer (95).

The *BCL2* (B-cell leukaemia/lymphoma 2) gene, which is located on chromosome 18q21 and encodes a 26KDa protein, is an important regulator of apoptosis. The cellular location of bcl2α is on intracellular membranes including nuclear membranes and that of bcl2β is cytoplasmic. Abnormalities of the *BCL2* gene were first discovered in human follicular lymphoma in which the gene is translocated to the immunoglobulin heavy chain locus of chromosome 14 (96). As a result, the *BCL2* oncogene is activated which enhances cell survival, rather than promoting proliferation. Bcl2 is now recognized as a survival factor for many types of cells. In T cells, BCL2 confers resistance to apoptosis normally induced by glucocorticoids, radiation and other agents. The expression of Bcl2 is widespread during embryogenesis but is restricted to long-lived cells in adults. A critical mediator of

bcl2 regulated apoptosis is interleukin–1 β-converting enzyme (ICE), a cysteine protease that processes IL-1β during the inflammatory response. Over-expression of ICE in mammalian cells causes apoptosis that is inhibited by bcl2. In general, the activity of a family of ICE-related genes appears critical in driving apoptosis. A schematic diagram of the bcl2 protein structure is shown in figure 3.6.

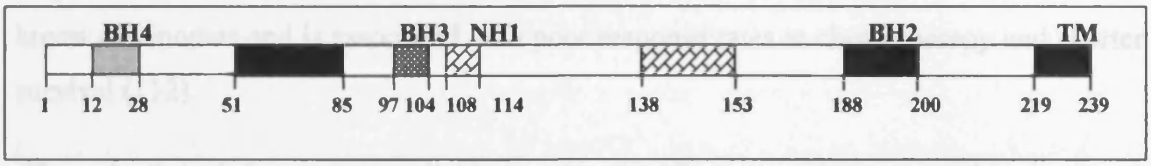


Figure 3.6; Schematic diagram of the bcl2 protein structure

BCL2 is a member of a multigene family, highly conserved in evolution. Other proteins of the family, bclXs, bax, bad, bak and bik antagonize inhibition of apoptosis by binding to bcl2. Hence the balance of expression of various members of the bcl2 family determine the extent to which cell death is promoted or prevented. Heterodimerization of bcl2 and bax proteins plays a pivotal role in regulating the fate of individual cells co-expressing these proteins (97;98). Excess of bcl2 promotes cell survival by inhibiting apoptosis, whereas excess of bax accelerates cell death.

Expression of the bcl2 protein product has been documented in a variety of normal human tissues including breast epithelium (99;100). Bcl2 protein is also expressed in a variety of haematological and solid tumours including prostate, colorectal, squamous cell lung, bladder, breast and nasopharyngeal carcinomas. In invasive breast carcinoma, bcl2 protein expression is associated with well-differentiated tumours and positive oestrogen receptor (ER) status (101-104). Among breast cancer patients treated with adjuvant chemotherapy or tamoxifen, those with bcl2 protein positive tumours, had significantly better survival rates (105). In bladder carcinomas, however, a high level of bax expression relative to that of bcl2 is a good prognostic indicator (106). One consequence of bcl2 expression is that cells expressing *MYC* in the absence of appropriate growth factors that would normally undergo apoptosis, are prevented from doing so.

Bax is a 21KDa protein with extensive amino acid homology with bcl2 protein (107;108). The protein is encoded by six exons and has been shown to undergo alternative splicing leading to at least two cytoplasmic forms (107;109). Bax has been shown to form

heterodimers with bcl2 and the ratio of bcl2 to bax determines the survival or death of cells following an apoptotic stimulus such as removal of growth factor (107;110). Stimulation of bax synthesis also appears to be a result of wild-type but not mutant p53 activity (111). It has been suggested that deregulation of apoptosis due to imbalances in bax/bcl2 levels may contribute to the pathogenesis of breast cancer (109). Bax is normally expressed in several epithelia including those in breast, small intestine, colon, prostate, respiratory tract and skin. Reduced expression has been detected in 34% of metastatic breast carcinomas and is associated with poor response rates to chemotherapy and shorter survival (112).

There is little information available on the significance of bax expression in ductal carcinoma in situ of the breast (DCIS) and its various histological grades or in invasive ductal carcinoma (IDC) and its histological grades.

Growth arrest and apoptosis can be induced by a variety of cytokines including the TGFβ family. P53 and TGFβ both can downregulate *BCL2* expression, but only p53 has the capacity to activate *BAX*.

Expression of the tumour suppressor gene *P53* that can arrest proliferation, may also promote apoptosis. Thus p53 levels increase in response to a variety of DNA–damaging treatments, and in addition to transactivating *BAX*, wild type p53 can repress *BCL2* expression. *P53* also appears to be able to induce apoptosis by transcription and translation–independent mechanisms. Wild-type p53 can induce cell cycle arrest in G_1 and apoptosis within the same cell but susceptibility to these processes is cell-type specific and apoptosis may occur without detectable arrest or, cells may arrest in G_1 without undergoing apoptosis. P53-independent apoptosis also occurs and is particularly important during normal development.

DNA REPAIR AND MICROSATELLITE INSTABILITY:

Errors that arise during DNA replication and are not corrected by the proof-reading activity of DNA polymerase may be rectified by a process called mismatch repair. Six human DNA mismatch repair genes have been identified, *MSH2, MSH3, MSH6, MLH1, PMS1* and *PMS2*. Mutations in these genes were first detected in hereditary non-polyposis colon cancer (HNPCC). Mutations in mismatch repair genes lead to an overall increase of the mutation rate, predispose to cancer development and are associated with a

phenotype of length instability of microsatellite loci. Microsatellites are short sequences of DNA (up to 6bp), repeated between 10 and 50 times, that are stably inherited, vary from individual to individual and have a relatively low inherent mutation rate. Microsatellite instability was first identified in colorectal tumours (113), showing that genomic instability is a very early event in the development of these tumours. It was shown that colorectal tumour DNA in some cases showed a substantial change in repeat length, often heterogeneous in nature, or a minor change (typically two base pairs), compared to normal DNA from the same patient (113). It is known that microsatellite instability is caused by mutations in mismatch repair genes. Germline mutations in the mismatch repair genes are causative of HNPCC. Initially, some reports indicated the presence of microsatellite instability in breast cancer (114) as well. However, Anbazhagan et al analysed 267 breast carcinomas with a total of 104 primers for microsatellite repeats (115). In this study, which included more than 10,000 reactions, not one single case of microsatellite instability was detected. Therefore microsatellite instability does not appear to play a significant role in the development of breast cancer. The only exception may be the development of breast cancer in patients from HNPCC families, in which case, microsatellite instability could arise as a late event in tumour progression.

ANGIOGENESIS AND METASTASIS:

Angiogenesis:

Angiogenesis (neovascularization) is the sprouting of capillaries from pre-existing vessels and occurs during embryonic development but is almost absent in normal adult tissues. However, transient regulated angiogenesis occurs in adult tissues during the female reproductive cycle and during wound healing. Pathological angiogenesis is characterised by the persistent proliferation of endothelial cells and is a prominent feature of a number of different types of disease including rheumatoid arthritis, psoriasis and proliferative retinopathy. In addition, many tumours are able to attract blood vessels from neighbouring tissues and the induction of new blood vessel growth is necessary if solid tumours are to grow beyond $2mm^3$ size (116;117). It appears that angiogenesis is important for the development of metastasis (118). Many studies using semi-quantitative histological techniques, have found a significant association between high tumour vascular density and poor prognosis in several types of tumour (119;120), including

breast cancer (121-124). However, other groups have failed to demonstrate such a prognostic effect (125-128). Neovascularization has also been described around ductal carcinoma in situ (DCIS) of the breast (121). As DCIS is not capable of metastasising, this finding suggests that neovascularization may be an early event in tumour progression. Despite the controversies regarding the prognostic value of angiogenesis in breast cancer, there is increasing evidence demonstrating a predictive value of angiogenesis for response to anti-cancer therapies and the development of anti-angiogenic drugs as novel therapeutic strategies is underway (116;129).

A variety of factors stimulate angiogenesis including angiogenin, fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor alpha and beta (TGF α and β), tumour necrosis factor alpha (TNFα), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), placental growth factor, prostaglandins E_1 and E_2, heparinase, integrins, macrophage derived factor, fibrin, nicotinamide, oncogenes like *INT2* and *HST1* and others. Angiogenesis inhibitors include angiostatin, endostatin, wild type P53, chondrocyte-derived inhibitor, heparinases, interferons α and β, protamine, thrombospondin, matrix metalloproteinases and others. In animal studies, administration of anti-angiogenic factors resulted in reduced growth rate of fast growing tumours. An anti-angiogenic drug called TNP-470 has been tested in phase II trials in breast, pancreatic, renal and cervical carcinoma and Kaposi's sarcoma, with evidence of tumour regression (130). There are other drugs in clinical trials such as thalidomide, combrestatin A-4, endostatin and vitaxin that target endothelial cells. Vascular endothelial growth factor (VEGF) antagonists have also shown promising results in clinical trials.

Metastasis:

Metastasis is the spread of malignant cells through the body that have detached from a primary tumour to give rise to secondary tumours and it is the major cause of death from cancers. The transition from pre-invasive tumour to invasion occurs in parallel with tumour induced neovascularisation (angiogensis) which facilitates dissemination of tumour cells into the vascular system. The molecular events that determine whether a cell becomes metastatic may be divided into two categories, namely, intracellular signalling pathways that regulate proliferation, apoptosis and the synthesis of cytokines; and secondly, changes in cell surface proteins and secreted proteins involved in cell adhesion,

cell regulation and proteolysis. The first category may be regarded as those events which determine whether an individual cell evolves into a tumour clone. The second group refers to cellular properties that may directly determine whether tumour cells metastasise. Metastasis is a multistep process that requires many different tumour-host cell interactions. Crucial among these are the events associated with invasion of the basement membrane by the tumour cells. The development of a metastasis requires expression of cell surface adhesion molecules by the tumour cells and the expression of proteolytic enzymes involved in tissue degradation. The major families of such proteins are (a) the cadherins (b) the immunoglobulin superfamily (c) the integrins (d) CD44 (e) the matrix metalloproteinases (MMPs) (f) the tissue inhibitors of metalloproteinases (TIMPs) (g) the serine, cysteine and aspartic proteinases and heparanase and (h) the metastasis suppressor genes, *NME1* and *NME2* (also called *NM23*).

The cadherins are transmembrane glycoproteins that mediate calcium-dependent intercellular adhesion, cytoskeletal anchoring and signalling. E-cadherin and some other members of the family act as invasion suppressors. The role of E-cadherin in breast cancer is described later in the chapter.

The immunoglobulin superfamily, also called neural cell-adhesion molecules (NCAMs), mediate homophilic or heterophilic Ca^{2+} independent intercellular adhesion. NCAM expression modulates the adhesive phenotype of glioma cells.

The integrins are a family of cell surface proteins that mediate cell-substrate and cell-cell adhesion. They are heterodimers of non-covalently linked α and β subunits. Sixteen α and 8 β subunits have been identified that give rise to at least 22 distinct integrins. Loss and aberrant expression of various integrins has been detected in colon and breast carcinomas (131).

CD44 is a 90 KDa cell surface glycoprotein implicated in cell-cell and cell-matrix adhesion, lymphocyte activation and metastasis. Multiple isoforms are generated by alternative splicing of 10 'variant' exons that are differentially expressed in a specific manner in normal tissues in response to a variety of cytokines. Gross overproduction of alternatively spliced forms occurs in breast and colon carcinomas and is associated with a poor prognosis (132).

The MMPs are a big family of at least 14 members. They are synthesized by connective tissue cells and act synergistically to digest the major macromolecules of connective tissue matrices. They are activated by plasmin, which itself is generated by plasminogen activators, tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), both of which are serine proteinases. High expression of uPA has been detected in a variety of malignant tissues, including breast carcinomas, brain tumours and melanomas. uPA expression is a powerful prognostic indicator for breast cancer (133). Specific inhibitors to matrix metalloproteinases (MMPIs) have been developed and several are in advanced stages of clinical development.

The urokinase plasminogen activator (uPA) system consists of the serine protease uPA, its glycolipid-anchored receptor, uPAR and its 2 serpin inhibitors, plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2). These are present primarily in stromal cells in invasive breast carcinoma. Recent findings suggest that the uPA system is involved at multiple steps in cancer progression (134). In particular, uPA has been implicated in remodelling of the extracellular matrix, enhancing both cell proliferation and migration and modulating cell adhesion. Many groups have shown that high levels of uPA in primary breast cancers are independently associated with adverse outcome. Paradoxically, high levels of PAI-1 also correlate with poor prognosis in patients with breast cancer. The prognostic value of uPA/PAI-1 in axillary node-negative breast cancer patients was recently validated using both a prospective randomised trial and a pooled analysis which are 2 different level 1 evidence studies (134). Assay of uPA and PAI-1 may thus help identify low risk node-negative patients for whom adjuvant chemotherapy is unnecessary. Finally, preclinical studies show that either inhibition of uPA catalytic activity or prevention of uPA binding to its receptor reduces tumor growth, angiogenesis and metastasis.

The TIMPs regulate the activities of MMPs. TIMPs are present in normal bone and cartilage cells and are secreted by a variety of cells in culture. TIMPs are potent inhibitors of several steps in invasion and metastasis, including the proteolytic degradation of the extracellular matrix, invasion of surrounding connective tissue, extravasation and subsequent tumour growth (135). In vitro studies suggest that TIMPs may have tumour suppressor activity. However, their role is slightly more complex than this, since there is some suggestion that they may have additional functions as cytokines or growth factors.

TIMP-1 expression is increased in some non-Hodgkin's lymphomas and metastatic prostate cancer, and increased expression of TIMP-3 has been detected in breast carcinomas (136). The significance of these findings is unclear.

Cathepsin D is an acidic lysosomal aspartic proteinase, involved in intracellular protein turnover. It is over-expressed in most primary breast cancers, and shown to have an association with indicators of tumour aggression such as large tumour size, high histological grade and lymph node positivity (137;138). Immunohistological studies have demonstrated that cathepsin D can be identified not only in breast cancer cells, but also in accompanying stromal tissue. Some studies have shown it to be an indicator of poor prognosis in breast cancer (139).

NME1 (non-metastatic cells 1, expressed) and *NME2* (formerly *NM23*-non-metastatic 23) encode nucleoside diphosphate kinases expressed on the cell surface. NME2 expression is increased in some neoplastic tissues, although in ductal breast carcinomas, this increase does not appear to correlate with tumour size, oestrogen receptor or progesterone receptor expression, lymph node metastases or other prognostic factors (140).

SPECIFIC GENETIC ALTERATIONS IN BREAST CANCER:

In breast cancer, the main genetic alterations are amplification of approximately 10 oncogenes and inactivation of an unknown number of tumour suppressor genes (141). Translocations and point mutations are very rare and therefore do not seem to play an important role in the development of breast cancer (142). In addition, germline mutations in the *BRCA-1* and *BRCA-2* genes account for genetic predisposition to develop breast and ovarian cancer, and the breast cancer in these patients is often poorly differentiated ductal carcinoma. Some other histological tumour types have also been shown to be associated with the presence of specific genetic alterations, for example, inactivation of E-cadherin is specific for lobular breast cancer and *HER-2 (cerB-2)* gene amplification is associated with poorly differentiated ductal carcinoma (141).

There are a number of studies examining the various genetic alterations in breast cancer and their association with various clinical and pathological factors, especially prognosis. Some of the data presented in these studies indicate conflicting results. For almost every genetic alteration, correlation with poor prognosis was found by some authors, whereas

others stressed the lack of such correlation. The reasons for such conflicting results can be summarized as follows:

1. There are differences in the methods used to detect the genetic alterations.
2. Many studies contain relatively small numbers of patients, who are often heterogeneous with respect to clinical stage and treatment.
3. Almost all the genetic alterations are present in only a small percentage of tumours, resulting in low statistical power.

It is hoped that as analytical methods improve and larger series of patients are studied with more scientific rigour, the way in which the profile of genetic alterations in breast cancer affects clinical behaviour will become clearer. Most treatment decisions for breast carcinoma and other malignancies are currently based on clinical and pathological features of the tumours. It is likely that in future, treatment decisions will also be influenced by the genetic profiles of tumours.

Gene Amplifications in Breast Cancer:

Most initial studies of breast carcinomas were carried out using Southern blot analysis. Employing comparative genomic hybridisation (CGH) on large series of tumours has also resulted in the identification of regions of several chromosomes, which are frequently amplified in breast cancer. The structure of many of the amplicons in breast cancer has proven to be relatively complex and many of them appear to contain more than one region of amplified DNA. For many of these amplified regions, it is not clear which genes are the actual oncogenes, for which over-expression is selected.

As many studies include relatively small number of tumours, it is difficult to estimate the exact frequency of amplification of each region. A very large study by Courjal et al is helpful for providing estimates; these investigators analysed amplification of 26 different loci in 1875 breast carcinomas using Southern blot analysis (143). The main loci which were found to be amplified in breast cancer are as follows:

1) Amplification of a region on chromosome 1q31-32 is frequent and occurs in up to 50% of all breast carcinomas (144). The oncogene driving this amplification event has not yet been identified.

2) Amplification of a region on the chromosome 1p is less frequent. The recently identified *DAM1* gene has been found to be amplified in breast cancer, but it is not certain whether this gene is driving the amplification (145).

3) The *EGFR* gene on chromosome 7p13 encodes a growth factor receptor located on the cell membrane. Amplification of this gene is found in less than 3% of breast carcinomas. Elevated expression of the EGF receptor protein, in the absence of gene amplification, has been associated with oestrogen receptor negativity (146). EGFR expression correlates with that of p53 but is inversely correlated with *MYB* amplification, thus *EGFR* and *MYB* expression provide indicators of a poor or good prognosis respectively.

4) The *FGF-receptor 1(FLG)* gene on chromosome 8p12 encodes a fibroblast growth factor receptor located on the cell membrane and is amplified in approximately 10% of breast carcinomas (147). Amplification has been correlated with reduced disease-free survival, especially if the gene is amplified together with the *CYCLIN D1* gene (144).

5) The *c-myc* gene on chromosome 8q24 encodes a nuclear protein which is 62 KDa and found in the nucleus during the G_0 to G_1 phase of the cell cycle (148). It is involved in regulation of growth and apoptosis. A *c-myc* gene amplification is found in approximately 20% of breast carcinomas (149;150) and is associated with oestrogen receptor negativity (145), locally advanced disease, high histological grade of the tumour (151) and poor prognosis (152). The c-myc protein has a very short half-life, precluding the analysis of c-myc protein overexpression as a substitute for analysis of gene amplification.

6) Studies using CGH indicate that in addition to the *c-myc* gene, other portions of chromosome arm 8q from 8q12 to 8qter are present at an increased relative copy number in a broad range of solid tumours, including breast cancer. There appears to be at least one additional oncogene mapping to chromosome 8q12-22, which has not yet been identified (153).

7) The *FGF-receptor 2 (BEK)* gene on chromosome 10q26 encodes a receptor for fibroblast growth factor located on the cell membrane. This gene is amplified in approximately 12% of breast carcinomas (147).

8) The *HSTF1* and *INT2* oncogenes are members of the fibroblast growth factor (FGF)

family. These two, together with *BCL1*, are amplified in 22% of human breast carcinomas (36;39). However, there is no evidence that the protein products of *HSTF1* and *INT2* genes are expressed in breast cancer.

9) *CYCLIN D1* gene on chromosome 11q13 encodes a nuclear protein, involved in cell cycle regulation. Amplification occurs in 15% of breast carcinomas. It has been found that *CYCLIN D1* can bind to the ER, resulting in ligand-independent activation of the receptor (154). *CYCLIN D1* gene amplification is associated with ER positivity. In ER positive tumours, *CYCLIN D1* gene amplification is associated with a relatively poor prognosis (144;155). Generally, however, enhanced cyclin D1 expression correlates with increased overall survival, whereas the worst prognosis correlates with reduced levels (156). *CYCLIN D1* gene amplification is more frequent in lobular carcinomas compared with ductal carcinomas. Cyclin D1 overexpression can be detected using immunohistochemistry and has been identified in 80% of invasive lobular carcinomas, but it is not always accompanied by *CYCLIN D1* gene amplification (157).

10) A region on chromosome 16p11-12 has been found to be amplified by CGH in approximately 20% of breast carcinomas (158). More detailed analyses of this region are not presently available.

11) The *cerbB-2 (HER-2/neu)* gene on chromosome 17q12 encodes a transmembrane glycoprotein and is amplified in 20-30% of breast carcinomas. It is discussed in more detail in chapter 4.

12) *PS6K* gene on 17q23 is a frequent site of amplification in breast cancer as revealed by CGH (159;160).This gene encodes a serine-threonine kinase whose activation is believed to regulate a wide range of cellular processes involved in the mitogenic response including protein synthesis, translation of specific mRNA and cell cycle progression from G_1 to S phase.

13) Amplification of the *CAS* (cellular apoptosis susceptibility) and *AIB1* (amplified in breast 1) genes on chromosome 20q13. The *CAS* gene encodes a protein, which may have a function in apoptosis and cell proliferation (161). The *AIB1* gene encodes a coactivator of the ER (162). These genes are both located in a region on chromosome 20 that is found amplified in approximately 15% of breast cancers. It is currently unknown whether the *CAS* and the *AIB1* genes are important in breast carcinogenesis. The amplification pattern

at chromosome 20q is quite complex; three independent regions of amplification have been identified and their co-amplification is common (163). Amplification of *AIB1* has been found to be associated with ER positivity (164), but not with clinical outcome (144). Amplification of this region detected by FISH with a cosmid probe has been found to be associated with high histological grade and poor prognosis (165).

14) Using CGH, other regions have been observed to be amplified (166). However, it is not known which oncogenes are driving these amplifications.

Tumour Suppressor Genes in Breast Cancer:

In breast cancer, like other cancers, inactivation of tumour suppressor genes plays an important role. The classical mechanism of inactivation is functional loss of both alleles of the tumour suppressor gene. In many cases, one tumour suppressor gene allele is mutated by a relatively subtle mutation (point mutation, small insertion, small deletion), while the other allele is completely lost. The presence of these subtle mutations has made it possible to identify the tumour suppressor genes that are presently known. In addition to the known tumour suppressor genes, a relatively large number of regions have been identified where frequent loss of heterozygosity (LOH) is observed including 1p, 1q, 3p, 5p, 6q, 7q, 8p, 9q, 10q, 11p, 13q, 14q, 15q, 16q, 17p, 17q, 18p, 18q, 19p, 21q, 22q, Xp (167). Large efforts are being dedicated to identify the tumour suppressor genes in these regions. The tumour suppressor genes in breast cancer known to date are:

1) The *IGF-II-receptor* gene on chromosome 6q26-27, which encodes a receptor for IGF-II located on the cell membrane. In addition, this receptor functions as the mannose-6 phosphate receptor. Mutations in this gene have been identified in some breast cancers (168) but no larger studies are available to find out the frequency of this mutation; most probably it is low (<10%).

2) The *p16INK4a (p16)* tumour suppressor gene on chromosome 9p21 is frequently inactivated in several tumour types by homozygous deletion, point mutation or methylation of the 5'CpG island. The mechanism of action of p16 involves binding to and inactivating the cyclin D-dependent kinase 4 or 6 complex and making the retinoblastoma protein inactive. This effect blocks the transcription of important cell cycle regulatory proteins and results in cell cycle arrest. In breast cancer, few studies have looked at p16

inactivating mutations, the emerging evidence suggesting that inactivating alterations affecting the p16 are rare at this site (169;170).

3) *PTEN* is a tumour suppressor gene located on chromosomal band 10q23.3.This region displays frequent LOH in a variety of human neoplasms including breast carcinomas. Germline mutations in the *PTEN* gene are causative of Cowden disease (see glossary) and sporadic mutations in breast cancer have been reported. However, *PTEN* mutations are not thought to play a major role in breast cancer formation (171).

4) The *P53* gene on chromosome 17p13 encodes a nuclear protein, which binds to DNA as a tetramer and is involved in regulation of transcription, DNA replication and apoptosis. Mutations of *P53* are the most common molecular abnormalities found in human solid tumours (11;172) and are present in a high proportion of breast cancers (173;174). Inactivating *P53* mutations are found in approximately 20% of malignancies at this site. The presence of *P53* mutations has been associated with poor prognosis (175) and resistance to chemotherapy and radiotherapy. In DCIS, *P53* mutations are associated with high grade. Missense mutations in the *P53* gene result in increased stability of p53 protein, which can then be detected using immunohistochemistry. The level of wild-type p53 protein is too low to be detected in breast cancer. The majority of the studies to look at the prognostic value of p53 alterations have been done using immunohistochemistry. A much smaller number of studies have looked at *P53* gene mutations. In interpreting immunohistochemistry, it is important to bear in mind that nearly 20% of the *P53* mutations (especially non-sense and frame-shift) lead to a truncated protein, resulting in negative staining. As *P53* is involved in the apoptotic response to genomic damage, inactivation of this gene can be hypothesised to be involved in resistance to chemotherapy and radiotherapy (176). Only a few studies have shown this to be true in clinical situations. A recent meta-analysis has confirmed the association of *P53* gene mutations with poor disease-free and overall survival (42). Some studies have demonstrated an inverse relationship between p53 and BCL2 protein expression in breast cancer and other solid tumours (177-179). Some studies of *P53* mutations in exons 5-9 have shown an association with low oestrogen receptor (ER) content, high S-phase fraction, positive nodal status and *HER2/neu* amplification. Mucinous and papillary tumours, which have a good prognosis, have a lower frequency of *P53* mutations. However, medullary carcinomas, which also have a good prognosis, appear to have a high frequency of *P53*

mutations along with inactivation of *BRCA1* gene (180). Currently, there is some controversy whether medullary carcinomas really have a better prognosis. Therefore, the occurrence of *P53* mutations in medullary carcinomas could explain these findings.

5) The *E-cadherin* gene on chromosome 16q22.1 encodes a cell adhesion protein involved in adhesion between epithelial cells. In the majority of invasive lobular carcinomas, there is complete loss of plasma membrane-associated expression of E-cadherin. In most cases of loss of E-cadherin, mutations in the *E-cadherin* gene are identified (181). In ductal carcinomas, E-cadherin expression is usually normal or slightly reduced and hence, *E-cadherin* inactivation appears to be specific for lobular carcinoma. Inactivating mutations in the *E-cadherin* gene have also been demonstrated in lobular carcinoma in situ (182).

Well differentiated DCIS also frequently shows LOH on chromosome 16q, which is probably attributable to another tumour suppressor gene that may play a role in its development (183;184).

6) *BRCA1* and *2*. In approximately 5-10% of breast cancer patients, a genetic predisposition to develop breast cancer is present. It has been demonstrated that mutations in the *BRCA1* and *2* genes are associated with a dominantly inherited breast cancer risk (72). The expression of BRCA1 in tissue sections from breast carcinomas can be demonstrated using immunohistochemistry (185). In future, detection of the absence of BRCA1 and/or BRCA2 expression may assist in identifying patients with *BRCA1* or *2* germline mutations. The histopathological features of tumours from *BRCA1* and *2* germline mutation carriers differ from sporadic carcinomas and from each other. Both are of higher grade as compared to sporadic cases and an excess of medullary/atypical medullary carcinomas has been reported in patients with *BRCA1* mutations (186). Multifactorial analysis shows that the only features independently associated with *BRCA1* mutations are a high mitotic count, pushing margins and the presence of a lymphocytic infiltrate (187). It is interesting to note that breast carcinomas in *BRCA1* and *2* gene mutation carriers show an increased frequency of mutations in the *p53* gene (188). The chromosomal regions of *BRCA1* and *BRCA2* display LOH in 25-56% of sporadic breast carcinomas, whereas somatic mutation of *BRCA1* and *BRCA2* in sporadic breast carcinomas is an infrequent event with a frequency of 0-9% (189-191).

THE MULTISTEP MODEL OF BREAST CARCINOGENESIS:

It is not known how many mutations are required for genesis of a specific cancer but it is supposed that approximately 12 may be involved and in most cases, these are acquired somatically. Animal studies have shown that the genesis of tumours requires at least two main steps, 'initiation' and 'promotion'. 'Initiation' is irreversible whereas 'promotion,' which follows it, is reversible. These model systems also indicate that the initiating event in tumorigenesis is mutation and subsequent tumour progression may be mediated by either genetic or epigenetic mechanisms. In vitro studies generally support the idea that transformation is at least a two stage process, one oncogene being needed for immortalization and another for transformation. In addition to the initiating of growth promoting events, the loss of growth inhibitory activity of TGFβ is thought to contribute to the development of many types of tumours.

The multistep model of breast carcinogenesis suggests a transition from normal epithelium to invasive carcinoma via in-situ carcinoma. It is debatable whether typical and atypical hyperplasias are intermediates in this multistep model. Typical hyperplasia is found in over 50% of women without breast cancer. A multistep model for breast carcinoma development, similar to that for colon carcinoma, has not yet been fully elucidated. The hypothetical model reported by Schnitt is mentioned on page 331. It is probable that there are different types of breast cancer, each of which may be genetically different as suggested in (figure 3.7)(142):

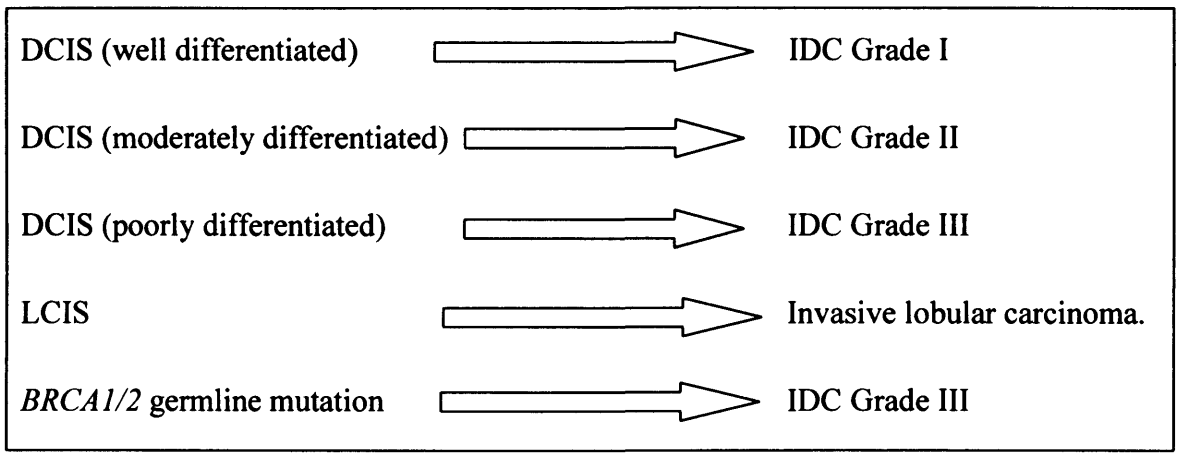


Figure 3.7; A hypothetical model describing the various grades of invasive carcinoma arising from carcinoma in situ

As DCIS and LCIS can co-exist in some cases and similarly invasive ductal and lobular carcinomas can show mixed patterns, this figure may be an oversimplification.

Members of the epidermal growth factor (EGF) family are widely expressed and are thought to play important roles in both mammary gland development and in tumorigenesis. Over-expression of at least two members of the family, amphiregulin and cripto-1, has been detected in breast tumours. Either can transform immortalized human and mouse mammary epithelial cells (192). Other members of the EGF receptor family (EGFR, HER2, HER3 and HER4) also frequently show abnormal expression in breast and other human cancers.

A few reports on LOH in DCIS have been published, regions with frequent LOH were found mainly on chromosomes 16 and 17, indicating that these are early events in breast cancer development that precede the tumour cells becoming invasive (183;193-195).It is interesting that normal epithelial cells adjacent to invasive carcinoma show LOH for chromosome 3p (196). Lakhani et al have described the dissection of 'normal' luminal and myoepithelial cell clones (197). Overall, LOH was found in normal cells in 50% of 10 breast cancer cases and in one of 3 reduction mammoplasty specimens. In one case, all luminal and myoepithelial samples exhibited loss of the same allele on chromosome 13q, suggesting the presence of a common stem cell for the two epithelial cell types. LOH has been demonstrated in normal tissues near and away from the carcinoma, suggesting that genetic alterations are likely to be more heterogeneous and widespread than previously thought, and probably occur very early in breast carcinogenesis. More oncogenes and tumour suppressor genes in breast cancer will be identified in the future and a complete catalogue of these genetic alterations is expected to be assembled in the next 5-10 years (108). Nearly, all the genetic alterations described in invasive breast cancer have been identified in carcinoma in situ, hence most of them are considered to be early events in breast carcinogenesis (167). One of the few changes that could be a late event in breast tumourigenesis is the amplification of c-myc gene, which has not been described in DCIS and is associated with locally advanced breast cancer.

It is a great challenge to complete this genetic modelling, to identify subgroups of breast cancers with distinct genetic alterations correlating with clinical behaviour and to utilize this knowledge to tailor therapy for individuals.

TECHNIQUES AVAILABLE FOR THE DETECTION OF GENETIC ALTERATIONS:

The following techniques are currently used to detect genetic alterations in breast cancer:
Karyotyping: Metaphase chromosomes can be isolated from cultured cells. These are subsequently stained and analysed under the microscope. In this way cytogenetic alterations have been detected in many tumour types, which have guided the positional cloning of several genes involved in cancer, especially in leukaemias and lymphomas. Unfortunately, the karyotypes of breast cancers and other solid tumours often are so abnormal that identification of recurrent abnormalities pointing to the locations of important genes has been difficult. It is also difficult to take breast carcinoma cells into tissue culture, which further hampers cytogenetic analysis.

Southern blot analysis:

For this technique, freshly frozen tumour material is required. DNA is isolated and digested with an appropriate restriction enzyme. The DNA is then size fractionated on an agarose gel, transferred to a filter and hybridized to a labelled gene probe. The technique is very labour-intensive and therefore not very well suited for use in daily clinical practice. The amplification of oncogenes in breast cancer has mainly been studied by this technique. Before the polymerase chain reaction became available, deletions of regions of DNA were also studied using this technique.

Comparative genomic hybridization (CGH):

Comparative genomic hybridization can be performed with DNA isolated from frozen material and from paraffin blocks (198), unless tumours have been fixed in acid-containing fixatives, such as Bouin's fixative. Differentially labelled tumour DNA and normal reference DNA are hybridized simultaneously to normal chromosome spreads. The hybridization is detected with two different fluorochromes. Regions of gain or loss of DNA sequences, such as deletions, duplications or amplifications, are seen as changes in the ratio of the intensities of the two fluorochromes along the target chromosomes. Comparative genomic hybridization of breast carcinomas has resulted in the identification of a number of chromosomal regions containing amplified sequences. CGH is technically difficult and time-consuming, precluding its use as a standard test. The advantage of CGH

over most other techniques is that the whole tumour genome can be analysed in one experiment.

However, the resolution of CGH on metaphase chromosomes is limited. Amplitude regions have to be 1-2 Mb in size before they can be detected. Recently, arrays of mapped sequences have been developed for CGH (199). Using these arrays, the copy number of smaller regions of DNA can be measured with high precision.

There is not always a correlation between loss of heterozygosity (LOH) picked up by PCR (see below) and deletions detected by CGH. This is due to the fact that LOH is often the result of loss of one allele in combination with duplication of the other allele.

Polymerase chain reaction (PCR):

This technique has revolutionized the analysis of genetic alterations in tumours. The basic principle is that oligonucleotide primers are used to amplify specific regions of DNA. These amplified regions can subsequently be further studied using additional techniques, including sequencing and single strand conformation polymorphism (SSCP) (see below). In breast cancer, PCR, using primers for repeated sequences of known chromosomal localization, has been used to study LOH. For many of these repeats there is heterogeneity within the population, resulting in polymorphisms. The paternal and maternal alleles can be distinguished if the patient is heterozygotic for such a polymorphism. By comparing DNA from normal cells with tumour DNA, LOH can be detected.

Fluorescent in-situ hybridization (FISH) and Bright field in-situ hybridization (BRISH):

The basis for this method is the hybridization of labelled DNA probes to cells. To obtain a detectable signal, the use of relatively large probes is required. Using FISH, it is possible to identify numerical chromosomal alterations in isolated nuclei or in tissue sections, usually using centromere probes. For this purpose, both frozen and paraffin-embedded material may be used. In addition, it is possible to detect gene amplification using this technique. A fluorescence microscope is required in order to perform FISH. It is also possible to visualise the binding of label using chromogens, which can be seen under a bright field microscope (bright-field in-situ hybridisation, BRISH) but this technique is less sensitive than FISH.

Single strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE):

SSCP and DGGE are rapid, simple and sensitive techniques for the detection of various mutations, including single nucleotide substitutions, insertions and deletions, in PCR-amplified DNA fragments. In breast cancer, these techniques have mainly been used for the detection of mutations in the *P53* gene, *BRCA1* and *BRCA2* genes.

Sequence analysis:

To confirm the presence of mutations detected by SSCP or DGGE, sequence analysis can be performed. It is also possible to look for genetic alterations in breast carcinomas by sequence analysis without pre-screening with the above-mentioned techniques. This is relatively labour-intensive, although further automation can make sequence analysis more feasible for the detection of genetic alterations in large sets of tumours.

Microarray analysis:

The technique of microarray analysis was developed at Stanford University and allows the study of the level of expression of up to10,000 mRNAs in a single experiment (200-202). Basically, cDNAs are microspotted onto glass slides. The mRNA from the cells to be analyzed is isolated and reverse transcribed to cDNA in the presence of red-fluorescent nucleotides. Fluorescent cDNA is then mixed with a green fluorescent-labelled standard and the mixture is hybridised to the microarray. Using a fluorescent scanner, the level of fluorescence is computerised and for each cDNA on the microarray the level of gene expression, relative to the standard is determined and transferred to a database. Using software specifically developed for microarray analysis, the level of expression for many thousands of genes can be compared between different tumour samples. The power of this approach for clinical research was recently illustrated by Alizadeh et al (203), who analysed RNA from 40 large B cell lymphomas for the expression of 17,856 genes using microarray analysis. Based on the pattern of gene expression, two distinct groups of tumours could be recognised within these otherwise indistinguishable B-cell lymphomas. One type expressed genes characteristic of germinal centre B-cells and the second type expressed genes normally induced during in-vitro activation of peripheral blood B-cells. Furthermore, it was shown that the germinal centre type B-cell lymphomas had a significantly better prognosis than the other group.

As a result of the Human Genome Project and the contributions of individual researchers, partial or complete nucleotide sequence is available for 60,000 genes of the estimated 80,000-100,000 genes that are expressed in human cells. Technically, with microarray analysis, it is possible to analyze all estimated 80,000-100,000 genes in the human genome for their expression. With the current density of 10,000 genes per array, it would only take 8-10 experiments to determine the level of expression of all genes within one tumour sample. At the moment, the availability of sequence confirmed cDNAs is the main limiting factor. With the rapid progression in gene cloning and sequencing, it is expected that larger microarrays will become available in the coming years and the expression of all genes expressed in humans will be assayed. Also the function of the proteins encoded by many of these genes will be elucidated in the coming years. Finally, the software required to analyse such large and complex datasets is also rapidly evolving. These developments make the application of microarray analysis in clinical research of great importance.

Immunohistochemistry:

Alterations in protein expression, resulting from genetic alterations, can often be detected using immunohistochemistry. It is a very accessible technique for most pathology laboratories. For many proteins, immunohistochemistry can be used on paraffin-wax sections. An important advance for the study of large series of tumours is the development of tissue arrays (204;205). As many as 1000 cylindrical tissue biopsies from individual tumours can be distributed in a single tumour tissue microarray. Sections of the microarray provide targets for immunohistochemistry and for parallel in situ detection of DNA and RNA. Consecutive sections allow the rapid analysis of hundreds of molecular markers in the same set of specimens.

CHAPTER 4: CerbB-2 IN BREAST CANCER:

INTRODUCTION:

The proto-oncogene *cerbB-2* has been localized on chromosome 17q12 and encodes a 185 kDa transmembrane glycoprotein which is a tyrosine kinase growth factor receptor (1-3). Figure 4.1 illustrates a schematic representation of the protein structure of cerbB-2.

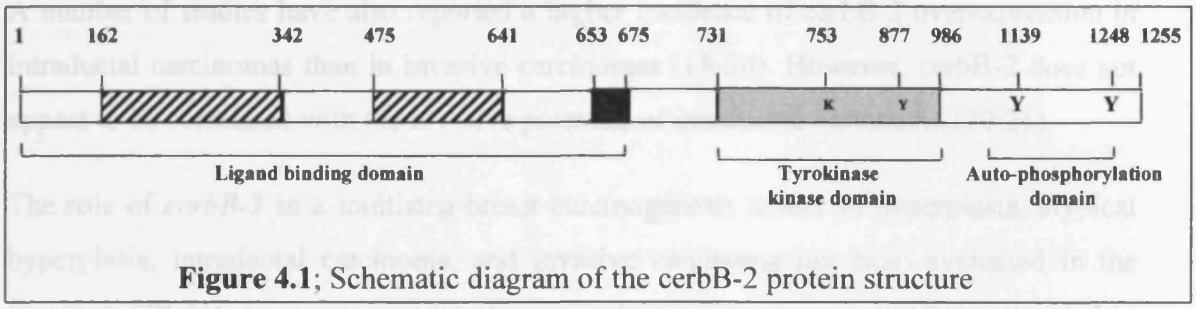


Figure 4.1; Schematic diagram of the cerbB-2 protein structure

The different groups who independently identified this gene have used differing terminology to describe the proto-oncogene (4;5). The name *HER-2* is derived from the term "human epidermal growth factor receptor" because *HER-2* has substantial homology with the epidermal growth factor receptor (6), but *cerbB-2* and *HER-2* both refer to an identical human homologue of the *Neu* oncogene in the rat.

The cerbB-2 protein belongs to a four-member family of closely related growth factor receptors that include HER-1 (erbB-1), HER-2 (erbB-2), HER-3 (erbB-3) and HER-4 (erbB-4). Direct ligands have been identified for HER-1, HER-3 and HER-4, but not for HER-2. The cerbB-2 receptor is known to mediate lateral signal transduction in all HER receptor family members (6;7). After ligand binding, EGFR, HER-3, and HER-4 can all heterodimerize with HER-2 leading to tyrosine phosphorylation of all the receptors (8;9). Though the function and regulation of the normal *cerbB-2* gene are not well understood, cerbB-2 overexpressed in cancer cells is thought to function as a growth factor and to play roles in cell differentiation, motility, and adhesion (10;11). The gene is widely expressed in epithelial cells of human fetal and adult tissues including breast tissue. Amplification of the *cerbB-2* gene and overexpression of the protein was first discovered in a human breast carcinoma cell line (12). Subsequently, *cerbB-2* amplification was reported in 30% of primary human breast cancers (13). To date, numerous studies have evaluated overexpression of cerbB-2 with or without gene amplification and found that

15% to 30% of breast carcinomas are positive for cerbB-2 (14). Generally, cerbB-2 protein overexpression correlates with gene amplification (15).

ROLE OF *cerbB-2* IN PATHOGENESIS OF BREAST CANCER:

Data from experiments using in vitro cell lines and in vivo with transgenic mice have suggested that *cerbB-2* amplification is an early event in breast carcinogenesis (14;16;17). A number of studies have also reported a higher incidence of cerbB-2 overexpression in intraductal carcinomas than in invasive carcinomas (18-20). However, cerbB-2 does not appear to be correlated with the invasive potential of intraductal carcinoma (20;21).

The role of *cerbB-2* in a multistep breast carcinogenesis model of hyperplasia, atypical hyperplasia, intraductal carcinoma, and invasive carcinoma has been evaluated in the literature (20-23). No gene amplification or protein overexpression has been reported in benign neoplasms or hyperplasias, and most of the reported cases of atypical duct hyperplasia have been negative for cerbB-2 overexpression (20;22;24). However, a "weak focal immunopositivity" has been reported in occasional cases of atypical hyperplasia (25).

In 1988, van de Vijver et al. (19) first reported a significant association between cerbB-2 positivity and a comedocarcinomatous pattern. Since then a number of other studies have all confirmed a significant correlation between cerbB-2 overexpression and biomarkers of poor differentiation such as high nuclear grade, lack of steroid receptors, high proliferative activity, and DNA aneuploidy (26-33). Moreover, cerbB-2 overexpression occurs in 70% to 90% of cases of mammary Paget's disease (34;35), a finding that has led some authors to suggest that cerbB-2 may function to promote the intraepithelial spread of carcinoma cells.

PROGNOSTIC SIGNIFICANCE OF *cerbB-2* IN INVASIVE BREAST CANCER:

More than a decade ago, the first study reporting cerbB-2 expression as a prognostic marker in breast carcinoma was published (13). In that initial study of 189 patients with both axillary lymph node-negative and axillary lymph node-positive breast cancer, Slamon et al, reported that *cerbB-2* gene amplification was strongly associated with shortened disease-free (DFS) and overall survival (OS) only in the lymph node-positive patients. Shortly after, this finding was verified by the same group in a larger patient

population (36). Since then, numerous retrospective studies involving thousands of patients have been published on this topic (36-74).

In general, most studies evaluating survival in patients with axillary lymph node-positive tumour conclude that cerbB-2 abnormalities determined by gene amplification or protein overexpression are associated with a worse clinical outcome (36;43;45;48;52;55;62;63;75). A number of studies have also demonstrated that the degree of amplification is important, i.e., the highest levels are associated with the poorest prognosis (13;54). However, the initial studies on patients with node-negative breast carcinoma reached different conclusions on the prognostic significance of cerbB-2. Some studies showed a similar trend towards poor prognosis in either all or some subsets of axillary node-negative patients whose tumours overexpressed cerbB-2 (36;42;50;52;54), while other studies found no significant correlation between cerbB-2 status of the tumour and clinical outcome (40;41;47;51;53). Some authors have explained the discrepant findings in node-negative breast cancer by citing poor study design, small study size, and lack of a standardized test for evaluation of cerbB-2 (76;77). Recently, however, it has been suggested that cerbB-2 is an independent prognostic factor in node-negative breast cancer also (78).

PREDICTIVE ROLE OF *cerbB-2* IN RELATION TO THERAPY IN INVASIVE BREAST CANCER:

It has been speculated that cerbB-2 overexpression might be a predictive marker of response to therapy (77;79) due to the fact that in most of the studies of cerbB-2 as a prognostic marker in node-positive breast cancer, the patients had adjuvant therapy. A number of studies have evaluated the relationship between cerbB-2 expression and response to therapy (chemotherapy, hormonal therapy, and radiation therapy).

Response to Chemotherapy:

Initial studies of the possible relationship between cerbB-2 overexpression and response to chemotherapy included patients treated with cyclophosphamide/methotrexate/5-fluorourcil (CMF), one of the most common chemotherapy regimens for breast cancer. Results of these studies suggested that CMF shows less benefit in those patients with cerbB-2 overexpressing tumours (50;52;58;80). The association between cerbB-2 overexpression and response to doxorubicin-containing regimens has also been studied

(81-83). These studies suggested that cerbB-2 overexpression may determine which tumours may be particularly sensitive to doxorubicin-containing regimens (82;83). Preclinical and clinical data suggest that cerbB-2 plays an important role in the chemotherapeutic response to paclitaxel (Taxol) and that antitumour activity of paclitaxel can be influenced by modulation of cerbB-2 in vitro and in vivo (84-86).

Response to Hormonal Therapy:

The relationship between cerbB-2 overexpression and resistance to antioestrogen therapy with tamoxifen has been evaluated in a number of studies (86-91). In several of these initial studies, cerbB-2 overexpressing tumours were resistant to antioestrogen therapy, which is expected since cerbB-2 overexpression has been reported to be associated with a lack of ER in breast carcinoma (86-90). However, in a recent study, Elledge et al found that cerbB-2 expression in ER-positive metastatic breast cancer was not associated with a poorer response to tamoxifen or a more aggressive course of disease (91). Leitzel et al reported that elevated serum levels of the extracellular domain of cerbB-2 have been associated with resistance to second line hormonal therapy (92). Another study reported a significant association between elevated circulating levels of cerbB-2 and resistance to antioestrogen therapy with doloxifine (93).

Response to Radiation Therapy:

The relationship between cerbB-2 overexpression and radiation therapy has also been studied. The results are not conclusive, but clinical studies suggest that cerbB-2 overexpression may confer radio-resistance on the breast cancer (94;95).

CerbB-2 AS A THERAPEUTIC TARGET:

The cerbB-2 receptor protein is an ideal therapeutic target because it is homogeneously expressed in both primary and metastatic tumours and its expression is quite low in normal tissues. Encouraging initial results obtained from in vitro studies using cell lines and xenografts have opened a new era in the treatment of breast cancer (96;97). The 4D5 anti-cerbB-2 antibody, which is directed against the extracellular region of cerbB-2, was humanized by inserting its murine antigen binding regions into the framework of a human immunoglobulin molecule (98;99). This recombinant humanized monoclonal anticerbB-2 antibody, called rhuMAb HER-2 (Trastuzumab, Herceptin) has been shown to have

significant tumour growth-inhibitory activity in breast cancer cell lines that overexpress cerbB-2. Clinical phase I and II trials demonstrated encouraging results when Herceptin was used as a single agent or in combination with chemotherapeutic agents (100;101). The efficacy of Herceptin plus chemotherapy was evaluated in phase III randomised clinical trials in which patients with stage IV metastatic breast cancers were randomised to receive Herceptin plus chemotherapy or chemotherapy alone. Patients who received the combination had a statistically significant longer median duration of response than patients who received chemotherapy alone (100-102). Herceptin was also evaluated as a single agent first-line therapy in patients with metastatic breast cancer (103;104). Therefore, National Institute for Clinical Excellence (NICE) has issued guidelines for the recommendations on the use of herceptin in metastatic breast cancer, both in combination with chemotherapy and as monotherapy (105). In trial setting, herceptin is being used in adjuvant treatment of breast cancer.

In addition to the use of anticerbB-2 antibodies, the use of *cerbB-2* as a target for gene therapy via the use of antisense probes and anti-cerbB-2 protein vaccination is under investigation (106;107).

METHODS OF EVALUATING *cerbB-2* STATUS:

Until recently, the greatest problem limiting the routine clinical use of cerbB-2 has been the lack of well-standardized assays and quality control programmes (108;109).

Methods that can be used to determine tumour *cerbB-2* status include assays of gene amplification (Southern blot hybridization, PCR, FISH), assays of protein overexpression (immunohistochemistry, ELISA, Western blot) and mRNA level evaluation (Northern blot).

There is no consensus about the reliability, reproducibility, and predictive value of each method and each has its advantages and disadvantages. The main limitation of using blotting techniques to assay *cerbB-2* gene amplification is the dilutional effect of nontumoural elements present in tissue samples. Furthermore, these techniques are quite expensive and require specialized molecular laboratories. The fluorescence in situ hybridization (FISH) technique allows simultaneous evaluation of gene amplification and morphology. This technique is more sensitive than Southern blot analysis for the detection of *cerbB-2* abnormalities in breast cancer (110). FISH can be performed on

small amounts of tissue and on cytological specimens (110;111). It is generally accepted to be a more discriminant test at the positive negative borderline, has greater ease of methodological standardisation and less observer variation.

Although FISH assays have been widely advertised in the literature as expensive techniques requiring special equipment and reagents, the recent development of commercially available sensitive probes now make the cost only slightly more expensive than immunohistochemical methods. The only special equipment required for FISH that may not be found in a routine laboratory is the fluorescence microscope.

In less than 5% of breast carcinomas, overexpression of cerbB-2 oncoprotein may be due to enhanced transcription without gene amplification (111). The majority of the studies evaluating cerbB-2 as a predictive factor for response to either conventional therapies or herceptin have used immunohistochemical methods. Immunohistochemistry (IHC) has been the predominant method used in clinical studies to determine overexpression of the cerbB-2 oncoprotein. In fact, immunohistochemical staining of frozen tissue is consistently found to be the most reliable technique in this regard (71). However, in order to be useful in routine laboratories the technique must be reliable in paraffin wax-embedded tissues. Generally, there is good concordance between FISH assay for *cerbB-2* and its detection by IHC (112).

The main technical issue for consideration in the case of IHC is the sensitivity and specificity of the antibodies used for immunohistochemistry. Busmanis et al reported a wide range of detection rates and staining patterns when they used a panel of six different antibodies (113). A similar result was obtained by Press et al, who used 21 monoclonal and 7 polyclonal antibodies to assay cerbB-2 protein overexpression on paraffin-wax embedded tissue sections of breast cancer (77). They found that the sensitivity and specificity of the antibodies varied from 6-80%. Another issue is the use of antigen retrieval techniques and resulting false positivity. A third issue is the tissue fixation methods used, especially in the case of paraffin wax-embedded tissue sections. The type of tissue fixation, the length of fixation and the method of tissue preparation can all influence the immunohistochemical staining results. Prolonged storage can cause a decrease or loss of immunopositivity (114). Finally, the fourth important issue is the interpretation of the staining. A membranous staining pattern is generally considered to be specific for cerbB-2 overexpression and has been shown to correlate with gene

amplification. In contrast, the clinical and biological significance of cytoplasmic staining is controversial. Tetu et al specifically evaluated the pattern of staining in relation to prognosis in patients with node-positive breast cancer, they found that cytoplasmic staining did not correlate with clinical outcome, but that membranous staining was a significant predictor of a shorter disease-free survival (58). Another study using a different monoclonal antibody, however, showed that strong cytoplasmic staining did correlate with bad prognosis (115).

Like many other cell surface receptors, a soluble form of the extracellular domain of the cerbB-2 protein can be detected and quantitated in the serum by enzyme-linked immunosorbent assay (ELISA) (116;117). Not all cerbB-2 positive tumours shed the protein. Furthermore the clinical significance of circulating cerbB-2 is uncertain. Janiska et al found that circulating cerbB-2 protein may be a non-specific phenomenon and may not necessary correlate with the presence of a tumour (118). Recently, a systematic literature review on the clinical value of measuring elevated serum levels of HER-2/neu oncoprotein was published in clinical chemistry (119). This review summarised data from 55 peer-reviewed journals and included over 6500 patients. It concluded that elevated levels of serum cerbB-2 are indicative of a particularly aggressive form of breast cancer and therefore is a valuable clinical tool for assessing prognosis, predicting response to therapy and detecting early recurrence of breast cancer. In primary breast cancer, the prevalence of elevated serum cerbB-2 levels varied between 0-28%, whereas in metastatic disease elevated values were found in 23-80% of all cases. This review also showed that cerbB-2 positive tumours are more prevalent than generally known by tissue testing and that elevated serum levels of cerbB-2 predict a poor response to hormone therapy and some chemotherapy regimens but can predict improved response to combination of chemotherapy and herceptin.

In summary, although cerbB-2 expression in breast cancer has been widely studied, there are still a number of unanswered questions regarding its biological and clinical significance (120). There is adequate preclinical and clinical evidence that cerbB-2 may be a clinically useful prognostic and predictive marker and can be used as a therapeutic target, but the lack of a reliable and reproducible method of evaluation hampers the drawing of any definitive conclusions. Therefore, research efforts should be focused on

the development of standard methodologies for evaluating cerbB-2 expression in tumour tissues.

Recently, there has been interest in using the FDA (food and drug agency)-approved HercepTest as a predictor of response to Herceptin therapy (121). The Hercep Test is a semiquantitative immunohistochemical assay to determine HER-2 overexpression in breast cancer tissues routinely processed for histological evaluation. Currently, it is the most expensive assay kit used in diagnostic histopathology laboratories (121). Therefore, Grady et al (121) have applied tissue microarray technology to analyse HER-2 status in 20 samples in one experiment using the HercepTest. Similar work has also been done by Rampaul et al (122). Both groups concluded that HercepTest combined with tissue microarray method is accurate, economical and easily integrated into a routine laboratory. However, heterogeneity of tumours can be a problem. Nevertheless, developing this technique further could be the way forward for the future.

In the UK, there are now published guidelines which have been formulated to give advice on methodology and quality assurance for local testing to ensure that HER-2 testing results are accurate and reliable, regardless of the test used (123) and also see appendix II. These guidelines have been updated further. All laboratories utilising assays for cerbB-2 as predictive or prognostic marker are required to participate in an appropriate external quality assurance (EQA) programme such as the one run by the UK National External Quality Assessment Scheme (NEQAS) for Immunocytochemistry.

EGFR/erbB-1/HER-1:

EGFR encodes the receptor for epidermal growth factor (124;125). It is located on chromosome 7p13 and the cellular location of the receptor is at the plasma membrane. There are at least 15 *EGFR* ligands (126;127) including EGF, TGFα, amphiregulin, cripto-1, betacellulin, heparin-binding EGF-like growth factor α, heregulin, glial growth factor and others. EGFR can heterodimerize with each of the other members of the EGFR family (HER-2, HER-3 and HER-4) and individual ligands for this family give rise to distinct patterns of *trans*-phosphorylation (128). *EGFR* is amplified in 20% of primary breast carcinomas and some studies (129-132) have shown it to be strongly associated with early recurrence and death in lymph node-positive patients. However, a recent study

found no prognostic significance for EGFR expression in invasive breast cancer even after long-term follow-up (133).

ErbB-3/HER-3:

HER-3 is located on chromosome 12q13 and the cellular location of the protein is at the plasma membrane where it functions as a receptor tyrosine kinase. HER-3 can form heterodimers with EGFR, HER-2 or HER-4. Overexpression of HER-3 occurs in mammary tumour-derived cell lines (134), but the role in invasive breast cancer is not clear. One study found no significant associations with overall survival, disease-free interval, regional recurrence, the presence of distant metastases, age, menopausal status, oestrogen receptor status, histological grade, lymph node stage, vascular invasion and c-erbB-2 protein expression (135).

ErbB-4/HER-4:

HER-4 is located on chromosome 2q33 and the cellular location of the protein is at the plasma membrane. HER-4 can form heterodimers with EGFR, HER-2 or HER-3. HER-4 is expressed in a variety of mammary adenocarcinoma cell lines (136), but again like HER-3, the role in invasive breast cancer is not clearly defined.

CHAPTER 5: OESTROGEN RECEPTORS IN BREAST CANCER:

INTRODUCTION:

Hormonal therapy is important in the management of breast and prostate cancer. This is based on the fact that some cancers express receptors for sex hormones and that their growth is partly dependent on hormonal stimulation. Female sex hormones include oestrogens and progestogens. Naturally occurring oestrogens are oestradiol, oestrone and oestriol whereas synthetic oestrogens include ethinyloestradiol, mestranol and diethylstilboestrol. There are two main groups of progestogens, *progesterone and its analogues*, which include dydrogesterone, hydroxyprogesterone and medroxyprogesterone; and *testosterone analogues* which include norethisterone and norgestrel. The newer progestogens, desogestrel, norgestimate and gestodene, are all derivatives of norgestrel. Steroid hormones are mainly produced in the ovaries and testis, and to some extent in the adrenal glands. Under physiological conditions, oestradiol acts as a proliferative hormone on breast epithelial cells, whereas progesterone has no proliferative effects (1). The proliferative effects of oestradiol on breast epithelial cells appear to be mediated by the cyclin D1-CDK2/4-pRB pathway as a result of which hyperphosphorylation of pRB occurs (2). On the other hand, oestradiol also increases wild-type p53 levels indirectly via increased c-myc transcription. P53 in turn, induces p21 and inhibits proliferation. Therefore, the effect of oestradiol on breast epithelial cell proliferation is two-sided; firstly, increased proliferation via pRB hyperphosphorylation and secondly, decreased proliferation via p53 upregulation (figure 5.1).

The sex hormones bind with high specificity and affinity to intracellular receptors which belong to a "superfamily" of proteins whose function is to control the transcription of a repertoire of other cellular genes (3). Steroid receptors for oestrogen and progesterone (ER and PR) are located in the cell nucleus. Hormone is believed to be transported to the nucleus where a steroid-receptor complex is formed with receptor dimerization. This dimer binds to specific DNA response element sequences usually located in the promoter regions of regulated genes. Some of the genes regulated by steroid receptors are involved in controlling cell growth.

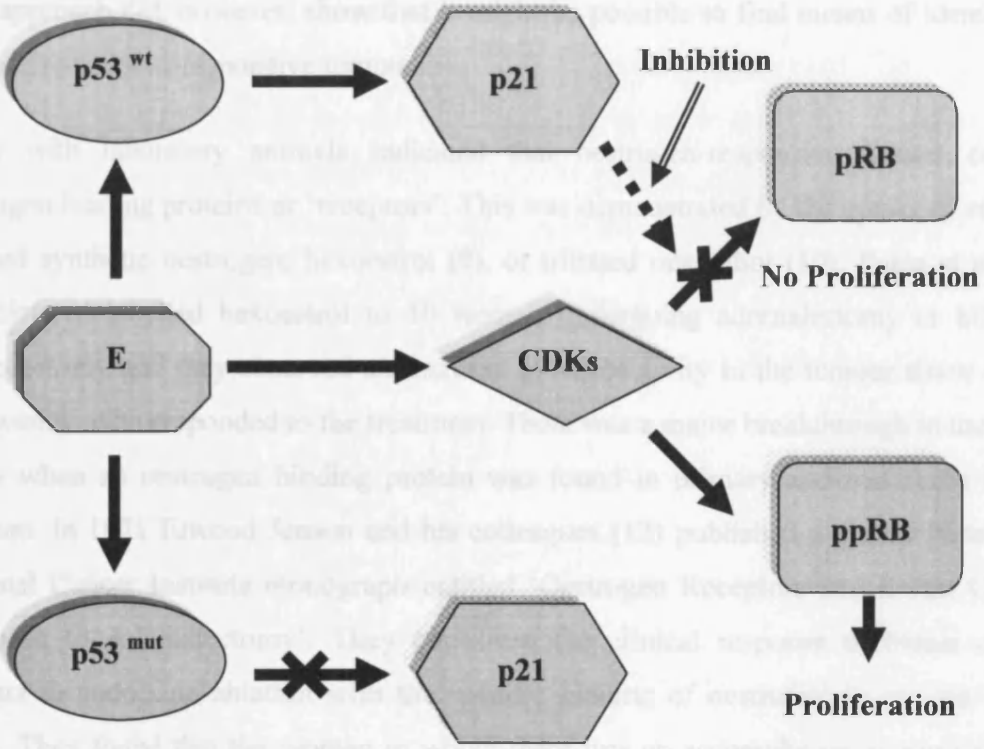


Figure 5.1; A hypothetical model describing relationship between oestrogen and the two tumour suppressors, p53 and pRB, where E = oestradiol

Since the end of nineteenth century it has been known that a proportion of breast carcinomas are hormone sensitive. It was Beatson (4) who showed that following oophorectomy, there was a regression of metastatic lesions in premenopausal women with breast cancer. More than 50 years later Huggins and Burgenstal (5) demonstrated that ablation of the adrenal glands could be similarly beneficial in post menopausal women. Shortly afterwards, it was shown by Luft and Olivecrona (6) and Pearson et al (7) that there was similar effect following hypophysectomy. Approximately 30% of women treated with endocrine ablation showed a dramatic improvement. Unfortunately, there was no way of distinguishing those who would benefit from those who would not. Therefore, there was considerable interest in finding ways of selecting women who would benefit from surgical endocrine ablation so that the others could be spared ineffective surgery. An early attempt to do this was made by Bulbrook and his colleagues in 1968 (8) when they measured urinary androgen and corticoid metabolites in women about to undergo adrenalectomy or hypophysectomy, but, although there was some correlation with the response, the association was not strong enough to allow it to be used clinically.

This approach did, however, show that it might be possible to find means of identifying responsive and non-responsive tumours.

Work with laboratory animals indicated that oestrogen-responsive tissues contain oestrogen binding proteins or 'receptors'. This was demonstrated by the uptake of either a tritiated synthetic oestrogen, hexoestrol (9), or tritiated oestradiol (10). Folca et al (11) administered labelled hexoestrol to 10 women undergoing adrenalectomy or bilateral oophorectomy and they observed an increase in radioactivity in the tumour tissue in the four women who responded to the treatment. There was a major breakthrough in the early 1970s when an oestrogen binding protein was found in primary and metastatic breast tumours. In 1971 Elwood Jensen and his colleagues (12) published a classic paper in a National Cancer Institute monograph entitled 'Oestrogen Receptors and Breast Cancer Response to Adrenalectomy'. They correlated the clinical response of breast cancer patients to endocrine ablation with the specific binding of oestradiol in tumour tissue slices. They found that the women in whom there was an accumulation of oestradiol in the tissues were more likely to respond to the treatment.

Afterwards, simple assays were developed using tumour tissue cytosols. Owing to the introduction of tamoxifen as an effective treatment for metastatic breast cancer (13) it was no longer necessary to identify the hormone-responsive women in order to spare the unnecessary surgery. Unless patients had rapidly progressing disease many clinicians were happy to use this relatively non-toxic drug in a therapeutic trial and assess response in vivo. Nevertheless, there has been continued interest in receptor measurement in order to refine treatment modalities for breast cancer, particularly in clinical trials relating to both treatments for advanced disease (14) and more recently as an adjuvant treatment in early disease (15;15;16).

ER – STRUCTURE AND FUNCTION:

The ER is subdivided into six functional domains A – F (figure 5.2). A/B domains are located at the N-terminal end of the receptor and contain transcription activator factor-I (TAF-I). The C-domain is highly conserved among members of the steroid receptor superfamily and mediates DNA-binding. The D-domain is the hinge region. E/F domains are at the C-terminus of the receptor and are responsible for high affinity ligand binding; these contain TAF-II. TAF-I and II can act independently or synergistically in a cell

specific manner (17-20). ER is a transcription factor and can be a target for signalling pathways. It can be modified post-transcriptionally at specific sites. The signalling pathways it can affect include the one with the "early response gene" AP-1. Reciprocally, these pathways can interfere, either positively or negatively, with the activity of ERs and can either directly modify the receptor or alter its ability to transactivate without apparent modification (17) (21-23)

Once bound by oestrogens, the ER undergoes a conformational change, allowing the receptor to bind with high affinity to chromatin and to modulate transcription via specific DNA elements called the ERE (oestrogen response elements) of target genes. ER exists as a non-transformed, non-DNA bound oligomeric complex in association with heat shock protein hsp 70 and hsp 90 in cells (18). Hsps function to help hold ER protein properly and to protect the hydrophobic hormone binding domain from inappropriate interactions. In one recent study (24), hsp 27 correlated positively with ER and predicted improved survival in breast carcinoma.

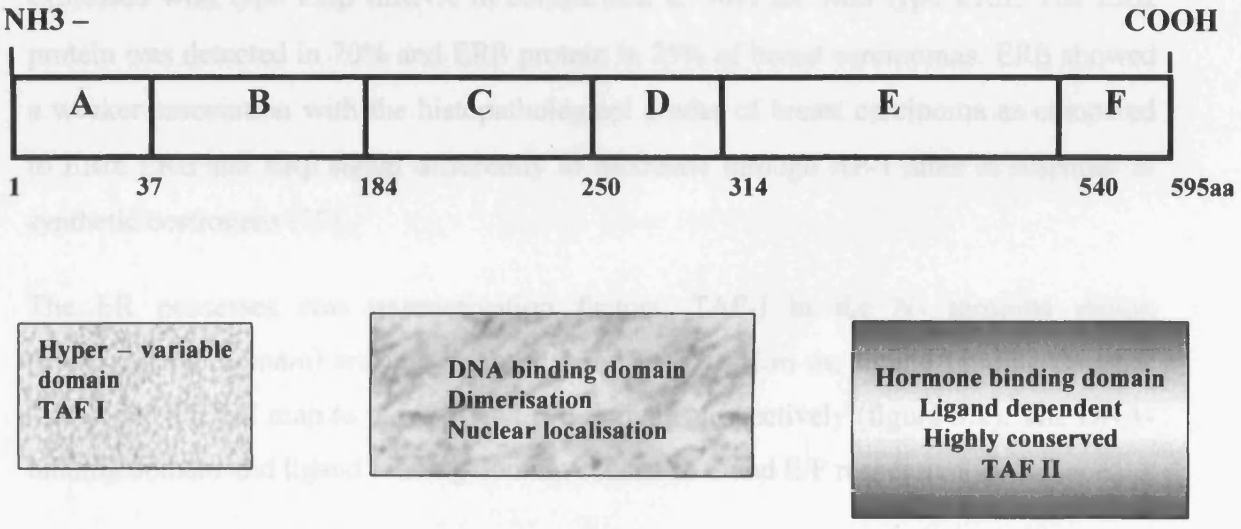


Figure 5.2; Schematic diagram of the ER protein structure

ER VARIANTS-ERα AND ERβ:

ER exists as two subtypes-ERα and ERβ (20;25). Until recently, only a single type of ER was thought to exist and mediate the genomic effects of oestrogen in mammalian tissues. However, the cloning of a gene encoding a second type of ER (ERβ) from mouse, rat and human has prompted a re-evaluation of the oestrogen signalling system (26;27). The classical ER is now referred to as ERα. The overall homology between the ligand binding domains of ERα and ERβ proteins is 55% and homology between N-terminal hypervariable domains is 22%. The DNA binding domain is virtually identical (19;28). ERβ binds oestradiol with an affinity similar to that of classical ERα but tissue distribution and relative levels of these variants are different. A recent study has looked at the cellular distribution of ERα and ERβ in normal human mammary gland employing immunocytochemistry (29). ERα was found to be restricted to the cell nuclei of epithelial cells lining ducts and lobules, whereas ERβ, in addition to epithelial cell nuclei was also detected in the cell nuclei of myoepithelial cells and some stromal and endothelial cell nuclei and some lymphocytes. Another study has looked at ERα and ERβ mRNA and protein in malignant and non-malignant breast tissue (30). Sixty six percent of tumours expressed wild type ERβ mRNA in comparison to 90% for wild type ERα. The ERα protein was detected in 70% and ERβ protein in 25% of breast carcinomas. ERβ showed a weaker association with the histopathological grades of breast carcinoma as compared to ERα. ERα and ERβ signal differently to modulate through AP-1 sites in response to synthetic oestrogens (17).

The ER possesses two transactivation factors, TAF-I in the N- terminal region (hypervariable domain) and the ligand dependent TAF-II in the ligand binding domain. TAF-I and TAF-II map to the A/B and E/F domains respectively (figure 5.2). The DNA-binding domain and ligand binding domain consist of C and E/F respectively.

H222 and 1D5 are anti-ERα specific monoclonal antibodies whereas 14C8 is anti-ERβ monoclonal antibody.

STEROID HORMONE RECEPTOR ASSAYS:

There has been a continuous development of the methods used to measure steroid hormone receptors over the last 25 years. The original assays were performed on tissue

slices but these were difficult to handle, therefore an alternative was sought by Gorski et al (31), who demonstrated the presence of ER in cytosols prepared from rat uterus. This led to the development of ligand binding assays using cytosols prepared from breast tumour tissue. Initially sucrose density ultracentrifugation was used to provide data relating the sedimentation velocity of the receptor protein to its molecular weight and density (32), however it was a cumbersome method of measuring steroid hormone receptor binding. An alternative was introduced involving the separation of unbound oestradiol from that bound to the receptors using dextran-coated charcoal (DCC) (33). Tritiated oestradiol was added, usually at several different concentrations and after allowing sufficient time for specific binding to the receptor to occur, DCC was added and centrifugation used to separate the unbound from the bound oestradiol. Scatchard analysis was used to calculate the amount of specific receptor binding and results expressed as femtomoles (fmol) of oestradiol bound per milligram of the cytosol protein or per gram of tissue. These assays had several disadvantages: ER is a thermolabile, unstable protein and particular care has to be taken when handling the tissue from the time it is removed from the patient, throughout storage and during the preparation of the cytosol and all subsequent stages of the assay. Furthermore, ligand binding assays do not take in to account the receptors which are already occupied by ligand – an important consideration particularly in prememopausal women (34). Other disadvantages are that these assays are time consuming, requiring an overnight incubation and they need approximately 500 mg of tissue, which is not always available. Furthermore, the cellularity of carcinomas and contamination with non-malignant tissue can affect the results.

It took a long time before specific oestrogen receptor antibodies could be developed (35). In 1986, Jensen et al (36) first described the use of both an Enzyme Immunoassay (EIA) for measuring ER in tumour cytosols and an immunohistochemical assay measuring ER in frozen sections (37). The antibodies used in these studies were H222 and H226, marketed by Abbott laboratories. The results of immunohistochemical assays were compared against EIA in a study reported in Supplement of Cancer Research (38). The ER immunohistochemical assays were discussed in detail by Pertschuk in 1990 (39). In another study, Pertschuk et al also showed (40) that there was a better correlation between ER content and response when measured by the immunohistochemical assay than the DCC assay.

The search continued for alternative antibodies, particularly those which could be used on formalin fixed paraffin-wax embedded tissues. In the late 1980s, studies started to appear in the literature describing ER immunohistochemical assays on formalin fixed paraffin-wax embedded sections using various enzyme digestion techniques including trypsin (41), pronase (42) and D-nase (43) with the Abbott H222 antibody. Initially the D-nase technique was quite popular but later the use of pronase in conjunction with overnight incubation with H222 antibody was found to give more consistent and satisfactory results (44). Improvements continued (45) and a new ER antibody ID5 (46) entered the market available in the UK from Dako Ltd. This produces good results (47). The success of these newer antibodies has been considerably helped by the coincidental development of antigen retrieval methods using heat, produced either by microwave (48) or pressure cooker (49). The use of H222 has declined following the development of antibodies such as ID5, which is more successful on fixed tissues. Combined with the microwave technique, it does not require overnight incubation. It gives an evaluation of ER status, which correlates well with the assessment made using Abbott H222 on both frozen and formalin fixed paraffin-wax embedded tissue (50).

EVALUATION OF ER STAINING IN IMMUNOHISTOCHEMICAL ASSAYS:

Immunohistochemistry revealed that the ER was located in the nucleus of breast cancer cells (51) although early research workers thought the protein to be in the cytoplasm. It is now known that the majority of receptor is only loosely bound in the nucleus and is easily extracted. Only a very small amount is so tightly bound to DNA that even the most rigorous methods fail to extract it. ER can shuttle between the nucleus and the cytoplasm, but is mainly found in the nucleus and immunohistochemistry fails to detect it in the cytoplasm (52).

Despite the increasing popularity of immunohistochemical methods, there is still no general agreement about the best way of scoring ER staining in tissue sections. The nuclear staining varies in both intensity and extent and the major problem is to determine how it should be evaluated. Kinsel et al described a method called histo-score (53). In this method, the staining intensity of each part of the section is assessed and allocated a value of 0 (nil), 1 (weak), 2 (distinct) and 3 (strong). The percentage of positive cells in each of the staining categories is multiplied by the intensity value and sum of these products in then added together to give the H score. The maximum score is 300. A score of 75 or

more is considered positive. This method is time consuming and gives a numerical value to something which is a subjective assessment.

An alternative immuno-reactive score (IRS) suggested by Remmele and Stegner (54) is widely used in Germany. In this method, the average percentage of stained cells over the entire section is assessed and allocated a value of 0 (no staining), 1 (<10%), 2 (11-50%), 3 (51-80%) and 4 (>80%). The average staining is assessed as 0 (no staining), 1 (weak), 2 (moderate) or 3 (strong) and the values are then multiplied, giving a score ranging from 1 to 12.

A variation of IRS method is the quick score (55) in which the value for intensity is added to that for the proportion of stained cells, giving a maximum number of 7. A modification of this (modified quick score) has been recommended by the National Co-ordinating Committee on Breast Screening Pathology in UK, see appendix II (56).

Another easy and quick alternative scoring method is to assess the overall appearance of the staining and to classify it as negative, weak, moderate or strong. The moderate and strong stains can be seen under the low power (x10) objective of the microscope. Weak staining is only evident at higher magnifications. This is called the 'category score'. It is purely descriptive and does not attempt to give a numerical value thus avoiding the danger of giving the result undue significance. Some researchers use only the percentage of positively stained tumour cells.

The validity of any assessment of the strength of staining, even, when great care is taken over monitoring consistency between batches of staining, is sometimes questioned. In the case of cerbB-2, however, Slamon et al (57;58) have shown that using diaminobenzidine (DAB) as the chromogen, a pale golden colour corresponds to both a low copy number of *cerbB-2* gene and a low level of protein expression as assessed by Western blotting. Whereas, tumours in which the staining is a very dark brown colour, have a high level of gene amplification and high levels of protein. While similar direct comparisons have not been made for ER, there is a suggestion that tumours with very dark staining nuclei on immunohistochemsitry may have higher DCC and EIA values. However, there is emerging evidence that there is a closer association between oestrogen receptor status and response to hormonal treatment using immunohistochemistry as compared to ligand

binding and enzyme immunoassays (59;60), probably because of lack of influence of tumour cellularity and type.

In one study, researchers have examined (61) the relationship between scoring methods and response to endocrine treatment in a series of patients with metastatic breast cancer. The immunohistochemical ER status was determined retrospectively on primary tumours from 134 patients who were treated at Guy's hospital between 1975 and 1990. All the patients subsequently received first line tamoxifen treatment for metastatic disease. The diagnosis and date of recurrence were determined in a standard manner according to the criteria of Hayward et al (62) and the response to treatment was assessed by UICC criteria (63). Four different methods of evaluating the staining were undertaken and comparisons were made to find which method produced a result most closely related to the clinical course of the disease. The methods used were; 1) the histo-score, 2) the consistency of staining i.e., whether negative, heterogenous or homogenous, with no attention being paid to the intensity of staining 3) the category score with negative, weak, moderate or strong and 4) the simplified category score with negative and weak staining combined and classified as negative and moderate and strong staining classified as positive.

The relationship between various methods of evaluation and the duration of response to tamoxifen (i.e., from start of treatment to time of progression) was calculated using log rank analysis and secondly the relationship between various methods of evaluation and type of response, complete or partial (responders) vs static and progressive disease (non responders) was calculated using the x^2 test. Results showed that when used to predict duration of response, all four methods of evaluation gave significant results but the most statistically significant result was for the simplified category score. As regard to comparison between the different methods of evaluation and the type of response to tamoxifen, all four methods were again highly significantly associated with response, however the subjective category score gave the most significant information. These results suggest that the information obtained by the category score is superior to that gained by the more time consuming histo-score.

EVALUATION OF ER STAINING AND IMAGE ANALYSIS:

Some groups have attempted to use image analysis in order to improve the consistency of the evaluation of immunohistochemical assays for ER. Bacus et al (64) developed the

CAS (Cell Analysis System) specifically for measuring the ER but the system has not been widely adopted in the UK. The lack of general acceptance of image analysis is because there is no really good system at a reasonable price, which can accurately and reproducibly recognise the stained tumour nuclei and express them as a percentage of non-stained tumour nuclei. Their use is usually as time consuming as counting the nuclei by human eye using a graticule. Esteban et al (65) described the use of SAMBA 4000 cell image analysis system to evaluate staining of ER using the ID5 antibody. They found that the choice of cut off point was critical, but even when a point was chosen which showed a correlation between positive staining for ER and survival, the correlation was not very strong. At present, general consensus is that image analysis has not yet reached the stage of development where it can be used reliably and efficiently. However, a recent study (66) has found significant correlation between semiquantitative hormone receptor scoring using immunoreactive score (IRS) and quantitative computer-assisted image analysis.

INCIDENCE OF ER IN BREAST CANCER AND PREDICTION OF RESPONSE TO TREATMENT:

When oestrogen receptors were first measured in primary breast cancers, about 65% of the tumours were found to be ER positive by the ligand binding assay using a cut off point of more than 5 fmol of oestradiol bound per milligram of protein to define positivity. Approximately, 33% of patients with metastatic disease respond to endocrine therapy, and 50% of patients with ER-positive tumours are hormone responsive in contrast to <10% of patients with ER-negative tumours. In general, patients with higher levels of ER are more likely to respond to endocrine therapy (67). Oestrogen receptors are essential for oestrogen action. Prediction of response can be further refined by combining oestrogen receptor assay with progesterone receptor (PR) assay (68). ER+PR+ tumours have a 78% response, ER+PR- a 55% response, ER-PR+ a 45% response and ER-PR- a 10% response (68). These results relate to ligand binding assay systems.

ER IN RELATION TO CLINICOPATHOLOGIC PARAMETERS AND TUMOUR BIOLOGY:

ER and Patient Age:

There is a steady increase in the ER content of mammary tumours with increasing patient age. Relatively low levels are seen in premenopausal women (69). These findings were

also confirmed in a large study of over 5000 patients by Romain et al (70) using EIA. However, a similar effect is not seen with progesterone receptors (69).

ER in Relation to Histological Grade and Tumour Type:

ER is always present in normal breast tissue although in normal pre-menopausal breast, ER^+ cells are distributed singly (71). Well differentiated tumours are more likely to be ER positive than poorly differentiated tumours and they are more likely to be hormone responsive (72). This association was clearly shown by Millis et al (73) in a study of 369 cases of infiltrating ductal carcinoma, 85% of the 47 well differentiated grade I, 82% of 193 moderately differentiated grade II and 57% of 129 poorly differentiated grade III carcinomas were ER positive. Other studies (74;75) confirmed this relationship. Infiltrating lobular carcinomas are very frequently ER positive while medullary carcinomas with lymphoid stroma are usually negative (74).

ER and Tumour Stage:

There is no relationship between ER status and tumour stage, which is time related and reflects the stage of the disease at diagnosis (73;76). The likelihood of early recurrence is much more dependent on tumour burden at the time of initial treatment than it is on ER status. This is reflected in the conflicting data relating ER positivity to disease free interval, some studies (77) showing ER to be an independent prognostic marker for recurrence, while others not (78;79).

ER and Ductal Carcinoma In Situ (DCIS) of the Breast:

There is no agreement in the literature about the proportion of ER positive DCIS. Using a variety of methods it has been reported as being as low as 32% by Poller et al (80) and as high as 81% by Zafrani et al (81). Burr et al (82) found ER expression in 91% of non comedo DCIS and 57% of comedo DCIS. In contrast lobular carcinoma in situ is nearly always ER positive (83). ER positivity is associated with small nuclear size, higher proliferation fraction (S phase fraction) and lack of cerbB-2 staining (80;84).

ER of Primary Tumour and Metastasis:

Initially the ER assays were performed on recurrent skin nodules or metastatic lesions, which were easy to excise. However, this was not always possible, for example, in

women whose metastases were in bone, lung or liver. Early studies showed that the ER content of metastases generally resembled that of the primary tumour (85). Therefore, it became common practice to measure ER in primary lesions. The results were recorded for use in the event of systemic recurrence.

ER status can give an indication of the likely site of metastases. ER positive tumours are most likely to spread to bone and ER negative tumours to lung and liver (86). There is generally good concordance between the receptor status of the primary tumour and metastases found in lymph nodes at the time of primary diagnosis (86).

ER and Tumour Proliferation:

Direct comparisons between ER status and proliferative activity have been made in various studies employing different methods to measure the rate of proliferation including thymidine labelling (87), bromodeoxyuridine (BRDU) labelling (88), and flow cytometry (89). The development of monoclonal antibodies raised against proliferation associated antigens, such as Ki67 (90), KiSI (91) and MIB1 (92) has made available immunohistochemical methods. Meyer et al (87) found that ER positive tumours have a low rate of proliferation. However, there is a small number of ER positive tumours with a high rate of proliferation and it is suggested that these are the ones which fail to respond to hormone treatment (93;94).

ER and Other Biological Markers:

Int-2 gene: *Int-2* gene is amplified in approximately 15 to 20% of mammary carcinomas and a very close association between ER positivity and *int-2* amplification has been reported (95).

CerbB-2: An inverse relationship exists between cerbB-2 and ER (96).

p53: There is a significant inverse relationship between p53 protein and ER positivity (97).

BCL2: There is a significant positive correlation between bcl2 and ER (98).

ER and Prognosis:

The availability of ER assays led to studies of the relationship between hormone receptor status in primary breast cancer and prognosis. A highly significant association was found between prolonged overall survival and the presence of ER in the primary tumour (99). The association between receptor status and disease free interval, first shown by Knight et al (100), is less strong, while the most profound effect of receptor positivity is seen in survival after relapse, when women with ER positive tumours live considerably longer than their ER negative counterparts (101).

ER and Male Breast Cancer (MBC):

Both ER and PR have been identified in a high proportion of MBC using the DCC method, ELISA and ICC (102-104). Most studies have reported that ER is expressed in at least 80% of MBC and PR in up to 75% of cases, which is in excess of the figures for female breast carcinoma. The reason for this is not entirely clear.

OESTROGEN – INDUCED PROTEINS:

Progesterone Receptors (PR):

Progesterone receptor (PR) is directly modulated by ER transcription protein and the level of PR expression therefore closely mirrors that of ER. Not all ER positive tumours are hormone responsive, and it has been suggested that other hormone induced proteins are possible markers of response. Horwitz et al (105) suggested progesterone receptor as one of them. They hypothesized that in unresponsive tumours ER, although present, was not functional. When studies of both ER and PR proteins were carried out (106;107), it was found that approximately 50% of tumours are positive for both, 25% are negative for both, 20% are ER + PR- and 5% are ER – PR+. Tumours co-expressing ER and PR have an 80% likelihood of response (107). However, there are still 20% of tumours positive for both receptors, which do not respond. Also, double negative tumours are highly unlikely to respond. At present, there seems to be little additional clinical benefit in determining PR status in addition to ER status in breast carcinoma (108).

Cathepsin D:

Westley and Rochefort (109) described a 52 kDa protein induced by oestradiol in the oestrogen dependent, hormone responsive MCF7 cell line. It was later identified as a precursor of cathepsin D which is a lysosomal protease (110). Tandon et al (111) suggested that cathepsin D measured in tumour cytosols might be a predictor of poor prognosis. However, immunohistochemical studies have shown it to be present in macrophages, in the stroma as well as in tumour epithelial cells and this may reduce the validity of the cytosol assay. Therefore, immunohistochemical studies generally do not consider cathepsin D an independent prognostic marker (112).

pS2:

Chambon et al (113) discovered pS2 protein produced by MCF7 cells following oestrogen stimulation. An immunohistochemical study on 178 breast carcinomas by Dookeran et al (114) found both cytoplasmic and membrane positivity in the majority of cases but no relationship was found between pS2 expression and other established prognostic markers including ER, or overall survival.

HORMONAL TREATMENT OF BREAST CANCER:

Hormonal therapeutic agents fall into three broad categories. The first includes drugs such as Zoladex, which is a potent gonadotrophin releasing hormone agonist and acts centrally to inhibit the release of gonadotrophins from the pituitary by a process of desensitization. The second category includes inhibitors of steroid synthesis, such as the aromatase inhibitor aminoglutethamide, which reduces oestrogen synthesis. The new generation of aromatase inhibitors include anastrozole and letrozole, which are non–steroidal and exemestane which is a steroidal aromatase inhibitor. The third category includes anti-oestrogens or SERMs (selective oestrogen receptor modulators). SERMs are compounds used as therapeutic agents which bind with high affinity to ER and mimic the effects of oestrogens in some tissues but act as oestrogen antagonists in others depending on the tissue (115). There are two types of SERMs, pure ER antagonist ICI 182,780 (Faslodex) and partial ER (ant)agonist tamoxifen. Tamoxifen is the most widely used agent currently and acts predominantly by inhibiting the action of oestrogen hormone in its target tissues by binding to ER. There is evidence that it stimulates the production of tumour suppressor cytokines such as insulin-like growth factor-1 (IGF-1) and transforming growth factor

beta (TGF β) (116;117), therefore, its mechanism of action is not just via the oestrogen receptor.

SUMMARY:

In general, the measurement of ER status and the part it plays in patient management have evolved and changed considerably over the years. ER and PR status can now be readily assessed on histological material and the assay is therefore available to any laboratory skilled in immunohistochemistry. Patients with an ER negative tumour have a less than 10% chance of responding to hormone treatment for advanced disease. Thus, measurement of receptors is important in young women who may be considered for treatment by oophorectomy and for women of all ages with rapidly progressing disease when rapid response to treatment is essential and therefore, a trial of treatment is inappropriate. Some laboratories have already combined the use of ER with other markers such as cerbB-2 and p53 with the aim of refining prediction of response to particular treatment regimes for metastatic disease and in selecting suitable adjuvant therapy for early disease.

CHAPTER 6: DUCTAL CARCINOMA IN SITU OF THE BREAST:

INTRODUCTION:

Ductal carcinoma in situ (DCIS) of the breast is defined as the proliferation of epithelial cells with cytological features of malignancy within ductal structures of the breast and distinguished from invasive carcinoma by the absence of stromal invasion across the basement membrane.

Until recently, DCIS was a relatively uncommon disease, representing only about 1% of all newly diagnosed cases of breast cancer (1). It was usually regarded as a single disease entity with a single treatment, namely, mastectomy. With wide use of mammography, the number of cases has increased dramatically. DCIS now accounts for 20-25% of detected cancers (2). Most patients with DCIS (95%) now present with clinically occult, non-palpable lesions (3). Furthermore, DCIS is no longer considered a single disease but recognised as a heterogeneous group of lesions with a diverse malignant potential (4-6). As understanding of the disease has evolved and the range of treatment options has widened, the process of making decisions about management has become more complex and controversial. DCIS has become so common and confusing that the first textbook devoted solely to the disorder was not published until 1997 (7).

DCIS is frequently associated with invasive carcinoma (45% of cases) (8), is conventionally regarded as the principal precursor of invasive breast cancer and is distinct from the other putative precursor, lobular carcinoma in situ (LCIS). LCIS is regarded as a high risk factor for development of invasive breast cancer but because it is frequently multifocal, pure LCIS without coexistent DCIS is generally managed differently from DCIS. At the 2nd European Organisation for the Research and Treatment of Cancer (EORTC) DCIS consensus meeting in September 1991, it was agreed by the participants on the basis of existing evidence that:-

1 DCIS is nearly always unicentric.
2 Detection of DCIS has increased with mammographic screening.
3 Recurrences of DCIS occur almost invariably at the site of previous excision.
4 The incidence of axillary lymph node involvement by metastatic carcinoma in DCIS is negligible (<1%), thus obviating the need for axillary lymph node dissection in cases of pure DCIS.

5 DCIS was felt to be heterogeneous, both histologically, radiologically and biologically (3).

The increased use of mammography as a tool for the detection of early breast cancer and population mammographic breast screening has led to an increase in the detection of ductal carcinoma in situ, particularly small localised lesions (9). This changing pattern of disease incidence (in reality disease detection) has highlighted many deficiencies in the understanding of DCIS and has produced a new range of problems. Difficulties arise in determining the criteria for diagnosis of DCIS as compared to atypical ductal hyperplasia (ADH), LCIS or micro-invasive breast carcinoma (MIC).

Some subtypes of DCIS are difficult to classify and rarer subtypes of DCIS can be difficult to recognise and diagnose. Other currently debated topics include the validity of pathological subclassification systems for DCIS, the question of what constitutes adequate pathological excision margins and the use of molecular markers in DCIS. The question of whether DCIS is a real 'cancer' or better regarded as a marker for patients with a high risk of subsequent invasive breast cancer is open (10). The use of adjuvant therapies (e.g., radiotherapy or endocrine therapy) is also an area of considerable disagreement among experts and some trials trying to answer these questions are mentioned later in this chapter (page 114).

HISTORICAL BACKGROUND:

The history of DCIS has been well reviewed by Fechner (10). DCIS was first described by Bloodgood in 1893 (11). Subsequently Lewis and Geschickter (12) described comedo carcinoma although they did not distinguish invasive carcinoma from in-situ carcinoma. Later, lobular cancerisation in DCIS was highlighted by Azzopardi (13). Page et al (14) identified cribriform, micropapillary and other types of non-comedo DCIS as high risk factors for the development of ipsilateral invasive breast cancer. They deduced that approximately 28% of women with DCIS could be expected to develop invasive breast carcinoma over a period of 15 years if left untreated.

CLINICAL PRESENTATION AND DIAGNOSIS:

DCIS may be categorised on clinical (symptomatic, screening detected, associated with Paget's disease, location of lesion within the breast), radiological (radiological size,

pattern of calcification, parenchymal deformity) or on pathological grounds (microscopic size of lesion, margin status, nuclear grade, architecture, cell size, presence or absence of necrosis, cerbB-2 expression, oestrogen (ER) or progesterone receptor (PR) expression, *P53* gene mutation or p53 protein expression, the presence of associated lesions). In practice, the optimum combination of these various criteria for patient management is not entirely clear. DCIS is not usually visible to the pathologist by naked eye inspection, unless comedo-type necrosis is present or there is marked periductal fibrosis associated with the lesion.

During 1997, more than 36,000 new cases of ductal carcinoma in situ, representing 17% of all new breast cancers were diagnosed in the United States (15). Most of these cases were diagnosed by mammography. High quality mammography is capable of finding a range of asymptomatic non-invasive lesions that cannot be palpated. These are often smaller, of low nuclear grade, and show much more subtle changes than the lesions detected with less advanced mammographic equipment in the past. The most common mammographic finding is microcalcification (80% of cases) (16;17), but some lesions may present as ill-defined and spiculate or architectural distortions with or without microcalcifications. Approximately 25% of symptomatic lesions are not apparent radiologically and present with nipple discharge, Paget's disease of the nipple or a palpable mass.

Pre-operative diagnosis of malignancy can be achieved using fine needle aspiration (FNA) cytology but this should be used as part of a 'triple approach' to diagnosis in combination with radiological and clinical findings (18). The cytological heterogeneity of DCIS suggests that a definitive diagnosis of DCIS should not be attempted on FNA cytology alone as it is impossible to discriminate DCIS reliably from ADH and invasive carcinomas (19). FNA cytology can be used for assessment of ER, PR and cerbB-2 status.

Some groups have promoted the use of multiple image guided needle core biopsies for diagnosis (20). Radiographs of such biopsies can be taken to confirm the presence of representative foci of calcification allowing radiological and histological correlation and a greater ability to distinguish DCIS from invasive cancer.

Prior to the advent of FNA and triple assessment, intra-operative frozen sections were used to detect the presence of either comedocarcinoma or invasive carcinoma, in which

case immediate mastectomy could be carried out, obviating the need for a second surgical procedure. However, the use of frozen sections is no longer considered necessary in the diagnosis of breast lesions detected by screening, as margins cannot be adequately assessed, thorough sampling of lesions cannot be performed properly to exclude foci of invasion, and the pathological grade of DCIS or invasive breast carcinoma cannot be assessed.

CLASSIFICATION AND PATHOLOGY:

Until recently there was no generally accepted method of classifying DCIS. Traditionally, histopathologists have classified DCIS on the basis of the intraduct architecture and cytological features (21) without any evidence of the clinical relevance of this method. Most pathologists recognise two major subtypes of DCIS according to the presence or absence of comedo necrosis but there are variable degrees and types of necrosis and their significance is debatable. These two subtypes differ not only in pathologic features but also in clinical presentation, appearance on mammography and malignant potential. The comedo type of DCIS is diagnosed when at least one duct in the breast is filled and expanded by large, markedly atypical cells and has abundant central luminal necrosis. This necrotic material is usually partially calcified and may be recognised on mammography as linear and branching calcifications. Prominent periductal fibrosis is common and may render the lesion clinically palpable. All other forms of DCIS are of the non-comedo type and include cribriform, micropapillary and solid types although focal necrosis can be seen in these patterns too and different histologic patterns may co-exist. This morphologic classification does not account for the marked heterogeneity of DCIS in its clinical behaviour. Furthermore, the reproducibility of this method of assessment is poor.

In April 1997, a consensus conference on the pathology of ductal carcinoma in situ was convened in Philadelphia under the auspices of the Breast Health Institute. Although there was agreement on a number of basic issues, such as the need to achieve sufficiently wide excision margins, measure tumour extent, and note nuclear grade, histological architecture, and polarization, consensus on a single unified classification for ductal carcinoma in situ was not achieved (22).

At present, there are several classifications based on histological structure, nuclear grade, comedo type necrosis, cytonuclear differentiation or various combinations of these factors. Nuclear grade, comedo type necrosis, tumour size and the width of the surgical margins are all important predictors of the probability of local recurrence after breast conservation treatment for ductal carcinoma in situ (2;21;23-29).

Many grading systems have utilized nuclear grade as the basis for the histological classification of DCIS. Patchefsky et al (4) reported that high nuclear grade in DCIS correlated with the presence of micro-invasion and Lagios et al (26) found that high nuclear grade DCIS was significantly associated with the likelihood of recurrence. The median nuclear area in DCIS is slightly higher than that in invasive breast cancer. Using current criteria for diagnosis of DCIS, MOP Kontron image analysis of nuclear area was performed on an unselected series of cases of DCIS and invasive breast cancer. Nuclear area equated morphometrically to the nuclear grade in DCIS. The mean nuclear area of 65 cases of DCIS was 47.9μm^2 (range 22.5 – 92.9μm^2) as compared to 44.4 μm^2 (range 22.3 – 80.3 μm^2) for invasive ductal carcinoma (30).

The prototype system based on nuclear grading was that of Lagios and co-workers (6), which sub-classifies DCIS into three grades, namely high, intermediate and low nuclear grade. A four point nuclear grading system for DCIS, which also considers the presence or absence of comedo type necrosis, has been advocated by Page and co-workers (5). Another system utilizing nuclear grade has been proposed by the European Pathologists Working Group (EPWG) at the instigation of EORTC following the 2nd EORTC DCIS Consensus meeting (3). This system is based on criteria of Holland et al (31) who have subdivided DCIS into poor, intermediate and well differentiated based on cytonuclear and architectural differentiation.

The National Co-ordinating Group for Breast Screening Pathology (NCGBSP) UK. (32), have employed Holland's criteria to divide DCIS into three categories namely high, intermediate and low nuclear grades which are described below. Other systems have been advocated by the Edinburgh Group (21), Nottingham Group (33) and Ottensen et al (34). The Nottingham system has been further refined (Van Nuys System) (24). The Van Nuys classification (24) takes into account nuclear grade and necrosis. The only system considering size of DCIS lesion is that of Ottensen et al (34).

However, no matter which system is used, histological classification alone is inadequate for determining proper treatment. If DCIS is truly a localized lesion, then meticulous margin assessment is far more important than histological sub-classification in predicting disease recurrence (35). In one study, nuclear grades of DCIS correlated well with the Nottingham prognostic index (NPI) and grades of invasive breast cancer (36).

The following is the classification system recommended by NCGBSP (33) and has been utilized in this study:

Low Nuclear Grade (LNG) DCIS:

This is composed of monomorphic, evenly spaced cells with roughly spherical, centrally placed nuclei and inconspicuous nucleoli. The nuclei are usually, but not invariably, small. Mitoses are few and there is rarely individual cell necrosis.

The cells are generally arranged in micropapillary and/or cribriform patterns (figure 6.1) the latter is more common and tends to predominate. There is usually polarization of cells covering the micropapillae or lining the intercellular lumina. Less frequently, low grade DCIS has a solid growth pattern. When terminal duct lobular units are involved, the process can be very difficult to distinguish from lobular carcinoma in situ. Features in favour of DCIS are greater cellular cohesion, slightly larger cell size, cytoplasmic basophilia and lack of intracytoplasmic lumina. Occasionally, however, there may be a combination of both processes.

Figure 6.1; Photomicrographs of a case of LNG DCIS at different magnifications

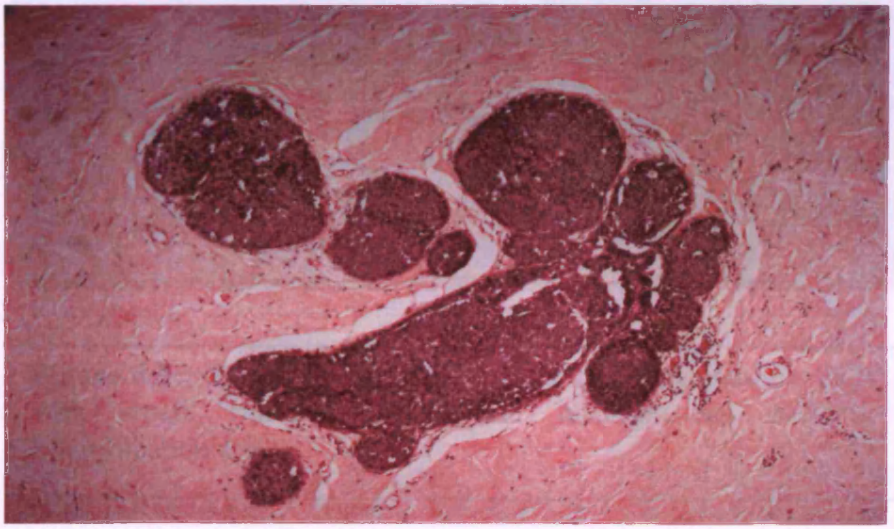


Figure 6.1a; LNG DCIS showing predominantly solid and cribriform growth pattern. Magnification x 50

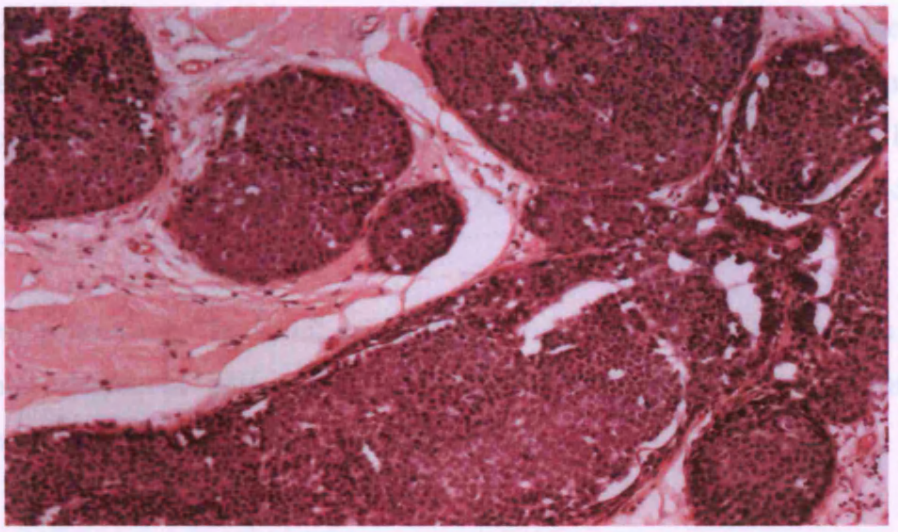


Figure 6.1b; Same case as in 6.1a, cells lining the intercellular lumina show polarization. Magnification x 100

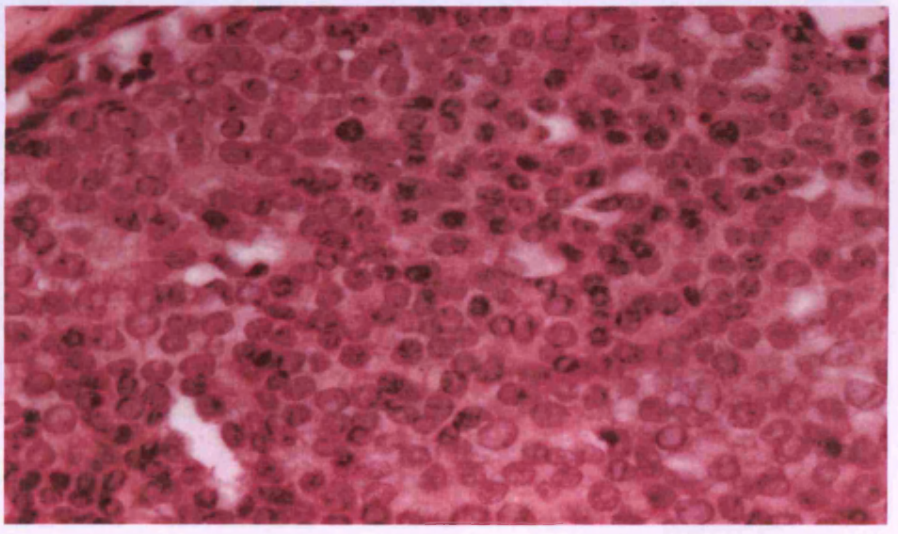


Figure 6.1c; Same case as in 6.1a, exhibiting monomorphic cells with small, rounded nuclei and inconspicuous nucleoli. Magnification x 400

Intermediate Nuclear Grade (ING) DCIS:

This group comprises those cases of DCIS, which cannot be assigned easily to the high or low nuclear grade categories (figure 6.2). The nuclei show mild to moderate pleomorphism, which is less than that seen in high grade DCIS but they lack the monotony of the small cell type. The nucleo-cytoplasmic ratio is often high and one or two nucleoli may be identified.

The growth pattern may be solid, cribriform or micropapillary and the cells usually exhibit some degree of polarization covering the papillary processes or lining the intercellular lumina although this is not as marked as in low nuclear grade DCIS.

Figure 6.2; Photomicrographs of a case of ING DCIS at different magnifications

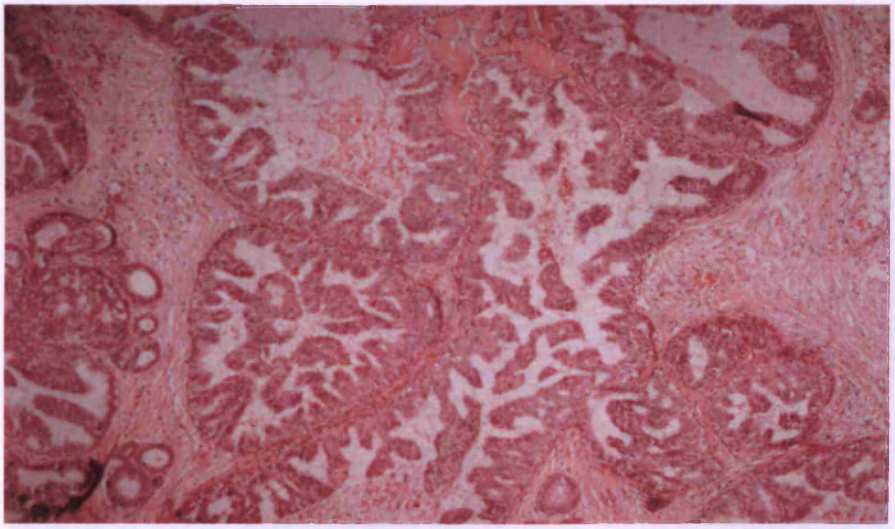


Figure 6.2a; ING DCIS showing cribriform and micropapillary growth pattern at a magnification x 50

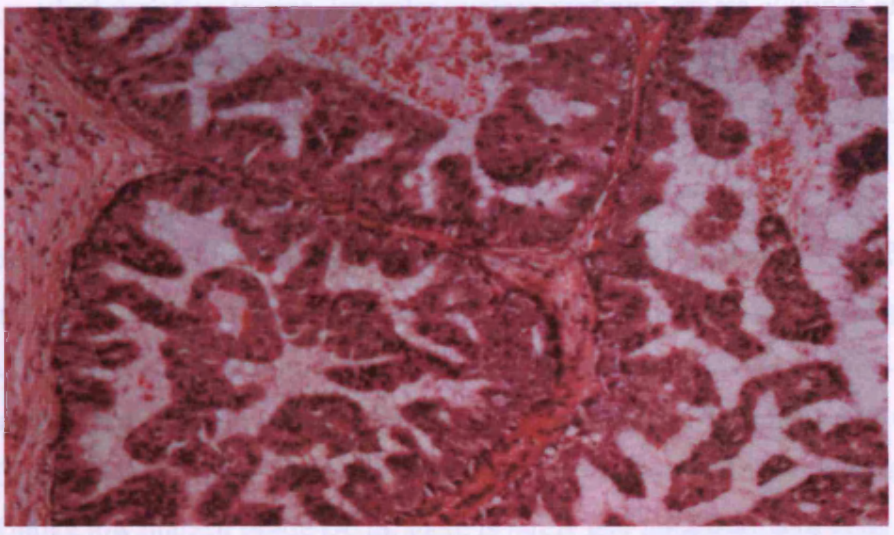


Figure 6.2b; Same case as in 6.1a, showing some degree of polarization covering the papillary processes. Magnification x 100

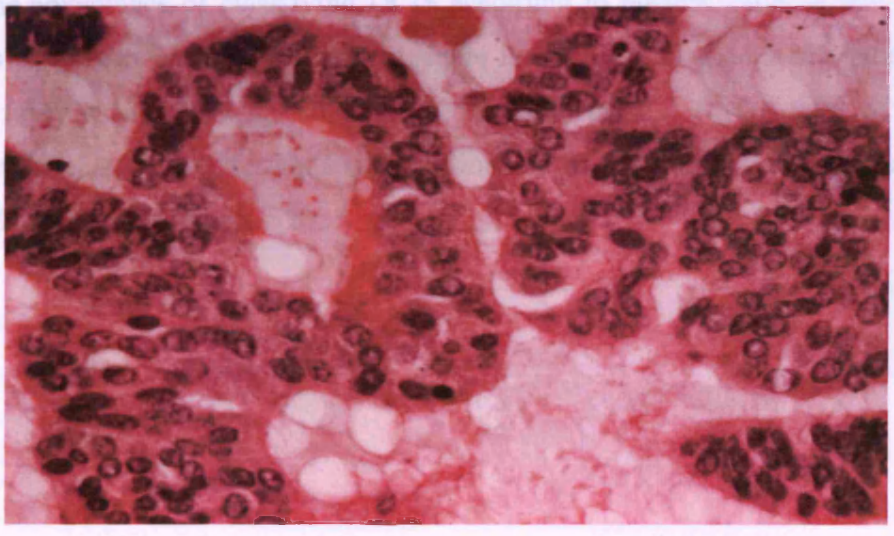


Figure 6.2c; Same case as in 6.2a, the nuclei show mild to moderate pleomorphism. Magnification x 400

High Nuclear Grade (HNG) DCIS:

This is composed of cells with pleomorphic, irregularly spaced and usually large nuclei exhibiting marked variation in size, irregular nuclear contours, coarse clumped chromatin and prominent, sometimes multiple nucleoli. Mitoses are frequently present and abnormal forms may be seen. A characteristic feature of this type of DCIS is the presence of individual necrotic cells and evidence of autophagocytosis.

High nuclear grade DCIS may exhibit several different growth patterns. It is often solid with central comedo-type necrosis (figure 6.3), which frequently contains deposits of amorphous calcification. Sometimes a solid proliferation of malignant cells fills the duct without necrosis but this is relatively rare. High nuclear grade DCIS may also exhibit a micropapillary or a cribriform pattern frequently associated with central comedo-like necrosis. Unlike low nuclear grade DCIS, there is rarely any polarization of cells covering the micropapillae or lining the intercellular spaces. Calcification is frequently present within the necrotic material and is amorphous in appearance. The involved ducts are frequently very distended with extensive periductal fibrosis and lymphocytic reaction. Cancerization of lobules is frequently present. Paget's disease of the nipple is almost exclusively associated with this type of DCIS.

One of the advantages of classifying DCIS according to nuclear grade is that although variations of growth pattern are frequent, there is usually a dominant cell type and the lesion is fairly easily classified into one of the main groups. Rarely cells of different nuclear grades may be seen within a single lesion and the case is then classified according to the highest nuclear grade observed.

Figure 6.3; Photomicrographs of a case of HNG DCIS

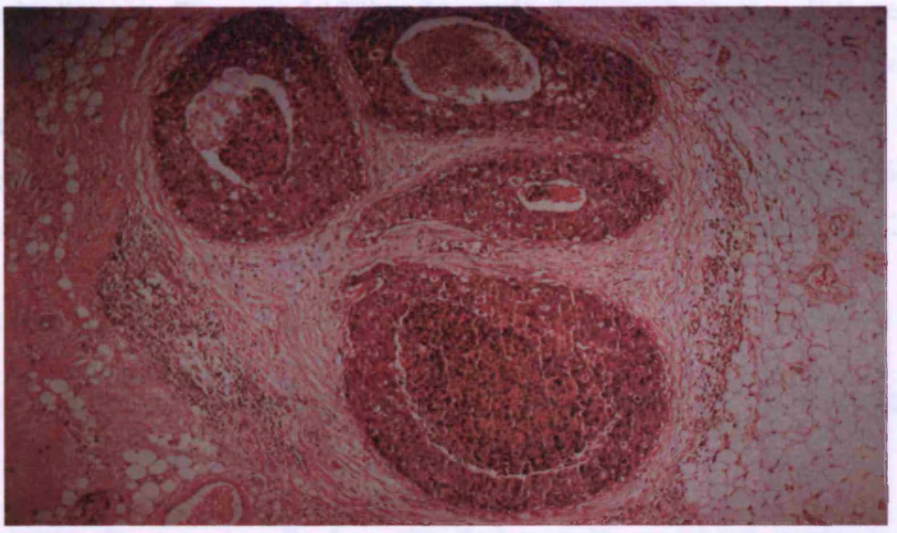


Figure 6.3a; HNG DCIS showing a comedo pattern with necrosis at a magnification x 50

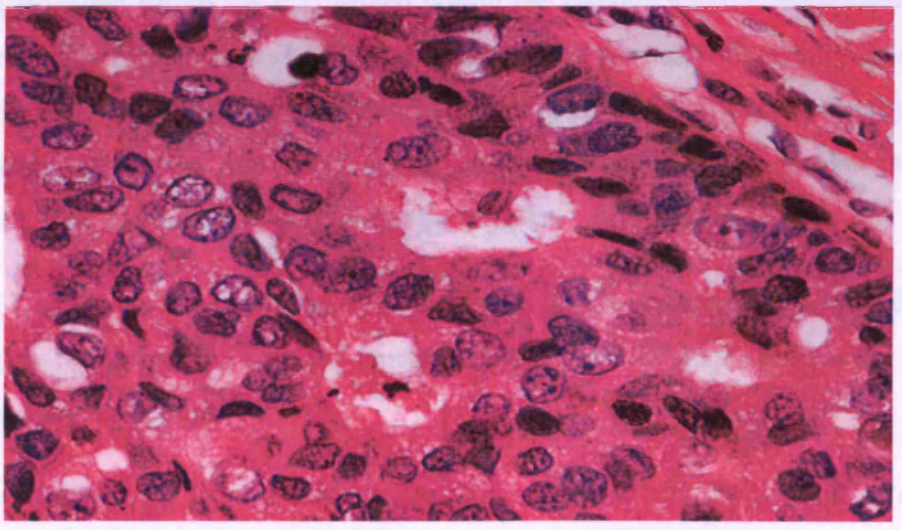


Figure 6.3b; Same case as in figure 6.3a, showing marked pleomorphism and frequent mitosis at a magnification x 400

Micro – invasive Carcinoma (MIC):

This is defined as a tumour in which the dominant lesion is non invasive but in which there are one or more clearly separate foci of infiltration, none of which measures more than 1mm in diameter. Tumours fulfilling these criteria are not common. If there is doubt about the presence of invasion, the case should be classified as DCIS as these lesions tend to behave like DCIS rather than invasive carcinoma (32). In one series of 115 DCIS specimens (26), the overall rate of micro-invasion was 14.7%, with an incidence of micro-invasion of 1.6% in lesions less than 25 mm and 29% in those larger than 26 mm.

Rare Subtypes of DCIS:

A variety of rare but morphologically distinct subtypes of DCIS is recognised. A complete account of these is given by Tavassoli (37).

Apocrine DCIS: The tumour cells show abundant granular cytoplasm, moderate to severe cytological atypia and central necrosis. Apical snouting (cytoplasmic protrusions) are not always seen. The cells may sometimes be highly atypical and no necrosis may be evident (38).

Encysted papillary carcinoma in situ: This type is more common in older women and carries an excellent prognosis if confined within the capsule without surrounding DCIS (39). It is usually circumscribed and accompanied by a fibrous wall, giving an encysted appearance. It has a papillary structure with fibrovascular cores, which are usually fine. It may be accompanied by micropapillary or cribriform DCIS. There is also frequently haemosiderin (or haematoidin) pigment in the adjacent fibrous tissue.

Clear cell DCIS: This is a proliferation of cells with optically clear cytoplasm and distinct cell margins forming cribriform structures. Central necrosis may be present. A solid pattern may also be present.

Signet ring DCIS: This is a very rare variant characterised by the proliferation of signet ring cells in solid or papillary growth patterns. The cytoplasm stains positively for diastase resistant-PAS or alcian blue (40) suggesting the presence of intracytoplasmic mucin.

Neuroendocrine DCIS: This lesion has an organoid appearance with prominent argyrophilia, resembling a carcinoid tumour. The neoplastic cells may be in a solid configuration or papillary, forming tubules, pseudorosettes, palisades or ribbons (41). These tumours may be truly neurosecretory and produce neuropeptide hormones including ACTH and adrenaline or noradrenaline.

Cystic hypersecretory DCIS: This is a variant of micropapillary DCIS (42;43). The cells produce mucinous secretions which distend the involved duct spaces giving a cystic appearance.

Male breast DCIS: This is a rare entity with only 90 or so cases reported in the World literature and accounting for slightly more than 7% of all male breast cancers in published series (44). It typically presents with a retroareolar mass and may require mastectomy.

Atypical ductal hyperplasia (ADH) and DCIS: The current guidelines for the UK Breast screening programme (32) define atypical ductal hyperplasia (ADH) as a rare lesion, often co-existing with fibrocystic change, a sclerosing lesion or a papilloma. The current definition rests on identification of some but not all of the features of ductal carcinoma in situ. It is commented in the UK guidelines that most difficulties are encountered in distinguishing ADH from low-grade variants of DCIS. These two lesions can co-exist. The guidelines are in keeping with Page's view that if cellular changes typical of DCIS occupy two separate duct spaces this should be regarded as DCIS, if only one duct space is involved, then the lesion should be considered as ADH (14). Others disagree with this interpretation. Tavassoli and Norris (37;45) argue that the overall geometric size of the DCIS lesion is more important than the number of duct spaces involved, with a lesion less than 2mm in overall size indicating ADH. Staining for alpha-smooth muscle actin, S100 protein and cytokeratin (especially CK 5/6 positive cells as they are lost in DCIS) has been suggested to be helpful in distinguishing ADH from DCIS (46). Similarly, antibodies directed against basal and luminal cell cytokeratins can be used to differentiate usual type epithelial hyperplasia from DCIS (47). Stains for alpha-smooth muscle actin can also be useful for the assessment of the presence or absence of microinvasion.

THE NATURAL HISTORY OF DCIS:

DCIS has been shown to arise within the terminal duct lobular units (TDLU) of the breast (48-51) although the size of the ducts suggests that extralobular ducts are also involved in

DCIS (52). The adult breast comprises 20 or more developmentally derived segments, which extend from the nipple radially outwards to the pectoralis fascia (see Chapter 1, figure 1.1). The TDLU connects anatomically via intralobular terminal ducts to extralobular terminal ducts, subareolar ducts and then to the nipple. The current belief is that DCIS originates in the terminal ducts and acini of the breast, grows and expands these ducts until, at a certain stage in its natural history, it develops invasive properties and penetrates the basement membrane. Autopsy based studies have demonstrated that 15% of young asymptomatic women may have DCIS (53;54). The lifetime risk of development of invasive breast cancer of between 1 in 8 and 1 in 12 in North America and Europe is consistent with these autopsy findings.

Using a three dimensional reconstruction technique, the serial sub-gross method of Egan and similar stereomicroscopic techniques, the distribution of DCIS has been mapped by Holland et al (49;50) as well as Faverly and co-workers (51;55). These studies show that the majority of cases of DCIS exist as a single lesion, 66% of which are in one quadrant (49;50) and that DCIS should, therefore, be regarded as a unicentric disease process. The growth of DCIS is segmental and not multicentric. Poorly differentiated DCIS (HNG) grows continuously and well differentiated DCIS (LNG) has a multi-focal growth pattern.

The view that DCIS is a single clonal process is further supported by the observation that in 12 cases of DCIS which were apparently multi-focal, all showed loss of heterozygosity (LOH) at the same chromosome locus implying that the foci of DCIS were derived from the same clone (56). Micro-dissection studies have shown LOH on chromosome 16 and 17 in both ADH and DCIS (56-60) suggesting that ADH is also part of the neoplastic spectrum of breast disease. It has been suggested that some forms of atypical hyperplasia undergo clonal evolution to DCIS and then to invasive cancer (61). This is supported by cytogenetic (56-60;62;63) and oncogene abnormalities discovered in DCIS, as well as by microscopic findings (5). A diagnosis of ADH indicates an approximately four fold increased risk for the development of invasive breast cancer (64;65). The incidence of invasive breast cancer after incisional biopsy showing DCIS has been reported as ranging from 14% to 75% in various published series (12;14;66-70).

MOLECULAR BIOLOGY OF DCIS:

Experimental Models of Breast Neoplasia:

Mice infected with the mouse mammary tumour virus (MMTV) show lesions morphologically identical to those seen in ADH/DCIS. Transgenic mice, which overexpress the G_1 phase associated cell cycle protein, cyclin D1 (also known as PRAD-1) show breast abnormalities with epithelial hyperplasia and development of invasive breast tumours (71). Similar tumour models have been described for cerbB-2 (72).

Interphase Cytogenetics:

Invasive breast cancer or breast carcinoma cell lines:

Documented abnormalities identified in invasive breast carcinoma include deletions (del) of Chr 1p13-36, del Chr 3p14-23, del Chr 6q21-27 and del Chr 16q21-24 (73) and amplification of Chr 1q21, 8p12, 8q24, 10q22, 11q13, 13q21, 13q31, 17q11, 17q12, 17q23, 17q24 and 20q12-13.2 in breast carcinoma cell lines (74). Genes implicated in breast cancer corresponding to these amplified chromosomal band regions include *cerbB-2* (17q12), *myc* (8q24), *FGFR1/FLG* (8p12), *FGFR2/BEK* (10q26), *PRAD-1/CYCD1* (11q13), *IGFR* (15q24-25). Further details of these genes have been discussed in Chapter 3.

DCIS and ADH:

The cytogenetic abnormalities present in DCIS and ADH have not yet been clearly defined. One fluorescent in situ hybridization (FISH) study (62) described true gains (aneuploidy) of Chr 1, Chr 17 and Chr 18 using pericentrometric probes for chromosomes 1, 16, 17, 18. Loss of heterozygosity in DCIS and ADH has been shown for chromosomes 16 and 17 (56-58). One other report also identified LOH in DCIS at Chr 3p, Chr 7q, Chr 16q and Chr 17p. A higher tumour proliferation fraction in DCIS was associated with LOH at 17p13.3 and 3p24-26 (63). Leal et al (75) determined aneuploidy by image analysis and observed this in 77.5% of DCIS cases mainly in the high and intermediate grade subtypes.

One study by Page et al has looked at BRCA-1 in 3 cases of DCIS, while in another study, LOH was also identified at one locus on Chr 17q in the vicinity of BRCA-1 in 10-

20% of informative cases of DCIS studied (76). Munn et al (77) found LOH for the BRCA1 gene in 74% of DCIS.

Oncogenes and Other Tumour Associated Proteins in DCIS:

Growth Factor Receptors and Growth Factors:

CerbB-2 (HER-2/neu): The reported incidence of cerbB-2 expression of DCIS in the literature is 45-60 percent. *CerbB-2* gene amplification and cerbB-2 protein expression is more frequent in high and intermediate nuclear grades of DCIS and those with comedo-type necrosis than in low nuclear grade and non-comedo DCIS. This observation was first reported in 1988 (78) and has subsequently been identified by others (30;79-89). *CerbB-2* gene amplification in DCIS has been confirmed by the differential polymerase chain reaction (PCR) technique in 13 (48%) of 27 cases of pure DCIS included in a study of both in situ and invasive breast carcinoma (88). CerbB-2 expression is very common in Paget's disease of the breast (90-93), reported incidence being 91 percent. At least some cases of invasive ductal carcinoma that express cerbB-2 seem to be derived from pre-existing DCIS (94).

EFGR (cerbB-1), cerbB-3, cerbB-4: Only limited studies are available on these growth factor receptors and there is at present insufficient evidence for them having any significant role in DCIS. In one study, cerbB-3 was shown to have an inverse correlation with cerbB-2, whereas no correlation was demonstrated between cerbB-3 positivity and type of DCIS, p53 and PR expression and proliferative activity (95).

TGF-β_1: In a study reported in the literature, 12 of 27 cases of DCIS showed some expression of TGF-β_1, with prominent immunohistochemical expression in 3 cases (96).

Cell Cycle Associated Genes and Proteins:

P53: The p53 protein is preferentially expressed in high nuclear grade DCIS and in cases of DCIS with comedo-type necrosis (80;87;97-103). The mechanism of this may be gene mutation, although one study (99) was unable to demonstrate *P53* gene mutations at exons 5 and 6, and only one case out of 39 (3%) showed mutation in exon 8 of *P53* gene when exons 8 and 9 were screened by a PCR/single strand conformation polymorphism

analysis (SSCP). Stabilisation of non-mutant p53 protein causing p53 protein expression or mutations in other exons of *P53* may explain this finding.

Another study of *P53* by SSCP or southern Blot which included 10 cases of DCIS found *P53* mutations in 2 of 17 (12%) intraductal carcinomas or carcinomas with a predominantly intraductal component (103). Conservation of *P53* mutations has been demonstrated in the progression of DCIS to invasive disease (98).

Rb1: Deletions of Rb1 in tumours with a 90% DCIS component have been described in one study (104).

Bcl2: Expression of bcl2 occurs at a higher frequency in in-situ than invasive carcinomas (105). Furthermore, bcl2 expression correlates inversely to worsening nuclear grades of DCIS that is from low to high nuclear grade (106).

Bax: Bax expression has been detected in 37/56 (66%) of DCIS after employing immunohistochemistry for its detection. It did not correlate to histological grades of DCIS or other molecular markers including Ki67, ER, p53 or cerbB-2. Positive correlation was seen with bcl2 (107).

Cyclin D1: Overexpression of cyclin D1 is present in 90% of malignant breast lesions, both DCIS and IDC (108).

p21 ras: A study by Going et al described how 14 cases of non invasive breast carcinoma showed a progressive increase in p21 ras immunostaining at the transition of normal epithelium to ADH and carcinoma in situ suggesting a putative role for p21 in disease progression (109).

Steroid Hormone Receptor and Related Proteins:

Oestrogen Receptor (ER): The literature suggests that ER expression is more common in lower nuclear grade and non-comedo variants of DCIS, although there is disagreement as to the exact overall frequency of ER expression in DCIS. The rate of ER positivity depends upon the grade mix and method of detection. In one study (110), immunohistochemically demonstrated ER expression was reported in 32% of cases of DCIS overall and positive tumour ER staining was related to non-comedo types of DCIS, small cell size, higher proliferation fraction (S-phase fraction) and lack of cerbB-2

staining. Other studies have found ER expression in a higher proportion of cases of DCIS (80;87;111-116). Burr et al (116) found ER expression in 91% of non-comedo DCIS and 57% of comedo DCIS.

Progesterone Receptor (PR): The frequencies of positivity range from 31% to 73% (80;87;115). In a study by Murphy et al, PR positivity was reported in 46% of cases using an immunohistochemical method with microwave antigen retrieval (117).

pS2: It has been reported that pS2 expression is more frequent in the extensive intraductal component of invasive carcinomas, being present in 54% of cases, in contrast with 27% for invasive carcinomas. The frequency of pS2 expression was greater in non-comedo than comedo DCIS (118). Another study showed intensive pS2 expression in comedo, solid, cribriform and micropapillary DCIS with significant positivity in 63-67% of cases. A progressive increase in pS2 expression from normal breast tissue to malignant disease was proposed (119).

Stromolysin 3: Stromolysin 3 expression has been examined in DCIS by Northern Blot analysis. Stromolysin 3 either was not found or only a trace level of expression was seen in 9 of 10 (90%) cases of DCIS compared with 62% of primary invasive or metastatic breast carcinomas (120).

Metallothioneins: Expression of metallothioneins is associated with high-grade comedo DCIS with necrosis. No evidence of p53 or metallothioneins co-expression was found using immunohistochemistry (121).

Metastasis Suppressor Proteins:

NM23: NM23 is a nucleoside diphosphate kinase gene product. Its expression was examined in two separate published studies reporting conflicting results (122;123). One of these reported greater expression of NM23 gene product in comedo DCIS as compared to non-comedo, the other described opposite results. Allelic loss of $NM23H_1$ has also been described in another study of invasive tumours with a 90% DCIS component (104).

Cell Adhesion Molecules:

β_1 and β_4 integrins: A study described by Hanby et al, looked at the β_1 and β_4 integrins in 40 cases of DCIS. β_4 integrin was generally found to be confined to basement membranes

with the suggestion made that loss of the β_1 integrin chain might be important in malignant progression (124).

Intercellular Adhesion Molecule – 1 (ICAM-1): A study by Bacus et al (125), described that in 39 cases of invasive breast cancer, a significant association was found with expression of the ICAM-1 in those cases of invasive carcinoma which had a prominent DCIS component.

Glutathione-S-transferase Pi (GST Pi): Bellamy et al (126) reported that 37 of 92 (40%) of DCIS cases were GST Pi positive. No correlation with histological variables, cerbB-2 expression or clinical outcome was found. Furthermore, there was no evidence that GST was a useful marker of prognosis in DCIS.

Angiogenesis:

Guidi et al (127), reported on 55 cases of DCIS, in which a diffuse pattern of increased micro-vessel density was associated with comedo necrosis, stromal desmoplasia, positive cerbB-2 expression, an increased proliferation fraction as assessed by KiS1 staining and the presence of lobular cancerisation. Two patterns of angiogenesis were identified, a diffuse increase of micro-vessels in the stroma surrounding lesions which correlated with comedo-type necrosis and micro-vessels in immediate apposition to the basement membrane of involved spaces in a "cuffing pattern", which did not correlate with any of the above factors. Another study of six cases of pure DCIS and 49 cases of DCIS associated with invasive breast carcinoma showed no correlation with histological type or nuclear grade of DCIS and angiogenesis, although there was less angiogenesis in benign proliferative lesions than in DCIS (128).

Proliferation Fraction:

Cases of DCIS with high nuclear grade and with comedo-type necrosis show high cellular proliferation fractions as assessed by Ki67/MIB1 staining of tumour nuclei, S-phase estimation using flow cytometric analysis of DNA, or by thymidine labelling or other indices of cell proliferation (30;33;80;82;87;129-131).

To conclude, although some molecular genetic data support the hypothesis that invasive breast cancer (IDC) usually arises from pre-existing in-situ lesions, this is difficult to

prove and more work needs to be done to help understand the relation between DCIS and IDC and the crucial progression step of one from the other.

MANAGEMENT OF DCIS:

Mastectomy versus Conservation:

For many years, the standard treatment for most patients with DCIS has been mastectomy. Although mastectomy is clearly an over-treatment for many cases, it results in an extremely low local recurrence rate and mortality from breast cancer with nearly 100% cure rates (1;23). During the last 10 to 12 years, interest in breast conserving surgery for patients with DCIS has been considerable. Data from the SEER Study (Surveillance, Epidemiology and End Results) showed that survival rates for women who had mastectomy and those who had lumpectomy or other breast conserving surgery, were the same or better than those expected for the general population (132). The size of the lesion determines whether breast conservation is technically feasible or not, the alternative being mastectomy with or without breast reconstruction. Lesion size is assessed by a combination of radiological and pathological factors. Most workers agree that DCIS greater than 25 mm in microscopic size is less amenable to breast conservation (lumpectomy) than smaller lesions.

At present histological subtypes of DCIS alone cannot be taken into account for management decisions until more substantive evidence from large randomised trials becomes available. A small, aggressive looking lesion (HNG) may be adequately treated by excision alone if the margins are widely clear, whereas a large, seemingly unaggressive (LNG) lesion with an excision margin that is not clear may be better treated by mastectomy and immediate reconstruction. Clearly other factors, in addition to the morphological appearance, must be considered when planning treatment (25;28).

Excision Alone versus Excision plus Radiotherapy – Protocol B-17:

There is a significant amount of published literature on DCIS treated by breast excision without radiotherapy (14;26;133-141) or with radiotherapy (133;137-139;142-151). One prospective randomised trial (139) known as "Protocol B-17", performed by the National Surgical Adjuvant Breast and Bowel project (NSABP) in the United States has addressed this issue. The results of protocol B-17 were updated in 1995 (29) and 1998 (152). In this

study more than 800 patients with DCIS that had been excised with clear surgical margins were randomised to two groups, excision only and excision plus radiotherapy. After eight years of follow-up, a significant decrease in local recurrence of DCIS and IDC was seen in the patients treated with radiotherapy. The overall local recurrence rate for patients treated with excision alone was 27% at eight years. For patients treated with excision plus irradiation, it was 12% at eight years (152). Radiotherapy resulted in a significant decrease in subsequent non-invasive DCIS as well as IDC.

Protocol B-17 has been criticised on a number of counts, including lack of analysis of different pathological subsets, lack of size measurements in more than 40% of cases in the initial pathological report, lack of mammographic-pathological correlation or specimen radiography, no uniform guidelines for tissue processing or size estimation; and the author's definition of what constitutes a clear excision margin (153;154). However the trial was designed more than 15 years ago, when researchers were asking a single broad question 'does radiotherapy benefit patients with DCIS treated with breast conservation?' It was not designed to answer the more sophisticated questions, which are asked nowadays namely 'exactly which subgroups of patients may benefit from radiotherapy and by how much?' Radiotherapy is expensive and in a few cases is followed by significant side effects, such as cardiac toxicity and pulmonary fibrosis (155). It changes the texture of the breast making mammographic follow-up more difficult, and may result in delayed diagnosis if there is a recurrence. Doctors must be satisfied that the benefits of radiation in terms of improved survival free of recurrence are greater than the side effects, complications, inconvenience and costs.

Current Approaches:

Current treatment for DCIS ranges from simple tumour excision, to various forms of wider excision (segmental and quadrant resections), to mastectomy with or without reconstruction. All treatments less than mastectomy may be followed by radiotherapy. The patient's preference is also taken in to account when planning management, therefore, no single approach is appropriate for all forms of the disease and treatment for each patient is individualised. If untreated, low nuclear grade DCIS may never cause a clinical problem since only about 40% of untreated low-grade lesions become invasive over a time span of approximately 20 to 30 years (156). A helpful guide in management decisions may be to employ the Van Nuys Prognostic Index as described below.

Van Nuys Prognostic Index:

As already mentioned, two of the prognostic factors in DCIS, namely, nuclear grade and comedo-type necrosis were used to develop the Van Nuys pathological classification (24). However, these two factors are inadequate as the sole guidelines in the treatment selection process, tumour size and margin width also being important (28). The Van Nuys prognostic index has been developed by combining all of these factors (25;157). Scores from 1 to 3 are given for each of the three different predictors of local breast recurrence (size of the tumour, width of the surgical margins and pathological classification). The scores for each predictor in each individual patient are combined to yield a total score ranging from a low of 3 (best prognosis) to a high of 9 (worst prognosis). Patients can be divided into three subgroups on the basis of their score (3-4, 5-7, 8-9). The probability of local recurrence is significantly different for each subgroup. Patients with scores of 3-4 can be considered for treatment with excision only as it has been shown that these patients show no difference in survival free of local recurrence at 12 years regardless of whether they have had radiotherapy (25). Patients with scores of 5-7 show a significant decrease in local recurrence rates with radiotherapy. Patients with scores of 8-9, if treated conservatively, can have unacceptably high local recurrence rates, regardless of irradiation treatment, and should be considered for mastectomy. The Van Nuys prognostic index is a numerical algorithm based on tumour features and recurrence data from a large series of patients with DCIS. It allows quantification of prognostic factors that are easily measured and the placing of patients into one of three clearly defined risk groups and thus can help with treatment planning. However, the Van Nuys Prognostic Index was developed in a retrospective study and ideally should be validated prospectively before serving as the basis for treatment recommendations. Therefore, there remains a clinical need for an accurate and reliable prognostication system for DCIS (158).

Axillary Node Management:

There is now uniform agreement that for patients with DCIS, there is no need to treat the axilla as the incidence of axillary node metastasis in DCIS is very low (159;160). For patients with large enough lesions to require mastectomy, a sentinel lymph node biopsy (SLNB) can be performed using a vital blue dye, a radioactive tracer, or both at the time of mastectomy (161-163) although the role of SLNB in DCIS is less clear. The sentinel

node can then be evaluated by haematoxylin and eosin (H&E) staining and if such routine stains are negative it can be followed by immunohistochemical staining for cytokeratin. Whether the significance of a microinvasive focus shown by immunostaining is the same as a larger metastasis detected on H&E, is not clear. However, in the case of node-negative IDC, occult axillary LN metastases are considered to be of no prognostic significance (chapter 2).

If invasion is documented in the mastectomy specimen, and is greater than 1 mm, then the lesion is no longer considered as DCIS but rather an invasive cancer with an extensive intraductal component (EIC). The lesion size under the circumstances is the maximum diameter of the largest invasive focus and not the diameter of DCIS.

Margin Width:

The most crucial information pathologists can currently provide is the accurate evaluation of the surgical margin status. An adequate pathologic evaluation requires proper orientation of the specimen during handling by the pathologist. In order to ensure this, the surgeons usually mark the specimen. The entire surface of the specimen is inked to facilitate microscopic recognition of the surgical edges. Margin width is the distance between DCIS and the closest inked margin and reflects the completeness of excision. Since DCIS is a non-invasive unicentric lesion, complete excision should produce a cure. Currently, the best way to assess completeness of excision is by determining the margin width. When the margin width exceeds 10 mm, the likelihood of residual disease is relatively small (164). There is little benefit from radiotherapy after excision of the carcinoma if the margins are greater than 10 mm (165), regardless of the nuclear grade (166) or the presence of comedo-type necrosis (167).

Researchers from Nottingham (168) have reported a local recurrence rate of 6% in a group of 48 patients treated with excision alone and with the margins greater than 10 mm. These results were updated by Blamey et al at the fourth Consensus Conference of the European Organization for Research and Treatment of Cancer (EORTC) held in 1998. They described how 20 mm or more is a 'clear' margin, with 10-20 mm indicating an 'uncertain' margin status and less than 10 mm an 'incomplete' tumour excision (169;170).

Adjuvant Therapy:

Opinions about the relative merits of adjuvant radiotherapy or tamoxifen treatment vary. Organisations such as the UKCCCR (United Kingdom Co-ordinating Committee on Cancer Research) under the auspices of the UK National Breast Screening Programme, The European Organisation for Research and Treatment of Cancer (EORTC), Scandinavian Groups and the National Surgical Adjuvant Breast and Bowel Project (NSABP) have instituted randomised clinical trials. The early results from the NSABP trial (139) indicate that, with a median 3.5 years follow-up of 818 patients, the rate of local recurrence of DCIS is lower in patients treated with local excision and radiotherapy (15.6%) as compared with local excision alone (26.2%). Approximately half the tumour recurrences were as invasive carcinoma in the excision alone group while only 28% of the tumour recurrences were invasive in the excision and radiotherapy group. The results were similar to these when the patients were followed-up for a further time period, up to a total of eight years (152). Similarly, the EORTC DCIS study has shown (174) that post-operative radiotherapy for DCIS reduces both invasive and non-invasive recurrences in the ipsilateral breast. However, unlike NSABP-B17, the EORTC study showed an increase number of contralateral breast cancers in patients randomised to receive adjuvant radiation.

Trials with tamoxifen as adjuvant therapy are also underway at present. Two studies have tried to evaluate the role of tamoxifen in the management of DCIS. The NSABP-B24 trial is comparing tamoxifen with placebo after breast conserving therapy (171) and a European study (UKCCCR-DCIS Phase III-90001) is comparing tamoxifen alone with tamoxifen and radiotherapy (172). The UKCCCR published their results in the Lancet in July 2003 of a randomised controlled trial enrolling 1701 patients (171). They showed that tamoxifen reduced the recurrence of ipsilateral DCIS but not invasive disease whereas radiotherapy reduced the incidence of ipsilateral invasive disease as well as ipsilateral DCIS. There was no reduction in the incidence of contralateral disease.

OUTCOME AFTER RECURRENCE:

The local recurrence rate for DCIS is 10-15%, but it depends on the length of follow-up. Retrospective studies have indicated a recurrence rate of 22-30% without RT and 8-15% with RT (34;138). Approximately 50% of the recurrences after excision and radiation for

DCIS are as invasive breast cancer (15;23;27) and nearly all of them occur in the region of initial biopsy in the ipsilateral breast (131;136). A study by Solin et al (173;174) has reported on 272 patients with DCIS; 42 patients (15.4%) developed recurrence after excision and irradiation, 45% of which were DCIS and 55% were invasive carcinoma. Six of these 42 patients subsequently developed metastatic breast cancer. Forty percent of these recurrences occurred between 5-10 years after treatment and 12% after 10 years. In another study by Silverstein et al (175) of a series of 707 patients, there were 74 (10%) recurrences, 39 of which (53%) were non-invasive and 35 (47%) were invasive. All of the patients with non-invasive recurrence did well. None developed distant disease and there were no deaths from breast cancer. Among the 35 patients with an invasive recurrence, half presented with stage I disease and the other half with stage 2A or higher. Seven patients developed distant disease and five died of breast cancer. The median follow-up for the 35 patients with invasive recurrences was 9.3 years. The chances of an invasive recurrence at eight years were 7% and the probability of death from breast cancer was 1.4%.

The treatment for a patient with an invasive recurrence should be based on the stage of the recurrence. Patients treated initially by mastectomy generally require excision of the recurrence followed by radiotherapy to the chest wall and chemotherapy. Patients previously treated by excision and radiotherapy generally require mastectomy, followed by chemotherapy if the invasive recurrence is high grade, or greater than 10 mm in diameter, or the markers are those of a poor prognosis. Patients previously treated by excision alone can undergo re-excision. If clear margins are obtained, they can be considered for breast conservation with radiotherapy. The decision to add adjuvant chemotherapy should be based on tumour prognostic factors. Patients treated with breast conservation surgery should be followed-up closely, perhaps with physical examination every six months and a mammogram every six to twelve months.

SUMMARY:

The incidence of DCIS is increasing and the disease is diagnosed in an increasing proportion of asymptomatic patients. DCIS is a heterogeneous pathological process. With the use of more refined molecular analysis, the relation of DCIS to IDC can be better defined.

Until recently, our approaches to DCIS have been based on its morphology rather than its aetiology. The focus of investigation is now shifting to the genotype rather than the phenotype of carcinomas. Morphologically normal looking tissue surrounding areas of DCIS may show LOH similar to that in the tumour. It is highly likely that genetic changes precede morphological evidence of malignant transformation. Medicine must learn how to recognise these genetic changes, how to exploit them, and how to prevent them. DCIS is a lesion in which the complete malignant phenotype of unlimited growth, angiogenesis, invasion and metastasis has not been fully expressed. With sufficient time, most non-invasive lesions will develop the ability to invade and metastasise (66). We must learn how to prevent this.

The goal in the treatment of patients with DCIS is to control local disease and prevent subsequent development of invasive cancer. Breast conserving therapy is an effective option in the management of DCIS. The use of radiotherapy after lumpectomy significantly decreases the rate of recurrence. Axillary lymph node dissection is not routinely recommended in the management of DCIS. There is no defined role for hormonal therapy at present. Most patients with recurrent disease can be treated effectively.

CHAPTER 7: MATERIALS AND METHODS:

HYPOTHESIS:

DCIS grades are not only morphologically different, but are also biologically and chemically distinct entities as well and this may be reflected in molecular marker expression and chemical structural differences. The nuclear grades of DCIS are biologically related to grades of IDC. Bax protein may play a role in pathogenesis of breast cancer.

AIMS AND OBJECTIVES OF THE STUDY:

The main aims and objectives of the study were:

1- To determine the clinical relevance of the DCIS classification based on nuclear grades and that recommended by NCGBSP (1), by correlating the different nuclear grades of pure DCIS with established markers of prognosis in invasive ductal carcinoma (IDC) of the breast. These established markers of prognosis are bcl2, cerbB-2, p53, ER and Ki67.

2- To compare the expression of the above mentioned molecular markers in the three DCIS grades versus three IDC grades, to see if any of these markers would reliably distinguish between DCIS grades, IDC grades, and between DCIS and IDC. This would help in understanding the pathogenesis of the disease. The DCIS grades have not been compared against IDC grades directly in terms of molecular marker expression in any previous study.

3- To investigate bax protein expression in DCIS and to correlate it to DCIS grades and other molecular markers. There is very little information available on bax protein expression in DCIS. For the above three objectives, immunohistochemistry was employed.

4- To determine if dividing DCIS into three nuclear grades on the basis of nuclear area measurement by image analysis correlates with manual grading by light microscopy.

5- To amplify exons 5-8 of *P53* gene in DCIS and IDC cases by polymerase chain reaction (PCR) and subsequently to analyse these cases by direct sequencing in order to determine the incidence of *P53* mutation in DCIS and compare it with that in IDC. There is little data available on *P53* mutations in DCIS.

6- To find out if structural characterization by Fourier Transform Infrared (FTIR) and FT-Raman spectroscopic techniques can distinguish between DCIS grades, IDC grades, and between DCIS and IDC as whole groups. Again, there is scanty data on FTIR in DCIS grades and IDC grades.

MATERIALS:

This is a retrospective study performed on breast carcinoma cases at the Royal Free Hospital. Suitable cases were selected by searching on the Royal Free Hospital Histopathology Department database (SNOMED Diagnostic Retrieval System) with the SNOMED T codes for the breast and the appropriate M codes for DCIS, IDC (LN negative), IDC (LN positive) and the corresponding lymph nodes containing metastatic tumour. The data retrieved included the patient's name, date of birth (DOB), hospital number and laboratory number. In order to ensure confidentiality, laboratory numbers rather than patients' names have been used. A total of 3-25 blocks per case were reviewed and cases were selected according to availability of enough tumour tissue. Haematoxylin and eosin (H&E) stained histological archive slides of selected cases were reviewed by light microscopy to confirm the pathological features and select a suitable block for further study. In case of any disparity between personal observations and the recorded pathological diagnosis, clarification was obtained with a specialist pathologist (JC) at a conference microscope. Histological reports of the cases were used along with medical records in order to access further clinical details. Thereafter, all research records were kept separate from clinical records to safeguard individual subjects under the Data Protection Act. This study was performed before the Pearson report on Alder Hey and the Caldicott report on patient confidentiality.

Formalin-fixed, paraffin-wax embedded blocks of the selected cases for 1985-1995 were retrieved from the archives of the Histopathology department, Royal Free Hospital. A total of 118 samples from 103 patients was studied for bcl2, Ki67, ER, p53 and cerbB-2 proteins by immunohistochemistry. These 118 specimens included 50 DCIS, 38 IDC (LN

-ive), 15 IDC (LN +ive) and the 15 corresponding lymph nodes containing metastases from the same patients as in the previous group. This is shown in table 7.1 which also gives details of the number of cases in different subgroups within these categories.

Table 7.1; Cases studied for bcl2, Ki67, ER, p53 and cerbB-2

Group I (DCIS)			Group II (IDC, LN -ive)			Group III (IDC, LN +ive, any grade)	
Group A	**Group B**	**Group C**	**Group D**	**Group E**	**Group F**	**Group G**	**Group H**
LNG n=18	ING n=17	HNG n=15	G I n=6	G II n=20	G III n=12	IDC n=15	Lymph Node Metastasis n=15
Total = 50 patients			Total = 38 patients			Total = 30 samples from 15 patients	
Total = 118 samples from 103 patients							

Within these 118 samples, 30 normal breast tissues adjacent to IDC and 24 DCIS associated with IDC were also studied, making a total of 172 samples.

Out of the 118 samples, 94 were studied for bax. These 94 specimens were from 81 patients and included 32 DCIS, 36 IDC (LN-ve), 13 IDC (LN+ve) and 13 corresponding lymph nodes from the same patients as in the previous group. This is shown in table 7.2 which includes numbers in subgroups.

Table 7.2; Cases studied for bax

Group I (DCIS)			Group II (IDC, LN -ive)			Group III (IDC, LN +ive, any grade)	
Group A	**Group B**	**Group C**	**Group D**	**Group E**	**Group F**	**Group G**	**Group H**
LNG n=12	ING n=10	HNG n=10	G I n=6	G II n=19	G III n=11	IDC n=13	Lymph Node Metastasis n=13
Total = 32 patients			Total = 36 patients			Total = 26 samples from 13 patients	
Total = 94 samples from 81 patients							

DCIS cases (Group I) were only included in the absence of any associated invasive component (pure DCIS) in either the diagnostic biopsy material or in the tissue removed as part of a second stage definitive treatment procedure and also in the absence of past history of breast carcinoma in either ipsilateral or contralateral breast. All group II patients were either clinically or histologically proven lymph node negative. This

highlights the fact that in spite of a meticulous search for histologically negative lymph node cases, it was not possible to have a histological proof in all cases of group II. As there can be high clinical false negative rate in the final analysis when LN negative cases were compared against the LN positive cases, analysis was performed twice. Once with total numbers of LN negative cases and then with histologically proven LN negative cases against LN positive.

After selection of appropriate paraffin-wax blocks, cutting of histological sections was performed with a microtome at 3μm thickness, taking an appropriate number of serial sections from each block to correspond with the number of markers to be stained as well as including sections for negative controls, and one section from each block for haematoxylin and eosin (H&E) staining. Sections from appropriate positive control wax blocks were also cut. Tissues used as positive controls are shown in table 7.3 on page 125, negative controls were provided by omission of primary antibody.

All the sections were mounted on 3-aminopropyl-triethoxysilane (APES) coated slides, the preparation of which is described in appendix 1. Sections were then incubated overnight at 37°C and stored until staining could be performed in batches. Haematoxylin and eosin staining was done on one section from each block and staining for each marker was performed by employing streptavidin-biotin immunoperoxidase (ABC) immunohistochemical technique which is described in the section of immunocytochemistry below.

HISTOPATHOLOGICAL ASSESSMENT:

Cases of DCIS were classified as low nuclear grade (LNG), intermediate nuclear grade (ING) and high nuclear grade (HNG) according to the published criteria of Holland et al (2) as described in chapter 6. In cases where more than one histological grade was identified, DCIS was graded according to the highest nuclear grade present. In addition to grade, histological type of DCIS was also recorded, as well as the presence or absence of comedo-type necrosis.

IDC cases were classified according to the grading system described by Elston and Ellis (3) described in chapter 2, as Grade I, Grade II and Grade III. The histological type of IDC was also recorded, as well as the presence or absence of lymph node metastasis.

IMMUNOCYTOCHEMISTRY (ICC):

Introduction:

Immunocytochemistry (ICC), also known as immunohistochemistry (IHC), was developed by Coons et al (4) and is defined as the identification of a tissue constituent in situ by means of a specific antigen-antibody reaction tagged by a microscopically visible label. Immunocytochemical techniques have given us the potential to localize antigens in cells and tissues with improved diversity, selectivity and specificity of staining reactions, as compared to classical staining methods for the demonstration of tissue components (5-8). Provided that a suitable antibody can be produced and the antigen preserved, there is no limit to the substances that may be localized immunocytochemically. The antigen can be whole micro-organisms (viruses, bacteria, fungi) or their parts, whole eukaryotic cells or sub-fractions. Antibodies that are raised against these antigens, may be polyclonal (usually raised in rabbit or goat) or monoclonal (usually raised in mouse or rat). The introduction of monoclonal antibodies in 1975 (9) has revolutionized much of ICC. Initial immunocytochemical methods employed fluorescent dyes as the labels. Later, the search for labels visible by ordinary light microscopy resulted in the use of enzymes. Nakane and Pierce (10), first described the use of peroxidase as a label for antibody localization and in the same year Graham et al used diaminobenzidine (DAB) as a substrate (11).

Applications:

ICC has a wide range of applications, as it can be performed on cell and tissue cultures (useful for localization of cell surface and intracellular antigens), cell lines, cytological preparations, paraffin-wax-embedded tissue sections and frozen sections. ICC has been used to study the localization of onco-proteins (src, ras, raf, myc, cerbB-2 etc.), intrinsic amines (dopamine, adrenaline, noradrenaline, serotinin, histamine), peptides including neuro-peptides (enkephalins) and neuro-endocrine peptides (substance P, vasoactive intestinal polypeptide, somatostatin, bombesin, gastrin, insulin, glucagon etc.), intermediate filament proteins (cytokeratins, vimentin, desmin, glial fibrillary acidic protein etc), neuro-filaments, pituitary hormones, tumour markers [carcino-embryonic antigen (CEA), human chorionic gonadotrophin (HCG), alpha-fetoprotein (AFP), calcitonin, thyroglobulin, adrenocorticotrophic hormone (ACTH), antidiuretic hormone]

and micro-organisms. ICC can be used to aid the diagnosis of organ-specific autoimmune disorders, non-organ-specific autoimmune disorders, transplant rejection, thyroid diseases, lymphoreticular tumours, leukaemias, glomerular disease and dermatological disorders. In the case of tumours, immunohistochemical staining for a combination of markers taken together with the morphology may help in determination of the site of origin of an occult primary tumour presenting with metastases, in differential diagnosis, functional classification of malignant disease, prognosis, and in marker prediction for immunolocalization and tumour targeting with antibodies ligated to toxic agents.

Immunohistochemistry is a reliable, reproducible and relatively simple technique to perform. It allows visualization of different protein products in paraffin and as well as frozen sections with accurate localization. However, the immunohistochemical expression of protein product in carcinoma cells does not always correlate with the abnormality in the corresponding gene.

Methods:

The various methods that can be employed for ICC in combination with light microscopy include:-

1. Direct methods. In these methods, a labelled primary antibody is applied directly to the tissue preparation.

2. Indirect methods. In these methods, the primary antibody is unlabelled and is identified by a labelled secondary antibody raised to the immunoglobulin of the species providing the primary antibody. These methods are more sensitive than the direct methods. Indirect methods include indirect immunofluorescence, in which secondary antibody is labelled with a fluorescent marker and the reaction is examined with an ultraviolet light; and, indirect immunoperoxidase method, in which the secondary antibody is labelled/conjugated with peroxidase and a conventional light microscope is used. The immunoperoxidase technique has the advantage of an insoluble permanent reaction product, so that morphological detail is retained. In the case of immunofluorescence, with time and photographic light exposure, the fluorescence fades.

3. Unlabelled antibody-enzyme methods. An unconjugated bridging secondary antibody is used between the primary antibody and the labelled detecting reagent, which is usually an enzyme-anti-enzyme complex [peroxidase-anti-peroxidase (PAP) or alkaline phosphatase-anti-alkaline phosphatase (APAAP)] or an avidin-biotin-enzyme complex (ABC).

In the case of the PAP method (6;12), the first layer is a primary antibody (e.g. rabbit) which is directed against the tissue antigen, the second layer being the unconjugated secondary antibody (e.g. goat anti-rabbit IgG) and the third layer is the rabbit PAP complex. The increased amount of label allows a high dilution of the primary antibody to be used thereby reducing unwanted background staining. The APAAP method works on the same principle as the PAP method but is mainly used with mouse monoclonal primary antibodies (13).

Avidin-Biotin Peroxidase Complex (ABC) Method - Principle and Technique:

The ABC method was described by Hsu et al (14). This method has gained the highest popularity and ensures maximal sensitivity. The ABC method requires incubation of tissue with primary antibody (1st layer), followed by incubation with biotinylated secondary antibody (2nd layer) and finally the addition of a pre-formed avidin-biotin-peroxidase complex (3rd layer) (figure 7.1). The avidin-biotin-peroxidase complex consists of many biotinylated horseradish peroxidase molecules cross-linked by avidin to form a three dimensional complex. In the complex, at least one of the four reacting sites on the surface of avidin is free to bind a biotin residue on the antibody molecule. This complex remains stable for several hours after formation.

Avidin is a basic glycoprotein present in large amounts in egg white. It consists of four subunits which behave as specific binding sites for four biotin residues (15). The specificity of ICC can be further increased by replacing avidin with streptavidin, which is a protein extracted from the culture broth of streptomyces avidinii. Biotin is a water-soluble vitamin (vitamin H) present in egg yolk.

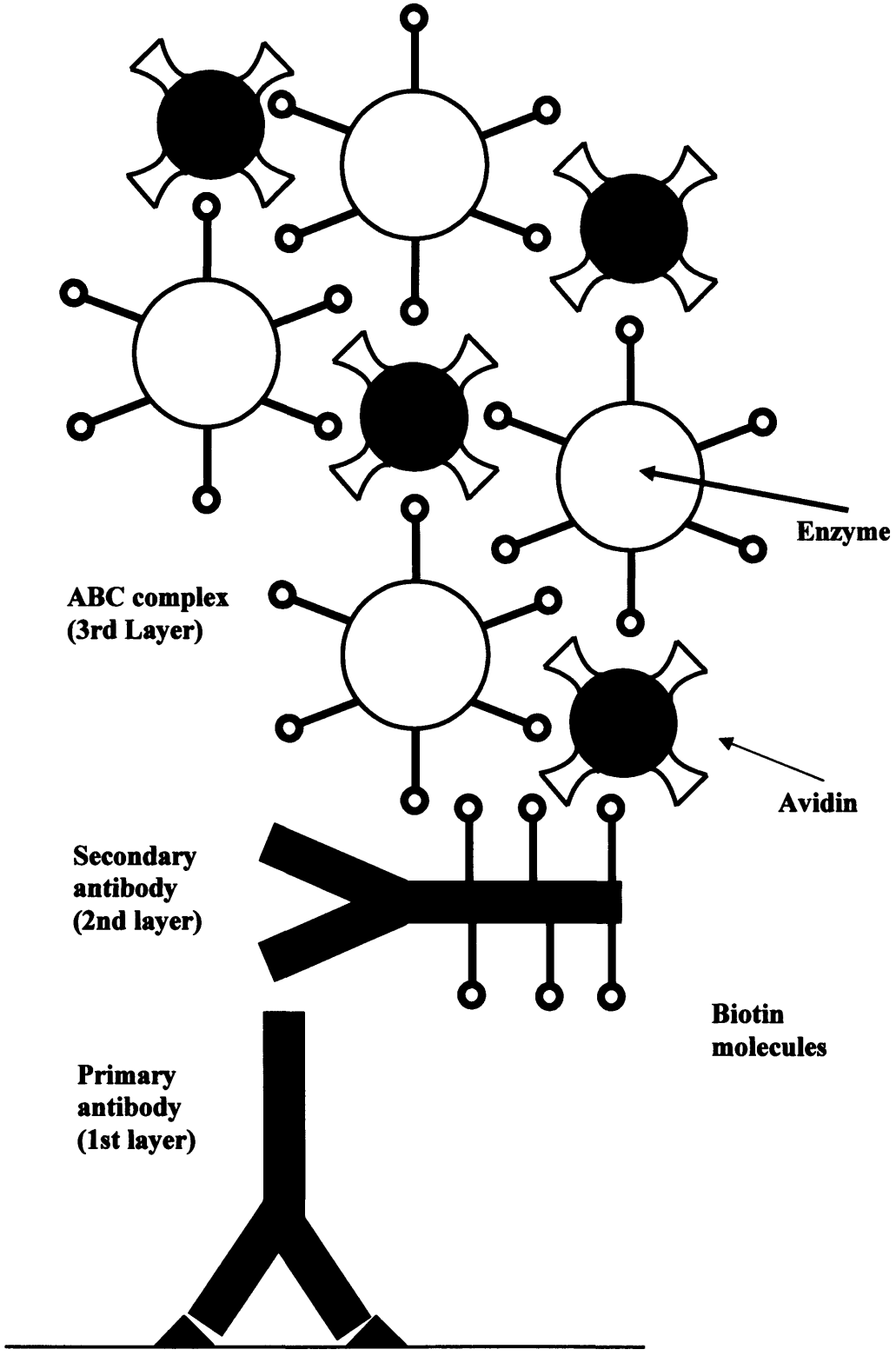


Figure 7.1; The ABC method

Because of its reliability, the ABC technique has been employed in this study to look at bax, bcl2, Ki67, ER, p53 and cerbB-2 in breast tissue and the detailed protocol of the technique is given below:

1. Sections were cut at 3μm thickness, mounted on APES coated slides, as described above and detailed in appendix 1.
2. Sections were de-paraffinised (de-waxed) in xylene and rehydrated in graded alcohols in the following way:

(i)	Xylene I	5 minutes
(ii)	Xylene II	5 minutes
(iii)	Xylene III	5 minutes
(iv)	Alcohol I (100%)	2 minutes
(v)	Alcohol II (70%)	2 minutes
(vi)	Alcohol III (50%)	2 minutes
(vii)	Double distilled water	2 minutes.

3. The slides were transferred to a humidity chamber at room temperature and covered with freshly prepared 0.5% H_2O_2 in water for 10 to 15 minutes in order to block the endogenous peroxidase activity. The composition of hydrogen peroxide solution is described in appendix 1 under the section 'blocking endogenous peroxidase activity'.

4. This step was followed by washing the slides with de-ionized or distilled water for 2 minutes each, changing the water between washes.

5. Antigen retrieval was performed by pressure cooking for bax and microwaving for bcl2, Ki67, ER, p53 and cerbB-2. The time for heat pre-treatment (microwaving or pressure cooking) for each marker is shown below:

Bax	90 sec (pressure cooking)
Bcl2	10 minutes (microwaving)
Ki67	10 minutes (microwaving)
ER	20 minutes (microwaving)
p53	10 minutes (microwaving)
CerbB-2	20 minutes (microwaving)

For cerbB-2, 20 cases were repeated without microwaving as it increased the sensitivity of the assay.

The detail of heat pre-treatment with microwave is given in appendix 1.

6. The slides were transferred to a humidity chamber and the sections were covered with two changes of Tris buffered saline (TBS) (pH 7.6) for 5 minutes each. The composition and preparation of TBS is described in appendix 1.

7. Slides were incubated with normal goat serum (NGS) from DAKO at a dilution of 1/10 for 15 minutes at room temperature to block non-specific binding of protein.

8. Sections were drained and covered with primary antibody, optimally diluted with TBS for 1 hour at room temperature. The primary antibodies to the markers studied, their source, clone, type, dilution and the positive control used is shown in table 7.3. Optimum conditions (working dilutions) were determined by titration of each antibody on receipt from the supplier using control tissue sections.

Table 7.3; Primary antibodies used in the study

Antibody	Source	Clone	Type	Dilution	Positive Control
Bax	Novabiochem	----------	Rabbit polyclonal	1/20	Colon
Bcl2	DAKO	124	Mouse monoclonal	1/50	Tonsil
Ki67	Immunotech	MIB1	Mouse monoclonal	1/50	Tonsil
ER	DAKO	ID5	Mouse monoclonal	1/100	Known positive breast cancer
P53	DAKO	DO-7	Mouse monoclonal	1/50	Known positive prostate cancer
CerbB-2	DAKO	----------	Rabbit polyclonal	1/400	Known positive breast cancer

9. In the case of negative controls, primary antibody was replaced by TBS. Sections were rinsed in TBS twice for 3 minutes each.

10. Sections were incubated with biotinylated goat anti-rabbit/anti-mouse secondary antibody mixture at a dilution of 1/200 for 30 minutes. This reagent is supplied by DAKO (as a part of the StreptABComplex/HRP Duet).

11. Sections were once again rinsed in TBS twice for 3 minutes each.

12. Sections were incubated with StreptABComplex from DAKO, prepared according to the manufacturer's protocol, for 15 minutes.

13. Sections were rinsed in TBS twice for 3 minutes.

14. Staining of the sections was developed with freshly prepared 3,3diaminobenzidine (DAB) solution. See appendix 1 for preparation of DAB.

15. Sections were washed in distilled water for 3 to 5 minutes.

16. Counter staining was done with Mayer's haematoxylin for 2 to 5 minutes.

17. This was followed by rinsing in water and differentiating in 0.5% acid alcohol and "blueing" in the blueing solution for 5 minutes.

18. Finally, the slides were dehydrated, followed by cleaning and mounting in DePeX (BDH Limited, Poole, England) and then covered with coverslips.

Sites of immunoperoxidase activity showed as brown colour. Assessment of the staining is discussed below.

Staining Characteristics and Assessment of Staining in ICC:

Ki67, ER and p53 staining was nuclear and the percentage of positive tumour cells with these markers was determined by counting 1000 cells per section using a computer-assisted semi-automatic image analysis system (Quantimet 500, Leica, Cambridge, UK) further described below. Cases were considered positive for ER and p53 when more than 10% of tumour cells were labelled as it is well established in the case of p53 that if more than 10% cells are positive for p53, then these cases are highly likely to have a p53 mutation (16). In the case of ER, the positivity cut-off has been determined in one study by correlating IHC with the biochemical assay (17). Rudas and colleagues have used a cut-off of 10% for ER, PR and p53 in DCIS (18). Quinn et al (19) have also applied 10% as a cut-off value to define positivity with ER, cerbB-2, bcl2 and p53 in DCIS. However, for the purpose of statistical analysis, the actual percentages were used to avoid missing any information. In the case of Ki67, it is not known from previous studies whether there is any biologically meaningful cut-off value, therefore the actual percentage of positively

stained Ki67 nuclei was used and this was referred to as Ki67 labelling index (LI). Also, the range for percentage of labelled cells as well as the median was calculated for each patient group and subgroups. This was done because the histograms did not show normal distribution of Ki67 in patient groups and parametric tests could not be applied. Bobrow et al (20) have arbitrarily used a cut off value of 10% for scoring KiS1 as positive or negative and found that KiS1 positivity was highly correlated to poorly differentiated (high nuclear grade) DCIS. Ringberg et al used a 10% cut-off for Ki67 positivity in DCIS as described in chapter 8 (21).

Elkablawy et al (22) have used a weighted score (WS) for evaluating bcl2 and p53 immunoreactivity in colorectal carcinomas based on the percentage of positive tumour cells and the staining intensity, previously described by Sinicrope et al (23). In this method a score of 0 – 4 is assigned according to the percentage of positively stained tumour cells as follows: 0 = <5% positively staining cells, 1 = 5 – 25%, 2 = 25 – 50%, 3 = 50 – 75%, 4>75%. Another score is assigned according to the intensity of staining as: 1 = weak, 2 = moderate and 3 = intense. Finally, the percentage and intensity scores are multiplied to produce a weighted score (WS). A WS of 6 or more was considered to represent a positive case with these markers.

The use of H score for the assessment of ER immunostaining similarly allows detailed correlation between hormone receptor levels and morphology but it is time-consuming.

CerbB-2 staining was membranous while bax and bcl2 staining was cytoplasmic (24). Labelling with these markers was assessed semi-quantitatively as: negative = 0, weak = 1, moderate = 2 and strong = 3. Cases were considered positive when they were either moderately or strongly positive (score 2 or 3) as in the case of cerbB-2 it is well established that a moderate to strong staining corresponds to increase in *cerbB-2* gene copy number (25). However, for the purpose of statistical analysis, actual score numbers were used in order to make use of all the available information. Furthermore, when cases were repeated for cerbB-2, the recommended scoring system in published UK guidelines (mentioned in chapter 4) was used, which assesses staining similarly as 0-3, but regards 3 as positive and 2 as borderline.

In most of the cases, the tumour staining was homogenous (i.e. uniform), but in some tumours a more heterogenous pattern (patchy staining) was observed. The assessment of

immnostaining was done blind without any prior knowledge of the histopathological grade. However, as some indication of the grade is usually apparent from the immunostained slides, therefore in most cases, assessment of immunostaining was done by a histopathologist as well (JC) who did it without the knowledge of the researcher's assessment for the purpose of obtaining double reading.

Factors Affecting ICC:

Main factors that need to be considered during ICC are tissue factors, antibody factors and the type of label used. Tissue factors include fixation, antigen retrieval and section thickness. Antibody factors include the dilutions of antibodies used, storage and the specificity.

Specificity Controls in ICC:

Positive controls: A positive control, known to contain the antigen being investigated, was included in every batch of immunostaining to confirm that all the reagents were working properly.

Negative controls: A negative control in which primary antibody was omitted, was also used with every batch of immunostaining to exclude non-specific staining.

Quality Control in ICC:

In view of the need for quality control in ICC, the factors affecting the staining (i.e., tissue and antibody factors) were kept as constant as possible. The tissue fixation was done with 10% buffered formalin in all cases; antigen retrieval times and techniques were constant for the different antigens at all times, section thickness was kept constant at 3μm. The antibodies were aliqouted on arrival and stored frozen at –20°C according to manufacturer's instructions. With every new batch of all the antibodies used, different dilutions were tried on positive control first to determine an optimum dilution. To reduce the chances of variability in the quality of staining, some sections were stained repeatedly on different occasions and the quality of staining ascertained to be the same before proceeding to the staining of all cases. Initially, for a few cases, the number of sections stained per block was more than one and the number of blocks per case was also more than one until the quality of staining was considered satisfactory enough to be comparable

in different cases. Finally cerbB-2 analysis was repeated in 20 cases as initial analysis suggested that some normal tissues were stained positive as well.

STATISTICAL ANALYSIS:

All statistical analyses were performed using the SPSS 10.0 statistical software package (SPSS Inc., Chicago, U.S.A.). The coefficient of variation (CV) was calculated to assess the variation between immunostaining of at least 10 sections, randomly selected, done twice on separate occasions. The relationships between the molecular markers studied - bax, bcl2, Ki67, ER, p53 and cerbB-2 were evaluated using the Spearman's rank correlation test which is a non-parametric test, as the histograms revealed distribution of these markers was not normal. The Kruskal-Wallis test, also a non-parametric test, was used to compare the distributions of the above-mentioned molecular markers among the different histological grades of DCIS and IDC. A p value of less than 0.05 was considered significant.

IMAGE ANALYSIS (IA):

Introduction:

Image analysis (IA) is about quantification, the classification of images and of objects of interest within images. IA enables quantification of parameters such as size, number, shape, area, position and optical density of identifiable parts of an image. Semi-automatic and automatic computer-assisted image analysis has made it possible to carry out a quantitative morphological characterization of immunostained cells and tissues. Computer-assisted image analysis has been used to quantify ER content in immunostained sections of breast carcinoma as described in chapter 5. Comparable results have been observed between the image analysis and flow cytometry to quantitate DNA content in breast (26) and other carcinomas. Furthermore, p53 immunoreactivity has been quantitated using a CAS-200 image analysis system in a large series of epithelial ovarian cancers and the results showed that larger the p53 positive nuclear area estimated by IA, poorer the prognosis (27). In another study (28), manual and image analysis estimation of PCNA by CAS-200 showed strong positive correlation.

Applications:

IA is used in a wide range of areas including autoradiography, angiography, chromosomal analysis, karyotyping, cytology, dental radiography, dermatology, fibre analysis, geology, histology, industrial inspection and quality assurance, kinetics, marine biology, motility studies, materials testing, muscle cell analysis, neurology, paint and pigment quality, paper quality, particle sizing, photographic emulsion monitoring, printing quality, robotics, tobacco analysis and wood structure.

The use of image analysis has the advantage that it provides an objective means of assessment for nuclear size (area) and an accurate means of counting nuclei stained positively by ICC. Once a protocol for counting nuclei has been established then it is less time consuming than conventional light microscopy. Other advantages apart from quantification and objectivity include consistency, reproducibility and speed of operation. The analysis is not completely automated, as the analyser is unable to identify two overlapping nuclei, counting them as one instead of two separate units. Therefore, analysis is semi-automated, being operator dependent. i.e., the operator has to identify the nuclei and instruct the image analyser to do the counting.

Technique:

The principal steps in IA include image capture, segmentation, object detection, measuring and analysis. A semi-automatic computer-assisted image analyser (Quantimet 500 by Leica, Cambridge Limited) was employed for the calculation of nuclear areas in the 50 cases of DCIS to classify cases into low, intermediate and high nuclear grades. H&E stained tissue sections of the cases studied were used. System calibration was done with light and threshold adjustment. The IA system consists of an IBM compatible computer, an image processor and a CCD colour video camera (Sony Corporation, Tokyo, Japan) attached to a standard Olympus BH-2 microscope (Olympus Optical Company, Hamburg, Germany), which was used to record images with a x40 objective. This gave a final magnification of x400. During measurement, areas of tumour containing stroma, blood vessels, inflammation or necrosis were excluded by the software editing mode. In this way, only tumour nuclei were entered into analysis. The IA was used for two purposes: (1) for nuclear area determination in all 50 cases of DCIS on H and E sections and (2) for counting of Ki67, p53 and ER positive nuclei in all 118

immunostained sections to determine percentage positive cells with these markers. The other three markers: cerbB-2, bcl2 and bax immunostaining was assessed semi-quantitatively as mentioned already because the software of the image analyser used was not developed enough at the time to allow quantification of total stained area for membranous and cytoplasmic stains.

In the case of DCIS, the mean of the nuclear areas of at least a 100 nuclei was calculated for each case. This was because determination of nuclear areas of 100 nuclei gave the most consistent and reproducible results as verified by a cumulative frequency plot similar to the one shown below. The criteria used for the classification of DCIS were as follows:

1- Low nuclear grade cases had a mean nuclear area of less than $40\mu m^2$.
2- Intermediate nuclear grade cases had a mean nuclear area between $40\text{-}45\mu m^2$.
3- High nuclear grade cases had a nuclear area of greater than $45\mu m^2$.

The problem with classification of DCIS by image analysis is that it is rather arbitrary and well-differentiated apocrine tumours may be mis-graded as high nuclear grade due to their large nuclei. Therefore, it is probably better used in adjunct to traditional morphological classification rather than replacing it.

In the case of immunostained sections, percentage of positive cells labelled with Ki67, p53 and ER was calculated, by counting at least a thousand cells in each section (20-30 high power fields). The figure of 1000 was derived from a cumulative frequency plot as shown in figure 7.2 for Ki67. Similar graphs, not shown here, were plotted for ER and p53 as well as nuclear areas.

Factors Affecting IA:

A large number of factors can affect measurement of objects in IA especially in histological image processing, such as thickness of the sections, colour and uniformity of the stain, level of illumination, image noise and image magnification. Absolute measurements on an image are difficult to make. It is preferable to make measurements relative to a standard. Absolute measurements involving colour images are especially difficult and require careful choice of camera and attention to calibration and quality control.

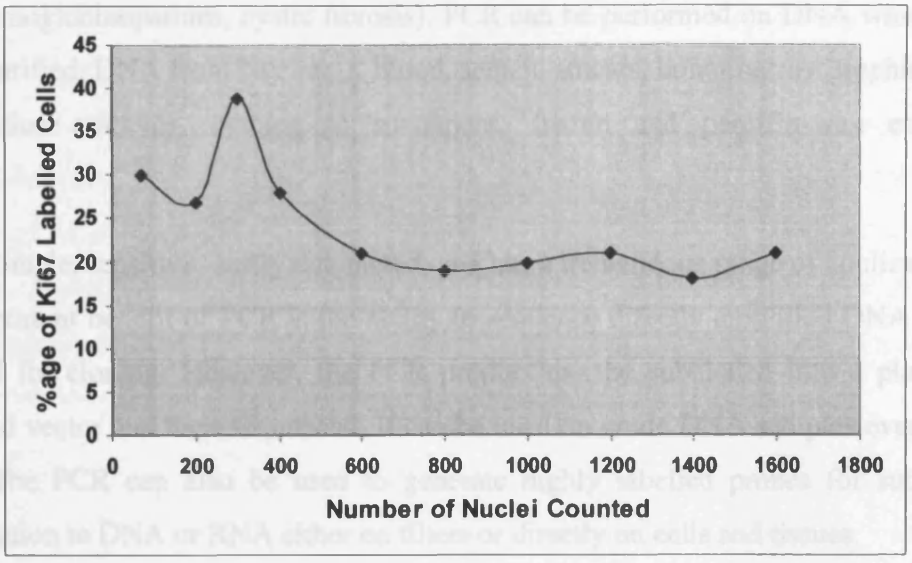


Figure 7.2; Cumulative frequency plot showing optimum number of cells counted to get a consistent result in a case with 20% cells labelled with Ki67.

POLYMERASE CHAIN REACTION (PCR):

Introduction:

Since its introduction in 1985, the polymerase chain reaction (PCR) has revolutionized molecular biology by providing a method for amplifying a small quantity of DNA (29;30). PCR allows millions of copies of any specific DNA sequence to be generated within a few hours. PCR has now become a valuable tool for the molecular biologist and can be used in a variety of contexts, including DNA sequencing, analysis of RNA transcription, mutagenesis, development of probes for genetic studies and screening of cDNA libraries.

Applications:

The PCR has a wide range of applications including detection of viruses [e.g. human papilloma virus (HPV) in cervical smear cells, HTLV-1 in T cell lymphomas], detection of chromosomal translocations in haematological malignancies [e.g. t(14;18) in follicular lymphomas, t(9;22) in CML and ALL], detection of point mutations (e.g. point mutations in *ras* oncogene, detection of tumour suppressor genes (e.g. p53, Rb), detection of amplification of an oncogene (e.g.*cerbB-2, MYC*), prenatal diagnosis of genetic disorders

(e.g. haemoglobinopathies, cystic fibrosis). PCR can be performed on DNA which is not highly purified, DNA from hair roots, blood, semen, smears, bone-marrow trephines, pre-implantation embryos, cytological specimens, frozen and paraffin-wax embedded sections.

PCR is simple, sensitive, easily automated, and has a tremendous range of applications. A very important benefit of PCR is the ability to sequence directly amplified DNA without the need for cloning. However, the PCR product can be subcloned into a plasmid or phagemid vector and then sequenced. It can be used on crude DNA samples even of low purity. The PCR can also be used to generate highly labelled probes for subsequent hybridization to DNA or RNA either on filters or directly on cells and tissues.

The PCR technique can be easily modified to amplify RNA. In this situation, extracted RNA is copied into double stranded cDNA using the retroviral enzyme reverse transcriptase, and the PCR is then performed on the cDNA copies.

The disadvantages are that PCR is not quantitative, it is notoriously sensitive to contamination and artifact resulting in false positives, therefore it is imperative that an appropriate range of controls is present in every assay and stringent laboratory procedures are followed. DNA replication during PCR has a significant error rate.

Principle:

The PCR is an *in vitro* technique that relies on the ability of DNA polymerases, in the presence of a mixture of deoxynucleotide triphosphates (dNTPs are dATP, dCTP, dGTP, dTTP), to copy a DNA strand using a short complementary DNA fragment as an initiating template. A pair of short DNA fragments called oligonucleotide primers, which are complementary to DNA sequences on opposite strands of the DNA flanking the fragment to be amplified, are used in the process (figure 7.3). The PCR involves repeated cycles of heat denaturation of the DNA (usually done at 90-94°C), annealing of the primers to their complementary sequences (usually done at 37-60°C) and extension of the annealed primers with DNA polymerase (usually done at 72-74°C by a heat-resistant DNA polymerase called Taq polymerase derived from *Thermus aquaticus*).

The extension products of one primer can serve as templates for the other primer, so that each successive PCR cycle doubles the amount of DNA synthesized in the previous one.

This chain reaction results in the exponential accumulation of the specific DNA target fragment to approximately 2^n, where n is the number of cycles. The amplified DNA can then be detected by agarose or polyacrylamide gel electrophoresis followed by direct visualization as a distinct band with ethidium bromide, which lights up the DNA when illuminated with ultraviolet (UV) light. The specificity and sensitivity of the PCR can be further increased by subsequent hybridization or DNA sequencing. The PCR requires rapid temperature changes, which can easily be achieved using commercially available thermal cyclers.

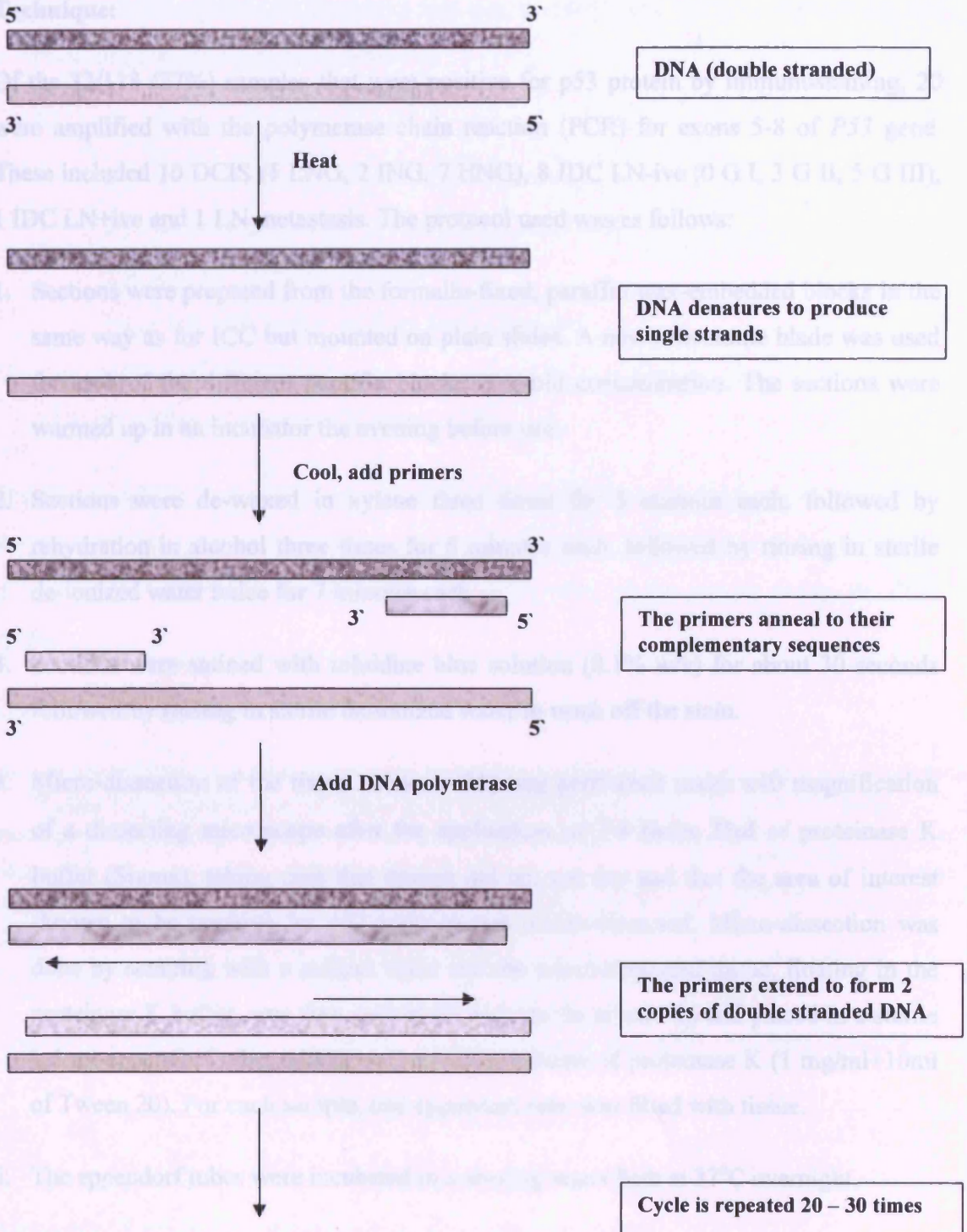


Figure 7.3; Polymerase chain reaction

Technique:

Of the 32/118 (27%) samples that were positive for p53 protein by immunostaining, 20 were amplified with the polymerase chain reaction (PCR) for exons 5-8 of *P53* gene. These included 10 DCIS (1 LNG, 2 ING, 7 HNG), 8 IDC LN-ive (0 G I, 3 G II, 5 G III), 1 IDC LN+ive and 1 LN metastasis. The protocol used was as follows:

1. Sections were prepared from the formalin-fixed, paraffin wax-embedded blocks in the same way as for ICC but mounted on plain slides. A new microtome blade was used for each of the different paraffin blocks to avoid contamination. The sections were warmed up in an incubator the evening before use.

2. Sections were de-waxed in xylene three times for 5 minutes each, followed by rehydration in alcohol three times for 5 minutes each, followed by rinsing in sterile de-ionized water twice for 7 minutes each.

3. Sections were stained with toluidine blue solution (0.1% w/v) for about 30 seconds followed by rinsing in sterile de-ionized water to wash off the stain.

4. Micro-dissection of the tissue sections (31) was performed under x40 magnification of a dissecting microscope after the application of 2-4 times 25µl of proteinase K buffer (Sigma), taking care that tissues did not get dry and that the area of interest (known to be positive for p53 protein) was micro-dissected. Micro-dissection was done by scraping with a scalpel blade and the micro-dissected tissue, floating in the proteinase K buffer, was then sucked up with sterile pipette tip and placed in a sterile 1.5 ml eppendorf tube, adding an equivalent volume of proteinase K (1 mg/ml+10ml of Tween 20). For each sample, one eppendorf tube was filled with tissue.

5. The eppendorf tubes were incubated in a shaking water bath at 37°C overnight.

6. Proteinase K was destroyed by incubating the eppendorf tubes at 95°C for 10 minutes.

7. The eppendorf tubes were centrifuged at 3500 rpm (rate per minute) for 10 minutes.

8. The supernatant was removed from each of the centrifuged eppendorf tubes and placed in new sterile eppendorf tubes and stored at 4°C until the PCR reaction was performed.

9. For the PCR reaction, PCR master mix was prepared. The reagents utilized for one PCR tube (one sample) are as follows:

    73µl PCR water (Sigma)
    2 µl upstream primer (5 nmol/ml) (Cruachem)
    2 µl downstream primer (5 nmol/ml) (Cruachem)
    2 µl dNTPs (10 mM) (Bioline)
    10 µl 10x NH_4 buffer, with no Mg^{2+} (Bioline)
    6 µl Mg^{2+} (Bioline)
    1 µl Taq polymerase (Bioline)

This 96 µl of PCR master mix was prepared for each DNA sample and 4 µl of each DNA sample from the eppendorf tubes was added to it. This was followed by the addition of 40 µl of mineral oil to each sample to prevent evaporation of the sample during the PCR cycles.

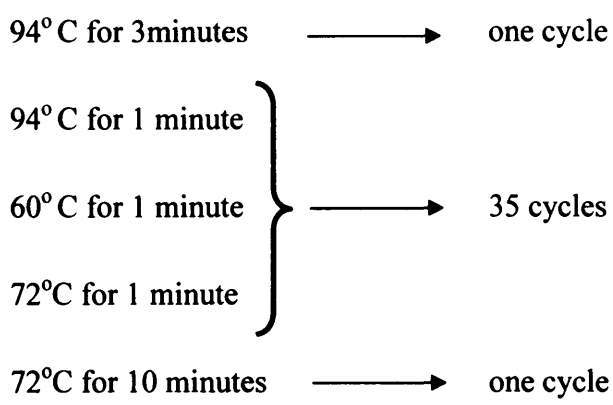
The sequences of the primers used are shown in table 7.4.

Table 7.4; Sequences of primers used in the PCR for exons 5 – 8 of *P53* gene.

Primers	Sequences (5'-3')	Nucleotide position	Bases
E5U	GGAATTCTGTTCACTTGTGCCCTGACTTTCAAC	13004-13030	33
E5D	GCAACCAGCCCTGTCGTCTCTCCA	13254-13267	24
E6U	CCAGGCCTCTGATTCCTCACTGATTG	13287-13312	26
E6D	AGGGCCACTGACAACCACCCTTAAC	1346713491	25
E7U	ACAGGTCTCCCCAAGGCGCACTGG	13947-13970	24
E7D	GGGCACAGCAGGCCAGTGTGCAG	14130-14152	23
E8U	AGGTAGGACCTGATTTCCTTACTGCC	14397-14422	26
E8D	GGAATTCTGAGGCATAACTGCACCCTTGGTCT	14619-14645	32

Explanation: E = exon, U = upstream of exon, D = downstream of exon.

10. The sample tubes were placed in PCR machine (Techne PHC) and run at the following programme. The same programme was followed for all four exons of p53.



11. The amplified mixture (5 µl) was run on an agarose gel and the rest was sent for sequence analysis. For the preparation of the agarose gel (32), 200 ml of Tris Borate EDTA (TBE) buffer (Sigma) (pH 8.3) was taken and 0.9 grams of agarose (Sigma) was added to 30 mls of this buffer, microwaving for 20-30 seconds, then allowing it to cool. Afterwards, 1 µl of ethidium bromide (Sigma) was added to this mixture and it was transferred to a gel electrophoresis kit. While this mixture cooled and solidified in the kit, 5 µl of gel loading solution (Sigma) was taken in sterile eppendorf tubes, the number of tubes corresponding to the number of samples, plus a positive and a negative control and one for the hyperladder. To one of the tubes, 5 µl of 100 bp DNA ladder (GIBCO BRL^{R}) was added and to the rest of the tubes, 5 µl each of the amplified DNA sample was added. When the gel solidified, 5 µl of each sample was loaded into each of the wells of the gel and the electrophoresis apparatus run for half an hour at a voltage of 100-200 volts. Afterwards, the gel was removed from the apparatus and visualized using an ultraviolet (UV) transilluminator, with the pictures taken at the same time to record the results. Positive and negative controls were used every time the PCR procedure was performed.

Factors Affecting PCR:

While the overall specificity of the PCR is determined by the two oligonucleotide primers, other factors influencing PCR specificity include (i) the times and temperatures of the annealing and extension steps, (ii) the magnesium concentration in the reaction buffer, and (iii) the concentration of Taq polymerase.

SEQUENCE ANALYSIS:

Introduction:

DNA sequencing is the determination of all or part of the nucleotide sequence of a specific DNA molecule. DNA sequencing is of major importance in molecular biology (33;34). The rate at which new sequence information is determined has increased rapidly over the last 20 years, to the extent that the entire human genome sequence of approximately $3x10^9$ base pairs will be determined within the next few years (35).

Applications:

The applications of DNA sequencing include confirmatory sequencing to check a particular construct or mutant (in the case of DNA whose sequence is already known), to sequence PCR products and strategies for sequencing large fragments of uncharacterised DNA.

The semi-automated sequencers do not require the use of radioisotopes as compared to manual sequencing and the time from running the gel to getting the sequence information is less than sequencing with radioactive labels, as the 12-24 hr autoradiography step is eliminated. It requires much less template DNA, typically 50 fmol, as compared to conventional or manual sequencing, which requires 500-1000 fmol of DNA.

With ABI 377, which was used in this study, the sequencing reaction is loaded onto a single lane of the gel, which allows a large number of samples to be run on the same gel, maximizing throughput. Up to 64 sets of sequencing reactions can be run simultaneously.

The disadvantages are that the data obtained are not of such high quality as those obtained by autoradiography. Furthermore, the 'hands-on' time is generally increased by the additional manipulations required. Semi-automated sequencers are very sensitive to contaminants of the polymerases used. Semi-automated sequencing is still generally less consistent and reliable than conventional sequencing.

Principle:

There are two sequencing techniques in current use. These are the 'chain termination' and 'chemical degradation' methods, also known as the 'Sanger' and 'Maxam and Gilbert'

methods respectively, after their original inventers. The chain termination method (or dideoxy chain termination method) depends on the enzymatic (by DNA polymerase) synthesis of labelled DNA, using specially modified nucleotides called dideoxynucleotides (ddNTP) to terminate the elongating strand. ddNTPs can be incorporated by DNA polymerases into a growing DNA chain through their 5' phosphate groups, just like dNTPs. However, they lack the 3'-OH group necessary for phosphodiester bond formation and chain elongation so the chain terminates at the precise point at which the ddNTP is incorporated. Four sets of reactions are performed on each template, differing only in which of the 4 ddNTP is added. The dNTP:ddNTP ratio is carefully selected so that the resulting labelled strands form a nested set of molecules up to several thousand bases long, each terminating at a specific base. These are separated according to size by high-resolution denaturing gel electrophoresis. This gives a 'ladder' of bands from which the DNA sequence can be read. The chemical degradation method is based on the base-specific chemical cleavage of a DNA molecule labelled at one end to generate a nested set of labelled molecules, each terminating at a specific base. Following high-resolution denaturing gel electrophoresis and detection of the labelled fragments, typically by autoradiography, the sequence of the original DNA can be read from the resulting sequencing 'ladder', just as in the chain termination method. The method in most common use is the dideoxy chain termination method. One of the limitations of the standard chain termination method is that only a single labelled DNA molecule is produced from each primer-template complex. The sensitivity of the method is limited therefore by the molar quantity of DNA template that can be used in the reaction. This is a major problem when sequencing large DNA templates or purified DNA fragments, for example PCR products. This limitation can be overcome by performing the sequencing reactions, denaturing the template DNA and repeating the reactions. Repeated denaturation is performed easily by thermal cycling on a PCR machine. This is called cycle sequencing. Cycle sequencing works on the same general principles as PCR, though the template DNA is amplified linearly rather than exponentially.

Semi-Automated Sequencers:

Semi-automated sequencing machines detect and analyse fluorescently labelled DNA with sophisticated detection systems and analysis software. The genome sequencing projects rely on the high throughput and reduced operator time of these instruments.

However, these are expensive, between 40,000-100,000 pounds. The ABI 377, which was the sequencer used in this technique, can simultaneously detect fluorescence at four different wavelengths, set to coincide with the emission of four different fluorescent dyes. As each DNA fragment moves past the detector, the base-specific dye is excited by the laser. The dye emits light at its characteristic wavelength. This fluorescent emission is separated according to its wavelength by a diffraction grating. The signal is detected by a charged couple device (CCD) camera and recorded and analysed by computer. The software separates the signal into four channels, each corresponding to one of the dyes. Use of a diffraction grating allows all four channels to be monitored simultaneously. Each dye is used to label strands terminating in one of the four possible dideoxynucleotides. The reaction mix is run in a single lane so that the colour of each band passing the detector represents the DNA sequence. The key advantage of automated sequencing is the automated data collection. The real-time data collection means that there is no opportunity to adjust for variable signal strength, therefore sequencing reactions must be carefully optimized. Furthermore, the extremely sensitive detectors can detect even minute levels of contamination by extraneous fluorochromes. The raw data from ABI 377 is in the form of lanes, each lane is a ladder of coloured bands, each representing a single nucleotide. This raw data is processed by the software to give the best known output, called an electropherogram. Values are assigned to each peak, so that the final output is the DNA sequence in text format. For a typical double-stranded DNA template, ABI 377 can read about 450-500 nucleotides at 99.8% accuracy. Five out of 20 samples that were amplified for each of the 4 exons of p53 were sent to MWG Biotech (Ebersberg, Germany) for sequence analysis. They performed cycle sequencing on an ABI 377 machine after purification of the samples.

Factors Affecting Sequencing by Semi-Automated Sequencers:

Factors, which diminish sequence quality, are impure template or primer preparations, inappropriate template or primer concentrations and inefficient removal of unincorporated labelled ddNTPs or primers. The real-time detection in these systems implies that the above mentioned factors need to be optimized prior to gel electrophoresis.

Problems that can arise in DNA sequencing can be template-specific or systematic. Template specific problems are commonly caused by either insufficient template DNA, contaminated template DNA, strong secondary structure in the template or no primer

binding site in the template. Sytematic problems are commonly caused by a defective reagent or suboptimal gel electrophoresis.

FOURIER TRANSFORM INFRARED (FTIR) MICROSPECTROSCOPY, CHEMICAL IMAGING AND FT-RAMAN SPECTROSCOPY:

Introduction:

At present the biochemical basis of human disease is a central tenet of modern medicine. Biochemical changes within tissue may either initiate disease or occur as a result of the disease process. The qualitative analysis of such changes provides important clues in the search for a specific diagnosis, and the quantitative analysis of biochemical abnormalities is important in measuring the extent of the disease process, designing therapy and evaluating the efficacy of the treatment. The conventional method for tissue analysis is histopathological examination of biopsy samples, but this method utilizes morphologic abnormalities rather than biochemical differences. Furthermore, pathological analysis requires tissue to be removed, with undesirable consequences. For example, in instances such as the arterial disease, the removal is extremely difficult or perhaps not possible. In cases where the tissue removal is permitted, the accuracy of diagnosis can be limited by factors related to random sampling and handling of tissue. In addition, conventional histopathology has limitations to its capability for providing immediate feedback and the precision to quantify the extent of the disease, particularly in the early stages. For these reasons, techniques that can provide real time and precise information about the tissue biochemistry are being developed as adjuncts to histological examination of biopsy specimens.

A number of other analytical techniques used for structural and compositional analysis of natural tissues include electron microscopy, X-ray diffraction and chemical analysis. However, in preparation for such analyses, the tissues can be subjected to processes which will alter their structure and/or composition. The ideal situation would be one in which minimal tissue preparation is required and physiological conditions are maintained as closely as possible with the possibility of in situ tissue analysis being particularly valuable.

Applications:

Recently, spectroscopy has emerged as one of the major tools for biomedical applications and has made significant progress in the field of clinical evaluation. Research has been carried out on a number of natural hard and soft tissues using Fourier Transform Infrared (FTIR), FT-Raman and Nuclear Magnetic Resonance (NMR) spectroscopic techniques. These vibrational spectroscopic techniques are non-destructive to the tissue and only very small amounts of material (micrograms to nanograms) are required. In addition, FTIR and FT-Raman spectroscopy also provides molecular level information allowing investigation of functional groups, bonding types and molecular conformations. Spectral bands in vibrational spectra are molecule specific and provide direct information about the biochemical composition. These bands are relatively narrow, easy to resolve and sensitive to molecular structure, conformation and environment. FTIR analysis has been reported on a number of biological tissues including bone (36), cornea (37), colon (38), lung (39), breast (40), heart (41) and liver (42).

The clinical management of breast cancer patients is guided by several clinical and pathological measures of tumour growth (43). These indicators include tumour size, histological type, grade and stage. However, an increasing proportion of cases now present at an earlier stage as axillary node negative or pre-invasive in situ disease, where determination of the likelihood of local or distant recurrence becomes even more dependent on assessment of the inherent biology of the tumour (44). Improvement in the accuracy of this assessment may in part come from better methods for the determination and integration of known prognostic factors. Amongst these tumour grade has been shown by some to be an excellent indicator of biological potential (3). However, reliable assessment of tumour grade has been hindered in the past by difficulties in determining reproducible criteria and the problem of interobserver variability (45).

In order to find a method of analysis which could measure characteristics within tumour tissue that would allow accurate and precise assignment in to grade categories, FTIR and FT-Raman spectroscopy techniques were employed to analyse breast tissue.

Principle:

FTIR spectroscopy is based upon the absorption of infrared light by covalent bonds as they vibrate. The frequency of light that is absorbed depends upon the nature of the bond

between the atoms, for example, C – C, C = C, C – H, C = O etc. The absorbed peaks for the vibrating atoms depend on their structure. Similarly FT-Raman spectroscopy depends upon the change in the polarisation of these molecules and the technique provides complimentary information alongside FTIR spectroscopy. Therefore, FTIR and FT-Raman spectra of a sample provide information about its chemical structure and composition. In biological systems, functional groups that strongly absorb infrared light and scatter the Raman source to initiate change in polarity of molecules include C = O, N – H, C – H and P – O groups.

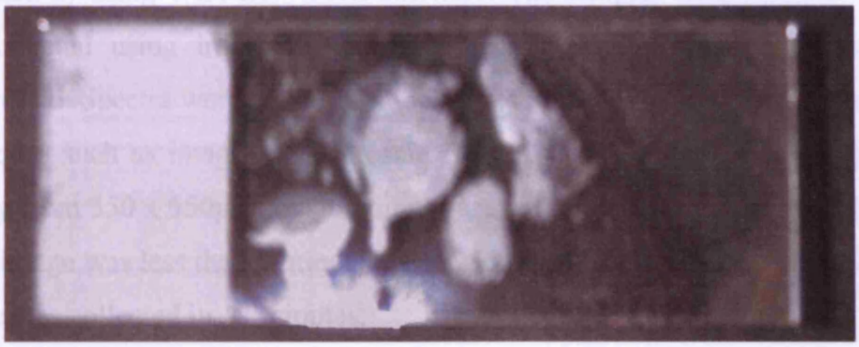
Therefore, intensity of the absorption bands in the infrared spectrum and scattering radiation in the Raman spectrum of a tissue provide information concerning lipid, protein, and nucleic acid contents of the sample, whereas the frequency of the absorption provides information relating to structure / conformation and intermolecular interactions. In other terms, FTIR and FT-Raman spectra of breast tissues provide information regarding the biochemistry of the tissue.

If the FTIR and FT-Raman spectra of the tissue can give information that reflects tissue biochemistry, then the changes in tissue biochemistry accompanying a disease process should be reflected in the changes in the obtained spectra of the diseased tissue. Therefore, it is reasonable to speculate that tumours of differing grades will also show different spectroscopic features, in principle allowing analysis of tumour spectra by grade.

Materials Analysed:

FTIR micro-spectroscopy, FTIR chemical imaging and FT-Raman spectroscopic techniques were employed to analyse a selection of the breast carcinoma cases. A total of 67 cases, which included 30 DCIS (10 LNG, 10 ING, 10 HNG), 30 IDC (10 GI, 10 GII and 10 GIII) and 7 normal cases were analysed. Details of the cases analysed are tabulated in table no. 8.35. Less common subtypes of DCIS for instance apocrine, signet-ring etc were not included in the analysis to minimize heterogeneity and the cases for each grade analysed were typical of the respective grade. Formalin-fixed paraffin wax-embedded sections from each case were 20μm thick and were dewaxed in xylene and rinsed in graded alcohols by immersion in 50, 70 and 100% absolute alcohol for 5 minutes each. For each section 15 spectra were recorded to evaluate the accuracy and

reproducibility. These 15 spectra were obtained from 3 nuclei (5 spectra per nucleus) in each case to obtain a spectrum of nuclear DNA and then these 15 spectra from each case were superimposed to obtain one representative spectrum from each case.



Figure, 7.4; Cancerous tissue sample mounted on a glass slide

FTIR Analysis of Breast Tissue:

Infrared spectra were obtained using a Nicolet 800™ spectrometer in conjunction with a Nicplan™ microscope equipped with a liquid nitrogen cooled MCTA detector. An Attenuated Total Reflectance (ATR) slide on an objective equipped with diamond crystal (Spectra-Tech™, USA) was mounted on the microscope. This ATR objective provides two modes of operation; one is for viewing of the specimen and the other for infrared (IR) analysis. The view mode allows viewing of the area of interest and the IR mode is for spectral data acquisition. A zoom on aperture was used to mask areas of interest. Spectra were obtained at $4cm^{-1}$ resolution, averaging 256 scans.

Three nuclei were analysed for each specimen as mentioned above, taking fifteen spectra for each tissue. FTIR spectrophotometer was calibrated by using polystyrene standard at $4cm^{-1}$ resolution. To evaluate the accuracy and precision of the technique, spectra of cancer tissues were overlaid and evaluated for peak positioning.

FTIR Chemical Imaging:

Recently, an infrared chemical imaging technique has been developed which also provides very useful information regarding the chemical structure of the tissues. In this study, a total of 67 samples were analysed using this technique. Details of the samples analysed are tabulated in table no. 8.35.

Spectra were obtained using a Digilab rapid-scan FTIR spectrometer Excalibur FTS 3000 equipped with the UMA-600 infrared microscope equipped with a liquid nitrogen cooled MCTA detector and a MCT focal plane array detector. The obtained spectra were processed by using WIN-IR™ PRO software. The samples were mounted on glass slides and collected using infrared reflectance techniques. A gold mirror was used as a background. Spectra were collected at $8cm^{-1}$ resolution, averaging 256 scans. A variety of techniques such as imaging and mosaic images were employed for samples with areas varying from 350 x 350μm to a maximum area of 1.4 x 1.4 mm. The collection time for a single image was less than 5 minutes with 256 scans. For the larger image at 64 scans, the mosaic was collected in 17 minutes.

FT-Raman Analysis of Breast Tissue:

FT-Raman spectra of the 67 breast tissue samples were recorded using a Nicolet 910 spectrophotometer, equipped with a Nd:YVO_4 near-infrared laser which eliminates the problems of sample fluorescence and photo-decomposition. The low power near-infrared laser in the FT-Raman spectrophotometer makes it possible to analyse biological tissues without the loss of sensitivity due to fluorescence of organics.

Prior to analysis of the tissues, FT-Raman spectrophotometer was calibrated using sulphur, diamond and cyclohexane as standards at $4cm^{-1}$ resolution. Spectra of all the samples analysed were checked against these standards to monitor the accuracy and precision of the technique by checking the peak positioning.

The tissue samples were analysed mounted on glass slides and attached to a metal post (sample holder). Scattered radiation was collected and collimated by 180^{o} reflective optical geometry. The spectra were obtained by using 450mW of laser power, 256 number of scans (depending upon samples) and $4cm^{-1}$ resolution. Data processing was performed using a 680D spectral workstation. Three nuclei were analysed for each specimen taking fifteen spectra for each tissue.

CHAPTER 8: RESULTS:

CLINICOPATHOLOGICAL FINDINGS:

All the 103 breast cancer cases studied were diagnosed at the Royal Free Hospital between 1985 and 1995. Two further cases were also studied, making a total of 105; one of these was diagnosed in 1969 and the other one in 1971. These were included for the purpose of comparison and both of these were low nuclear grade DCIS. However, these were not included in the final statistical analysis as the fixatives and the processing regimes used were probably different three decades ago. Furthermore, there was no control over delay in fixation at the time. It is well known that different fixatives affect the result of ICC (1), and also does delay in fixation (2). A short delay (up to 1 hour) in fixation results in significant decrease in mitotic count, with the potential for alteration of histological grade (2). A decrease in intensity and percent nuclear staining with p53 also occurs, MIB1 labelling or ER staining are not profoundly affected (2;3). From 1985-1995, duration of fixation was fairly uniform at the routine histopathology laboratory.

Of the cases studied, 50/103 (49%) were pure DCIS, 53/103 (51%) were IDC including 38/103 (37%) LN negative (non-metastatic) and 15/103 (15%) primary breast carcinomas with their metastatically involved lymph nodes. Age at diagnosis ranged from 26-88 years, with a median of 55 years. The 53 IDC patients tended to be younger than the DCIS patients, with an age range at diagnosis of 26-84 and a median of 52 years as compared with the 50 DCIS patients who had an age range at diagnosis of 33-88 with a median of 55 years. All except one of the patients were female, the one male had a grade II IDC, LN negative. This incidence of 1% is in agreement with that in the published literature on male breast cancer (4-6). In a more recent study, male breast cancer has been shown to be even less common (7). This case was not included in the final statistical analysis in view of the possible differences in tumour biology in males as compared to females (8).

All patients were treated by mastectomy or local excision with or without radiotherapy. 57/103 (55%) had their tumour in the left breast, 43/103 (42%) in the right breast, 1/103 (1%) presented with bilateral breast lumps and in 2/103 (2%) cases, the side was not recorded. 2/103 (2%) were post-chemotherapy, but apart from that no other patient had

received any prior treatment. All cases were sporadic, except one whose twin sister also had breast carcinoma.

Histological grading, typing and LN status of DCIS cases:

The 50 DCIS cases were all pure, that is without any prior history of breast disease in either ipsilateral or contralateral breast and also without any associated invasive component, although 1/50 (2%) had a focus of micro-invasion (this was an HNG). It is known that micro-invasive carcinoma (MIC) behaves clinically like DCIS rather than an established invasive carcinoma (8). However, since biologically it is not strictly DCIS, it was not included in the final statistical analysis.

Of the DCIS cases, 18/50 (36%) were LNG, 17/50 (34%) were ING and 15/50 (30%) were HNG. The distribution of DCIS in this study is unusual and suggests that the case mix is not typical. Histological type was recorded as: 9 comedo, 8 cribriform, 5 solid, 4 papillary, 4 mixed cribriform and papillary, 4 mixed comedo and cribriform, 3 mixed cribriform+solid+papillary, 3 comedo+cribriform+solid, 3 mixed comedo and solid, 2 micropapillary and clinging, 2 cribriform and clinging, 1 cribriform and solid, 1 apocrine and 1 mixed lobular and ductal in situ carcinoma. Comedo-type necrosis was found in 16/50 (32%) of DCIS and was more frequent in HNG; as 10/14 (71%) of HNG, 5/18 (27%) of ING and 1/17 (6%) of LNG had comedo-type necrosis. 15/50 (30%) of DCIS had associated benign changes including cystic change, fibrosis, apocrine metaplasia, focal epithelial hyperplasia, sclerosing adenosis and a papilloma. 3/50 (6%) of DCIS had associated atypical ductal hyperplasia (ADH). 3/15 (20%) of HNG DCIS had associated Paget's disease of the nipple.

Lymph node status was unknown in 24/50 (48%) DCIS, histologically proven LN-ive in 25/50 (50%) DCIS and 1/50 (2%) had LN micrometastasis, this particular case was the one with microinvasion and was a high nuclear grade. Although occult axillary lymph node metastases are considered insignificant clinically (9), this case as mentioned above, was excluded from the statistical analysis.

Histological grading, typing and LN status of IDC cases:

The 53 IDC cases included 38/53 (72%) LN negative (non-metastatic) and 15/53 (28%) LN positive (metastatic). Of 38 LN-ive cases, 32/38 (84%) were histologically proven LN

negative and 6/38 (16%) were clinically LN-ive, without any documentation of histological LN status. During final statistical analysis, when LN –ive cases were compared against LN+ive, the analysis was carried out twice. Firstly, including all LN-ive cases, and then only histologically LN-ive cases to eliminate the factor of poor sensitivity of clinical assessment.

Within 15 metastatic IDC cases, 10/15 (67%) had between 1-3 involved LN, 2/15 (13%) had 4-7 and 3/15 (20%) had more than 7 LN involved. Within the total 53 IDC, 7/53 (13%) were G I, 26/53 (49%) were G II and 20/53 (38%) were G III. Elston and Ellis (10) found G I to be 19%, G II 34% and G III were 47% in a series of 1830 patients. Among 38 LN negative patients, 6/38 (16%) were G I, 20/38 (53%) were G II and 12/38 (32%) were GIII. Among 15 metastatic cases, 1/15 (7%) was G I, 6/15 (40%) were G II and 8/15 (53%) were G III. The association of a higher histological grade with positive lymph node status is shown in table 8.1. Metastatic cases were most often grade III (8/15 or 53%) whereas only 12/38 (32%) of non-metastatic were grade III. However, a chi-squared test did not show this association to be statistically significant, with a p value of 0.25.

Table 8.1; Correlation of IDC grades with lymph node status

	Histological grade of IDC cases			
LN status	**G I**	**G II**	**G III**	**Total**
LN negative	6	20	12	38
LN positive	1	6	8	15
Total	7	26	20	53

Histological typing revealed that 36/38 (94%) of non-metastatic IDC were invasive ductal, 1/38 (3%) was lobular (G I, LN-ive) and 1/38 (3%) was mixed lobular (G II, LN-ive). All 15/15 (100%) of metastatic IDC were invasive ductal in type.

Some normal surrounding breast tissue was found in 30/53 (57%) of total IDC and 24/53 (45%) had associated DCIS. All G I IDC had either LNG or ING DCIS associated with them, none had HNG. G II IDC had all three grades of DCIS associated with them and G III had only HNG associated with them. This suggests that perhaps GII is not a pure disease, some cases are similar to LNG and GI and others may be biologically similar to HNG and GIII. The correlation of IDC grades with the grades of associated DCIS is

tabulated in table 8.2. A chi-squared test showed this association to be significant with a p value of <0.05.

Table 8.2; Correlation of IDC histological grades with associated DCIS grades				
	Histological grades of IDC			
DCIS grades	**G I**	**G II**	**G III**	**Total**
LNG	2	3	0	5
ING	2	6	0	8
HNG	0	6	5	11
Total	4	15	5	24

NUCLEAR GRADING OF DCIS BY IMAGE ANALYSIS:

The grading of 50 DCIS cases was done with the help of an image analyser on the basis of mean nuclear area (MNA) and it correlated well with the visual grading by light microscopy as shown in table 8.3 (p value <0.0001 using a chi-squared test). The quantitation was carried out without prior knowledge of histopathological grade.

Table 8.3; Correlation between light microscopy and IA for DCIS grading				
	Visual DCIS grading by light microscopy			
Mean nuclear area (MNA) measurement with range of MNA in brackets	**LNG**	**ING**	**HNG**	**Total**
38 (< 40 μm^2)	16	2	0	18
42 (40 – 45 μm^2)	0	17	0	17
49(> 45 μm^2)	0	1	14	15
Total	16	20	14	50

RESULTS OF IMMUNOSTAINING:

The assessment and scoring of bax, bcl2, cerbB-2, p53, ER and Ki67 was done as described in chapter 7. Results of the scoring are tabulated in tables 8.4-8.11. Tables 8.4-8.6 show the immunoprofiles of LNG, ING and HNG cases respectively. Tables 8.7-8.9 show the immunoprofiles of G I, G II and G III cases (LN negative) respectively. Table

8.10 shows the immunoprofiles of metastatic IDC and table 8.11 shows the immunostaining results of their corresponding lymph nodes. The empty boxes under the bax column indicate cases that were not immunostained with this particular marker. Note some immunoprofiles in each group suggest mixed profiles.

Table 8.4; Immunoprofiles of LNG cases

No.	Histology	Bax	Bcl2	Ki67	ER	p53	cerbB-2
1	LNG		2	1	63	0	1
2	LNG	2	2	2	96	0	1
3	LNG		2	3	15	20	1
4	LNG		2	2	51	0	2
5	LNG	2	3	8	70	0	2
6	LNG	3	0	5	0	0	0
7	LNG	0	0	8	17	0	2
8	LNG	1	0	7	97	0	0
9	LNG	2	2	8	94	0	0
10	LNG		2	5	49	0	1
11	LNG	3	2	5	52	0	0
12	LNG	2	0	22	0	0	0
13	LNG	2	0	4	30	0	0
14	LNG	0	0	2	13	0	0
15	LNG		2	8	54	0	1
16	LNG		1	6	48	0	1
17	LNG	3	2	1	26	0	1
18	LNG	2	2	12	53	0	2

Table 8.5; Immunoprofiles of ING cases

No.	Histology	Bax	Bcl2	Ki67	ER	p53	cerbB-2
1	ING		1	2	50	0	0
2	ING		0	3	1	1	0
3	ING		0	6	0	0	2
4	ING	0	1	10	75	1	1
5	ING		2	4	89	0	1
6	ING	2	3	8	78	0	0
7	ING	0	1	8	24	0	0
8	ING	1	1	14	46	0	1

Continued on next page.....

Table 8.5; Immunoprofiles of ING cases

No.	Histology	Bax	Bcl2	Ki67	ER	p53	cerbB-2
9	ING	2	0	13	0	6	2
10	ING	1	1	9	86	0	1
11	ING	2	2	6	91	10	0
12	ING		2	9	50	0	1
13	ING	2	2	7	63	0	2
14	ING		1	5	40	0	0
15	ING		2	32	75	10	2
16	ING	2	3	9	85	0	2
17	ING	0	1	4	83	0	1

Table 8.6; Immunoprofiles of HNG cases

No.	Histology	Bax	Bcl2	Ki67	ER	p53	cerbB-2
1	HNG	2	0	17	0	80	3
2	HNG	2	2	11	38	5	3
3	HNG	0	0	31	0	80	3
4	HNG		2	12	76	11	3
5	HNG		1	21	24	46	3
6	HNG	3	1	31	15	79	3
7	HNG		1	7	0	12	2
8	HNG	2	0	14	0	10	1
9	HNG	2	0	40	0	4	3
10	HNG	3	0	28	0	77	3
11	HNG	1	0	49	0	0	2
12	HNG	3	0	5	94	0	1
13	HNG		0	1	0	0	2
14	HNG		0	14	0	0	3
15	HNG	2	2	20	20	0	1

Table 8.7; Immunoprofiles of IDC GI, LN negative

No.	Histology	Bax	Bcl2	Ki67	ER	p53	cerbB-2
1	G I	2	1	21	50	2	1
2	G I	2	2	5	57	0	1
3	G I	0	0	2	50	0	1
4	G I	1	0	17	11	0	2
5	G I	2	2	18	39	0	1
6	G I	2	2	15	82	0	1

Table 8.8; Immunoprofiles of IDC GII, LN negative

No.	Histology	Bax	Bcl2	Ki67	ER	p53	cerbB-2
1	G II	2	0	24	37	0	0
2	G II	0	0	21	50	0	3
3	G II	1	1	80	45	30	2
4	G II	3	0	14	10	2	3
5	G II	2	1	16	0	60	2
6	G II	2	0	7	26	0	1
7	G II	3	3	12	80	0	1
8	G II	1	3	16	70	20	1
9	G II	2	1	15	50	0	1
10	G II	1	2	11	60	0	1
11	G II	2	3	9	50	1	1
12	G II	2	2	10	90	1	1
13	G II	1	0	23	0	1	1
14	G II	3	3	15	53	1	3
15	G II	1	2	12	40	0	1
16	G II	3	3	14	64	0	2
17	G II	1	2	2	30	0	1
18	G II	3	2	17	78	0	1
19	G II		2	12	37	1	2
20	G II	1	3	40	0	0	2

Table 8.9; Immunoprofiles of IDC GIII, LN negative

No.	Histology	Bax	Bcl2	Ki67	ER	p53	cerbB-2
1	G III	1	0	75	0	6	2
2	G III	0	0	26	0	0	2
3	G III	2	0	28	0	2	2
4	G III	2	0	39	0	2	2
5	G III	1	0	21	0	0	2
6	G III	1	0	25	50	0	1
7	G III	0	0	40	0	40	2
8	G III		0	50	0	1	1
9	G III	2	0	35	0	25	3
10	G III	2	3	32	81	50	0
11	G III	1	2	30	0	30	1
12	G III	2	1	50	0	70	1

Table 8.10; Immunoprofiles of IDC LN positive (metastatic)							
No.	Histology	Bax	Bcl2	Ki67	ER	p53	cerbB-2
1	G I		0	14	61	4	1
2	G II	2	2	15	34	0	2
3	G II		2	22	37	5	3
4	G II	2	1	9	69	15	1
5	G II	2	2	27	47	1	0
6	G II	1	1	32	72	70	2
7	G II	3	2	16	50	0	0
8	G III	2	1	33	20	10	1
9	G III	3	0	21	0	0	2
10	G III	1	1	28	43	30	3
11	G III	3	3	37	56	0	1
12	G III	1	0	27	0	50	2
13	G III	1	0	22	0	0	1
14	G III	1	2	18	43	10	0
15	G III	2	0	14	0	0	2

Table 8.11; Immunoprofiles of metastatic lymph nodes							
No.	Histology	Bax	Bcl2	Ki67	ER	p53	cerbB-2
1	LN		0	5	63	10	1
2	LN	1	1	25	40	0	2
3	LN		2	24	30	7	3
4	LN	2	2	3	40	25	2
5	LN	1	0	30	67	0	0
6	LN	1	0	24	53	70	2
7	LN	3	2	13	57	0	0
8	LN	1	0	37	20	20	1
9	LN	1	0	31	0	0	3
10	LN	1	0	36	50	40	1
11	LN	1	1	25	58	0	2
12	LN	0	0	38	0	50	2
13	LN	1	0	32	0	0	2
14	LN	1	2	22	53	20	0
15	LN	2	0	20	20	0	1

Table 8.12 shows the immunoprofiles of normal breast tissue that was near the cancerous tissue in 30/53 (57%) of cases.

Explanation:
N G I = normal tissue near grade I IDC, LN negative.
N G II = normal tissue near grade II IDC, LN negative.
N G III = normal tissue near grade III IDC, LN negative.
N GI M= normal tissue near grade I metastatic IDC.
N GII M= normal tissue near grade II metastatic IDC.
N GIII M= normal tissue near grade III metastatic IDC.

Table 8.12; Immunoprofiles of normal breast tissue

No.	Histology	Bax	Bcl2	Ki67	ER	p53	cerbB-2
1	N GI	2	1	5	60	0	1
2	N GI	2	0	1	57	0	1
3	N GI	0	0	1	50	0	1
4	N GI	3	0	6	48	0	2
5	N GI	2	2	4	67	0	2
6	N GI	3	1	1	30	0	1
7	N GII	3	0	10	53	0	0
8	N GII	2	0	1	55	0	2
9	N GII	2	2	4	50	0	1
10	N GII	3	2	1	51	0	1
11	N GII	2	3	1	48	0	1
12	N GII	3	0	1	0	0	0
13	N GII	0	2	1	34	0	0
14	N GII	3	1	5	30	0	0
15	N GII	1	0	1	51	0	0
16	N GIII	1	1	24	28	0	2
17	N GIII	3	0	9	33	0	2
18	N GIII	2	1	1	20	0	2
19	N GIII	1	0	3	0	0	2
20	N GIII	1	0	5	50	0	1
21	N GIII	0	1	10	24	0	1
22	N GIII		0	11	58	0	1
23	N GIII	1	3	2	50	0	1
24	N GIII	3	1	1	20	0	1
25	N GI M		2	2	53	0	1
26	N GII M		2	2	40	0	1
27	N GII M	2	2	1	70	1	1
28	N GIII M	3	1	1	2	0	0
29	N GIII M	2	1	4	50	0	1
30	N GIII M	2	1	3	20	0	0

Table 8.13 shows the immunostaining results of 24/53 (45%) DCIS associated with IDC.

Explanation:
LNG GI= lower nuclear grade associated with grade I IDC.
LNG GII= lower nuclear grade associated with grade II IDC.
ING GI= intermediate nuclear grade associated with grade I IDC.
ING G II= intermediate nuclear grade associated with grade II IDC.
ING GII M= intermediate nuclear grade associated with grade II metastatic IDC.
HNG GII=high nuclear grade associated with grade II IDC.
HNG GII M=high nuclear grade associated with grade II metastatic IDC.
HNG GIII=high nuclear grade associated with grade III IDC.
HNG GIII M=high nuclear grade associated with grade III metastatic IDC.

Table 8.13; Immunoprofiles of DCIS associated with IDC

No.	Histology	Bax	Bcl2	Ki67	ER	p53	cerbB-2
1	LNG GI	2	1	10	60	1	1
2	LNG GI	2	2	4	66	0	1
3	LNG GII	1	1	10	57	0	0
4	LNG GII	2	3	8	80	0	1
5	LNG GII	1	1	5	40	0	1
6	ING GI	0	0	6	48	0	2
7	ING GI	2	2	6	68	0	2
8	ING GII	2	0	13	46	0	0
9	ING GII	1	0	7	0	28	2
10	ING GII	2	0	6	26	0	1
11	ING GII	3	3	12	80	0	3
12	ING GII	1	3	16	70	16	1
13	ING GII M		2	10	40	2	2
14	HNG GII	1	0	17	50	0	3
15	HNG GII	2	1	16	0	60	2
16	HNG GII	0	2	14	0	0	1
17	HNG GII	3	3	15	58	0	3
18	HNG GIII	1	0	30	0	1	2
19	HNG GIII	2	0	35	0	35	3
20	HNG GII M	2	2	12	64	10	3
21	HNG GII M	2	1	20	50	0	0
22	HNG GIII M	2	1	25	10	10	1
23	HNG GIII M	3	1	19	1	0	2
24	HNG GIII M	1	1	24	43	27	3

5/24 (21%) were LNG, 8/24 (33%) ING and 11/24 (46%) HNG. The incidence of HNG was higher in this group of DCIS as compared to pure DCIS.

Twenty cases were repeated for cerbB-2 without antigen retrieval and gave the following results shown in table 8.14.

Table 8.14; Cases stained for cerbB-2 without antigen retrieval

Number	Histology	Result	% Positive cases
1	Normal breast	Negative	0/15 Normal
2	Normal breast	Negative	
3	Normal breast	Negative	
4	Normal breast	Negative	
5	Normal breast	Negative	
6	DCIS GI	Negative	2/5=40% DCIS
7	DCIS GII	Negative	
8	DCIS GII	Negative	
9	DCIS GIII	Positive	
10	DCIS GIII	Positive	
11	IDC GI	Negative	3/10=30% IDC
12	IDC GII	Negative	
13	IDC GII	Negative	
14	IDC GII	Negative	
15	IDC GII	Negative	
16	IDC GII	Negative	
17	IDC GIII	Negative	
18	IDC GIII	Positive	
19	IDC GIII	Positive	
20	IDC GIII	Positive	

The incidence of bax, bcl2, Ki67, ER, p53 and cerbB-2 expression in normal breast tissue adjacent to IDC and in DCIS and IDC grades, metastatic IDC and lymph nodes is shown in table 8.15 and also in figures 8.2, 8.4, 8.6, 8.8, 8.10, 8.12. For Ki67, the range of labelled cells is recorded with medians in brackets. For other markers, the actual number of positive cases is recorded with percentages in brackets.

Table 8.15; Incidence of bax, bcl2, Ki67, ER, p53 and cerbB-2 in normal and cancerous breast tissue

	Bax	Bcl2	Ki67	ER	p53	cerbB-2
Normal	**19/27 (70%)**	**9/30 (30%)**	**1-24 (2)**	**27/30 (90%)**	**0/30 (0%)**	**7/30 (23%)**
LNG	9/12 (75%)	11/18 (61%)	1-22 (5)	16/18 (89%)	1/18 (6%)	4/18 (22%)
ING	5/10 (50%)	7/17 (41%)	2-32 (8)	14/17 (82%)	2/17 (12%)	5/17 (29%)
HNG	8/10 (80%)	3/15 (20%)	1-49 (17)	6/15 (40%)	8/15 (53%)	12/15 (80%)
Total DCIS	**22/32 (69%)**	**21/50 (42%)**	**1-49 (8)**	**36/50 (72%)**	**11/50 (22%)**	**24/50 (48%)**
G I	4/6 (67%)	3/6 (50%)	2-21 (16)	6/6 (100%)	0/6 (0%)	1/6 (17%)
G II	11/19 (58%)	11/20 (55%)	2-80 (16)	17/20 (85%)	3/20 (15%)	8/20 (40%)
G III	5/11 (45%)	2/12 (17%)	21-75 (34)	2/12 (17%)	5/12 (42%)	7/12 (58%)
Total IDC	**20/36 (56%)**	**16/38 (42%)**	**2-80 (18)**	**25/38 (66%)**	**8/38 (21%)**	**16/38 (42%)**
IDC	8/13 (62%)	6/15 (40%)	9-37 (22)	11/15 (73%)	6/15 (40%)	7/15 (47%)
LN	3/13 (23%)	4/15 (27%)	3-38 (25)	12/15 (80%)	7/15 (47%)	8/15 (53%)
Grand Total*	**50/81 (62%)**	**43/103 (42%)**	**1-80 (17)**	**72/103 (70%)**	**25/103 (24%)**	**47/103 (46%)**
Grand Total**	**53/94 (56%)**	**47/118 (40%)**	**1-80 (15)**	**84/118 (71%)**	**32/118 (27%)**	**55/118 (47%)**

* result of all 103 cases
** includes all 103 cases plus 15 LN=118

Note; this was preliminary cerbB-2 analysis as on repeat, normal cases were negative and IDC positivity was 30% as tabulated in Table 8.14.

Bax immunostaining:

The bax staining localization was predominantly cytoplasmic but in several cases nuclear staining was also observed (figure 8.1a). Figure 8.1 (a-n) shows cases of bax positive normal breast, LNG, ING and HNG DCIS, G I, G II and G III IDC.

Normal breast tissue adjacent to tumour areas showed positive bax staining in 70% of cases. Overall, 62% of breast carcinoma cases were positive, with 69% of DCIS, 56% of non-metastatic IDC and 62% of metastatic IDC showing positive bax staining as depicted in table 8.15. Metastatic lymph nodes showed only 23% positivity. Figure 8.2 shows bax incidence compared in normal breast tissue, different grades of DCIS and IDC and lymph node metastases with their primary tumour. Normal breast tissue was also compared with DCIS and IDC as whole groups to enable comparison with the results in the literature.

Figure 8.1; Photomicrographs of bax immunostaining showing cases of normal breast tissue, LNG, ING and HNG DCIS, GI, GII and GIII IDC.

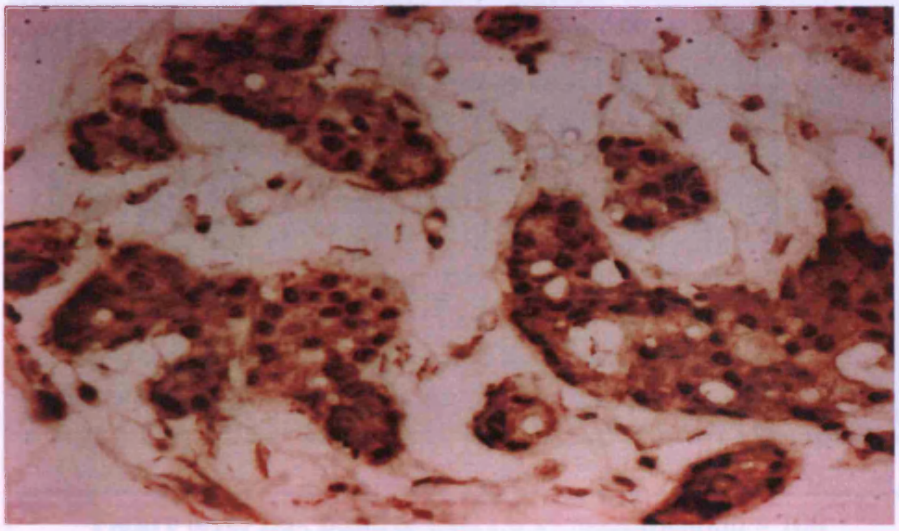


Figure 8.1a; Normal breast tissue positive for bax showing cytoplasmic and some nuclear staining at a magnification x 400

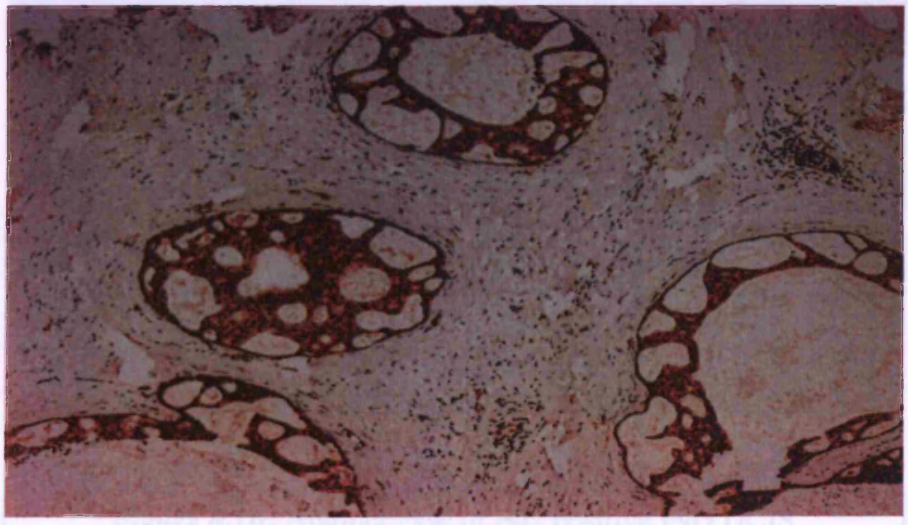


Figure 8.1b; Bax positive LNG DCIS magnification × 100

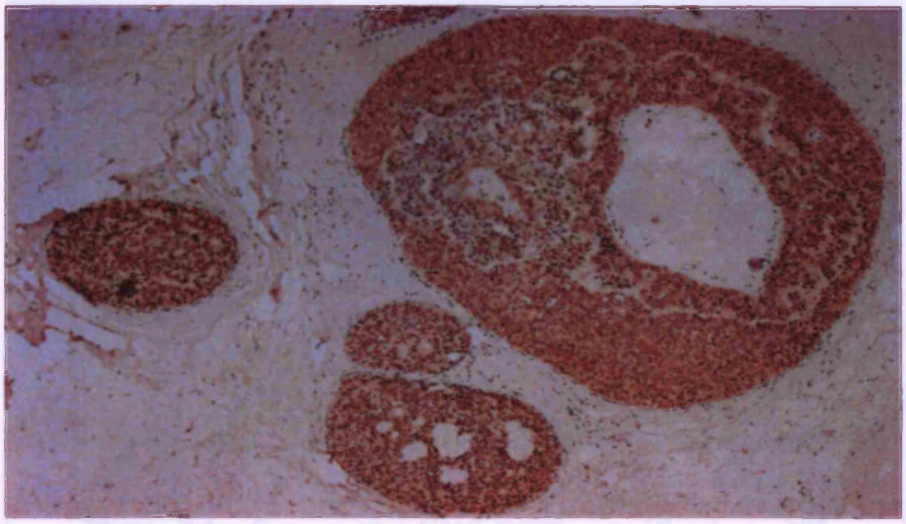


Figure 8.1c; Bax positive ING DCIS magnification × 100

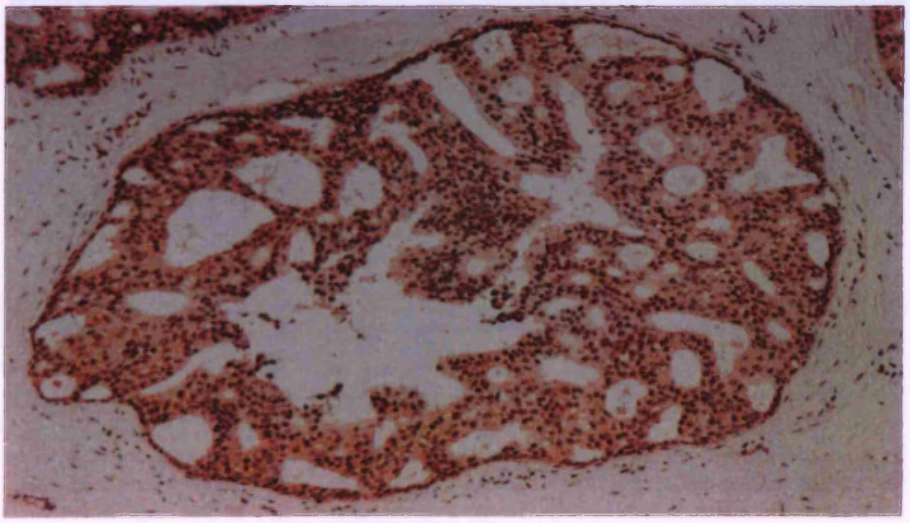


Figure 8.1d; Another case of Bax positive ING DCIS magnification × 100

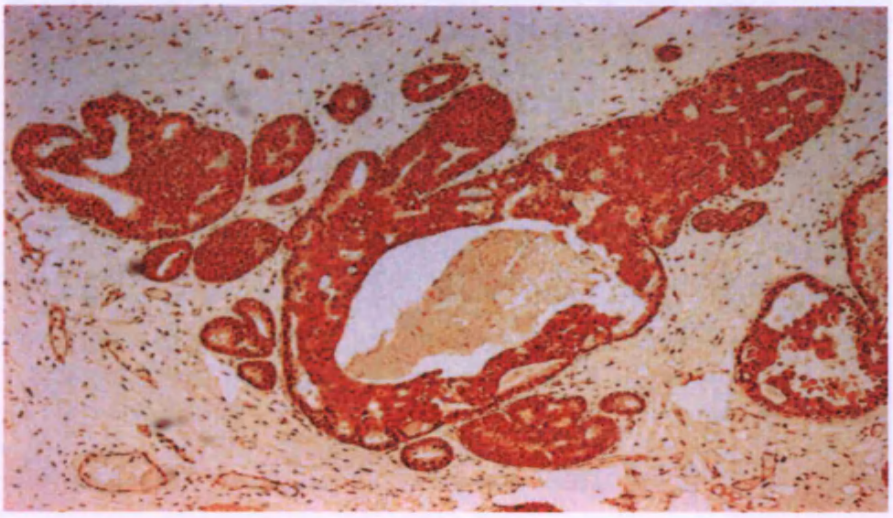


Figure 8.1e; Bax positive HNG DCIS magnification × 100

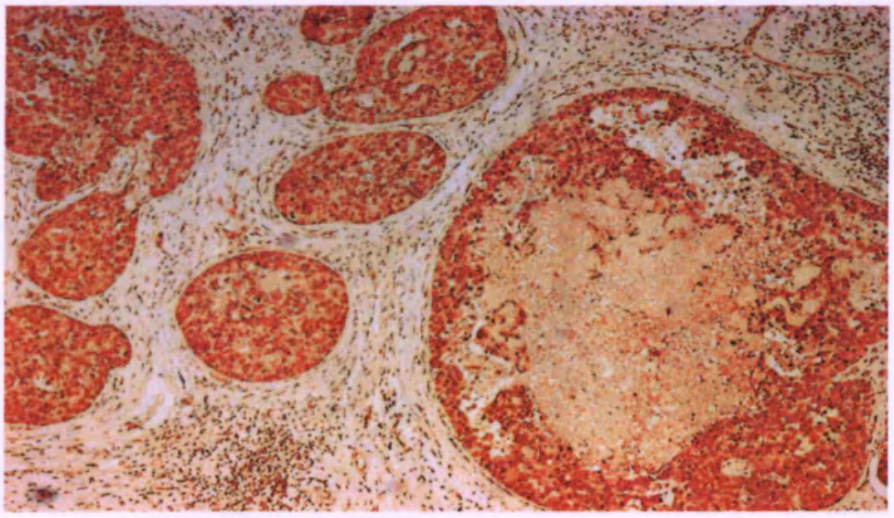


Figure 8.1f; Another case of Bax positive HNG DCIS magnification × 100

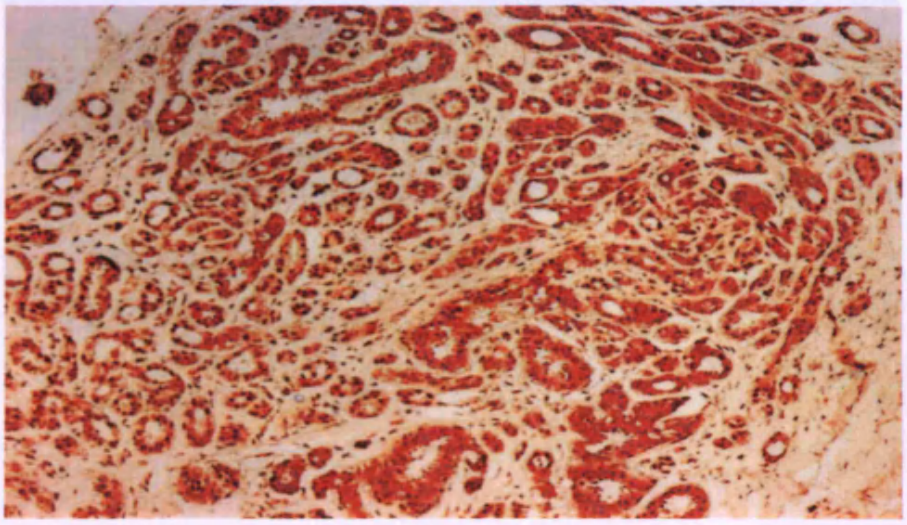


Figure 8.1g; Bax positive grade I IDC magnification × 100

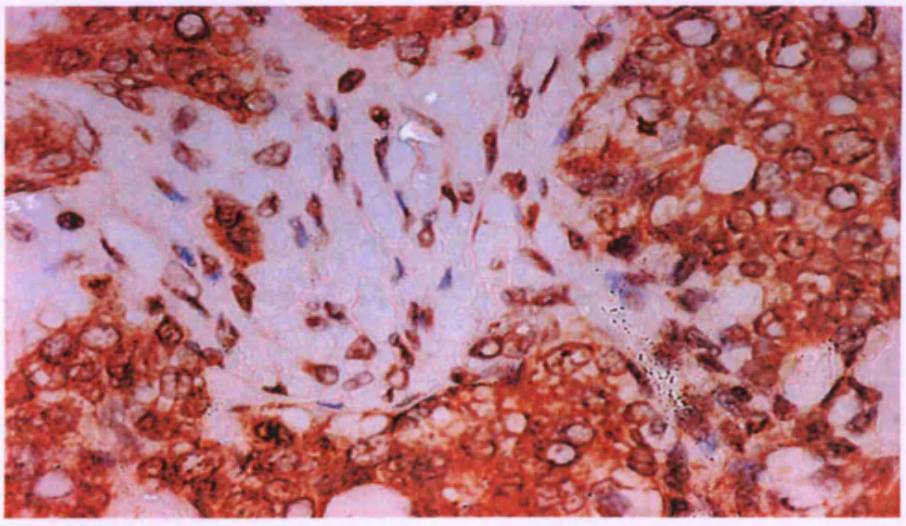


Figure 8.1h; Same case as in figure 8.1h at magnification × 400

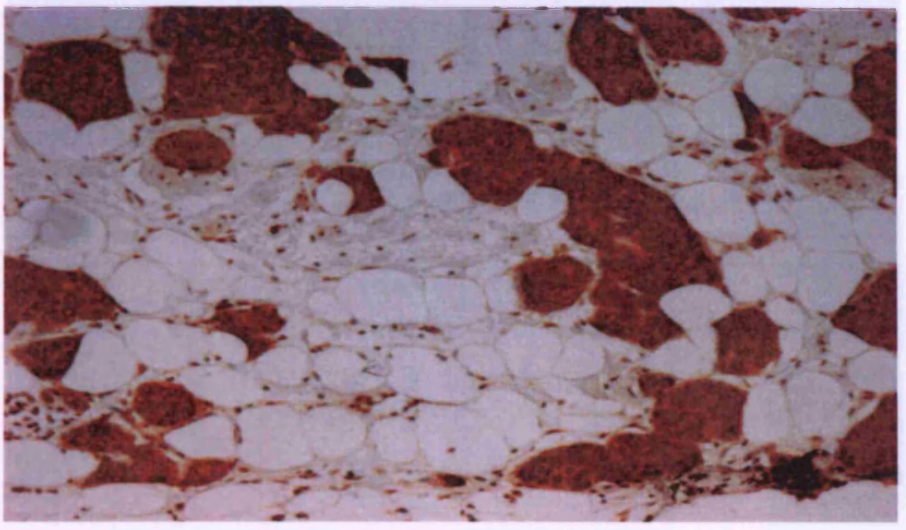


Figure 8.1j; Bax positive grade II IDC magnification × 100

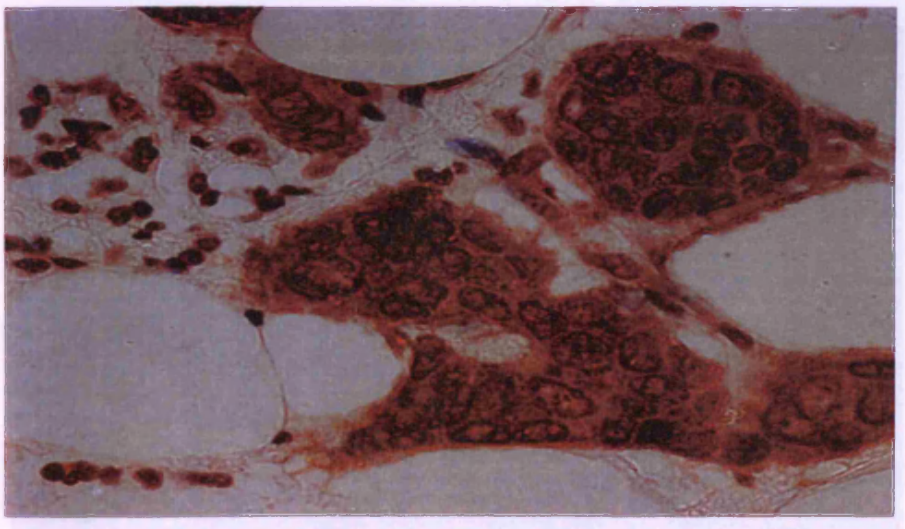


Figure 8.1k; Same case as in figure 8.1k at magnification ×400

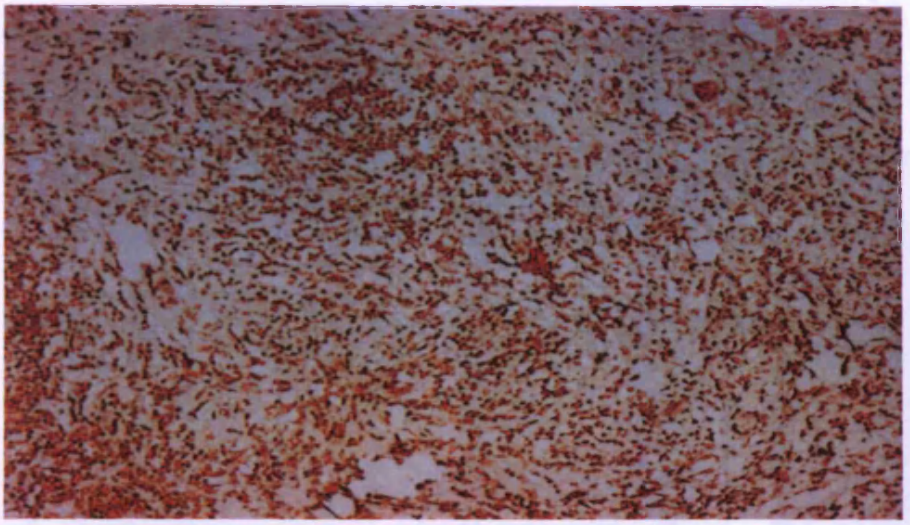


Figure 8.1m; Bax positive grade III IDC magnification ×100

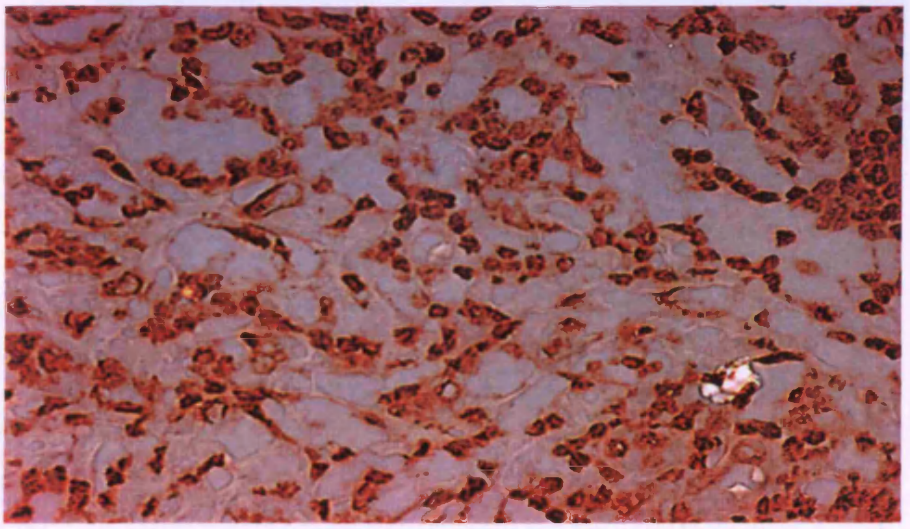


Figure 8.1n; Same case in figure 8.1n at magnification ×400

Figure 8.2; Bar graphs showing bax incidence in different groups of patients

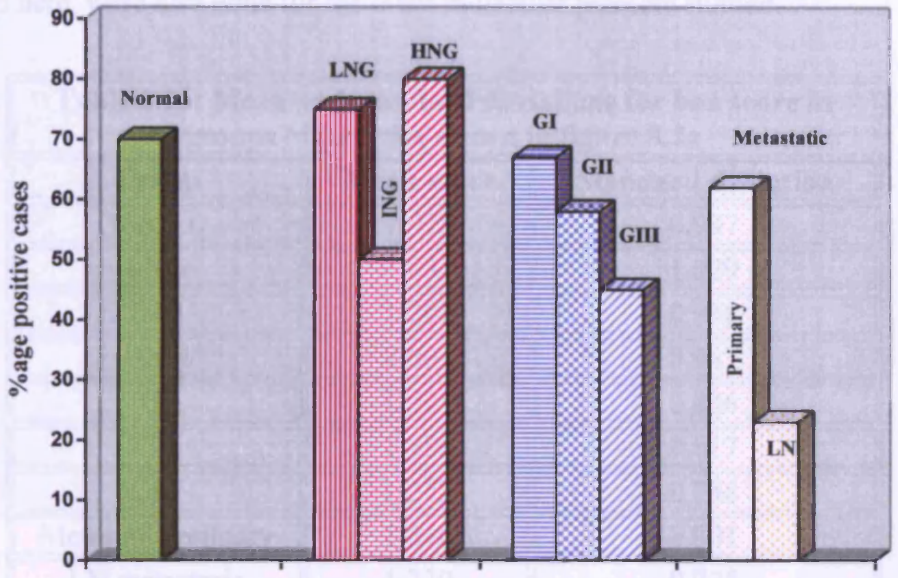


Figure 8.2a; Bax incidence in normal breast tissue, DCIS and IDC sub groups, metastatic primary and lymph node

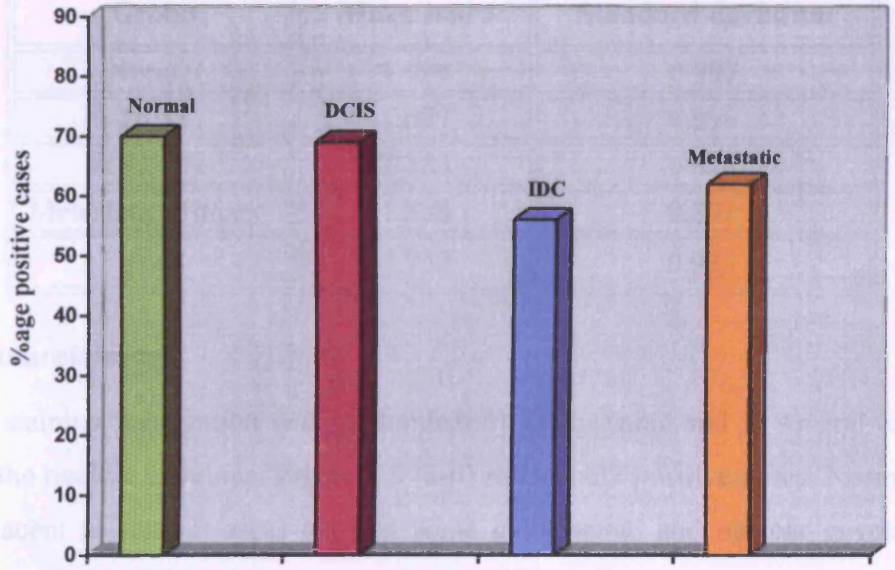


Figure 8.2b; Bax incidence in normal breast tissue as compared to DCIS as whole group, IDC lymph node negative and metastatic primary

The mean scores and standard deviations for bax for the different patient groups in figure 8.2 are shown in tables 8.16 and 8.17. These were done to assess variability in different groups. In this case, there was no significant difference statistically. Similar calculations, not shown here, were also done for the other molecular markers studied.

Table 8.16; Mean and standard deviations for bax score in groups of patients shown in figure 8.2a

Group	Mean score	Standard deviation
Normal	1.925	0.997
LNG	1.833	1.029
ING	1.201	0.918
HNG	2.001	0.942
GI	1.501	0.836
GII	1.789	0.917
GIII	1.272	0.786
Metastatic primary	1.846	0.801
LN metastasis	1.230	0.725
Total	1.677	0.923

Table 8.17; Mean and standard deviations for bax score in groups of patients shown in figure 8.2b

Group	Mean score	Standard deviation
Normal	1.926	0.997
DCIS	1.687	0.998
IDC, LN-	1.583	0.874
Metastatic primary	1.846	0.801
Total	1.732	0.933

Bcl2 immunostaining:

The bcl2 staining localization was predominantly cytoplasmic and in several cases also involved the nuclear envelope. Figure 8.3 (a-b) shows bcl2 positive cases. Normal breast tissue adjacent to tumour areas showed some cytoplasmic and nuclear envelope bcl2 staining in at least 19/30 (63%) cases and 8/30 (27%) of these were considered positive which served as internal positive controls in bcl2 negative tumours. Overall, 42% of breast carcinoma cases were positive, with 42% DCIS, 42% non-metastatic IDC and 40% metastatic IDC showing positive staining as shown in table 8.15 and figure 8.4. The metastatic LNs showed positivity in 27% of cases.

Figure 8.3; Photomicrographs of bcl2 positive normal breast and DCIS.

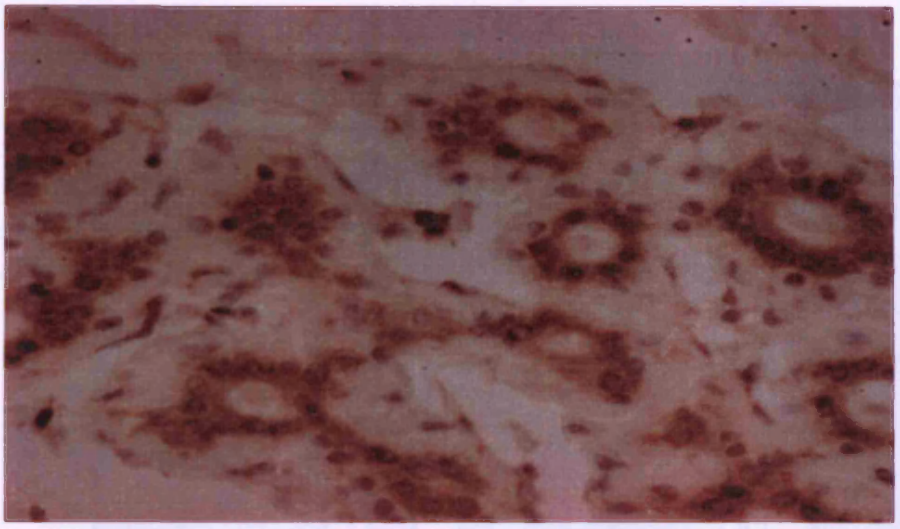


Figure 8.3a; Normal breast tissue positive for bcl2 magnification x 400

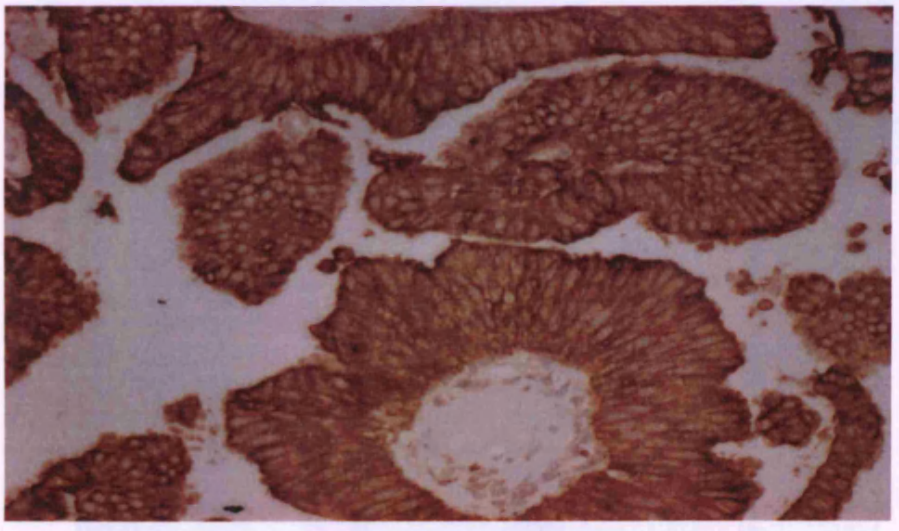


Figure 8.3b; A micropapillary ING DCIS showing bcl2 positivity at a magnification of x 100

Figure 8.4; Bar graphs showing bcl2 incidence in different groups of patients

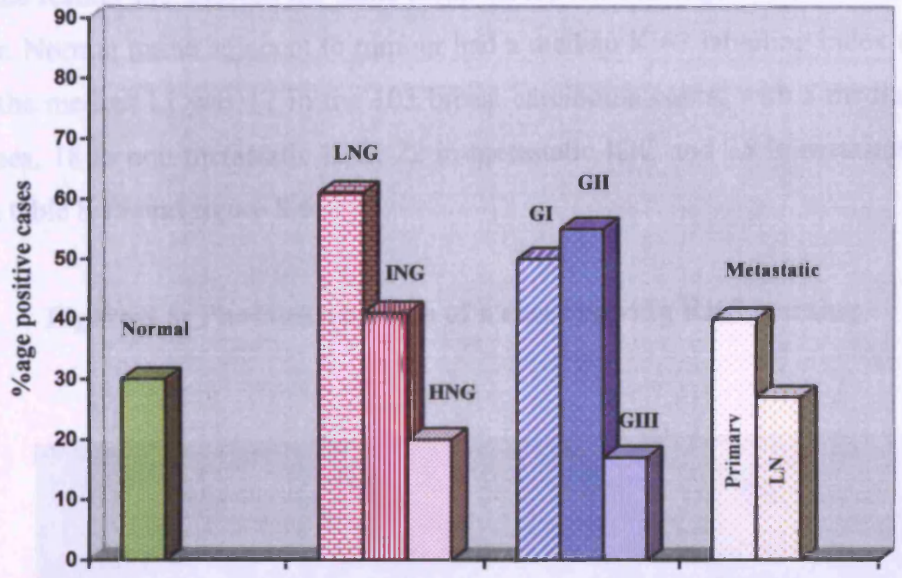


Figure 8.4a; Bcl2 incidence in normal breast tissue, DCIS and IDC sub-groups, metastatic primary and lymph node

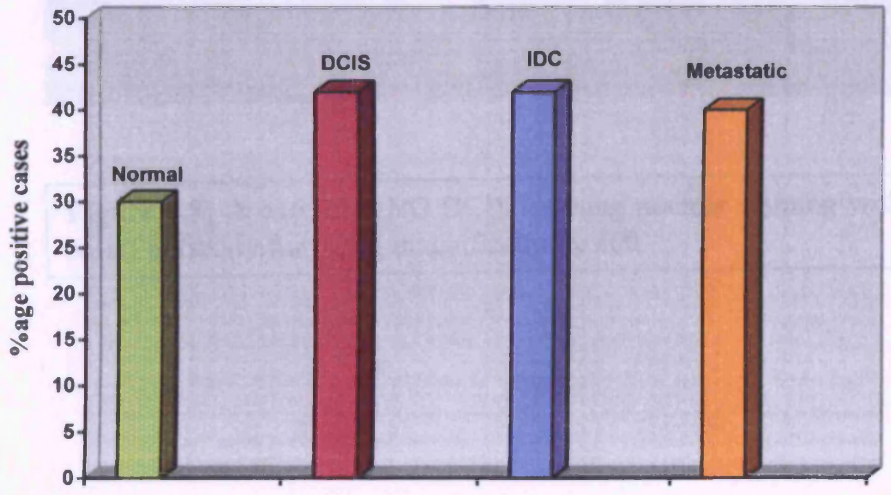


Figure 8.4b; Bcl2 incidence in normal breast tissue as compared to DCIS as whole group, IDC lymph node negative and metastatic primary

Ki67 immunostaining:

Ki67 immunostaining was nuclear as shown in figure 8.5. However 4/118 cases showed strong membrane staining including one HNG DCIS, one GIII non-metastatic IDC and one metastatic IDC with its corresponding LN. These were repeated at least 3 times to confirm the results and showed the same result on repeat. The significance of this finding is unclear. Normal tissue adjacent to tumour had a median Ki67 labelling index (LI) of 2. Overall, the median LI was 17 in the 103 breast carcinoma cases, with a median of 8 in DCIS cases, 18 in non-metastatic IDC, 22 in metastatic IDC and 25 in metastatic LN as shown in table 8.15 and figure 8.6.

Figure 8.5; Photomicrograph of a case showing Ki67 staining

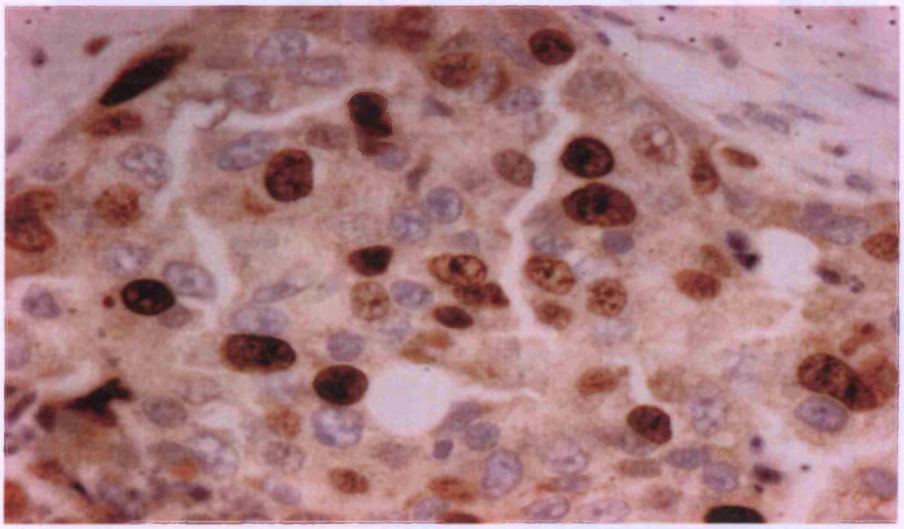


Figure 8.5; A case of HNG DCIS showing nuclear staining with MIB1 antibody for Ki67, magnification x 400.

Figure 8.6; Bar graphs showing median values for Ki67 labelling index in different groups of patients.

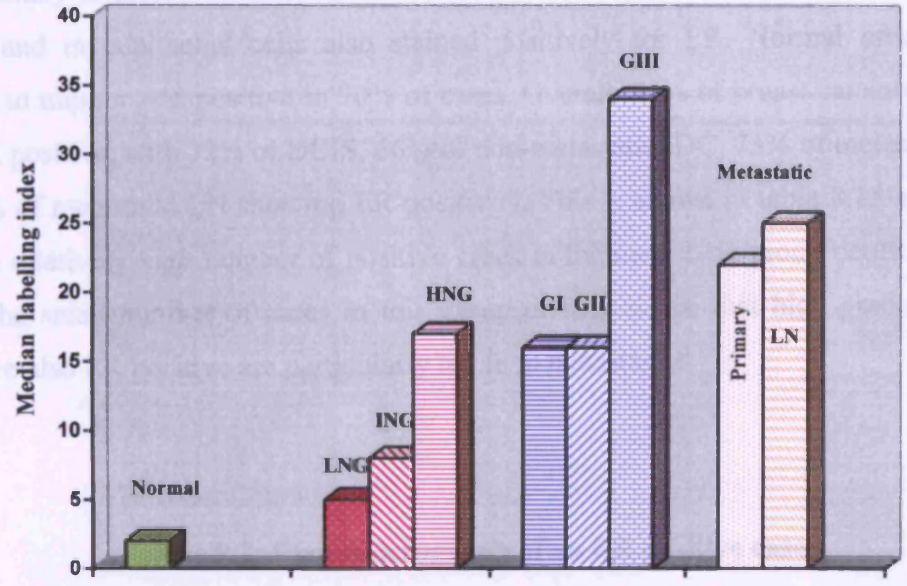


Figure 8.6a; Ki67 incidence in normal breast tissue, DCIS and IDC sub groups, metastatic primary and lymph node

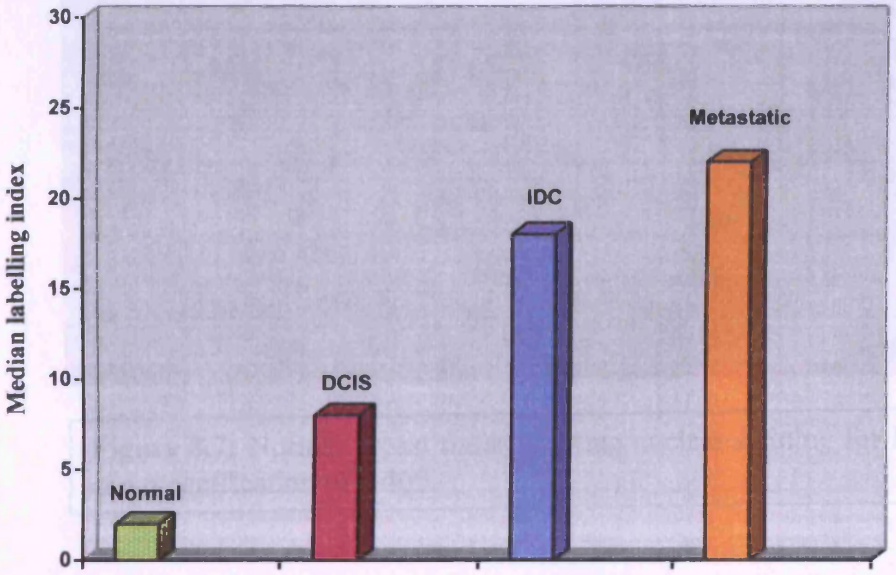


Figure 8.6b; Ki67 incidence in normal breast tissue as compared to DCIS as whole group, IDC lymph node negative and metastatic primary

ER immunostaining:

ER staining was nuclear as shown in figure 8.7. There was occasional pale cytoplasmic staining of tumour cells in some cases. The proportion of weakly staining nuclei as well as moderately to darkly staining nuclei was recorded in each case. In some cases, a few stromal and myoepithelial cells also stained positively for ER. Normal breast tissue adjacent to tumour was positive in 90% of cases. Overall, 70% of breast carcinoma cases were ER positive, with 72% of DCIS, 66% of non-metastatic IDC, 73% of metastatic IDC and 80% of metastatic LN showing ER positivity. This is shown in table 8.15 and figure 8.8. The relatively high number of positive cases in the metastatic group could either be due to the small number of cases in this group or it could be that high grade tumours which are also ER positive are particularly liable to metastasise.

Figure 8.7; Photomicrograph of an ER positive case

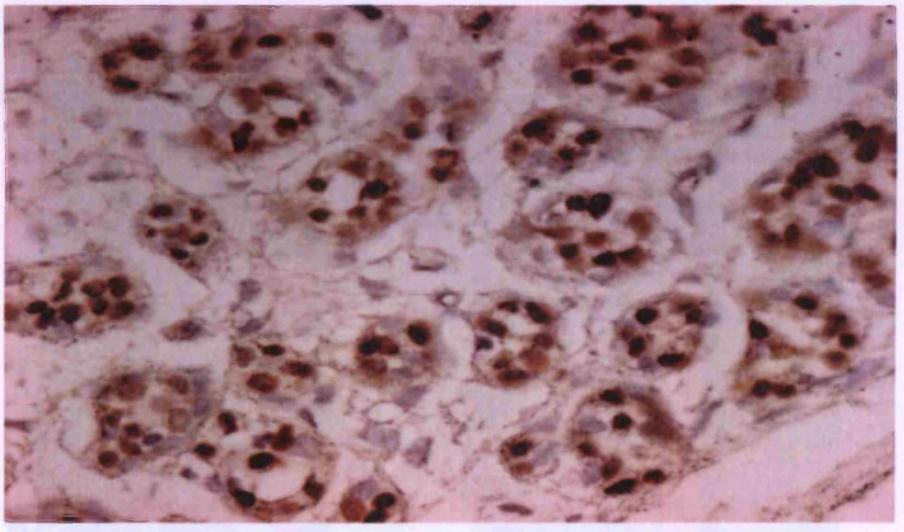


Figure 8.7; Normal breast tissue showing nuclear staining for ER at a magnification of x 400.

Figure 8.8; Bar graphs showing ER incidence in different groups of patients

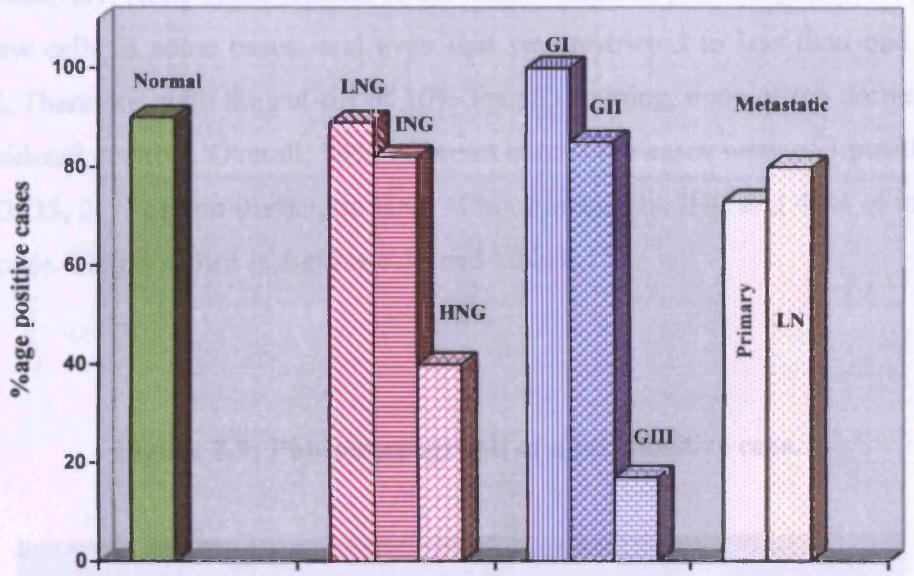


Figure 8.8a; ER incidence in normal breast tissue, DCIS and IDC sub groups, metastatic primary and lymph node

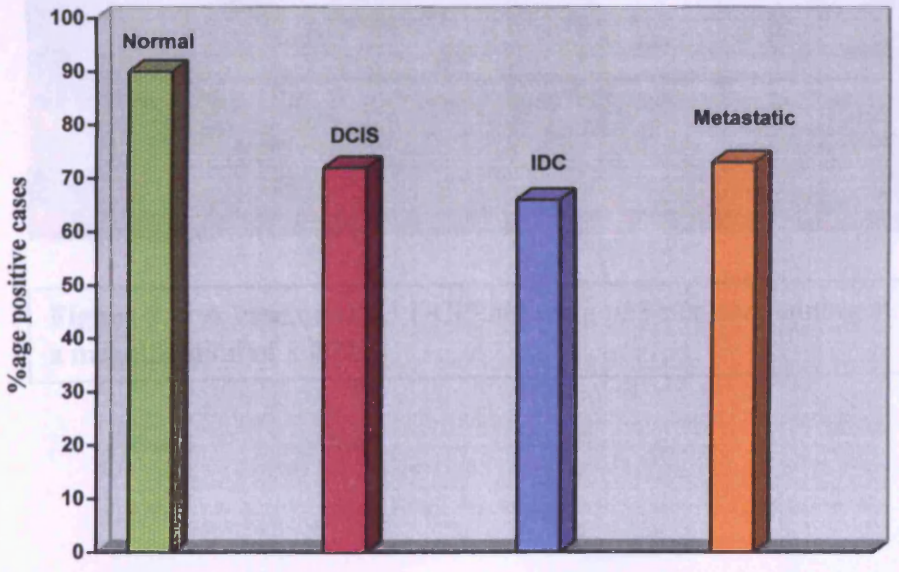


Figure 8.8b; ER incidence in normal breast tissue as compared to DCIS as whole group, IDC lymph node negative and metastatic primary

P53 immunostaining:

P53 immunostaining was nuclear as shown in figure 8.9. In some cases, stromal cells also stained positively. None of the normal breast tissue exhibited positive p53 staining except a very few cells in some cases, and even that was restricted to less than one cell per thousand. Therefore, with the cut-off of 10% for p53 staining, none of the normal tissues was considered positive. Overall, 24% of breast carcinoma cases were p53 positive, with 22% of DCIS, 21% of non-metastatic IDC, 40% of metastatic IDC and 47% of metastatic lymph nodes. This is shown in figures 8.10 and table 8.15.

Figure 8.9; Photomicrograph of a p53 positive case.

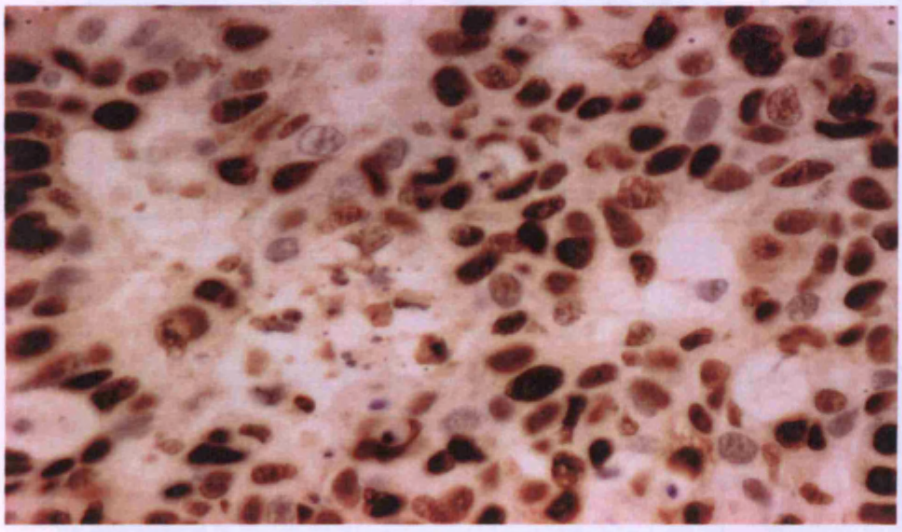


Figure 8.9; A case of HNG DCIS showing p53 nuclear staining at a magnification of x 400.

Figure 8.10; Bar graphs showing p53 incidence in different groups of patients.

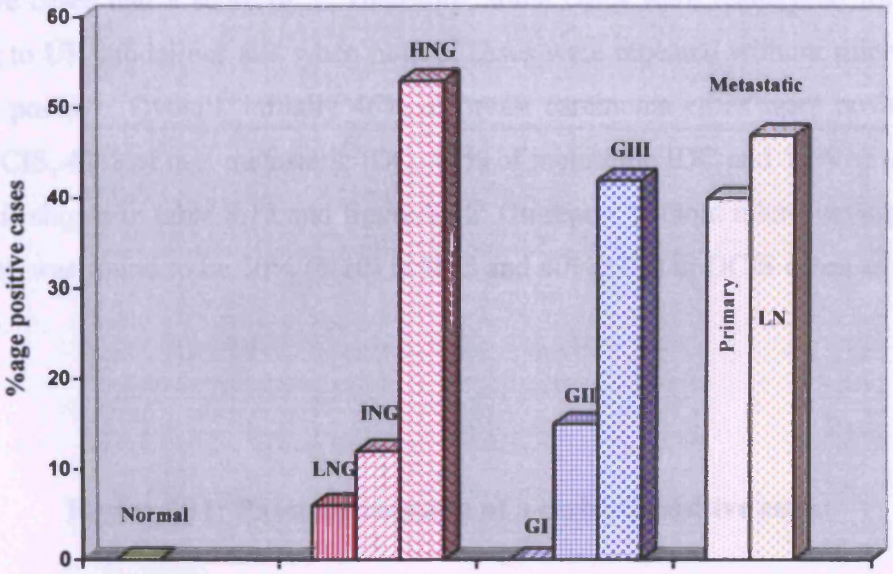


Figure 8.10a; P53 incidence in normal breast tissue, DCIS and IDC sub groups, metastatic primary and lymph node

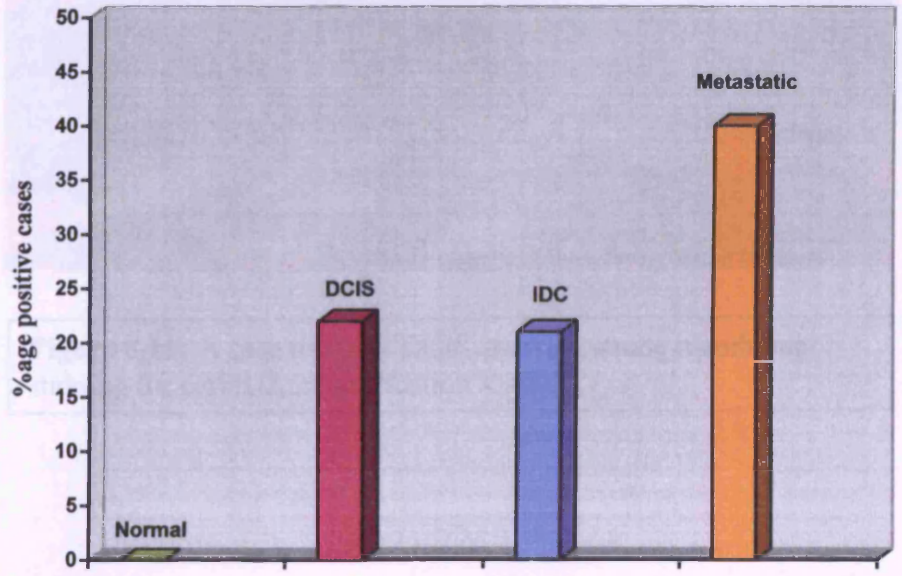


Figure 8.10b; P53 incidence in normal breast tissue as compared to DCIS as whole group, IDC lymph node negative and metastatic primary

CerbB-2 immunostaining:

CerbB-2 staining was membranous as shown in figure 8.11. In the initial analysis, 23% of normal breast tissues were positive. However, none of the normal tissues had a score of 3, all positive cases had a score of 2. Therefore, some cases were reanalysed for cerbB-2 according to UK guidelines and when normal cases were repeated without microwaving, none was positive. Overall, initially 46% of breast carcinoma cases were positive, with 48% of DCIS, 42% of non-metastatic IDC, 47% of metastatic IDC and 53% of metastatic LN. This is shown in table 8.15 and figure 8.12. On repeat without microwaving, cerbB-2 positivity was found to be 30% (3/10) in IDC and 40% (2/5) in DCIS cases as shown in table 8.14.

Figure 8.11; Photomicrograph of a cerbB-2 positive case.

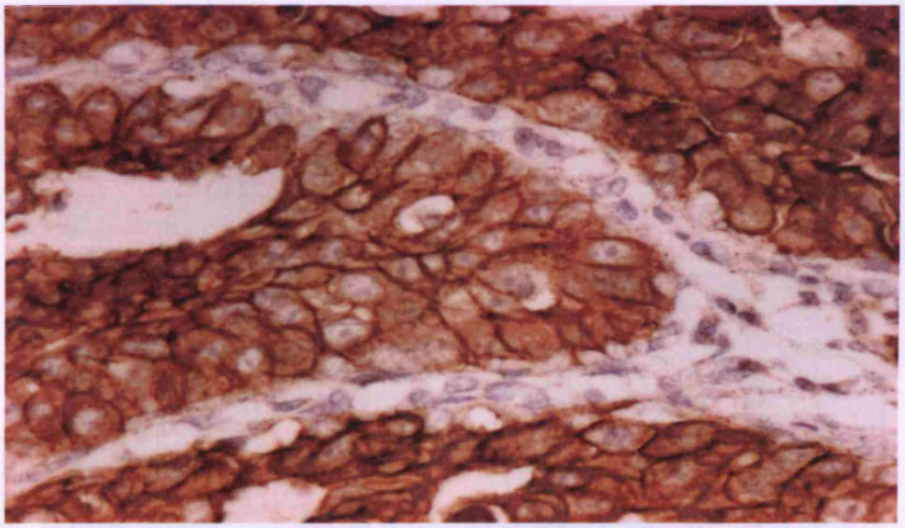


Figure 8.11; A case of HNG DCIS showing strong membrane staining for cerbB-2, magnification x 400.

Figure 8.12; Bar graphs showing cerbB-2 incidence in different groups of patients

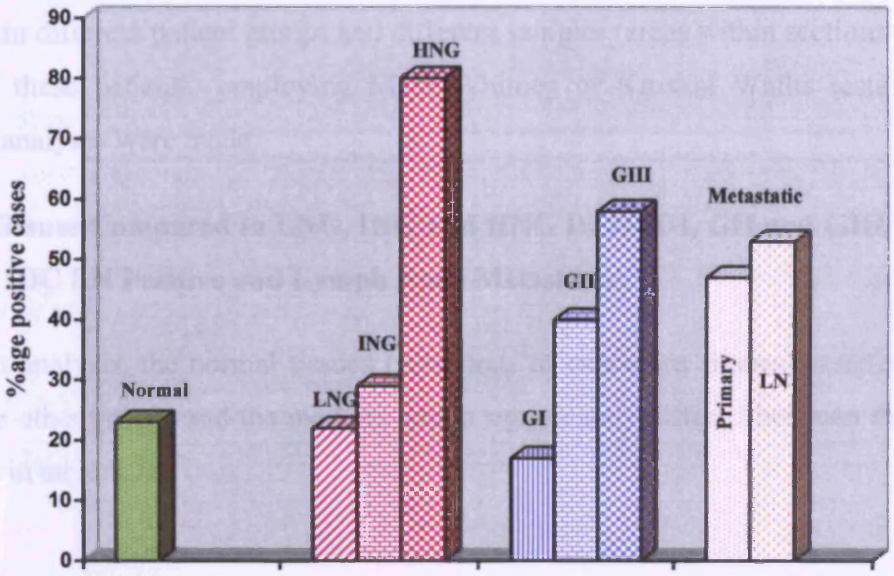


Figure 8.12a; cerbB-2 incidence in normal breast tissue, DCIS and IDC sub groups, metastatic primary and lymph node

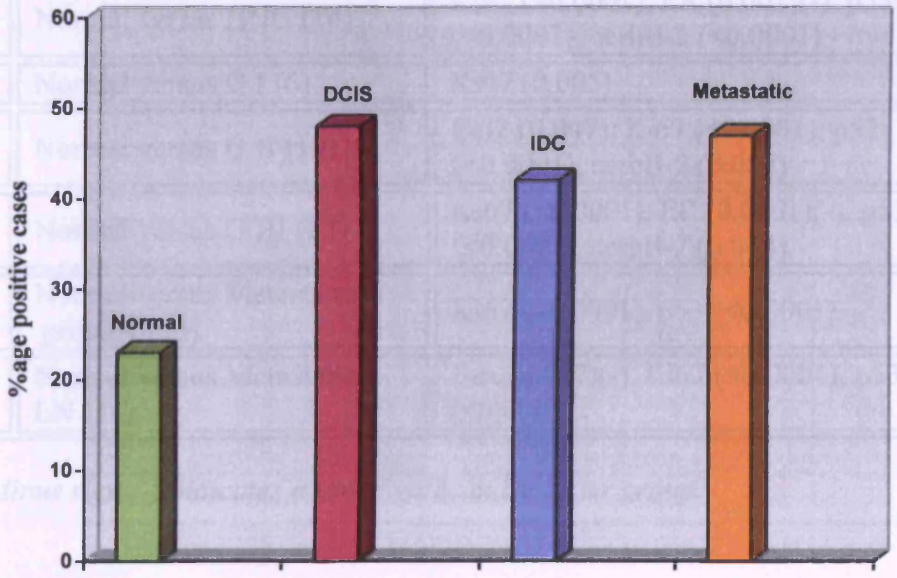


Figure 8.12b; cerbB-2 incidence in normal breast tissue as compared to DCIS as whole group, IDC lymph node negative and metastatic primary

RESULTS OF STATISTICAL ANALYSIS:
CORRELATIONS OF MARKERS WITH DIFFERENT GROUPS:

The values of molecular markers studied, bax, bcl2, Ki67, ER, p53 and cerbB-2 were compared in different patient groups and different samples (areas within sections from the tissues of these patients) employing Mann-Whitney or Kruskal Wallis tests and the following analyses were made:

Normal Tissues Compared to LNG, ING and HNG DCIS, GI, GII and GIII IDC LN Negative, IDC LN Positive and Lymph Node Metastases:

In the first analysis, the normal tissues (surrounds of cancerous tissues) were compared against the other groups and the markers which significantly differed between the groups are shown in table 8.18.

Table 8.18; Groups compared against each other for molecular markers

No.	Group (number)	Molecular markers (p values)
1	Normal (30) versus LNG (18)	Ki67 (0.034)
2	Normal versus ING (17)	Ki67 (0.001), p53 (0.009)
3	Normal versus HNG (14)	Ki67 (<0.0001), ER (0.002)(-), p53 (<0.0001), cerbB-2 (<0.0001)
5	Normal versus G I (6)	Ki67 (0.005)
6	Normal versus G II (19)	Bcl2 (0.047), Ki67 (<0.0001), p53 (<0.0001), cerbB-2 (0.002)
7	Normal versus G III (12)	Ki67 (<0.0001), ER (0.0001)(-), p53 (<0.0001), cerbB-2 (0.044)
8	Normal versus Metastatic primary (15)	Ki67 (<0.0001), p53 (<0.0001)
9	Normal versus Metastatic LN (15)	Bax (0.027)(-), Ki67 (<0.0001), p53 (<0.0001)

Minus sign (-) indicates a lower value in the latter group.

Normal Tissues Compared to DCIS and IDC as Whole Groups:

In the next analysis, normal tissues (n=30) were compared against pure DCIS (n=49) and the three markers significantly higher in DCIS were Ki67, p53 and cerbB-2 (p values <0.0001, 0.002 and 0.003 respectively).

Normal tissues were also compared against IDC LN negative (n=37) with which they were associated and again the three markers which were significantly higher in the latter group were Ki67, p53 and cerbB-2 (p values <0.0001, <0.0001 and 0.01 respectively).

Comparison between DCIS Subgroups:

The subgroups within the pure DCIS group (n=49) were compared against each other as follows: LNG (n=18) versus ING (n=17) versus HNG (n=14) and the p values are shown in table 8.19 below:

Table 8.19; p values of molecular markers in DCIS subgroups

Markers	LNG versus ING (p values)	ING versus HNG (p values)
Bax	0.159	0.134
Bcl2	0.909	0.05 (-)
Ki67	0.05	0.001
ER	0.483	0.004 (-)
p53	0.02	<0.0001
cerbB-2	0.732	<0.0001

Negative correlations with the increasing grade of DCIS are indicated by minus (-) sign. Except bax, the rest of the markers were significantly related to the nuclear grades of DCIS. Bcl2 and ER showed a negative correlation with increasing nuclear grades of DCIS as expected; and Ki67, p53 and cerbB-2 showed a positive correlation.

Figures 8.13a-c show an LNG case positive for bax, bcl2 and ER respectively. Figures 8.14a-c show an HNG case positive for Ki67, p53 and cerbB-2 respectively and figures 8.15a-b show another HNG case positive for cerbB-2.

Figure 8.13; Photomicrographs of an LNG DCIS case positive for bax, bcl2 & ER.

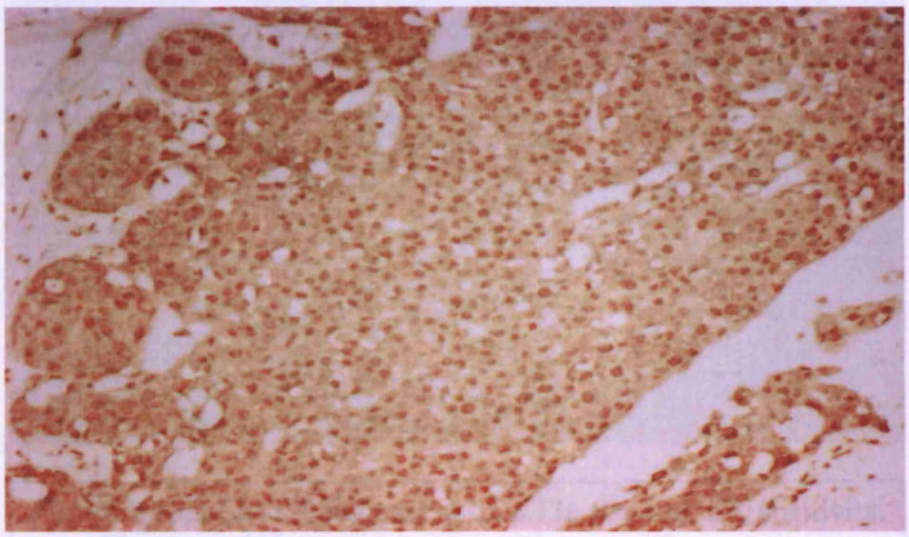


Figure 8.13a; Bax positive LNG, magnification x 200

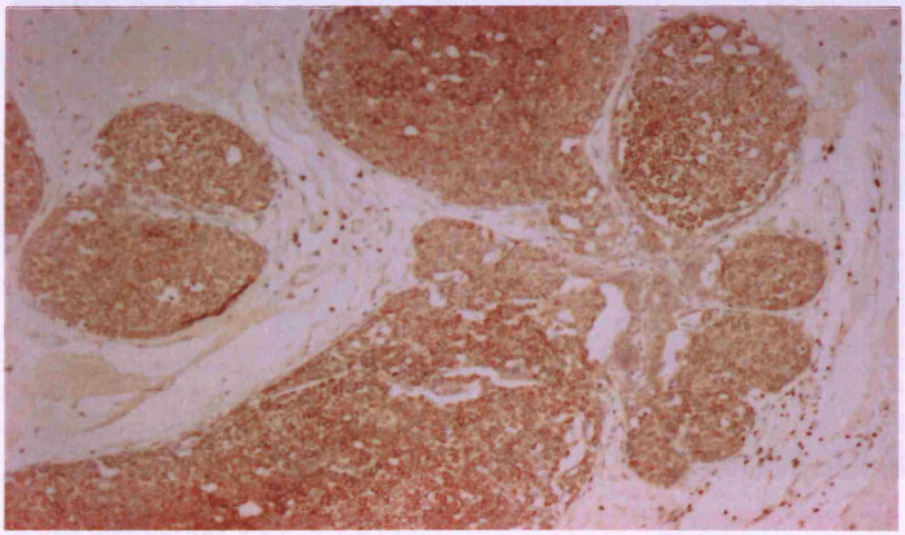


Figure 8.13b; Same LNG case as in 8.13a showing bcl2 positivity at a magnification x 100

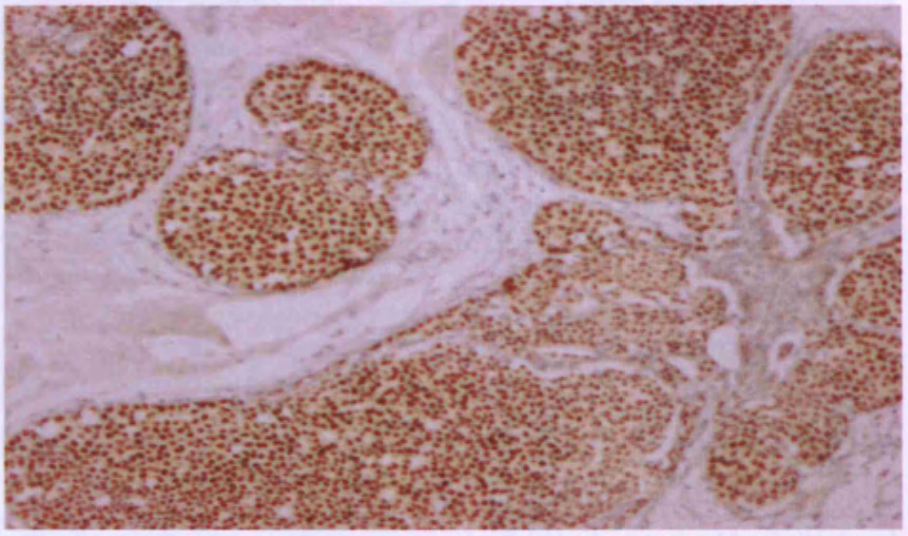


Figure 8.13c; Same LNG case as in 8.13a showing ER positivity, magnification x 100

Figure 8.14; Photomicrographs of an HNG DCIS case positive for Ki67, p53 and cerbB-2.

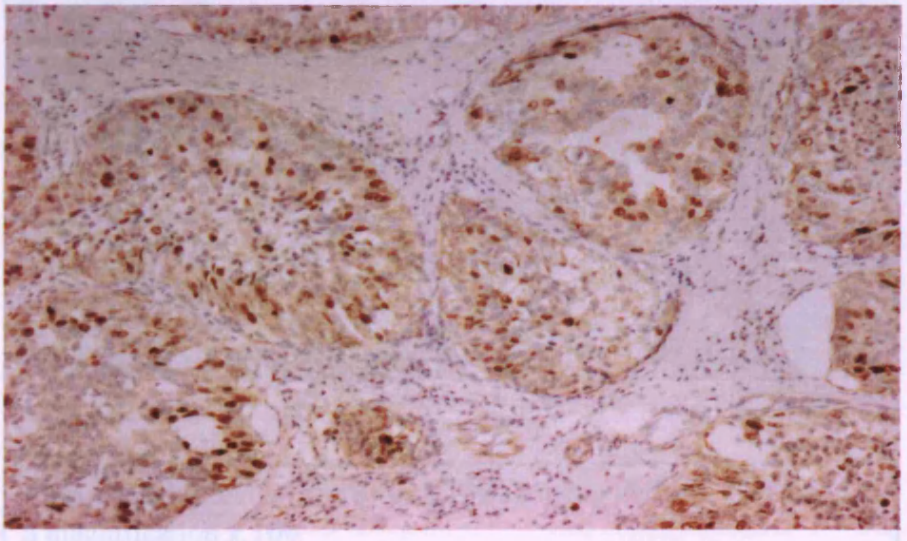


Figure 8.14a; Ki67 immunostaining in an HNG case at a magnification x 100

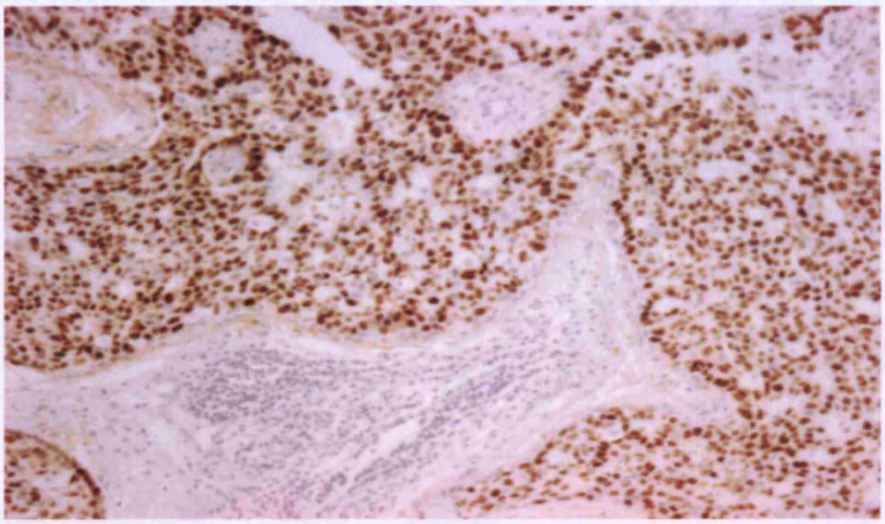


Figure 8.14b; Same HNG case as in 8.14a showing p53 positivity at a magnification x100

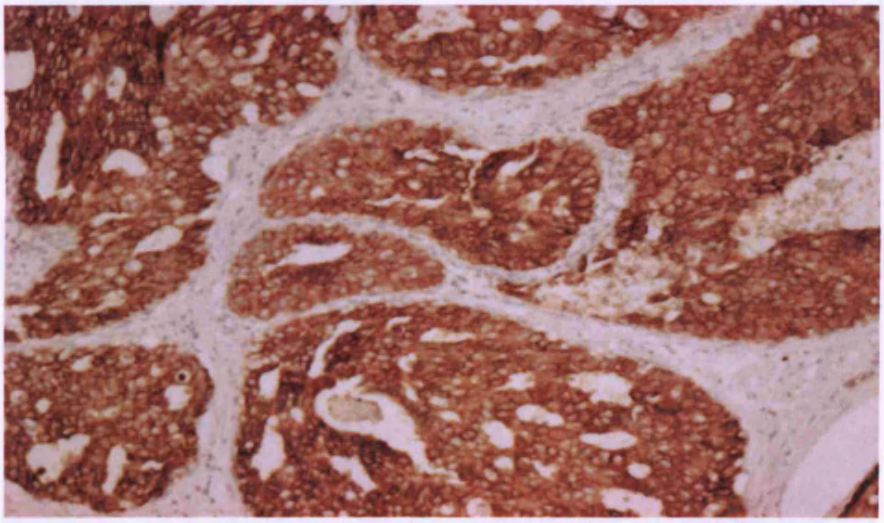


Figure 8.14c; Same case as in 8.14a showing cerbB-2 positivity at a magnification x 100

Figure 8.15; Photomicrographs of an HNG DCIS case positive for cerbB-2.

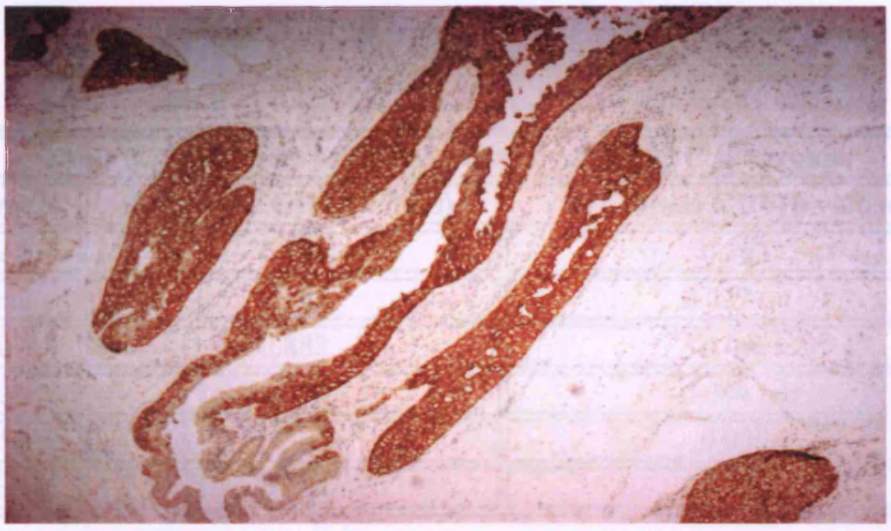


Figure 8.15a; Another HNG case positive for cerbB-2 showing the normal looking duct near the bottom of the picture to be cerbB-2 negative, magnification x 50

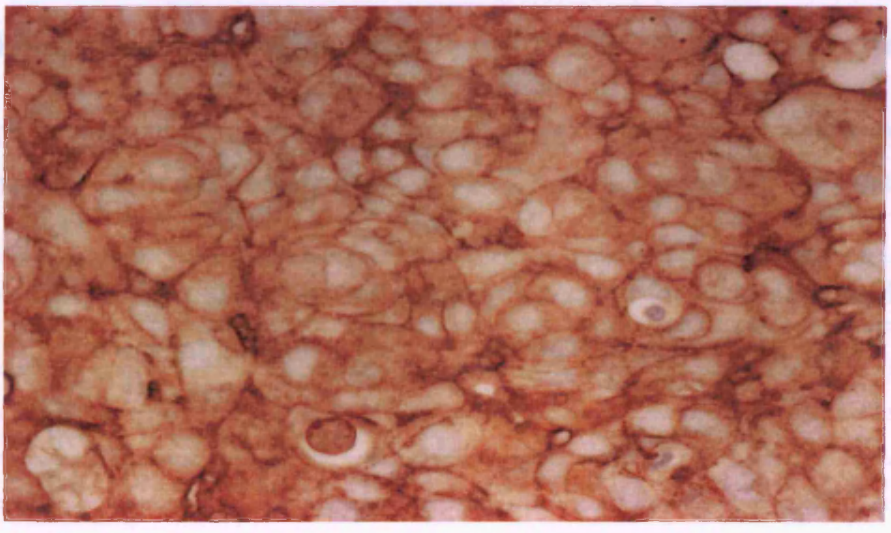


Figure 8.15b; Same case as in 8.15a at a magnification x 400

Comparison Between IDC (LN negative) Subgroups:

The subgroups within IDC LN-ve cases (n=37) were also compared against each other: G I (n=6) versus G II (n=19) versus G III (n=12) and the p values are depicted in table 8.20.

Table 8.20; p values of Molecular Markers in IDC subgroups

Markers	GI versus GII (p values)	GII versus GIII (p values)
Bax	0.642	0.363
Bcl2	0.355	0.027 (-)
Ki67	0.882	<0.0001
ER	0.656	0.004 (-)
p53	0.355	0.020
cerbB-2	0.324	0.042

Negative correlations with the increasing grade of DCIS are indicated by minus (-) sign. Once again, all the markers correlated significantly with the histopathological grades of non-metastatic IDC except Bax. Figure 8.16 shows a G I IDC case positive for Bcl2 and figure 8.17 shows the same case staining positively for ER. Figure 8.18 shows a G III IDC case positive for Ki67 and same case is positive for p53 as shown in figure 8.19.

Comparison between Comparable Subgroups of Pure DCIS and IDC LN Negative:

The subgroups of pure DCIS and non-metastatic IDC were compared against each other and the markers which differed significantly between the two are shown in table 8.21.

Table 8.21; Subgroups compared against each other for molecular markers

No.	Sub - group (number)	Molecular markers (p value)
1	LNG (18) versus GI (5)	Ki67 (0.09)
2	ING (17) versus GII (19)	Ki67 (<0.0001)
3	HNG (14) versus GIII (12)	Ki67 (0.003), cerbB-2 (0.021)(-)

In the above, Ki67 is higher in the latter groups, whereas cerbB-2 is significantly lower in GIII as compared to HNG indicated by a minus (-) sign.

Figure 8.16; Photomicrograph of a G I IDC case positive for bcl2.

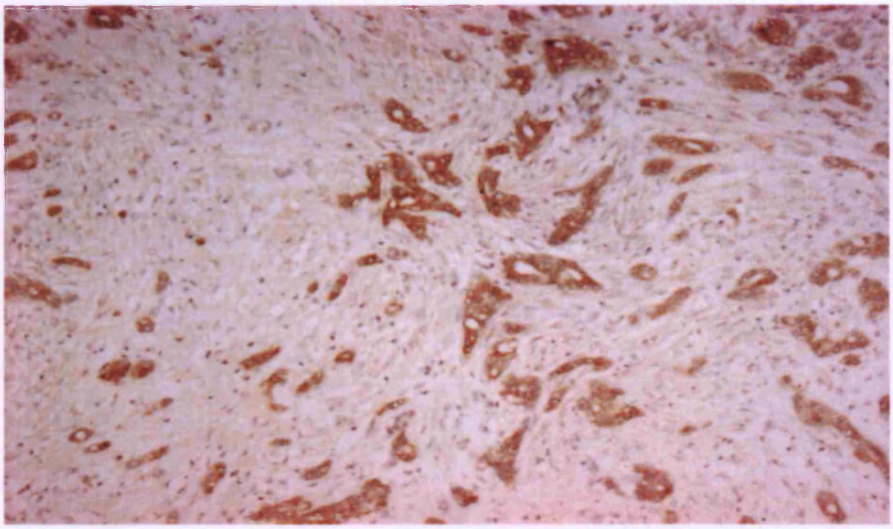


Figure 8.16a; G I IDC positive for bcl2, magnification x 50

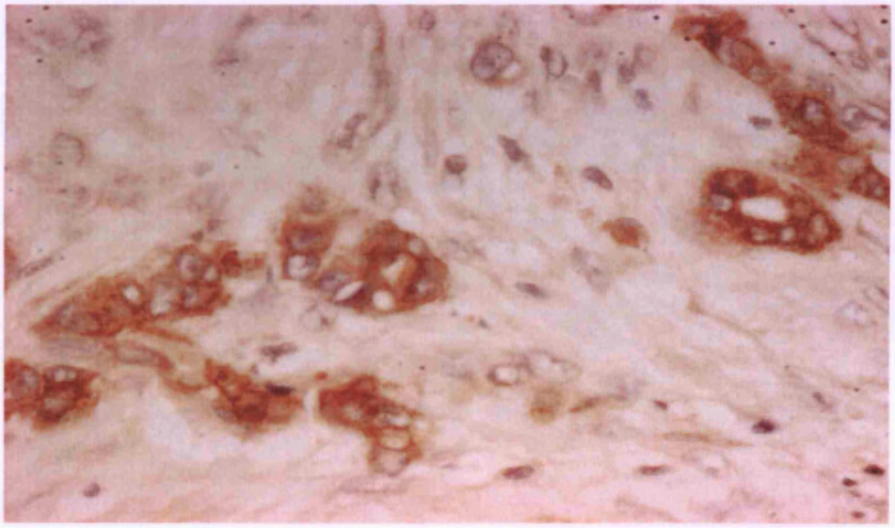


Figure 8.16b; Same case as in figure 8.16a, magnification x 400

Figure 8.17; Photomicrograph of a G I IDC case positive for ER.

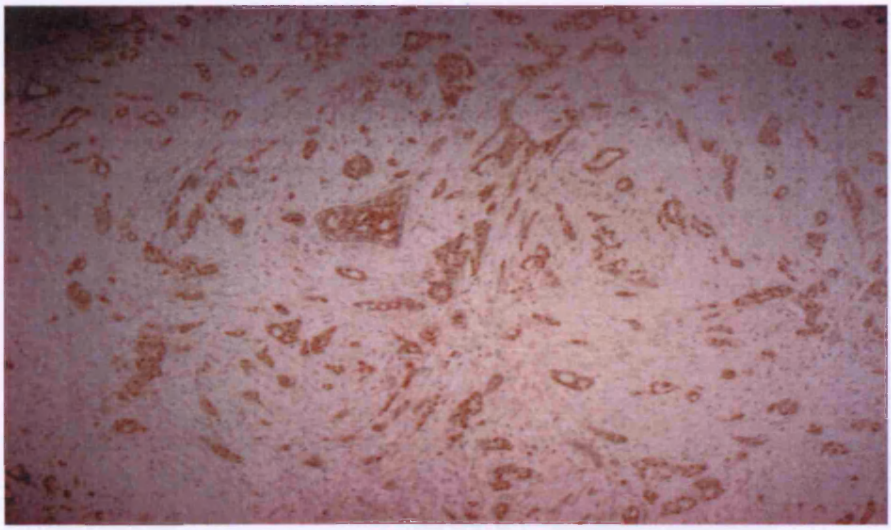


Figure 8.17a; Same GI IDC case as in figure 8.16, positive for ER, magnification x 50

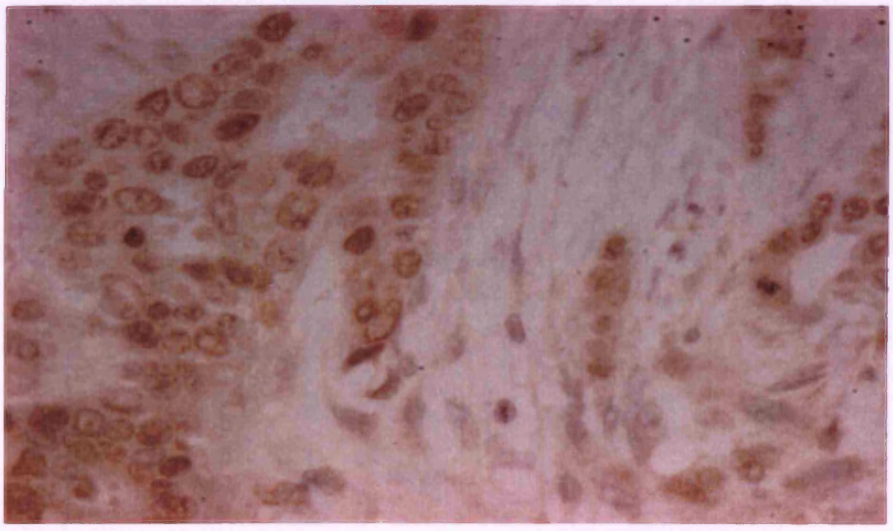


Figure 8.17b; Same case as in 8.16 and 8.17a, showing the different intensity staining of the nuclei for ER at a magnification x 400

Figure 8.18; Photomicrograph of a G III IDC case positive for Ki67.

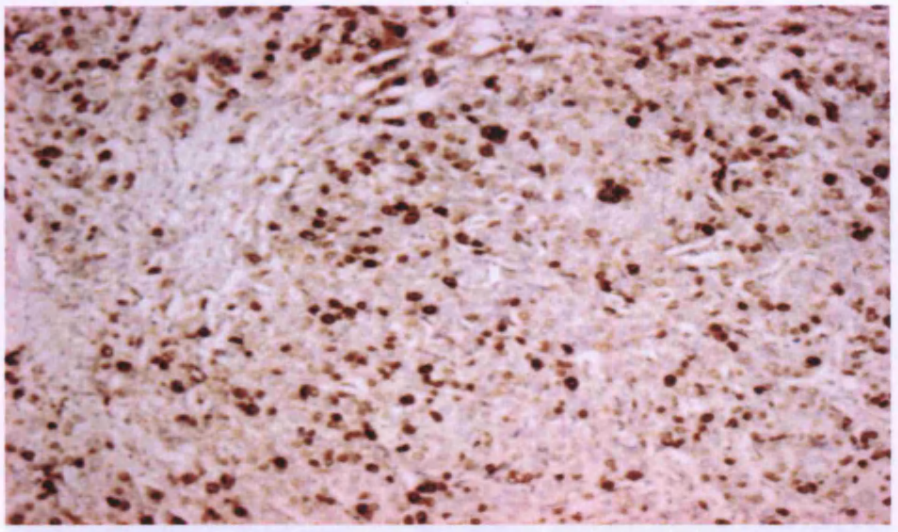


Figure 8.18a; GIII IDC showing Ki67 immunostaining at a magnification of x 100

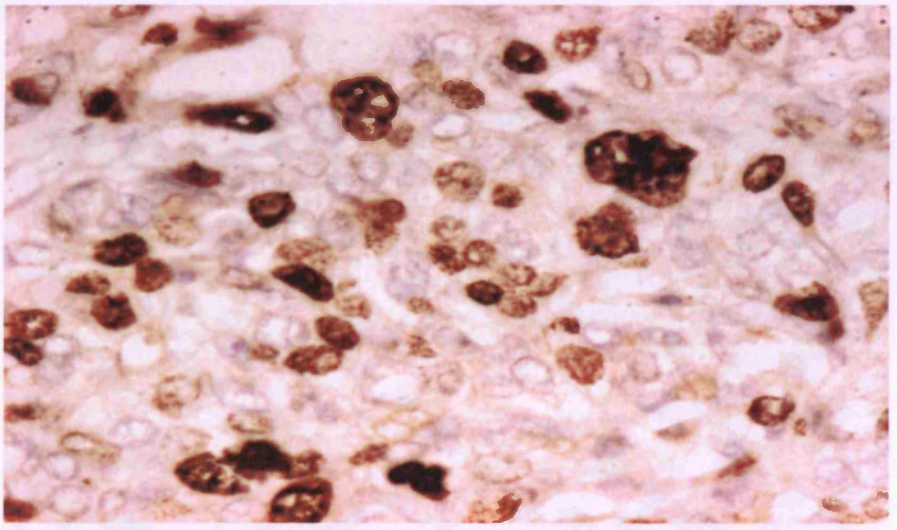


Figure 8.18b; Same case as in 8.18a, magnification x 400

Figure 8.19; Photomicrograph of a G III IDC case positive for p53

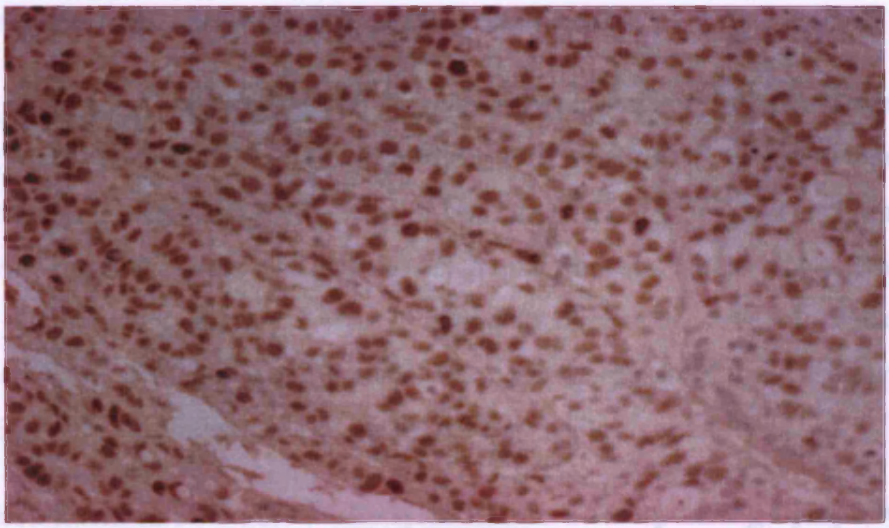


Figure 8.19a; Same case as in figure 8.18 showing p53 positivity at a magnification of x 200

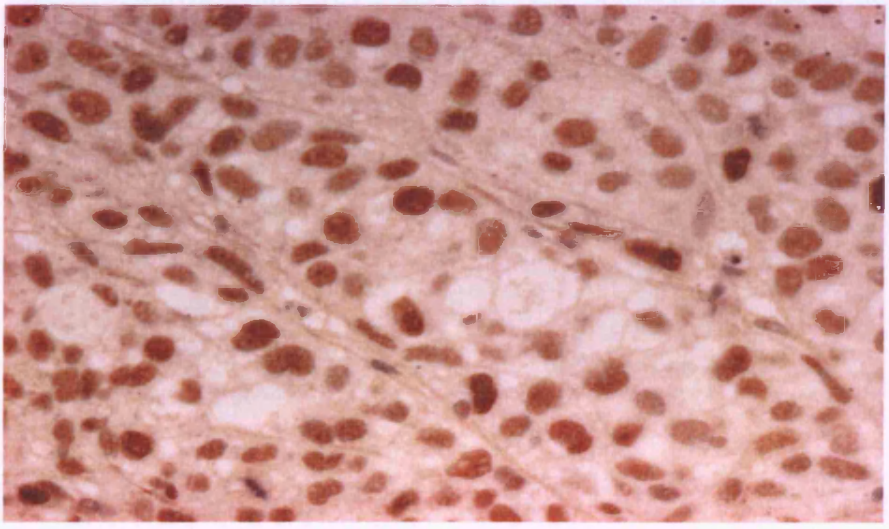


Figure 8.19b; Same case as in figures 8.18 and 8.19a showing p53 positivity at a magnification of x 400

Comparison Between Pure DCIS and IDC LN Negative:

Ki67 was significantly higher in LN negative IDC (n=37) as compared to pure DCIS (n=49), the p value being <0.0001.

Comparison Between Non-Metastatic and Metastatic IDC:

IDC LN negative (n=37) and IDC LN positive (n=15) cases were compared against each other, but no significant difference with respect to expression of molecular markers was found between the two groups. The analysis was carried out twice first including all the lymph node negative cases and second excluding the six cases (16%) which were clinically lymph node negative, in view of high clinical false negative rate. This could be partly due to the small number of metastatic cases or possibly due to other markers involved in the step from invasion to metastasis.

Comparison Between IDC (LN Positive) and Their Corresponding Lymph Nodes:

The 15 metastatic IDC were compared against their 15 involved lymph nodes and in this case only bax expression was significantly different between the two, being much reduced in the lymph node metastases, with a p value of 0.045. The expression of the rest of the markers was not significantly different. Figure 8.20 illustrates a LN metastasis showing immunostaining for various markers.

Figure 8.20; Photomicrographs of a metastasis-containing lymph node positive for (a) bcl2, (b) ER, (c) Ki67, (d) p53, (e) cerbB-2 and (f) bax

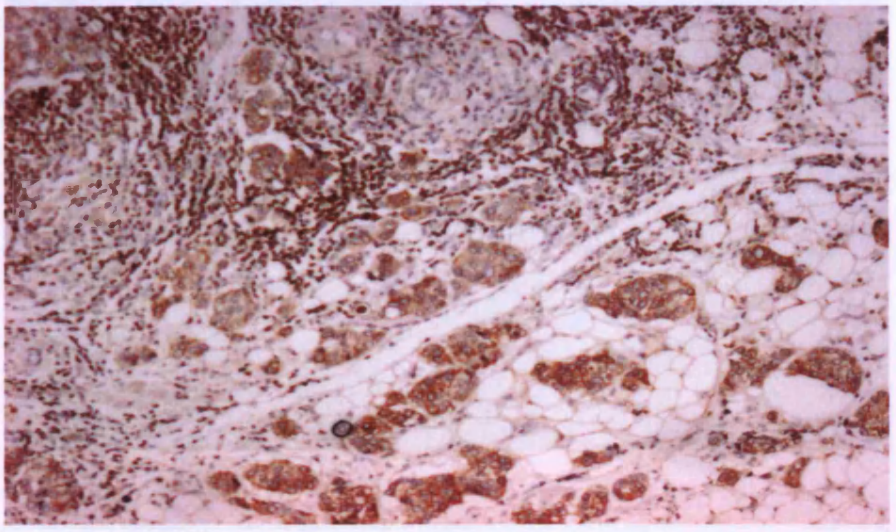


Figure 8.20a; Photomicrograph of a metastasis-containing lymph node positive for bcl2, magnification x 100

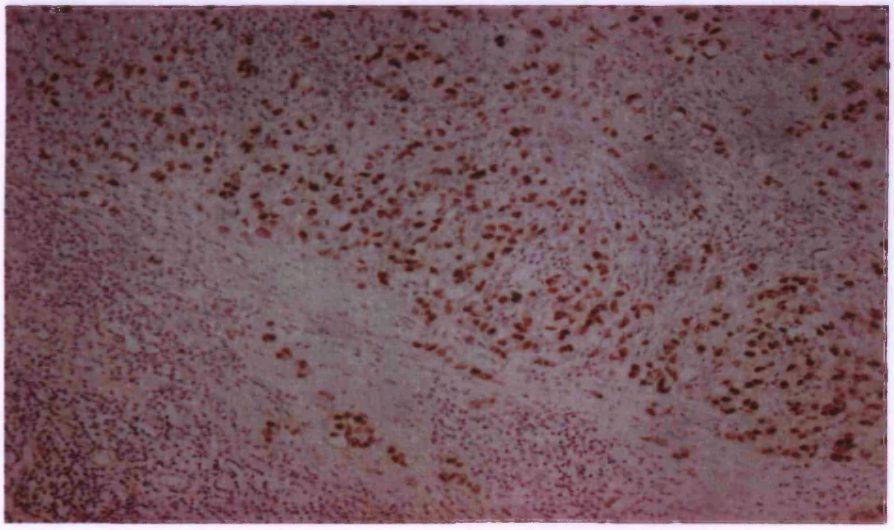


Figure 8.20b; Photomicrograph of a metastasis-containing lymph node positive for ER, magnification x 100

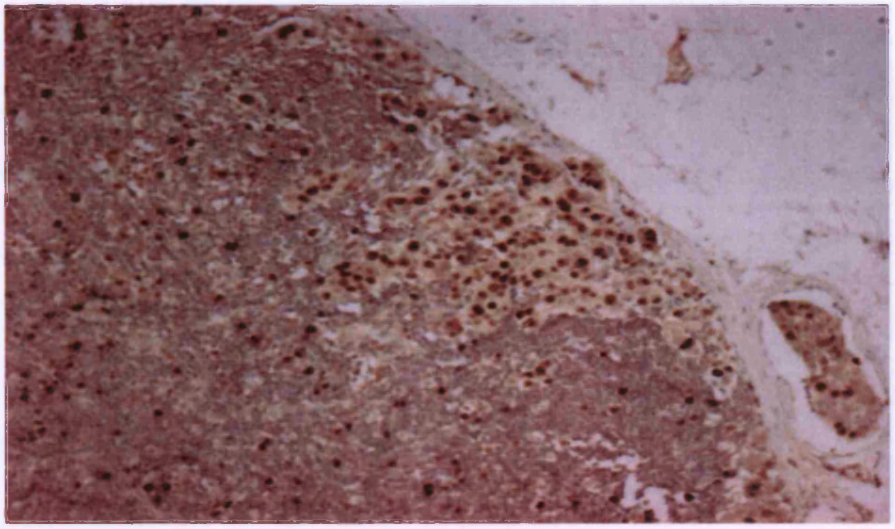


Figure 8.20c; Photomicrograph of a metastasis-containing lymph node positive for Ki67, magnification x 100

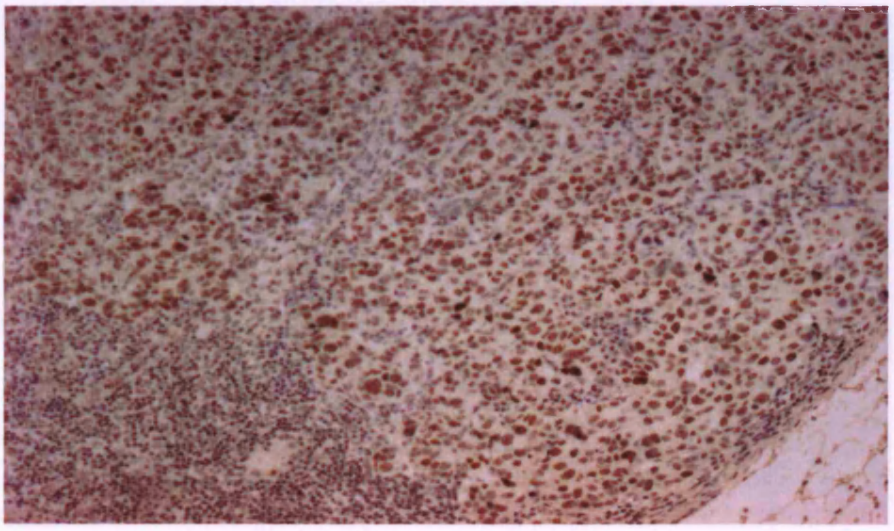


Figure 8.20d; Photomicrograph of a metastasis-containing lymph node positive for p53, magnification x 100

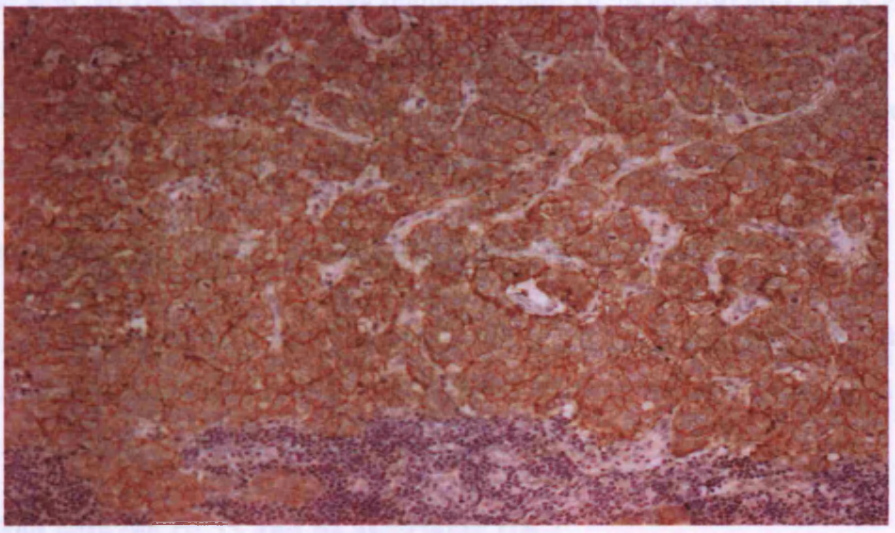


Figure 8.20e; Photomicrograph of a metastasis-containing lymph node positive for cerbB-2, magnification x 100

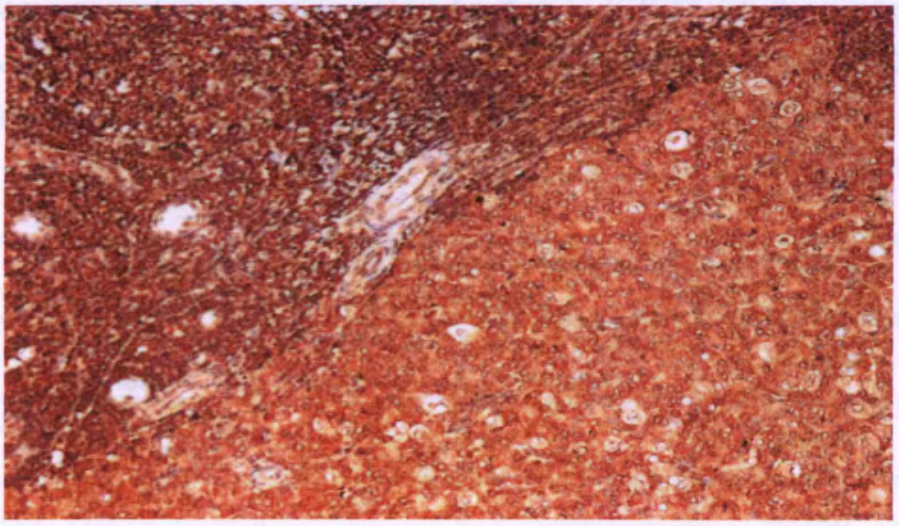


Figure 8.20f; Photomicrograph of a metastasis-containing lymph node positive for bax, magnification x 100

Comparison Between DCIS and IDC as Whole Groups:

In the next step, all DCIS (n=73) cases including pure DCIS (n=49) and DCIS associated with IDC (n=24) were compared against all IDC (n=52) cases including non-metastatic (n=37) and metastatic (n=15) and the only marker significantly different between these two groups was Ki67, being higher in the latter group, p value<0.0001.

Comparison Between Pure DCIS and DCIS Associated With IDC:

Pure DCIS (n=49) cases were also compared with DCIS associated with IDC (n=24) and in this case the only marker significantly different between the two groups was Ki67 (p value = 0.024), being higher in the latter group.

Comparison between DCIS and its associated IDC:

DCIS associated with IDC (n=24) were compared against IDC (n=37) with which these were associated. Once again, Ki67 was the differentiating marker between the two, being higher in IDC (p value = 0.015).

CORRELATIONS OF MARKERS WITH COMEDO-TYPE NECROSIS:

Comedo-type necrosis was significantly related to high Ki67 labelling index (>10% labelled cells) and to p53 and cerbB-2 positivity, the p values being 0.001, <0.001 and 0.01 respectively as shown in table 8.22.

Table 8.22; Distribution of molecular markers in cases with and without necrosis.

Molecular markers	Cases with necrosis	Cases without necrosis	p value
Ki67 high (>10%) Ki67 low (<10%)	11 5	5 20	0.001
p53+ p53-	9 7	2 23	<0.001
cerbB-2+ cerbB-2-	11 5	8 17	0.01

CORRELATIONS BETWEEN MARKERS:

The correlations between all the markers- bax, bcl2, Ki67, ER, p53 and cerbB-2 were studied using Spearman's rank correlation test, which is a non-parametric test, as the histograms revealed distribution of these markers was not normal. These correlations were studied within the following groups:

1- All 101 patients including 49 pure DCIS cases, 37 non-metastatic IDC and 15 metastatic cases (table 8.23).
2- All 170 samples including the above 101 cases, plus 15 metastatic LN, 30 normal breast tissue areas associated with IDC and 24 DCIS associated with IDC (table 8.24).
3- All 73 DCIS cases including 49 pure DCIS and 24 DCIS associated with IDC (table 8.25).
4- All 52 IDC cases including 37 non-metastatic and 15 metastatic (table 8.26).
5- All 23 LNG DCIS including 18 from pure DCIS group and 5 associated with IDC (table 8.27).
6- All 25 ING DCIS including 17 from pure DCIS group and 8 associated with IDC (table 8.28).
7- All 25 HNG DCIS including 14 from pure DCIS group and 11 associated with IDC (table 8.29).
8- All 7 G I IDC including 6 non-metastatic and 1 metastatic (table 8.30).
9- All 25 G II IDC including 19 non-metastatic and 6 metastatic (table 8.31).
10- All 20 G III IDC including 12 non-metastatic and 8 metastatic (table 8.32).
11- All 15 metastatic LN (table 8.33).

Negative correlations are indicated by a minus (-) sign. Some boxes have been left blank to avoid repetition of the same correlations.

Table 8.23; Nonparametric correlations-all cases (101), p values are shown

	Bax	Bcl2	Ki67	ER	p53
Bax					
Bcl2	0.010*				
Ki67	0.400	0.061(-)			
ER	0.261	<0.0001**	<0.0001**(-)		
p53	0.837	0.302	<0.0001**	0.008**(-)	
cerbB-2	0.911	0.165	<0.0001**	0.001**(-)	<0.0001**

*Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

It can be seen from the above table that significant positive correlations were found between bax and bcl2, bcl2 and ER, Ki67 and p53, Ki67 and cerbB-2, and p53 and cerbB-2. Significant negative correlations were seen between ER and Ki67, ER and p53, and ER and cerbB-2. There was a trend towards negative correlation between bcl2 and Ki67.

Table 8.24;Nonparametric correlations-all samples (170), p values are shown

	Bax	Bcl2	Ki67	ER	p53
Bax					
Bcl2	0.002**				
Ki67	0.018*(-)	0.019*(-)			
ER	0.122	<0.0001**	<0.0001**(-)		
p53	0.208	0.364	<0.0001**	0.005**(-)	
cerbB-2	0.682	0.246	<0.0001**	0.001**(-)	<0.0001**

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

In all the 170 samples, once again there were positive correlations demonstrated between bax and bcl2, bcl2 and ER, Ki67 and p53, Ki67 and cerbB-2, and p53 and cerbB-2. Significant negative correlations were observed between bax and Ki67, bcl2 and Ki67, Ki67 and ER, ER and p53, and ER and cerbB-2. Therefore, by increasing the number of samples, two additional negative correlations, that is between bax and Ki67 and between bcl2 and Ki67, became apparent.

Table 8.25;Nonparametric correlations-all DCIS (73), p values are shown

	Bax	Bcl2	Ki67	ER	p53
Bax					
Bcl2	0.066				
Ki67	0.937	0.199			
ER	0.811	<0.0001**	0.006**(-)		
p53	0.893	0.189	<0.0001**	0.002**(-)	
cerbB-2	0.468	0.823	<0.0001**	0.032*(-)	<0.0001**

*Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Within all DCIS, positive correlations were observed between bcl2 and ER, Ki67 and p53, Ki67 and cerbB-2, and p53 and cerbB-2. There was a trend towards positive correlation between bax and bcl2. There were significant negative correlations between Ki67 and ER, ER and p53, and ER and cerbB-2.

Table 8.26; Nonparametric correlations-all IDC (52), p values are shown					
	Bax	**Bcl2**	**Ki67**	**ER**	**p53**
Bax					
Bcl2	0.014*				
Ki67	0.136	0.073(-)			
ER	0.052	<0.0001**	0.001**(-)		
p53	0.397	0.721	0.001**	0.342	
cerbB-2	0.735	0.132	0.324	0.029*(-)	0.369

*Correlation is significant at the.05 level (2-tailed).
**Correlation is significant at the.01 level (2-tailed).

The significant positive correlations within IDC cases were between bax and bcl2, bcl2 and ER, and Ki67 and p53. The significant negative correlations were between Ki67 and ER and cerbB-2 and ER. There was a trend toward positive correlation between ER and bax; and a trend toward negative correlation between Ki67 and bcl2.

Table 8.27; Nonparametric correlations LNG cases (23), p values are shown					
	Bax	**Bcl2**	**Ki67**	**ER**	**p53**
Bax					
Bcl2	0.099				
Ki67	0.541	0.597			
ER	0.811	0.012*	0.505		
p53	0.832	0.926	0.843	0.633	
cerbB-2	0.814	0.014*	0.943	0.577	0.646

*Correlation is significant at the 0.05 level (2-tailed).

The two significant positive correlations in this subgroup were between bcl2 and ER and bcl2 and cerbB-2. There was a trend towards positive correlation between bax and bcl2.

Table 8.28; Nonparametric correlations ING cases (25), p values are shown					
	Bax	**Bcl2**	**Ki67**	**ER**	**p53**
Bax					
Bcl2	0.171				
Ki67	0.656	0.189			
ER	0.644	<0.0001**	0.882		
p53	0.652	0.967	0.115	0.525	
cerbB-2	0.358	0.525	0.125	0.860	0.564

** Correlation is significant at the 0.01 level (2-tailed).

In ING cases, there was a positive correlation between bcl2 and ER.

Table 8.29; Nonparametric correlations HNG cases (25), p values are shown					
	Bax	**Bcl2**	**Ki67**	**ER**	**p53**
Bax					
Bcl2	0.484				
Ki67	0.388	0.118			
ER	0.162	0.004**	0.138		
p53	0.935	0.644	0.150	0.259	
cerbB-2	0.864	0.816	0.366	0.690	0.015*

*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).

In HNG cases, there was a positive correlation between bcl2 and ER and p53 and cerbB-2. It is interesting to note that in LNG cases, there was a positive correlation between bcl2 and cerbB-2, whereas in HNG cases, there was a positive correlation between p53 and cerbB-2.

Table 8.30; Nonparametric correlations GI cases (7), p values are shown					
	Bax	**Bcl2**	**Ki67**	**ER**	**p53**
Bax					
Bcl2	0.022*				
Ki67	0.305	0.618			
ER	0.396	0.554	0.379		
p53	0.550	0.394	0.631	0.525	
cerbB-2	0.353	0.322	0.661	0.139	0.582

*Correlation is significant at the 0.05 level (2-tailed).

The significant correlation in GI cases was between bax and bcl2.

Table 8.31; Nonparametric correlations GII cases (25), p values are shown					
	Bax	**Bcl2**	**Ki67**	**ER**	**p53**
Bax					
Bcl2	0.287				
Ki67	0.271	0.402			
ER	0.262	0.057	0.459		
p53	0.600	0.499	0.234	0.904	
cerbB-2	0.825	0.639	0.589	0.740	0.265

There was a trend toward positive correlation between bcl2 and ER in GII cases.

Table 8.32; Nonparametric correlations GIII cases (20), p values are shown					
	Bax	**Bcl2**	**Ki67**	**ER**	**p53**
Bax					
Bcl2	0.307				
Ki67	0.628	0.618			
ER	0.485	0.001**	0.735		
p53	0.672	0.076	0.041*	0.781	
cerbB-2	0.693	0.006**(-)	0.964	0.029*(-)	0.813

*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).

The significant positive correlations within GIII cases were between bcl2 and ER and Ki67 and p53, whereas negative correlations occurred between bcl2 and cerbB-2 and ER and cerbB-2.

Table 8.33; Nonparametric correlations-metastatic LN (15), p values are shown					
	Bax	**Bcl2**	**Ki67**	**ER**	**p53**
Bax					
Bcl2	0.102				
Ki67	0.001**(-)	0.033*(-)			
ER	0.414	0.386	0.077(-)		
p53	0.260	0.728	0.728	0.872	
cerbB-2	0.286	0.976	0.486	0.033*(-)	0.908

*Correlation is significant at the.05 level (2-tailed).
** Correlation is significant at the .01 level (2-tailed).

Within the lymph nodes with metastatic tumour, all significant correlations were negative; between bax and Ki67, between bcl2 and Ki67 and between ER and cerbB-2. There was a trend towards negative correlation between ER and Ki67.

RESULTS OF PCR:

Of the 32/118 (27%) samples that were positive for p53 protein by immunostaining, 18 were amplified with the polymerase chain reaction (PCR) for exons 5-8 of the *P53* gene. Two other cases, which had more than 5% cells labelled with p53 were also amplified, making a total of 20 cases. These included 10 DCIS (1 LNG, 2 ING, 7 HNG), 8 IDC LN-ive (0 G I, 3 G II, 5 G III), 1 IDC LN+ive and 1 LN metastasis. Figures 8.21-8.29 show the pictures of ethidium bromide stained gels of cases 1-20 amplified for exon 5-8, where L = 100bp DNA ladder, + = positive control and – = negative control. All the pictures contain wells in which samples were loaded, at the top. The direction of the arrow along x-axis indicates the direction in which samples were loaded and the direction of arrow along y-axis shows the direction in which DNA molecules migrated. The smallest DNA molecules move fastest and hence 100 bp molecules within the DNA ladder migrate the most. The exons 5 – 8 of *P53* gene were all between 100 – 300 bp.

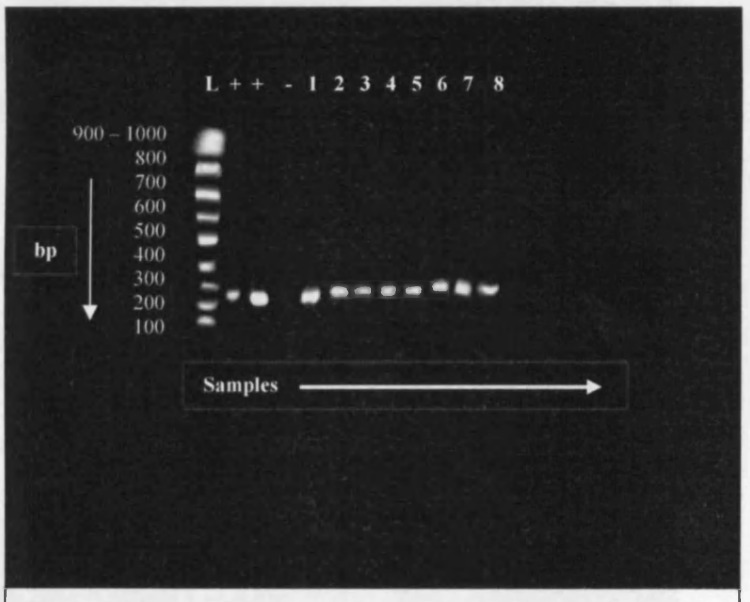


Figure 8.21; Amplification products of exon 5 of *P53* for cases 1 – 8, showing all cases contain DNA between 100 – 300 bp

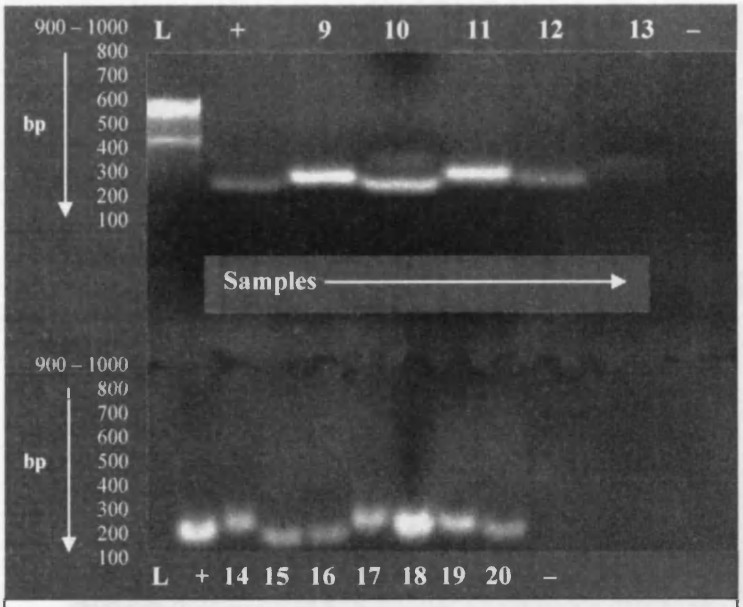


Figure 8.22; Amplification products of exon 5 for cases 9 – 13 on the top side of the picture and cases 14 – 20 at the bottom of the picture showing all cases except no. 13 contain DNA between 100 – 300 bp. The ladder at bottom of the picture is not clearly visible

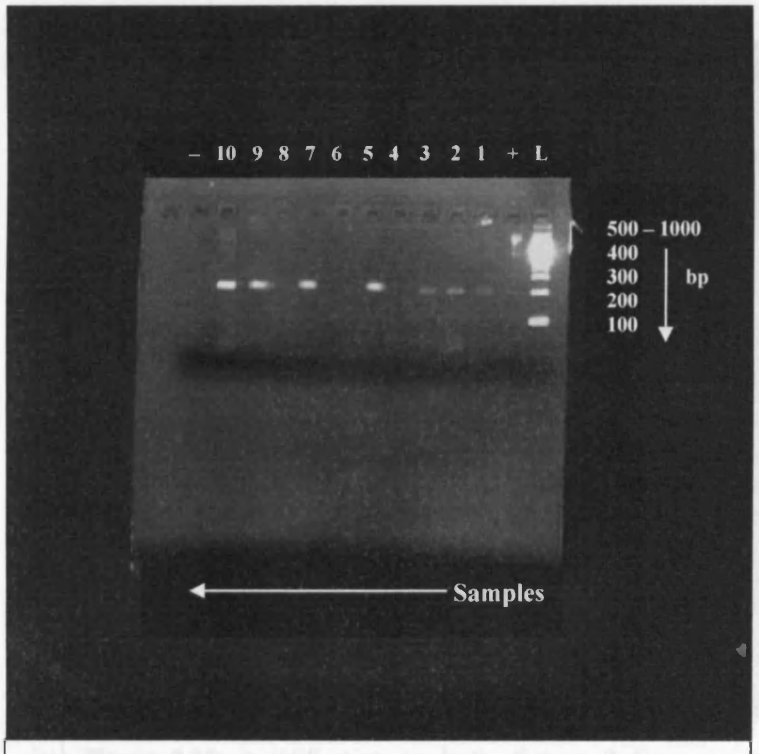


Figure 8.23; Amplification products of exon 6 for cases 1 – 10 showing cases no. 1, 2, 3, 5 7, 9 and 10 contain DNA between 100 – 300 bp

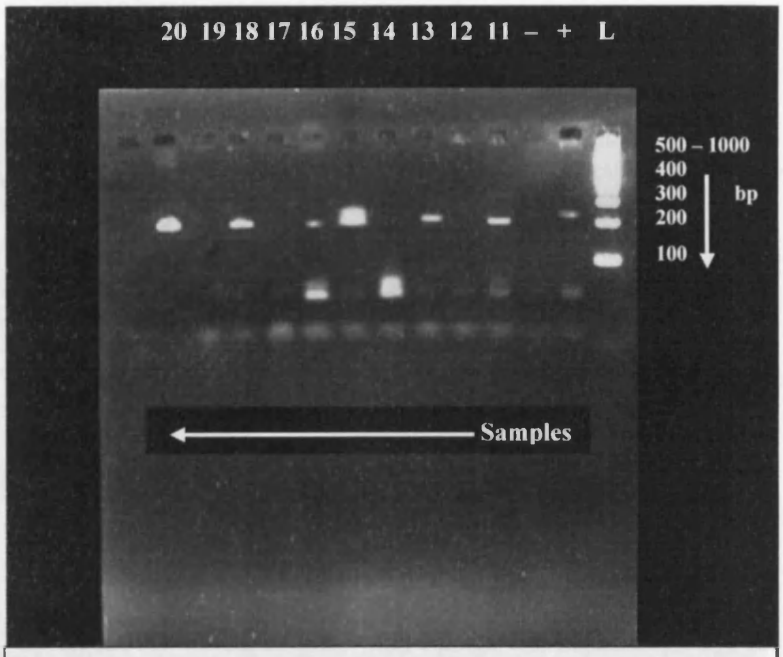


Figure 8.24; Amplification products of exon 6 for cases 11 – 20 showing cases no. 11, 13, 15, 16, 18 and 20 contain DNA between 100 – 300 bp, in cases 14 and 16 additional DNA fragments smaller than 100bp can be seen migrating further downwards

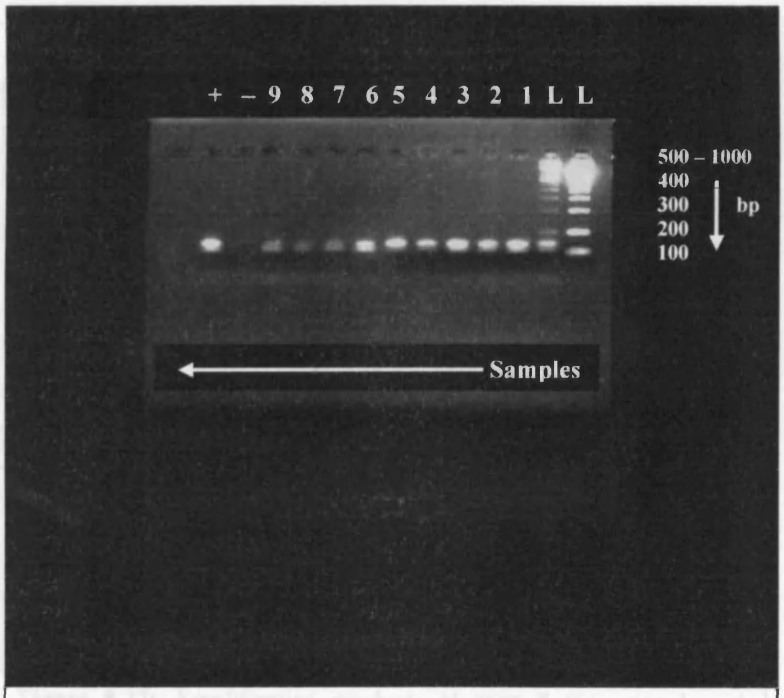


Figure 8.25; Amplification products of exon 7 for cases 1 – 9 showing all cases contain DNA between 100 – 300 bp. The 100 bp DNA ladder was accidentally loaded twice

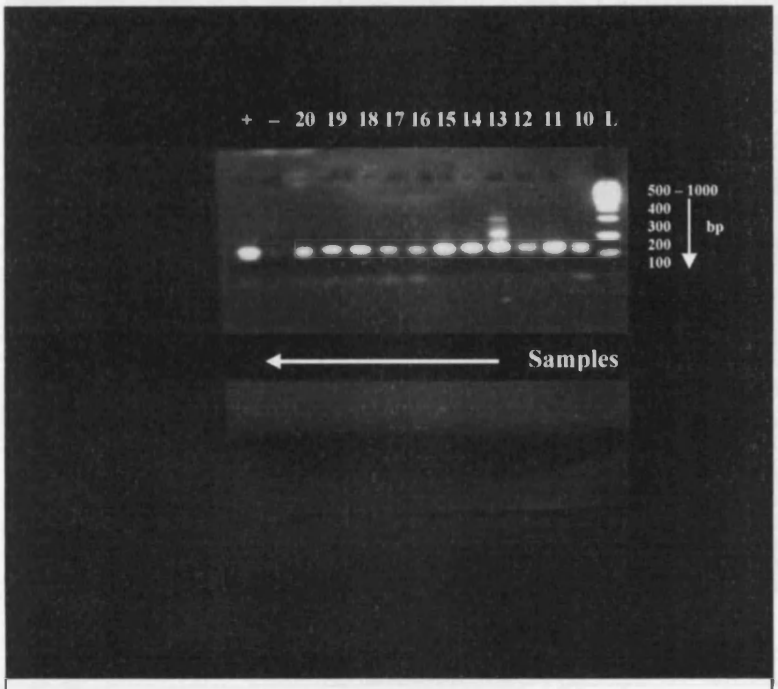


Figure 8.26; Amplification products of exon 7 for cases 10 – 20 showing all cases contain DNA between 100 – 300 bp

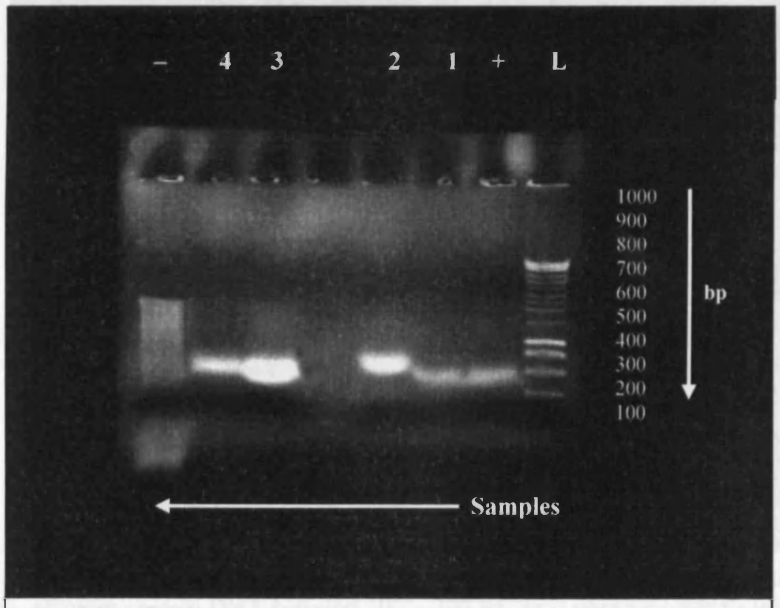


Figure 8.27; Amplification products of exon 8 for cases 1 – 4 showing all cases contain DNA between 100 – 300 bp. There is some smudging effect seen in the case of negative control possibly due to primer effect. The empty lane between 2 and 3 indicates that it was not loaded with a sample as the well was broken

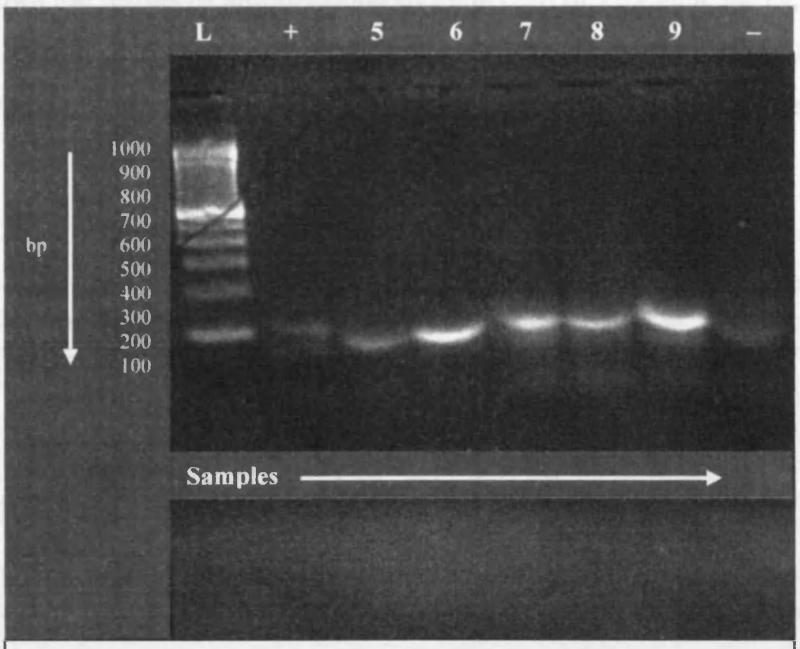


Figure 8.28; Amplification products of exon 8 for cases 5 – 9 showing all cases contain DNA between 100 – 300 bp

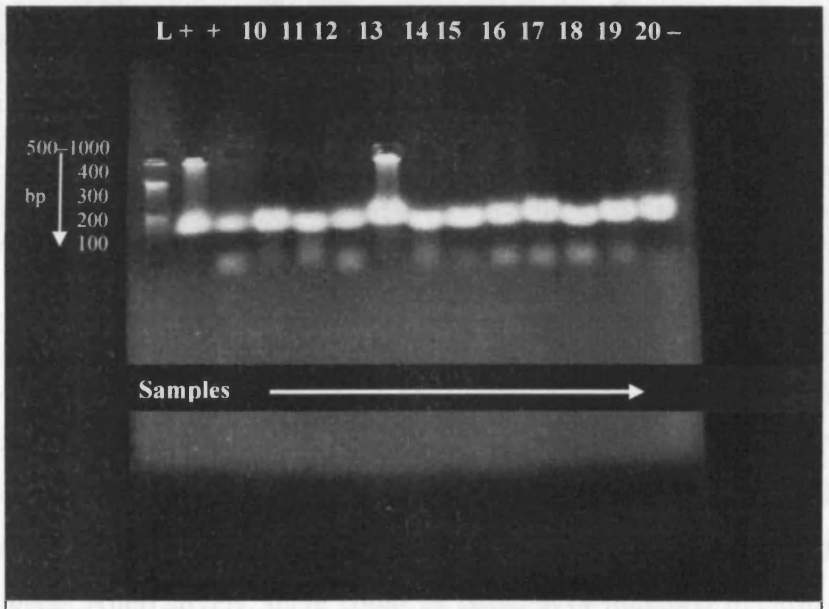


Figure 8.29; Amplification products of exon 8 for cases 10 – 20 showing all cases contain DNA between 100 – 300 bp. Two positive controls were used with these cases

Table 8.34 shows the results of amplification of exons 5-8 of *P53* gene by PCR for the 20 cases and the results of p53 immunostaining expressed as percent positive cells for the same cases. Plus and minus signs indicate the detection or absence of a particular exon respectively.

Table 8.34;Cases positive for p53 by immunostaining and amplified for exon 5-8 of *P53*

No.	Histology	p53 (%)	Exon 5	Exon 6	Exon 7	Exon 8
1	LNG	20	+	+	+	+
2	ING	6	+	+	+	+
3	ING	10	+	+	+	+
4	HNG	80	+	-	+	+
5	HNG	5	+	+	+	+
6	HNG	46	+	-	+	+
7	HNG	79	+	+	+	+
8	HNG	12	+	-	+	+
9	HNG	10	+	+	+	+
10	HNG	77	+	+	+	+
11	G II	30	+	+	+	+
12	G II	60	+	-	+	+
13	G II	20	-	+	+	+
14	G III	16	+	-	+	+
15	G III	25	+	+	+	+
16	G III	50	+	+	+	+
17	G III	30	+	-	+	+
18	G III	70	+	+	+	+
19	G III LN+ive	10	+	-	+	+
20	Metastatic LN	20	+	+	+	+

Cases 4, 6, 8, 12, 14, 17 and 19 did not show the presence of exon 6 and case 13 was negative for exon 5. This seems probably to be due to some technical problem with exon 6 amplification, resulting in undetectable quantity of DNA. If there were true deletion of exon 6, then all other exons would also be absent in these cases resulting in deletion of whole of *P53* gene. Alternatively, there could be deletion or mutation in exon 6 in these cases. Case 13 probably did not have enough DNA in it to allow detection of exon 5. Generally, all cases positive for p53 by immunostaining had exons 5, 7 and 8 detectable by PCR and most cases also had exon 6. All the samples had repeat PCR performed by

MWG Biotech to double check results and they were similar. The next question was whether these had any p53 mutation as determined by DNA sequencing.

RESULTS OF SEQUENCE ANALYSIS:

Unfortunately, the samples could not be sequenced, the probable reason being that they had a secondary structure which meant that the sequencing primers could not work. The PCR was repeated by MWG Biotech to confirm that PCR worked, which was reported that it did. However, they were unable to sequence it. It is possible that cloning of the PCR product in a specific vector could help to sequence these samples.

FTIR ANALYSIS OF BREAST TISSUES:

In this study, FTIR–Attenuated Total Reflectance (ATR) microspectroscopy and FTIR Chemical Imaging were employed to study the biochemical changes taking place within the breast carcinoma. FTIR spectra of the normal breast tissue were compared with the malignant tissue. Different grades of DCIS (LNG, ING and HNG) and IDC (GI, GII and GIII) were analysed as shown in table 8.35.

Table 8.35; Number of cases studied with FTIR, FTIR Chemical imaging and FT-Raman

No.	Histology	Total number of Cases
1	Normal	7
2	LNG	10
3	ING	10
4	HNG	10
5	G I	10
6	G II	10
7	G III	10
Total Number		**67**

FTIR Spectroscopic Differences between Normal, DCIS and IDC:

The FTIR spectra of the normal breast tissue, a case of HNG DCIS and a case of GIII IDC are given in figure 8.30. The most noticeable feature of each spectrum is the complexity, with prominent absorption in almost all regions of the spectra. Significant differences exist both in the absolute and relative intensities of the absorption bands of the spectra. The significance is spectroscopic, not statistical as in spectroscopy, a difference

of 10 wavelengths is considered significant. The reason that DCIS and IDC of same grade gave different spectra as opposed to no difference suggested by the initial molecular marker study could be that by immunohistochemistry only 6 markers were studied, whereas an FTIR spectrum was a collective reflection of total number and types of proteins in each case, which could have been more than 100,000. Before the spectrum of cancer tissue is discussed in detail it is important to characterise the positioning of the peaks and their assignment for the normal tissue.

The FTIR spectrum of normal tissue has well defined peaks in the region of 3500 to 650cm^{-1} (see figure 8.30 and table 8.36). Assignment of the peaks is attributed to the previous infrared spectroscopic studies on natural tissues and on cellular organic macromolecules (11-21).

A broad band centred at 3285cm^{-1} indicates the presence of amino groups. Secondary amines and arylamines exhibit only a single characteristic infrared band in the N – H stretching region (3500 – 3100cm^{-1}). A weak infrared band present in the region of (3050 – 3070cm^{-1}) is characteristic of secondary amides.

The region between 3100 to 2700cm^{-1} is due to the presence of C – H vibrations of lipids, proteins and DNA. The peak centred at 2958cm^{-1} is due to CH_3 antisymmetric stretch. Peaks centred at 2920 and 2850cm^{-1} confirm the presence of CH_2 antisymmetric and symmetric stretches respectively.

The strong absorption band in the region of 1700 to 1600cm^{-1} is arising from (C = O) vibrations of the amide groups of polypeptide chains. In the infrared spectrum, (C = O) bands tend to be among the strongest and this intensity of the carbonyl band makes the detection of this group simpler.

The peak centred at 1656cm^{-1} is due to bonded (C = O) and band at 1538cm^{-1} confirms the presence of C – N – H moieties. Mono substitute amides usually exist with the NH and (C = O) bonds. The hydrogen bonded stretch is seen near 3290cm^{-1} with a weaker band near 3070 cm^{-1} is a Fermi resonance enhanced overtone of 1538cm^{-1} band. This band at 1538cm^{-1} involves both (C – N) stretch and (C – N – H) in-plane bend in the amide II band. This band is a characteristic for mono-substituted amides. Amide III bands absorb more weakly and are confirmed by the presence of peaks at 1310 and 1260cm^{-1}.

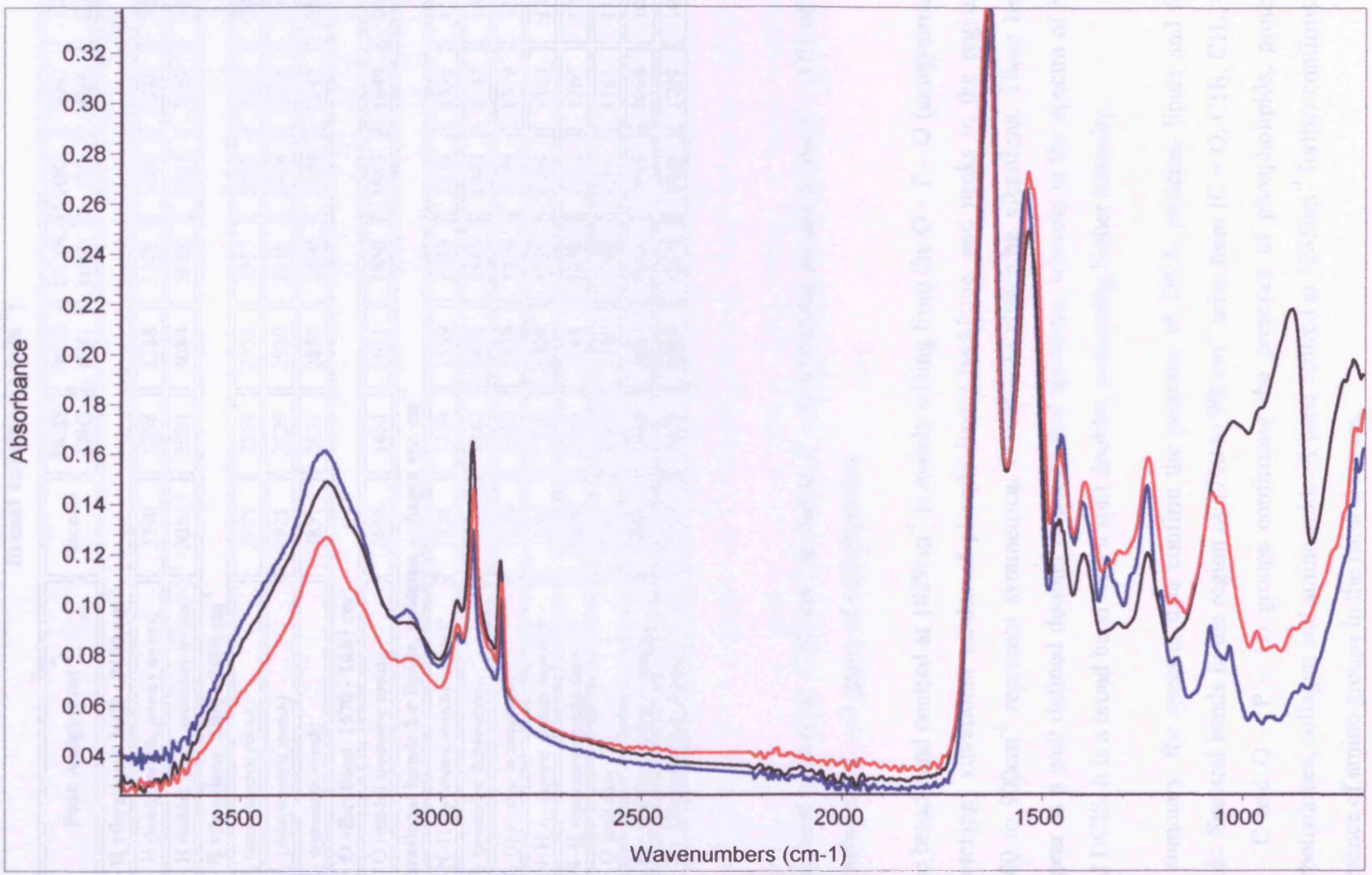


Figure 8.30; FTIR Spectra of Normal, DCIS (HNG) and IDC (GIII) Breast Tissues Where; Blue = Normal, Red = DCIS and Black = IDC

Table 8.36; Peak assignments of the FTIR spectra of normal breast tissue and breast carcinoma (cm^{-1})

Peak Assignment	Normal	DCIS LNG	DCIS ING	DCIS HNG	IDC GI	IDC GII	IDC GIII
N - H vibrations 3600 - 3100 cm^{-1}							
N – H (bonded stretch, primary amines)	3290	3288	3288	3288	3286	3286	3287
N – H (bonded stretch, secondary amines)	3086	3084	3084	3085	3083	3083	3083
C - H vibrations 3300 - 2800 cm^{-1}							
CH_3 (antisymmetric stretch)	2959	2958	2958	2957	2959	2957	2957
CH_2 (antisymmetric stretch)	2921	2920	2920	2919	2919	2918	2918
CH_2 (symmetric stretch)	2851	2851	2850	2850	2849	2849	2849
C = O vibrations 1670 - 1630 cm^{-1}							
C = O (amide I symmetric stretch)	1656	1651	1651	1650	1649	1649	1649
Vibrational bands for lipids, proteins, collagen etc. cm^{-1}							
C– N–H (symmetric stretch) Amide II	1538	1539	1539	1539	1539	1539	1539
CH_2 (symmetric deformations)	1452	1442	1442	1441	1442	1442	1442
CH_3 (symmetric deformations)	1379	1379	1379	1379	1379	1379	1379
C–N–H (symmetric stretch) Amide III	1310	1310	1309	1310	1309	1309	1309
C–N–H (symmetric stretch) Amide III	1260	1261	1261	1260	1261	1260	1260
C – O (stretching)	1161	1161	1160	1160	1161	1161	1161
O – P – O (antisymmetric stretching)	1067	1068	1067	1068	1068	1068	1068
O – P – O (symmetric stretching)	1029	1029	1029	1029	1029	1029	1028

The band centred at 1160 cm^{-1} is due to C – O stretching modes of the C – OH of cell proteins and C – O group of carbohydrates.

The broad band centred at 1029cm^{-1} is mainly arising from the O – P – O (antisymmetric stretching) vibrational modes of phosphodiester backbone, and peaks in the region of 1100 to 900cm^{-1} represent symmetrical O – P – O stretching vibrations. These bands appear as a well defined doublet in the normal spectrum, whereas in the spectra of IDC and DCIS, it is a broad band with a split doublet, possessing higher intensity.

In summary, the spectral data confirm the presence of DNA, proteins, lipids and fatty acids. Spectral bands in the region of 1700 to 900cm^{-1} arise from (C = O, CH_2, CH_3, C – O – C and O – P – O) groups confirming the presence of phospholipids, proteins, carbohydrates, collagen and amino acids. A band centred at 3285cm^{-1} further confirms the presence of amino groups in the natural tissues.

FTIR Spectroscopic Differences between IDC Grades (I, II, and III):

As mentioned earlier, significant spectroscopic differences between spectra of normal and invasive ductal carcinoma (IDC) nuclei are observed (figure 8.30). Spectra of normal and breast carcinoma nuclei are complex and posses well-defined and prominent spectral bands at 1700 to 900cm^{-1} and 3500 to 2700cm^{-1} region. Differences in the intensity and positioning of peaks in the spectra are attributed to the compositional changes between the normal and cancer tissue.

The increase in intensity of the C – H peaks may be attributed to the increase in the lipids, proteins and DNA. Similarly, increase in intensity and shifting of peaks in the area of 1700 to 1500cm^{-1} (carbonyl stretching and amide-bending vibrations in the DNA) confirm the changes in the chemical structure of normal tissue. An overall shift of peaks in the fingerprint region (1700 to 800cm^{-1}) is observed in the spectra.

Spectral area of 1250 to 960 cm^{-1} (peaks assigned to O – P – O, P – O and C – O stretching vibrations of the phosphodiester group) shows significant spectroscopic differences between the nuclear spectra of IDC grades, as peak positioning is also shifted in addition to change in absorbency of the peaks. This indicates that chemical structure is changing with the formation of new peaks suggesting the formation of new groups, as two small peaks in the region of 1200 to 1000cm^{-1} merge together to form a broad band. This band could be attributed to C – O of phospholipids and proteins and appears to be due to increase in these substances in the cancer cells.

A prominent difference is also observed in the bands appearing at 1080 and 1030 cm^{-1}. The grade I has a well-defined doublet, similar to the one observed in the FTIR spectrum of the normal tissue. The peak centred at 1029cm^{-1}, increases in intensity with the increasing grade (I to III), whereas the peak centred at 1068cm^{-1} reduces in intensity with the increasing grade (I to III) (figure 8.31).

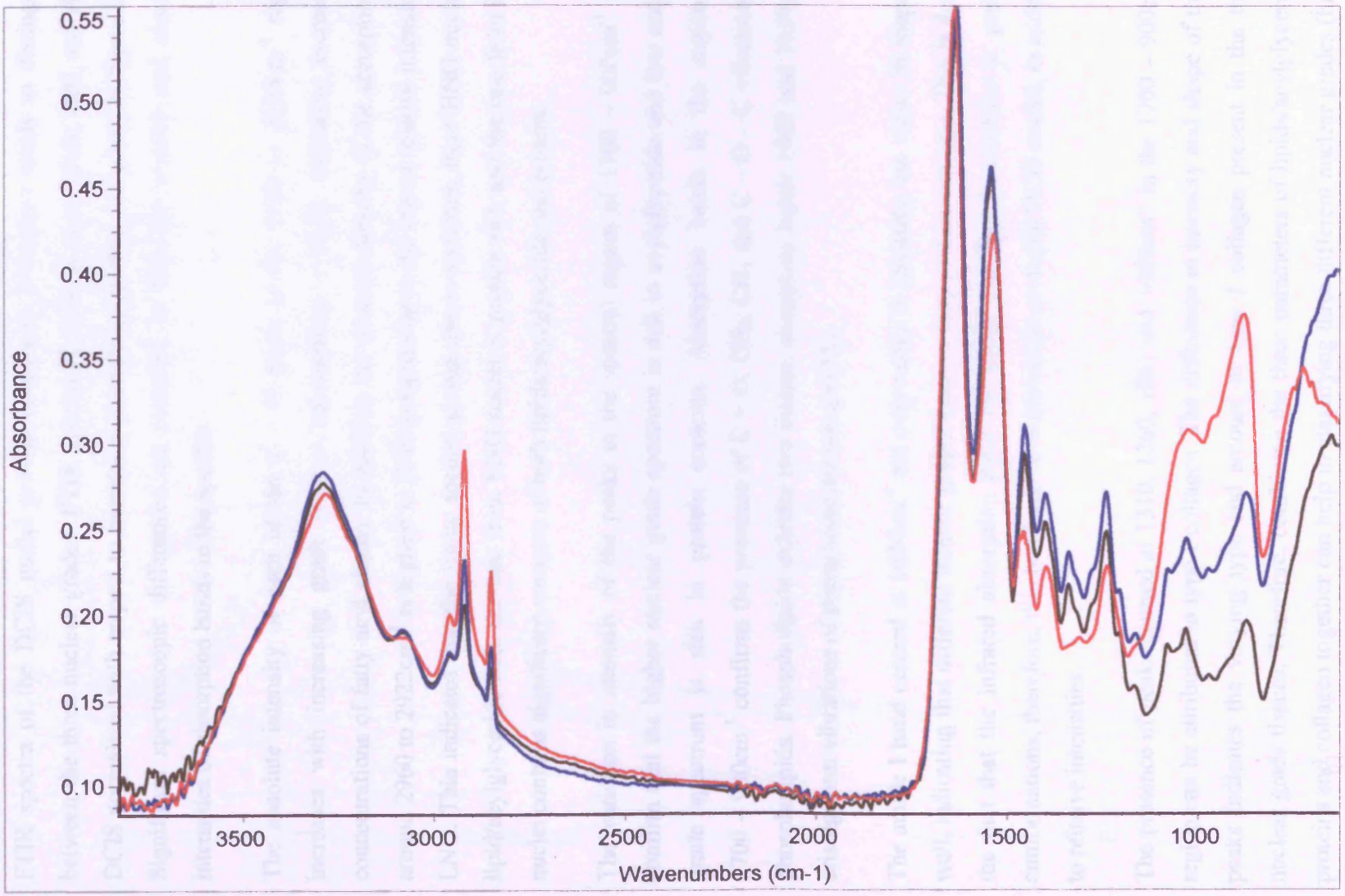


Figure 8.31; FTIR Spectra of different IDC grades, where Red = GIII, Blue = GII, and black = GI

FTIR Spectroscopic Differences between DCIS Grades (LNG, ING and HNG):

FTIR spectra of the DCIS nuclei provide prominent absorption bands to distinguish between the three nuclear grades. FTIR spectra of sections from LNG, ING and HNG DCIS normalised with respect to maximum absorption intensity are given in figure 8.32. Significant spectroscopic differences are observed in both the relative and absolute intensities of absorption bands in the spectra.

The absolute intensity of each of the C – H peaks in the 3500 to 2700cm^{-1} region increases with increasing grade (low – intermediate – high), indicating increasing concentrations of fatty acyl chains. In addition the relative intensity of the absorption at around 2960 to 2922cm^{-1} is highest in HNG progressing to the lowest relative intensity in LNG. This indicates that the tissue section giving rise to spectrum from HNG nuclei is lipid/acylglyceride rich, the one from LNG nuclei is protein rich and the one from ING nuclei contains significant amounts of both lipids/acylglyceride and protein.

The variation in intensity of the peaks in the spectral region of 1700 – 900cm^{-1} also confirm that the higher nuclear grade spectrum is rich in acylglyceride and low nuclear grade spectrum is rich in protein contents. Absorption bands in the region of 1700 – 900cm^{-1} confirms the presence of C = O, CH_2, CH_3 and C – O – C vibrations of phospholipids. Phospholipids exhibits two intense absorption bands 1067 and 1029cm^{-1} arising from vibrations of phosphodiester groups (15).

The amide I band centred at 1654cm^{-1} not only varies in intensity, but varies in shape as well, indicating that different tumour grades have varying protein contents. This is due to the fact that the infrared absorption band for amide I groups is sensitive to protein conformations, therefore, this band can be employed to grade the DCIS nuclei, in addition to relative intensities.

The presence of peaks centred at 1310, 1260, 1067 and 1029cm^{-1} in the 1700 – 900cm^{-1} region can be attributed to type I collagen. The difference in intensity and shape of these peaks indicates the varying type and amount of type I collagen present in the three nuclear grade tissues. Therefore, combining the three parameters of lipids/acylglyceride, proteins and collagen together can help in classifying three different nuclear grades (high, intermediate and low) more precisely and consistently.

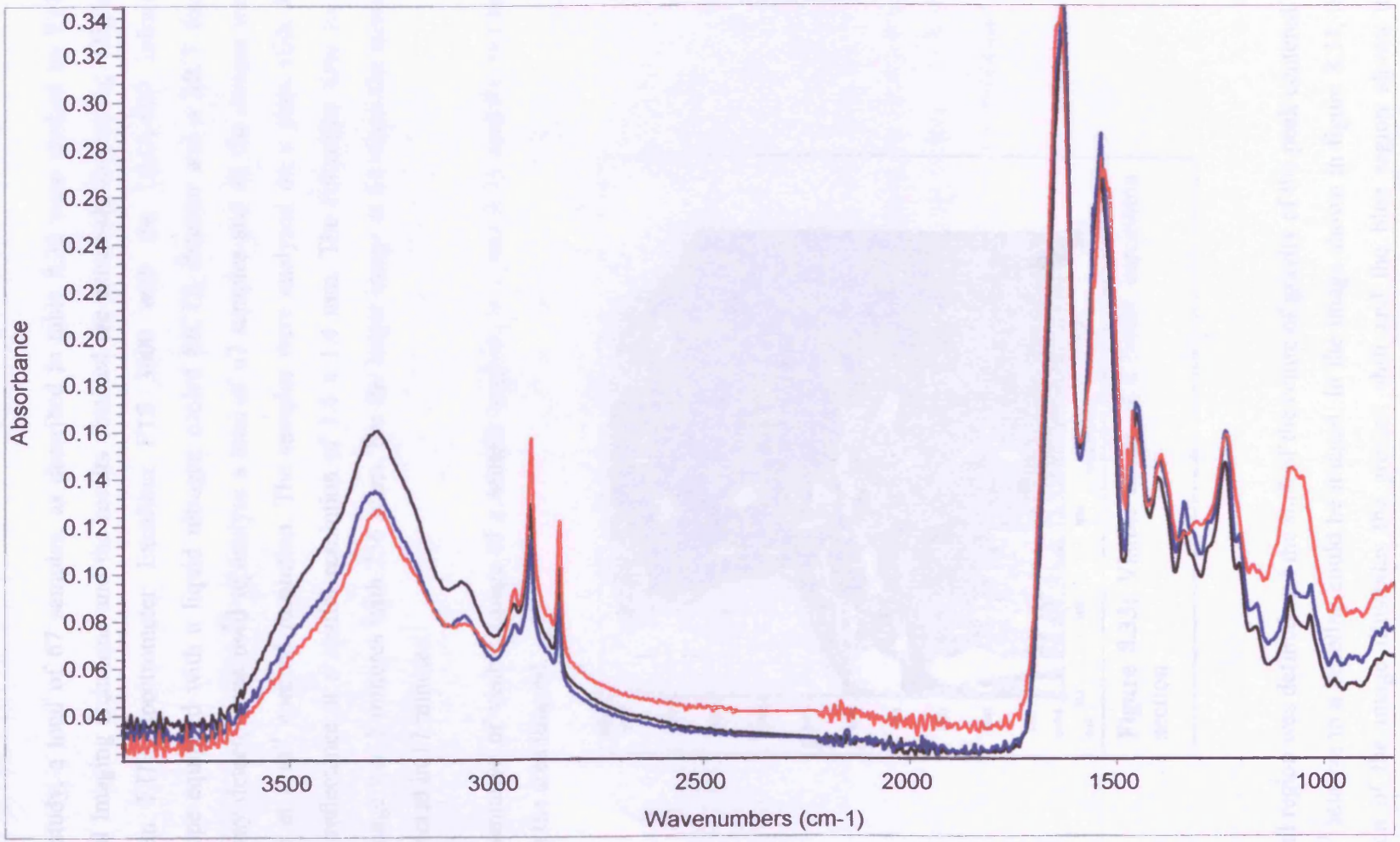


Figure 8.32; FTIR Spectra of different DCIS grades, where; Red = HNG, Blue = ING, and Black = LNG

FTIR Chemical Imaging:

In this study, a total of 67 samples, as described in table 8.35 were studied by FTIR Chemical Imaging technique and the results obtained are extremely interesting. Digilab rapid-scan FTIR spectrometer Excalibur FTS 3000 with the UMA-600 infrared microscope equipped with a liquid nitrogen cooled MCTA detector and a MCT focal plane array detector was used to analyse a total of 67 samples and all the images were collected at $8cm^{-1}$ spectral resolution. The samples were analysed on a glass slide by external reflectance at a spatial resolution of 1.4 x 1.4 mm. The collection time for a single image was 5 minutes with 256 scans. For the larger image at 64 scans the mosaic was collected in 17 minutes.

A representative of visible image of a sample is given in figure 8.33 and the red line indicates the area imaged.

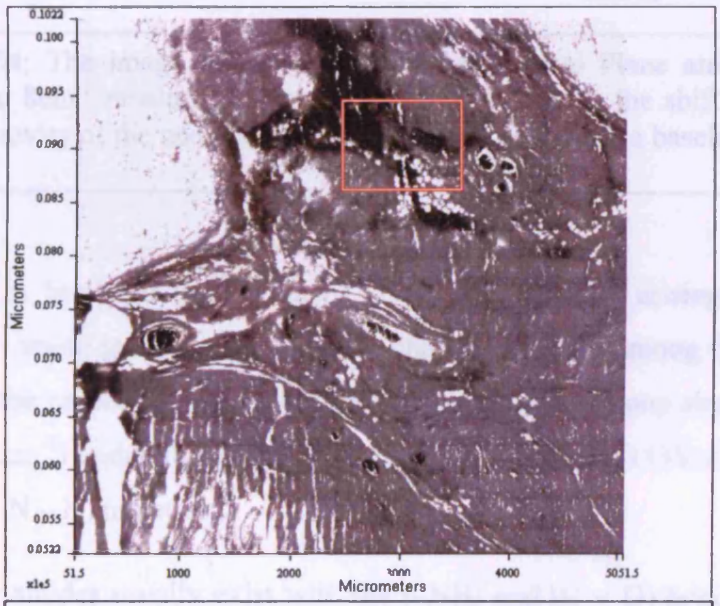


Figure 8.33; Visible image of a breast carcinoma section

A spectral region was defined and the shift of the centre of gravity of the peak centered at $1658cm^{-1}$ relative to a baseline could be imaged. In the image shown in figure 8.34, the red region of the image illustrates the greatest shift and the blue region shows the minimum shift.

The image in Figure 8.34 was collected using a 64 x 64 MCT focal Plane array and collected at 8cm^{-1} resolution. This technique was used for imaging all the normal and cancerous tissues.

Band Shift of Amide I band between 1700-1600cm^{-1}.

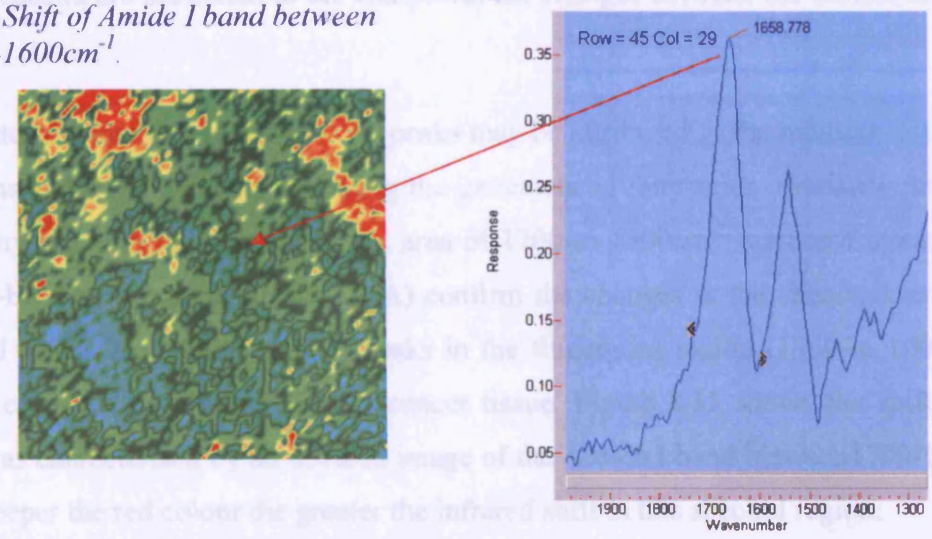


Figure 8.34; The image obtained 64 x 64 MCT focal Plane array and collected at 8cm^{-1} resolution. A spectral region indicating the shift of the centre of gravity of the peak centered at 1658cm^{-1} relative to a baseline.

Strong absorption bands in the region of (1700 to 1600cm^{-1}) arising from (C = O) vibrations of the amide groups of polypeptide chain, tend to be among the strongest and the intensity of the carbonyl band makes the detection of this group simpler. The Peak centred at (1632cm^{-1}) is due to bonded (C = O) and the band at (1538cm^{-1}) confirms the presence of (C – N – H) moieties.

Mono substitute amides usually exist with the (–NH) and (C = O) bonds. This band at (1538cm^{-1}) involves both (C – N) stretch and (C – N – H) in-plane bend in the amide II band. This band is a characteristic for mono-substituted amides. Amide III bands absorb more weakly and are confirmed by the presence of peaks at (1310 and 1235cm^{-1}). A Band centred at (1160 cm^{-1}) is due to (C – O) stretching modes of the (C – OH) of cell proteins and (C – O) group of carbohydrates.

Spectral bands in the region of (1700 to 900cm^{-1}) arise from (C = O, CH_2, CH_3, C – O – C and O – P – O) groups confirming the presence of phospholipids, proteins, carbohydrates,

collagen and amino acids. As described earlier, differences between normal and cancerous tissues' nuclear spectra are observed. Spectra of normal and breast cancer tissue are complex and possess well defined and prominent spectral bands at (1700 to 700cm^{-1}) & (3500 to 2700cm^{-1}) region. Difference in intensity and positioning of peaks in the spectra are attributed to the compositional changes between the normal and cancer tissue.

The intensity increase of the (C – H) peaks may be attributed to the increase in the lipids, proteins and DNA contents indicating the increment of fatty acids. Similarly, increase in intensity and shifting of peaks in the area of 1700 to 1500cm^{-1} (carbonyl stretching and amide-bending vibrations in the DNA) confirm the changes in the chemical structure of normal tissue. An overall shift of peaks in the fingerprint region (1700 to 1000cm^{-1}) is observed in the spectrum of breast cancer tissue. Figure 8.35 shows the shift in these bands as characterised by an infrared image of the Amide I band between1700-1600cm^{-1}. The deeper the red colour the greater the infrared shift in this spectral region.

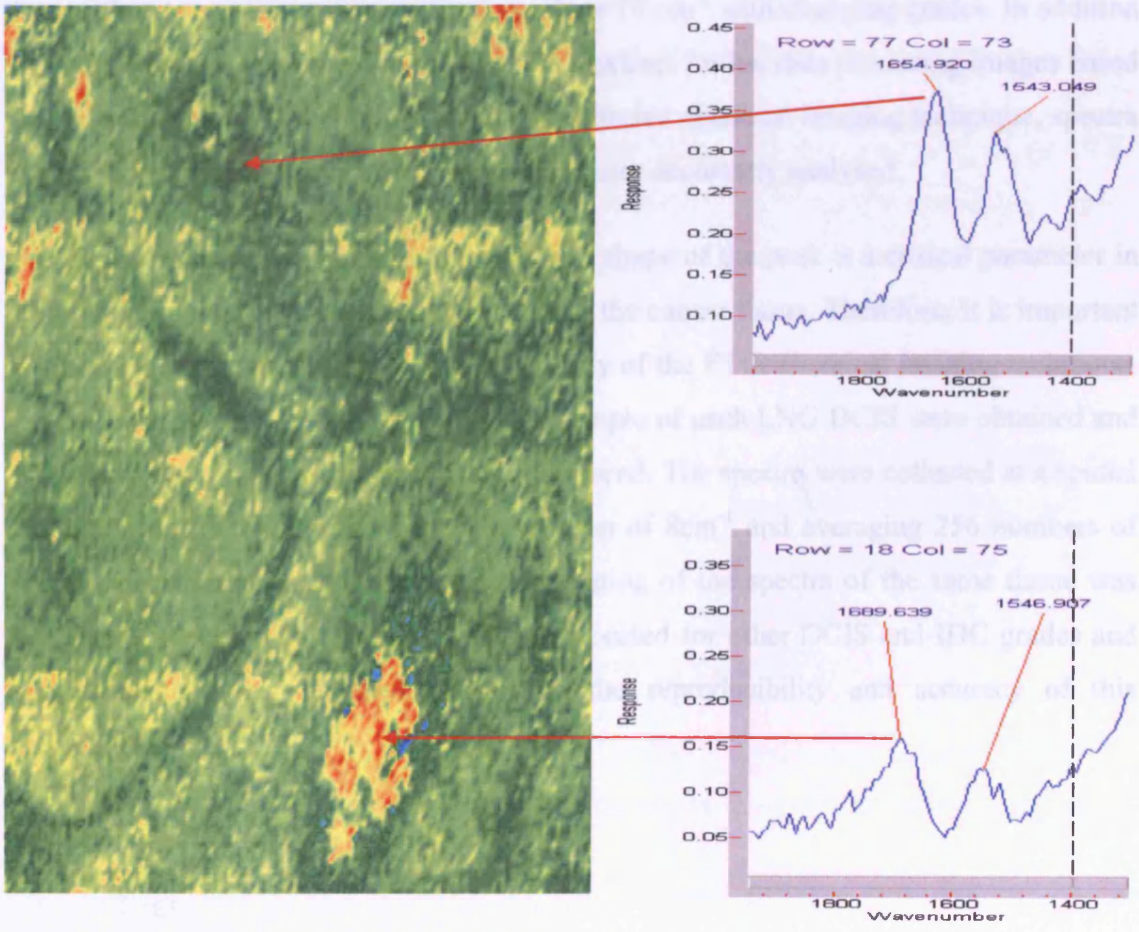


Figure 8.35; The image above obtained by a mosaic of an area 1.4 X 1.4 mm, Band shift of Amide I band between 1700 – 1600 cm^{-1}

Different colours in the image represent the varying nature of chemical structure present in the tissue. The technique allows the extraction of the IR spectrum from the required area of the IR image and comparison with the surrounding area, as illustrated in figures 8.35 and 8.36. By employing FTIR chemical imaging useful information pertaining to carbonyl stretching and amide-bending vibrations in the DNA can be extracted. This data can then be used to define infrared images that show the shift of amide bands between 1700 to1500cm^{-1}. The images extracted show that this shift is significant sometimes varying by as much as 10 cm^{-1} with changing grades. In addition PCA analysis can be applied to the images to extract further data producing images based on the principal components. Employing an infrared chemical imaging technique, spectra from the required image can be obtained and more accurately analysed.

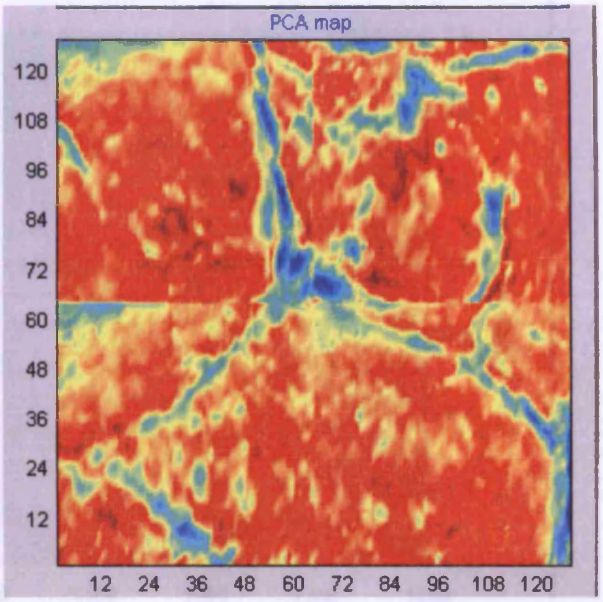


Figure 8.36; PCA map showing different colours based on varying principal components

As the wavenumber positioning, intensity and shape of the peak is a critical parameter in differentiating between the type and grades of the cancer tissue. Therefore, it is important to demonstrate the accuracy and reproducibility of the FTIR chemical imaging technique. To demonstrate this, 15 spectra within one sample of each LNG DCIS were obtained and the peak positioning and its shape were monitored. The spectra were collected at a spatial resolution of 0.5 x 0.5 mm. spectral resolution of 8cm^{-1} and averaging 256 numbers of scans. The difference within the peak positioning of the spectra of the same tissue was less than ± 0.1cm^{-1}. This process was also repeated for other DCIS and IDC grades and similar results were obtained confirming the reproducibility and accuracy of this technique.

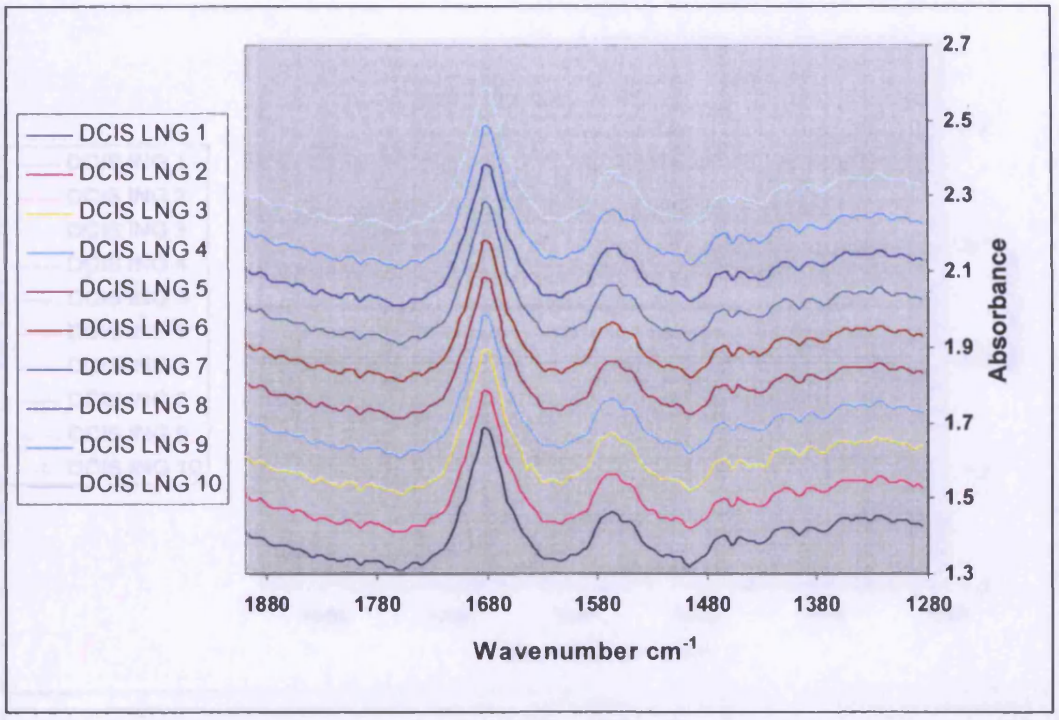


Figure 8.37a; FTIR spectra of DCIS LNG indicating that there were no major differences in shape and positioning of peaks within the fingerprint region

Once the variability within the same case was evaluated and reproducibility of the FTIR chemical imaging was established, it was important to establish variability within different cases of the same grade. FTIR nuclear spectra of 10 cases of LNG DCIS are shown in figure 8.37a, which indicate that there is no significant peak shift or change in the shape of the peaks relative to each other.

The difference within the peak positioning of the nuclear spectra of the different cases within same grade was less than ± 0.5cm^{-1}. Spectra in figure 8.37b and 8.37c are from ING and HNG DCIS, which also demonstrate that there is very little peak shift or change in the shape of the peaks relative to each other confirming that nuclear spectra of the same grade across the different samples did not possess major variability.

This was also checked for IDC grades (GI, GII and GIII) and results obtained confirmed that there is no significant spectral variability within the grades. Spectra are presented in figure 8.38a, 8.38b and 8.38c.

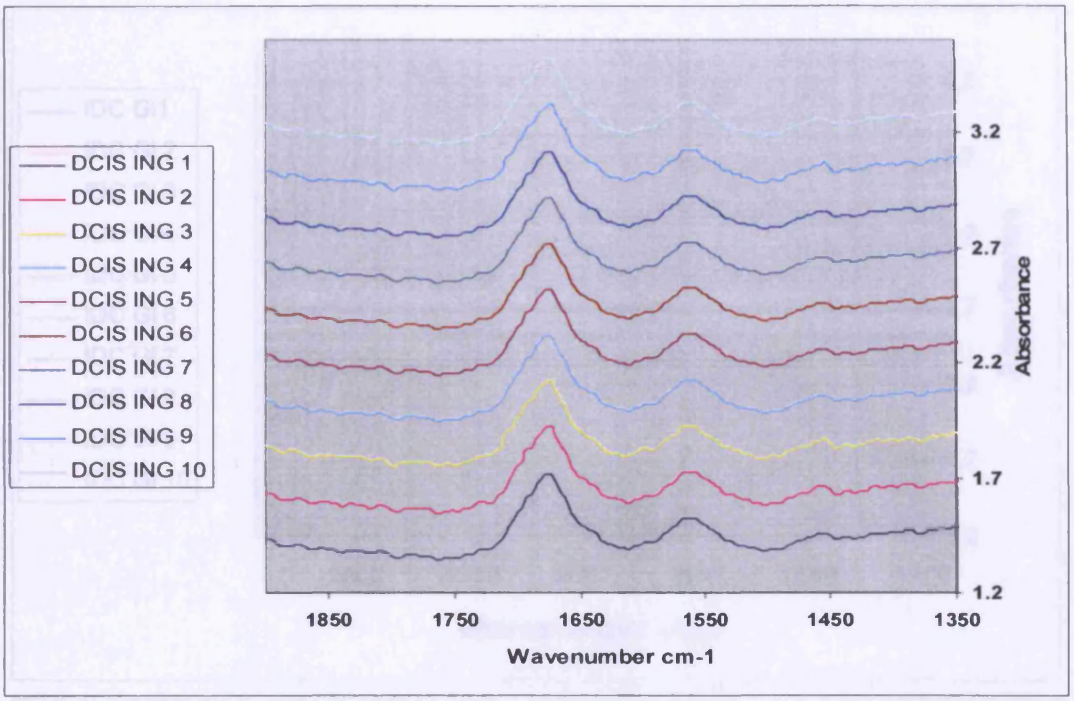


Figure 8.37b; FTIR spectra of DCIS ING indicating that there were no major differences in shape and positioning of peaks

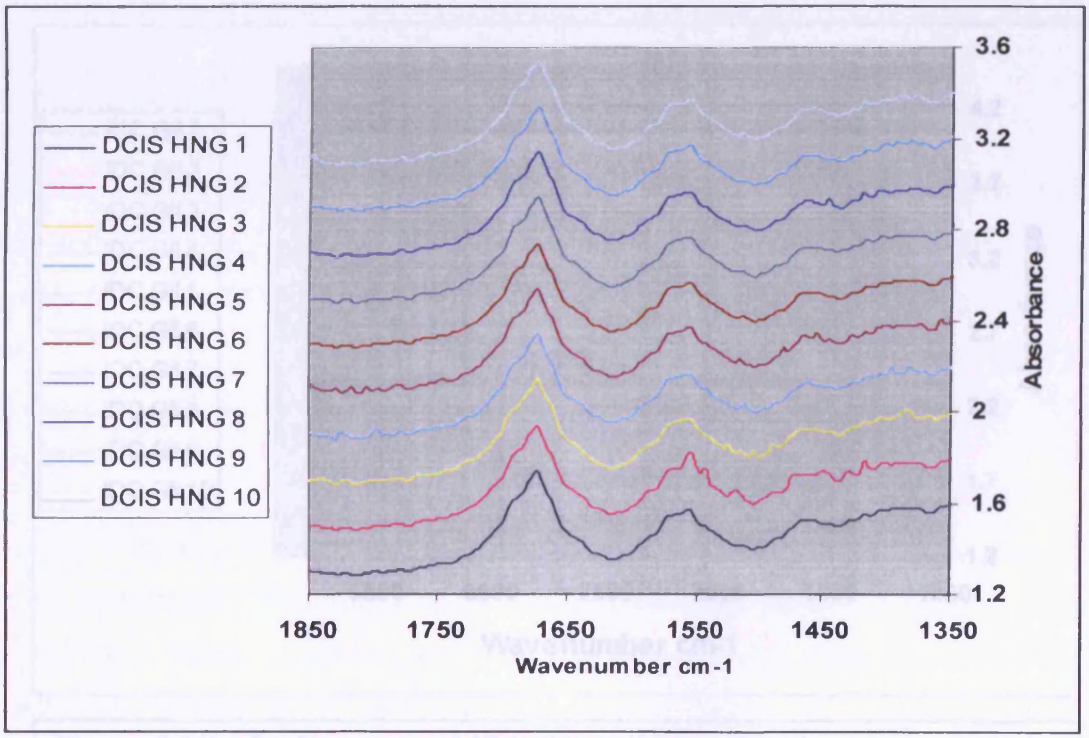


Figure 8.37c; FTIR spectra of DCIS HNG indicating that there were no major differences in shape and positioning of peaks

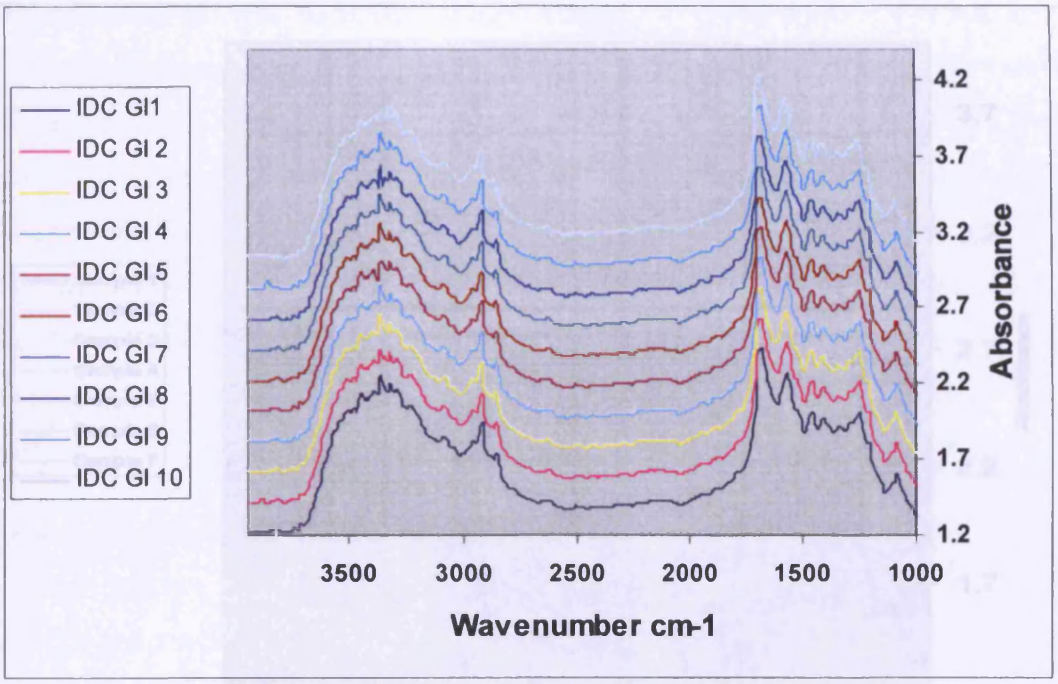


Figure 8.38a; FTIR spectra of IDC GI indicating that there were no major differences in shape and positioning of peaks

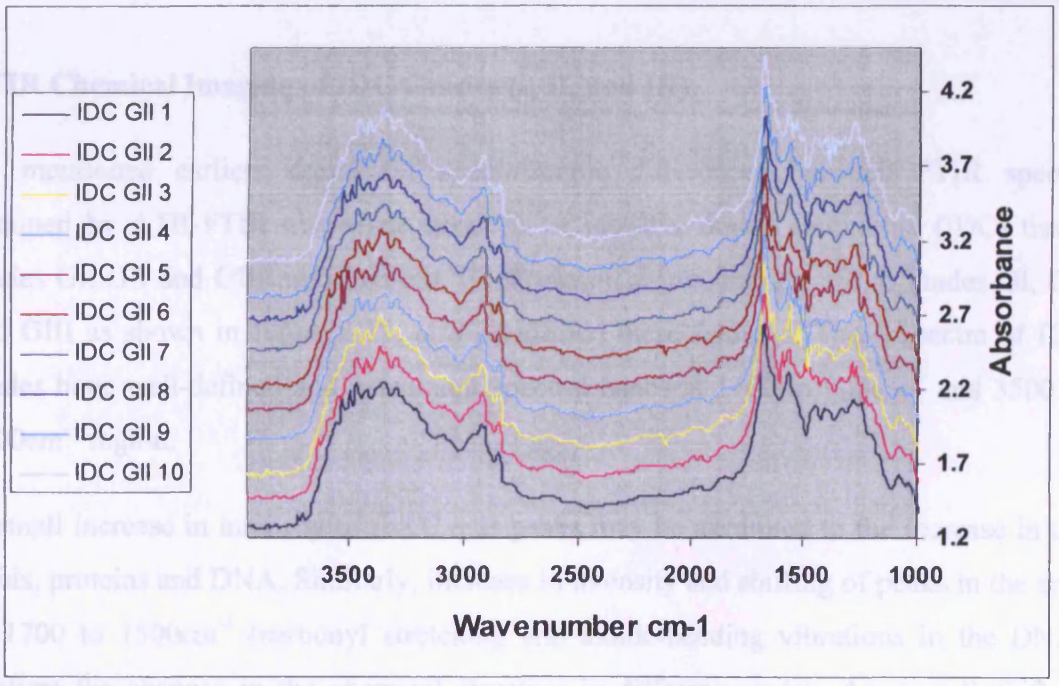


Figure 8.38b; FTIR spectra of IDC GII indicating that there were no major differences in shape and positioning of peaks

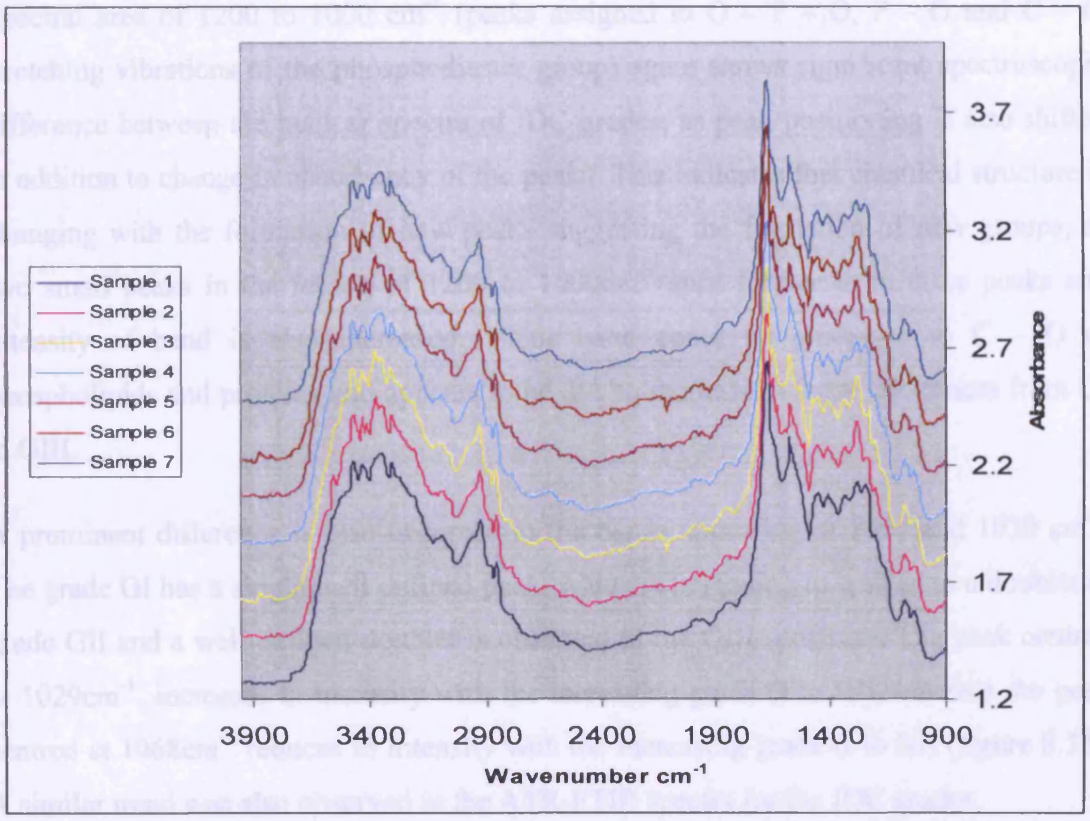


Figure 8.38c; FTIR spectra of IDC GIII indicating that there were no major differences in shape and positioning of peaks

FTIR Chemical Imaging of IDC Grades (I, II, and III):

As mentioned earlier, significant spectroscopic differences between FTIR spectra obtained by ATR-FTIR micro-spectroscopy of invasive ductal carcinoma (IDC) tissue grades GI, GII and GIII are observed. FTIR chemical imaging spectra of grades GI, GII and GIII as shown in figure 8.39, also confirmed these findings. These spectra of IDC grades have well-defined and prominent spectral bands at 1700 to 900cm^{-1} and 3500 to 2700cm^{-1} region.

A small increase in intensity of the C – H peaks may be attributed to the increase in the lipids, proteins and DNA. Similarly, increase in intensity and shifting of peaks in the area of 1700 to 1500cm^{-1} (carbonyl stretching and amide-bending vibrations in the DNA) confirm the changes in the chemical structure in different grades. An overall shift of peaks in the fingerprint region (1700 to 1000cm^{-1}) is also evident in the spectra of different IDC grades.

Spectral area of 1200 to 1000 cm^{-1} (peaks assigned to O – P – O, P – O and C – O stretching vibrations of the phosphodiester group) again shows significant spectroscopic difference between the nuclear spectra of IDC grades, as peak positioning is also shifted in addition to change in absorbency of the peaks. This indicates that chemical structure is changing with the formation of new peaks suggesting the formation of new groups, as two small peaks in the region of 1200 to 1000cm^{-1} split further in to three peaks and intensity of band is also increased. This band could be attributed to C – O of phospholipids and proteins and appears to be due to increase in these substances from GI to GIII.

A prominent difference is also observed in the bands appearing at 1080 and 1030 cm^{-1}. The grade GI has a single well defined peak, which is beginning to split in to a doublet in grade GII and a well defined doublet is observed in the GIII spectrum. The peak centred at 1029cm^{-1}, increases in intensity with the increasing grade (I to III), whereas the peak centred at 1068cm^{-1} reduces in intensity with the increasing grade (I to III) (figure 8.38). A similar trend was also observed in the ATR-FTIR spectra for the IDC grades.

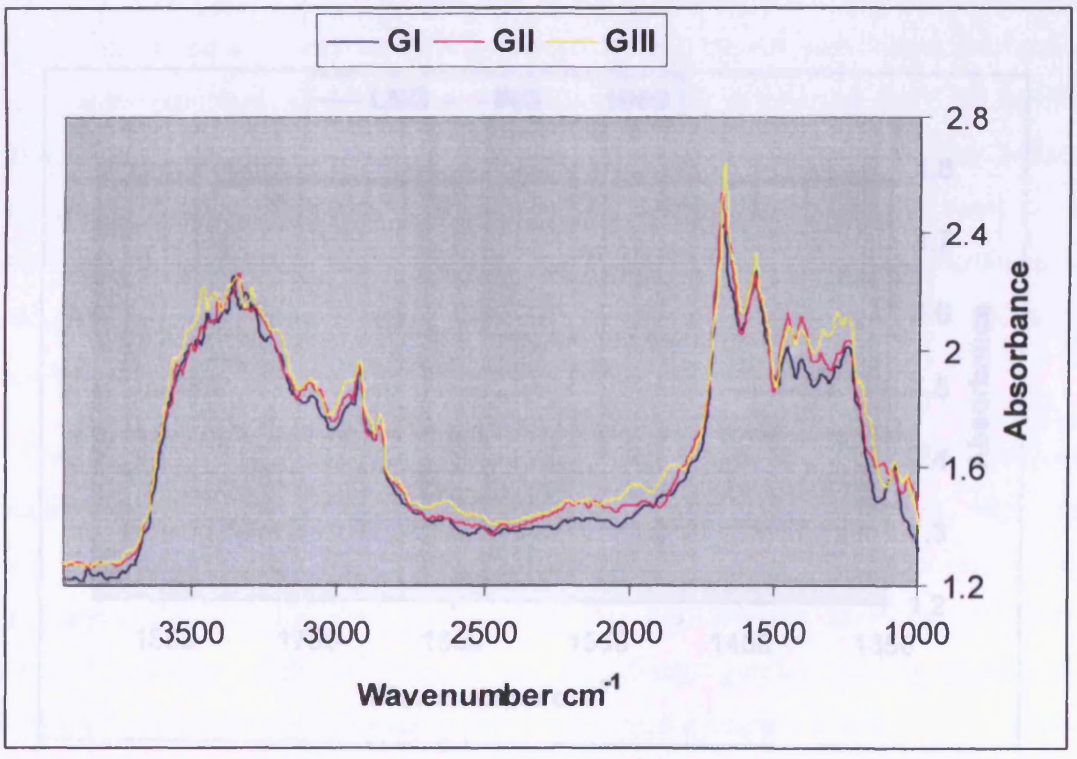


Figure 8.39; FTIR Spectroscopic Differences between IDC grades (GI, GII and GIII)

FTIR Chemical Imaging of DCIS Grades (LNG, ING and HNG):

FTIR chemical imaging spectra of the DCIS tissues provide absorption bands that are more or less similar to that obtained by FTIR ATR-micro-spectroscopy, but are slightly well defined due to the increased sensitivity of the technique. Spectra of sections from LNG, ING and HNG DCIS in the region of 1700 to 1300cm^{-1} are shown in figure 8.40. This region provides significant spectroscopic difference both in the relative and absolute intensities of absorption bands in the spectra and also shift in the positioning of the peaks.

The variation in intensity of the peaks in the spectral region of 1700 – 1300cm^{-1} also confirm that the higher nuclear grade spectrum is rich in acylglyceride and low nuclear grade spectrum is rich in protein contents. Absorption bands in the region of 1700 – 1350cm^{-1} confirms the presence of C = O, CH_2, CH_3 and C – O – C vibrations. The amide I band present between 1750 at 1600cm^{-1} not only varies in intensity, but varies in shape as well, indicating that different tumour grades have varying protein contents.

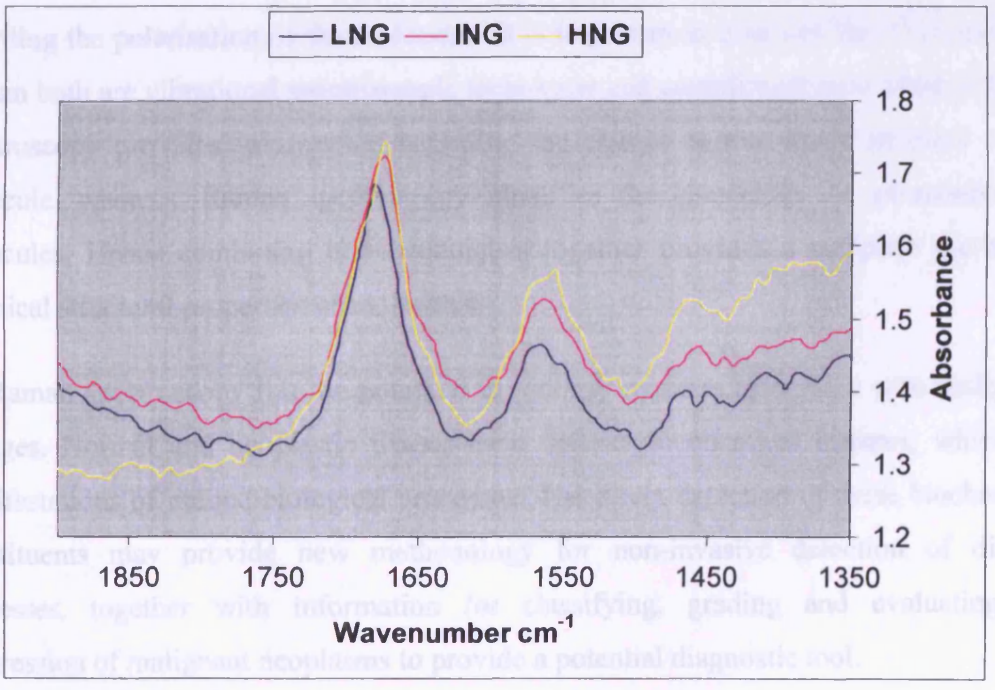


Figure 8.40; FTIR Spectroscopic Differences between DCIS Grades (LNG, ING and HNG)

This amide I vibrational mode exhibits significantly high sensitivity to conformational changes in the secondary structure, as a shift in the bands (4 to 8cm^{-1}) was also observed indicating the changing nature of chemical structure. The peaks of maximum absorption in the amide I stretching band region shifted to lower frequencies with increasing nuclear grades indicating stronger hydrogen bonding. This is due to the fact that the infrared absorption band for amide I groups is sensitive to protein conformations, therefore, this band can be employed to differentiate between the DCIS grades, in addition to relative intensities and wavenumber shift.

These results indicate that the composition of the bulk of proteins in breast tissues undergo considerable changes during carcinogenesis, including changes in the extent of hydrogen bonding between the amide groups in proteins, conformational differences such as increase in helical and decrease in turn secondary structure and differences in the relative content of collagen rich connective tissue for the different type of tissues.

FT-RAMAN ANALYSIS OF BREAST TISSUES:

In addition to the FTIR spectroscopic and imaging technique, FT-Raman spectroscopy was also employed to compliment the spectral data, as it provides additional information regarding the polarisation of the molecules. It is important to note that the FTIR and FT-Raman both are vibrational spectroscopic techniques and compliment each other. Infrared spectroscopy provides information regarding the change in the dipole moment of the molecule, whereas, Raman spectroscopy identifies the change in the polarisation of molecules. Hence combining both techniques together provides a complete picture of chemical structural properties of molecules.

FT-Raman spectroscopy has the potential to identify markers associated with malignant changes. Normal and neoplastic tissues have distinct biochemical features, which are manifestations of unique biological processes. The direct detection of these biochemical constituents may provide new methodology for non-invasive detection of disease processes, together with information for classifying, grading and evaluating the progression of malignant neoplasms to provide a potential diagnostic tool.

FT-Raman Spectroscopic Differences between Normal, DCIS and IDC:

FT-Raman nuclear spectra of a normal breast tissue, a case of DCIS HNG and a case of IDC GIII are given in figure 8.41. The most notable feature of each spectrum is the low fluorescence background, with well defined peaks in the regions of 1800 to 550cm^{-1}of the spectra. Obvious spectroscopic differences exist in both the absolute and relative intensities of the peaks in the spectra. Before the spectrum of cancer tissue is discussed in detail it is important to characterise the positions of the peaks and their assignment. These are shown in table 8.37. Assignment of the peaks is attributed to previous Raman spectroscopic studies on natural tissues and on cellular organic macromolecules (15;22-27).

The peak present at 1655cm^{-1} in the spectrum of normal breast tissue is due to a C = C stretch amide I band. The intensity of bands varies with the degree of fatty acid unsaturation and depends on the lipid to protein ratio. The spectral bands at 1455, 1442 and 1303cm^{-1} are due to CH_3 and CH_2 scissoring deformations. The peaks present at 970, 876 and 720cm^{-1} in the spectra are due to C – C stretch.

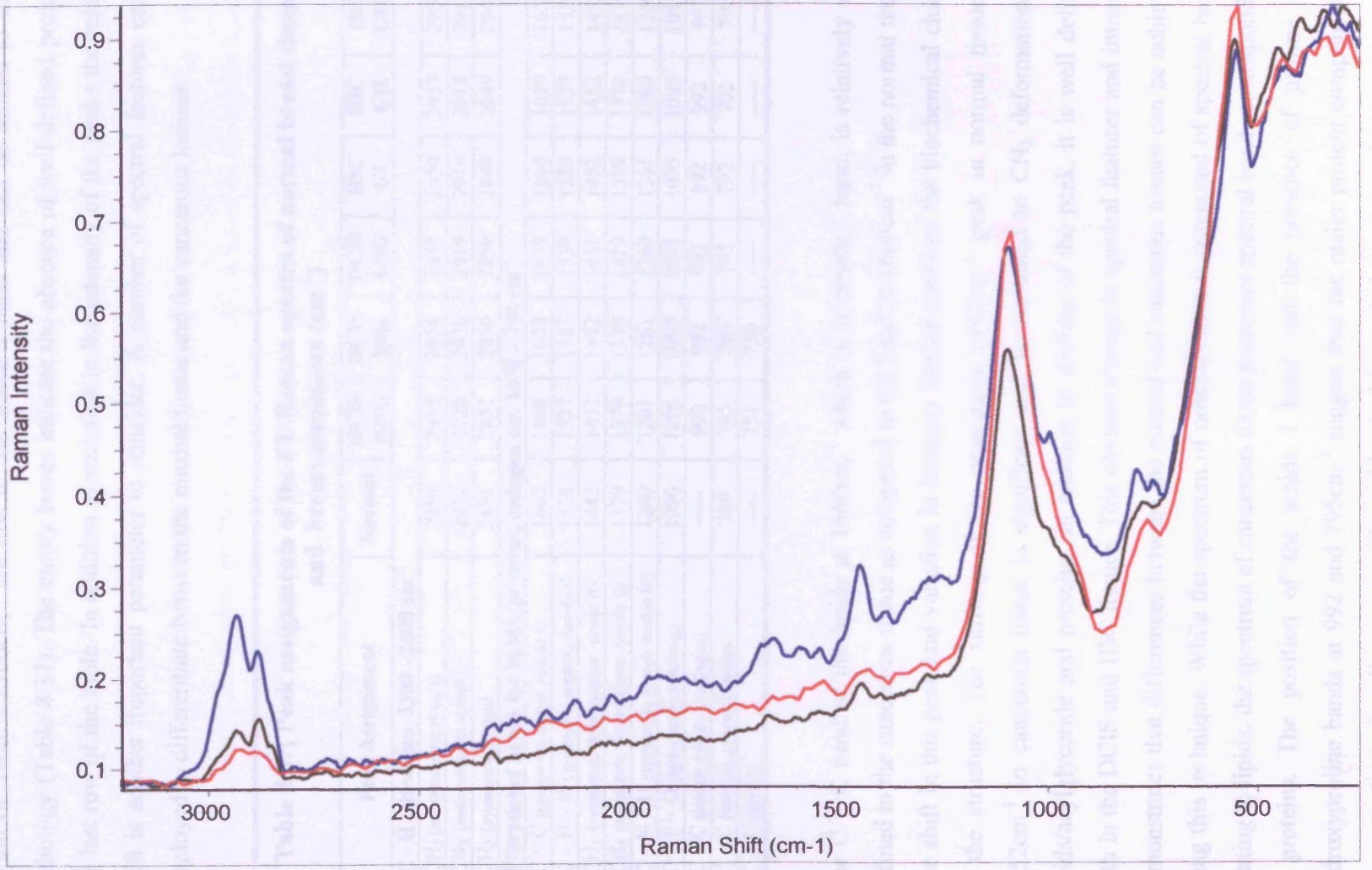


Figure 8.41; FT-Raman Spectra of normal, DCIS (HNG) and IDC (GIII) Where; Red = Normal, Black = IDC (GIII) and Blue = DCIS (HNG)

The changes in the relative intensities of the C – H bending mode, peaks present in the region of 2960 – 2700cm^{-1} are due to the amide I band and can be related to tissue pathology (Table 8.37). The empty boxes indicate the absence of well-defined peaks in the last row of the table. In addition to variation in the intensity of the peaks the relative shift is another important parameter to consider. A number of spectral features can be employed to differentiate between the normal tissue and the cancerous lesions.

Table 8.37; Peak assignments of the FT-Raman spectra of normal breast tissue and breast carcinoma (cm^{-1})

Peak Assignment	Normal	DCIS HNG	DCIS ING	DCIS LNG	IDC GI	IDC GII	IDC GIII
C - H vibrations 3300 - 2800 cm^{-1}							
CH_3 (antisymmetric stretch)	2959	2958	2958	2957	2959	2957	2957
CH_2 (antisymmetric stretch)	2921	2920	2920	2919	2919	2918	2918
CH_2 (symmetric stretch)	2851	2851	2850	2850	2849	2849	2849
Vibrational bands for lipids, proteins, collagen etc. 1670 – 750 cm^{-1}							
C = C (symmetric stretch Amide I)	1662	1660	1658	1658	1660	1659	1658
C – N – H (symmetric stretch) Amide II	1538	1537	1537	1539	1539	1537	1536
CH_2 (symmetric deformations Amide II)	1442	1452	1452	1451	1452	1452	1452
CH_3 (symmetric deformations Amide II)	1379	1379	1379	1379	1379	1379	1379
C – N – H (symmetric stretch Amide III)	1260	1261	1261	1260	1261	1260	1260
O – P – O (symmetric stretching)	1096	1096	1095	1095	1095	1095	1095
C – C (stretch Amide I, collagen)	-----	992	992	992	992	992	992
C – C (stretch), polysaccharides	780	795	795	795	792	792	792
C – C (stretch)	-----	751	750	-----	-----	-----	-----

The C = C band of the lipids at 1660cm^{-1} which is an amide I band, is relatively well defined in the cancerous tissue as compared to the band at 1660cm^{-1} in the normal tissue. The shift in this peak and variation in intensity further confirms the biochemical change of the structure. The shifting of the prominent 1442cm^{-1} peak in normal tissue to 1452cm^{-1} in cancerous tissue is significant and is attributed to CH_2 deformation of lipids/acylglyceride and proteins. In addition to shifting of the peak, it is well defined both in the DCIS and IDC tissues. This obvious change in spectral features and intensity demonstrates that differences between the normal and cancerous tissues can be achieved using this technique. While the spectrum of normal tissue is composed of spectral bands relating to lipids, the spectrum of cancerous tissue possesses spectral bands corresponding to proteins. The position of the amide I band and the presence of proline and hydroxyproline bands at 992 and 795cm^{-1} suggest that the major protein component in cancerous tissue is collagen, as evidenced by the histopathological examinations.

The lower intensity of the lipids/acylglyceride bands in the cancerous tissue spectrum implies a relative increase in the protein content.

The spectral differences in the region of 960 – 800cm^{-1} and 1400 – 1080cm^{-1} further confirm the increment of protein content and relative decrease in the lipids/acylglyceride content in the cancerous tissues. The bands at 1655, 1442 and 1262cm^{-1} are due to amide I, CH_2 bending and amide III vibrations of the proteins respectively. The presence of amide I bands further confirms the increment in protein content in the cancer tissue as compared to normal tissue.

FT-Raman Spectroscopic Differences between IDC Grades (I, II and III):

Figure 8.42 illustrates the differences between grades I, II and III of IDC. The FT-Raman spectrum of the normal tissue is weaker in intensity than that of the IDC tissue and the intensity of the peaks increases with increasing grades, as the grade III has the highest intensity, grade I possessing the minimum and grade II lying at an intermediate level.

The increase in intensity of the C – H peaks suggests change in the lipids, proteins and DNA contents. The absolute intensity of each of the C – H peaks in 3500 to 2700cm^{-1} region varies with increasing grade (I, II and III), indicating varying concentrations of fatty acyl chains.

Similarly, increase in intensity and shifting of peaks in the area of 1660 to 600cm^{-1} (carbonyl stretching and amide-bending vibrations in the DNA) confirm the changes in the chemical structure of breast tissue and this change in intensity provides useful information in differentiating between the IDC grades.

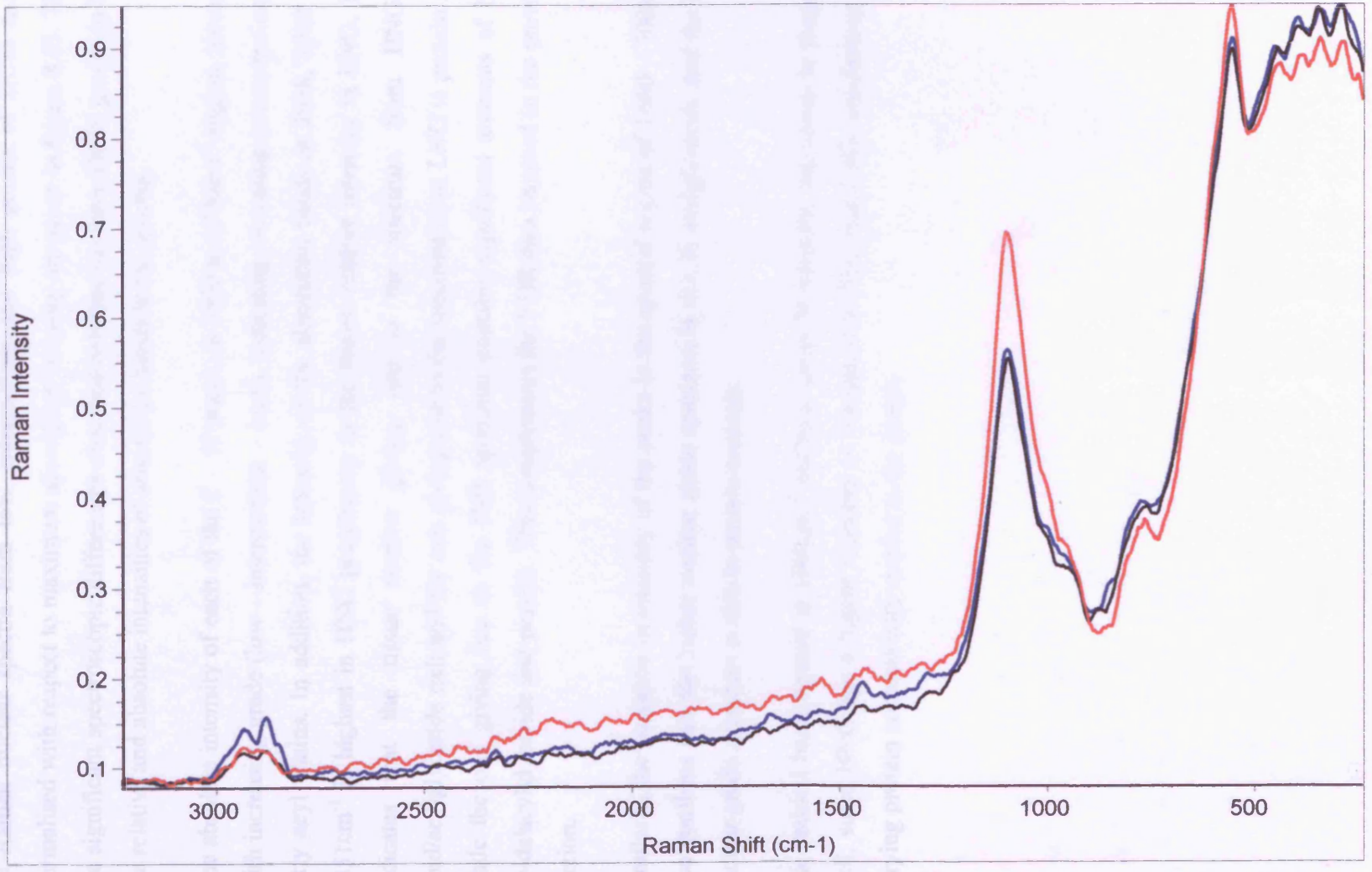


Figure 8.42; FT-Raman spectra of different IDC grades, where; Red = GIII, Blue = GII and Black = GI

FT-Raman Spectroscopic Difference between DCIS Grades (LNG, ING and HNG):

FT-Raman nuclear spectra from low, intermediate and high grades of DCIS tissues normalised with respect to maximum absorption intensity are given in figure 8.43. There are significant spectroscopic differences observed between the three DCIS grades in both the relative and absolute intensities of absorption bands in the spectra.

The absolute intensity of each of the C – H peaks, in 3500 to 2700cm^{-1} region, increases with increasing grade (low – intermediate – high), indicating increasing concentrations of fatty acyl chains. In addition, the intensity of the absorption bands at 2958, 2920 and 2851cm^{-1} is highest in HNG progressing to the lowest relative intensity in LNG. This indicates that the tissue section giving rise to the spectrum from HNG is lipid/acylglyceride rich and the one giving rise to the spectrum from LNG is protein rich while the one giving rise to the ING spectrum contains significant amounts of both lipids/acylglyceride and protein. This compliments the FTIR data reported in the previous section.

Similarly, the variation in intensity of the peaks in the spectral region of 1660 – 700cm^{-1} also confirms that the higher nuclear grade spectrum is rich in acylglyceride and the low nuclear grade spectrum is rich in protein contents.

The amide I band centred at 1660cm^{-1} not only varies in intensity, but varies in shape as well, since HNG has a higher intensity as compared to ING and LNG, confirming the varying protein contents with respect to the grades.

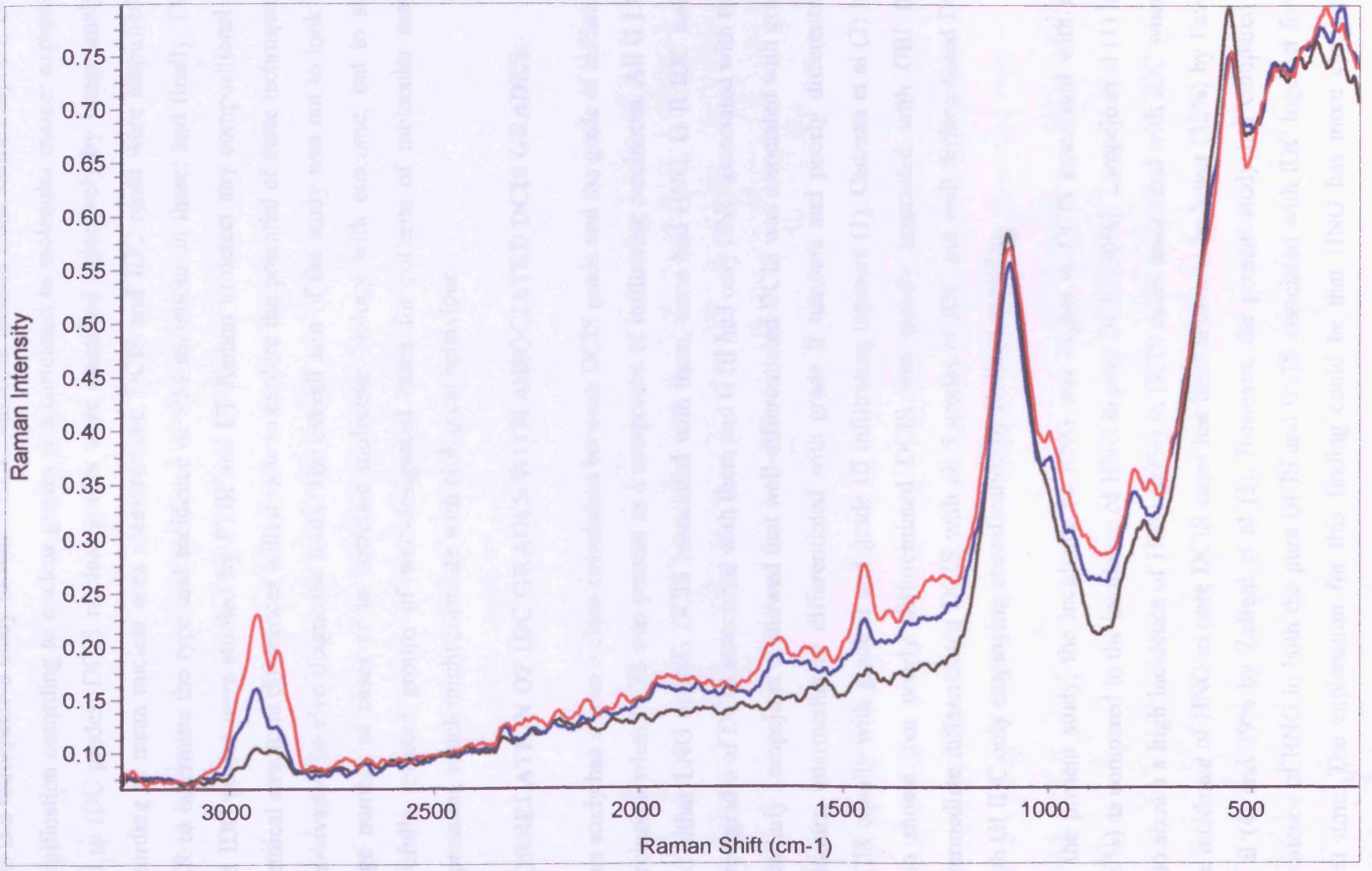


Figure 8.43; FT-Raman spectra of different DCIS grades, where; Red = HNG, Blue = ING and Black = LNG

CHAPTER 9: DISCUSSION:

This is a retrospective study in which DCIS has been studied from various aspects; DCIS classification according to nuclear grades was correlated to molecular marker expression and to IDC grades; DCIS nuclear grades were classified objectively by image analysis according to mean nuclear area measurement; DCIS and IDC cases were amplified by PCR to determine the type and incidence of *P53* mutations in these; and finally, DCIS and IDC grades were studied by FTIR and FT-Raman to detect any compositional and chemical structural differences with a view to explore the potential of these techniques as non-invasive in-vivo diagnostic tools. The overall aim of the study was not to look at a large number of cases or to correlate molecular biology with outcome, but to study carefully selected groups of well-designated cases for patterns of molecular marker expression, which might correlate with biological behaviour.

CORRELATION OF IDC GRADES WITH ASSOCIATED DCIS GRADES:

This study has shown a close correlation between DCIS grade and the grade of infiltrating carcinoma when DCIS was present as a component of infiltrating carcinoma. All G I IDC had either LNG or ING DCIS associated with them, none had HNG. G II IDC had all three grades of DCIS associated with them and G III had only HNG associated with them. Similarly Lampejo et al showed that well-differentiated DCIS was associated with grade I tumours, intermediately differentiated with grade II tumours and poorly differentiated DCIS equally with grade II and grade III infiltrating tumours (1). Cadman et al (2) have also shown that poorly differentiated DCIS was mainly associated with GIII IDC, intermediate differentiated DCIS with all 3 grades of IDC and well differentiated DCIS with GI IDC only employing classification by Holland et al(3).

In the present study, the incidence of HNG was higher in DCIS associated with IDC (46%) as compared to the incidence of HNG in pure DCIS (30%). Lampejo et al (1) have also shown a high incidence of HNG (69%) in DCIS cases associated with IDC whereas the incidence of HNG in pure DCIS cases has been shown to be lower (32%) by Faverly et al (4) and 39% by Zafrani et al (5). However, the present study has compared the incidence of HNG in both the pure DCIS and DCIS associated with IDC together for the first time. The explanation for this finding could be that HNG has more chances of

invasive carcinoma associated with it as shown by Patchefsky (6) and consequently less likely to be pure DCIS.

DCIS CLASSIFICATION IN RELATION TO MOLECULAR MARKER EXPRESSION:

At present, there is insufficient evidence to show that there is any correlation between the DCIS classification system which is currently in use in the UK and clinical behaviour of DCIS, although prospective trials are underway to determine the prognosis of patients with different DCIS grades. One study (7) has shown this classification based on nuclear grade as well as Van Nuys classification to predict recurrence in DCIS. The classification currently in use in the UK has been derived from the one by Holland et al, which is recommended by the European Pathologists Working Group (EPWG). The difference is that classification based on nuclear grade divides DCIS into LNG, ING and HNG and the one by Holland et al divides DCIS into well, intermediate and poorly differentiated DCIS. Furthermore, the classification by Holland et al takes into account polarity of cells in addition to nuclear grade. The advantage of classifying DCIS on the basis of nuclear grade is that the cytonuclear features on which the classification is based, are much more consistent throughout a lesion as compared to architectural pattern. Furthermore, the criteria for the classification are well defined and therefore, should result in better inter-observer reproducibility. In some studies (8-10), the reproducibility of this classification has been shown to be good.

The biological validity of the histological classification of DCIS based on nuclear grades was supported in the present study by significant association of histological grades of DCIS with bcl2, Ki67, ER, p53 and cerbB-2. Correlation of biological features with histological grades of DCIS employing Holland's classfication has also been reported by Bobrow et al (11) and Zafrani et al (5). Bobrow et al (11) studied 105 cases of pure DCIS for immunohistochemical expression of cerbB-2, p53, PR and KiS1. They considered any amount of staining with cerbB-2, p53 and PR antibodies as positive and for KiS1, they used an arbitrary cut-off of 10% or more labelled cells as representing high proliferation rate. They showed positivity of PR to be 45%, cerbB-2 45%, p53 33% and cases with high proliferative activity (>10% labelled cells) to be 54%. The pure DCIS they studied was selected on the basis of absence of an associated invasive component; however, they did not exclude cases with a past history of invasive breast cancer. Zafrani et al (5)

studied 97 cases of pure DCIS for expression of ER, PR, p53, cerbB-2 and Ki67 by IHC. They found that 81% cases were positive for ER, 73% for PR, 40% for p53 and 57% for cerbB-2. They also showed that 52% of tumours had a high proliferative activity as determined by Ki67 labelling using 10% as a cut off value. In these two studies, there was a significant correlation between poorly differentiated DCIS and the expression of cerbB-2 and p53, and a high level of proliferation. In contrast, well differentiated DCIS had a low level of staining with antibodies to proliferation markers and rarely expressed cerbB-2 oncoprotein or p53 protein. Biological features of the intermediate group showed an intermediate pattern or a combination of features of both of the other two types. Bobrow et al (11) noted a significant association between positive expression of PR and well-differentiated DCIS. In contrast, Zafrani et al (5) found no significant correlation between DCIS subtypes and either ER or PR expression. Inconsistencies in these two studies can be explained by the use of different antibodies to detect the markers and the different methodology to show them. Bobrow et al (11) noted the nuclear diameter of different histological grades of DCIS as follows: poorly differentiated > 20μm (3 RBCs), intermediate differentiated 15-20μm (2.5-3 RBCs) and well differentiated < 15μm (2 RBCs).

In neither of above-mentioned two studies were the histological grades of DCIS compared against histological grades of IDC with respect to the expression of molecular markers. Furthermore, there is no previous study comparing pure DCIS against DCIS associated with IDC in terms of molecular marker expression. Finally, bcl2 and bax were not included in these series (5;11). A study by Quinn et al, which found that bcl2 correlated negatively with increasing histological grades of DCIS employing Holland's (3) classification, similar to the present study, did not include pure DCIS cases (12). Furthermore, as the above mentioned two studies used positivity cut-off values for the markers studied, and much simpler statistical analysis, it was felt necessary to further confirm the biological validity of the classification by including actual score values and employing different statistical methods.

Radiological (3) and 3-dimensional (4;5) studies also support this classification system. There is evidence that mammographic appearance shows quite good correlation with this classification (3) in that poorly differentiated DCIS is generally associated with linear, branching or coarse-granular microcalcifications which can usually be easily recognized

as malignant. There is also good correlation between the extent of microcalcifications and the extent of poorly differentiated DCIS within the breast. Well differentiated DCIS with a non-solid growth pattern is usually associated with multiple clusters of fine-granular (sand-like) microcalcifications on mammography. Microcalcification is less common in the intermediate category of DCIS, but, when present, may produce either coarse-granular or fine-granular appearances on mammography.

Three dimensional studies (4) using a stereomicroscopic technique have shown correlation between types of DCIS and the pattern of growth within the mammary tree. It was shown by Faverly et al that practically all DCIS was segmental (unicentric), not multicentric and 17/19 (90%) of the poorly differentiated DCIS showed continuous growth whereas 19/27 (70%) of the well differentiated DCIS was discontinuous (multifocal) within a segment. Irrespective of the histologic grade, only 5/60 (8%) of total DCIS had a multifocal distribution.

A study by Gupta et al has compared three different classifications of pure DCIS (Holland, Van Nuys and Cardiff) in 102 cases in relation to expression of biological markers including p53, E-cadherin and MIB1 (13). All these classification systems showed good correlations with the markers studied, with well differentiated lesions expressing high E-cadherin, low p53 and low MIB1 labelling and poorly differentiated lesions showing the reverse. The authors concluded that of the three classification systems, the Cardiff classification correlated the best with the markers.

Scott et al (14) have suggested a different classification with which over 90% of DCIS can be easily classified into the following five categories: high grade (HG), intermediate grade (IG), low grade (LG), pure micropapillary (M), and pure apocrine (A). Mack et al (15) investigated whether there is a relationship between lesion size and the immunohistochemical expression of p53, cerbB-2, bcl2, and Ki67 with this categorization of DCIS. They studied 70 cases of DCIS with IHC. Of the 70 DCIS cases, 17 (24.3%) were HG, 23 (32.9%) were IG, 21 (30%) were LG, seven (10%) were pure micropapillary cases, and two (2.9%) were pure apocrine DCIS. The mean size of the DCIS for each subcategory was statistically significantly different ($p=0.008$). In particular, the micropapillary DCIS cases were largest (mean size, 17 mm). The mean immunohistochemical scores for cerbB-2 for each category were also statistically different ($p=0.007$), whereas the mean scores for p53 and Ki67 for each category showed

a trend toward significance (p=0.073, p=0.062, respectively). There were no significant differences between bcl2 mean scores and each subcategory. Because this combined histological cytological classification system was predictive of size and cerbB-2 positivity, they supported the clinical relevance of this particular classification system. However, apocrine DCIS can easily be assigned to one of the three categories based on the nuclear grade (16), therefore obviating the need for a time-cosuming exercise.

Ringberg et al investigated cell biological factors in 187 cases of DCIS employing the Van Nuys classification and showed that ER and PR negativity, cerbB-2 overexpression, low bcl2 expression, p53 accumulation, DNA non-diploidy and high Ki67 were strongly associated with high grade DCIS and comedo-type necrosis (17). They therefore proposed a summary cell biological index (CBI-7) based on a combination of these factors and found this index was a strong predictor for ipsilateral recurrence by univariate analysis after a median follow-up of 62 months and hence had prognostic value, whereas none of these markers had any individual prognostic significance. The antibodies to ER, p53, bcl2 and Ki67 that they used were same as in this study, however their anti-cerbB-2 antibody was different. They used a cut-off of 10% labelled cells to define a positive case for ER, PR, cerbB-2, bcl2, p53 and Ki67. The incidence of positive cases was 60% for ER, 43% for PR, 54% for cerbB-2, 56% for bcl2, 26% for p53, 42% for high Ki67 (>10% cells) and 60% for DNA non-diploidy.

In a recent trial by EORTC (protocol 10853) (18), the risk of distant metastases was significantly higher in poorly differentiated DCIS as compared to well differentiated. On the basis of the results of EORTC trial and the present study, classification based on nuclear grades is strongly recommended. The present study proves that there is a biological basis of DCIS classification based on nuclear grades and supports the hypothesis of different biological routes for different grades of DCIS. However, as there was insignificant difference in molecular marker expression between LNG versus ING as compared to ING versus HNG, this suggests that biologically LNG and ING may behave similarly.

Bax and Bcl2 in Breast Carcinoma:

There is scanty data available in the literature on bax incidence in DCIS, its relation to DCIS grades and the role of bax in the pathogenesis of breast carcinoma. In the present study, bax positivity was observed to be 62% in breast carcinoma cases with 69% of DCIS, 56% of LN negative IDC and 62% of LN positive IDC being positive. Lymph node metastases showed 23% positivity. It did not correlate to the histopathological grades of DCIS or IDC. However, bax protein expression was significantly lower in the metastatic lymph nodes, this novel observation suggests that reduced levels of bax protein may contribute to lymph node metastases. This is supported by the study by Krajewski et al (19) who found that reduced expression of bax is associated with poor response rates to chemotherapy and shorter survival in patients with metastatic breast cancer. Bax did not correlate to ER, p53 or cerbB-2. Lack of correlation with ER suggests that unlike bcl2, it is not hormonally regulated. It correlated positively with bcl2 in all cases and especially in well differentiated grades of DCIS and IDC. Bax correlated negatively with Ki67, especially in metastatic lymph nodes.

A retrospective study by Dimitrakakis et al (2) has looked at the expression of p53, bcl-2, bax and cerbB-2 genes in breast cancer patients to investigate their prognostic significance. Archival tissues from 121 patients treated between 1986-1992 were analysed and incidence of percent positive cases was 24%, 15%, 59% and 53% for cerbB-2, p53, bcl-2 and bax respectively. They found a significant correlation between bax expression and age >60 years ($p=0.04$) and also between bax expression and tumour size ($p=0.02$) as well as stage ($p=0.001$). Fifteen percent of patients with stage I cancers expressed the bax gene, compared with 58% and 65% with stage II and III respectively. There were no other correlations with clinical or histological parameters. None of the four markers proved to be independent predictors of five-year survival, in either univariate or multivariate analysis.

Rochaix et al studied bax expression in 110 IDC and found it to be expressed in 75% of cases (20). It did not correlate to tumour grade, ER or p53 expression, a finding similar to that of the present study. Lack of correlation with p53 could be explained by the mutation or inactivation of p53, the latter being unable to promote *BAX* gene expression. They found that bcl-X correlated positively with ER and concluded that bcl2 and bak are critical determinants of regulating apoptosis in breast carcinoma. However, unlike

Rochaix et al, the present study showed a positive correlation between the expression of bax and bcl2, which was more pronounced in well differentiated grades of DCIS and IDC. Similarly, positive correlation between bcl2 and bax has been reported by Krajewski et al in IDC (19).

Kapucuoglu et al (21) reported on bax protein expression in DCIS and its histological grades, which showed bax expression to be 67% in DCIS, similar to these results. However, in contrast to Kapucuoglu et al, this study did not show significant correlation of bax with poorly differentiated DCIS. They also found a positive correlation between bcl2 and bax in IDC, similar to the present findings and the findings of Krajewski et al (19), but their finding of a negative correlation between bcl2 and bax in DCIS was in contrast to the present findings.

Sturm et al (22) investigated the *BAX* gene in 68 breast cancer specimens for the presence of mutations in the coding sequence by SSCP-PCR and direct sequencing, and bax protein expression by IHC. They also screened for mutations in the exons 5-8 of the *P53* gene by SSCP-PCR. They found differential expression of bax in the breast cancer samples, but there was no mutation in the coding sequence of the *BAX* gene besides a polymorphism in exon 6. In contrast, they identified 23.5% tumours bearing mutations in the *P53* gene. Thus, there seem to be other not yet identified regulators apart from p53, involved in the regulation of bax protein expression. Similarly, the present study did not show any correlation between bax and p53 protein expression.

Bcl2 positivity in this study was 42% and bcl2 correlated significantly with well differentiated DCIS and IDC and also with bax and ER. The correlation with ER was particularly strong. This correlation has been reported previously (23;24). Bcl2 correlated negatively with Ki67 in all cases and especially in lymph node metastases. The correlation of bcl2 with well differentiated IDC is similar to the findings reported in literature (23;25;26). The relationship between bcl2 and differentiation grade has also been reported in DCIS (12;27). The finding of positive correlation between bcl2 and ER in both DCIS and IDC in this study relates well with the studies reported by Yang et al (23). They looked at bax, bcl2, p53, MIB1 and ER in 177 cases of IDC. They did not find any correlation between bax and ER in IDC, similarly the present study did not show any correlation between bax and ER in either DCIS or IDC. The fact that bcl2, but not bax, is hormonally regulated, was further confirmed in a recent study by Gompel et al (28). They

observed that oestradiol had an anti-apoptotic effect in normal breast and breast cancer cells, whereas antioestrogens and progestins had a proapoptotic effect. Employing quantitative RT-PCR, they showed that oestradiol increased bcl2 mRNA levels at 48 hours of treatment via a transcriptional mechanism, whereas none of the hormone treatments altered the pro-apoptotic protein levels, bax and bak. Furthermore, bcl2 displayed a strong cyclical variation in normal breast with the menstrual cycle and was considered the most hormone dependent member of the family.

Bcl2 in the present study did not correlate significantly to p53 or cerbB-2, which has also been reported by Quinn et al (12). However, in subgroup analysis, bcl2 correlated positively to cerbB-2 in LNG cases (n=23), and negatively in GIII cases (n=20). This seems to be a very interesting finding. A strong inverse correlation between bcl2 and proliferative activity as measured by MIB1 has been reported in breast cancer (23). The present data have confirmed this relationship as mentioned above. This supports the suggestion that apoptosis and proliferation are mechanistically linked (23).

P53 transactivates p21 and mdm2, and influences bcl-2 expression (29;30). In addition it has been shown that activation of the wild-type p53 direct signalling pathway leads to bax up-regulation and bcl-2 down-regulation, resulting in rapid apoptosis (31;32). Mutant p53 down-regulates bcl-2 expression in a mammary epithelial cell line (33). An inverse correlation between bcl-2 and p53 has been demonstrated in breast carcinoma (25), pancreatic cancer (34), thyroid cancer (35) and non-Hodgkin's lymphoma (36). However, bcl-2/bax regulated apoptosis can occur in a p53-independent manner (37). The lack of significant association between p53 and bcl-2 and also between p53 and bax in the present study could be due to p53-independent apoptotic pathways.

Wild-type p53 transactivates the *WAF1/CIP1/SDI1* gene in response to DNA damage. The *WAF1* gene encodes the p21 nuclear protein, a potent inhibitor of cyclin-dependent kinase (CDK) activity. P21 interacts with proliferating cell nuclear antigen (PCNA) to block PCNA-dependent DNA replication, leading to G_1 cell-cycle arrest (38;39). In normal cells, the transcription of mdm2 is dependent on p53 and the mdm2 protein regulates p53 by a negative feedback loop (30).

Bcl-2 expression was found to be inversely proportional to mitotic index and to be positively correlated with apoptotic index by Elkablawy et al (40). This observation could

be explained by the finding that bcl-2 can inhibit the transition of cells from resting (G_0) to a cycling phase and hence reduce their proliferation and turnover, possibly due to the presence of an anti-proliferative domain in bcl-2 (41-43).

Apoptosis is important for both tissue development and differentiation and its deregulation may contribute to tumourigenesis. The *BCL-2* family of genes has been shown to encode proteins, which can either suppress or accelerate apoptosis (44). The bcl-2 apoptotic pathway is involved in morphogenesis and differentiation. In human fetal tissues, bcl-2 protein has been demonstrated in most developing epithelia and is expressed in cells at different stages of differentiation (45). In normal adult tissues, bcl-2 protein is regularly present in ductal epithelial cells of all exocrine glands. Immunohistochemical studies have detected bcl-2 overexpression in hypertrophic, hyperplastic and dysplastic lesions from skin (46), uterine cervix (47) gastrointestinal (48) and breast (27;49) epithelia.

Overexpression of bcl-2 gives cells a survival advantage and this, together with the alterations of other oncoproteins, may lead to rapid and uncontrolled cell growth, which characterizes the development of cancer. Enhanced expression of bcl-2 has been reported in several lymphoid and non-lymphoid tumours (45). Complex and not well understood interactions exist between the proteins involved in apoptosis regulation. Bcl-2 and Bax seem to form homo- and hetero-dimers and the ratio between the two proteins determines cell death or survival (50).

Proliferative Activity in Breast Carcinoma:

The relationship of proliferative activity to prognosis in invasive breast cancer is well established (51;52). Proliferative activity in DCIS has also been reported (53;54). The evaluation of proliferation in tissue sections has been facilitated by the development of antibodies to proliferation antigens that can easily be used on archival material (55;56). Some groups (5;11) have used arbitrary cut off values of 10% to categorize high and low proliferative activity with immunostaining of antibodies against proliferation antigens (Ki67, KiS1) and found good correlation of proliferative activity with DCIS grades. Some researchers have used a cut off value for Ki67 in breast carcinomas as well (57;58).

In this study, proliferative activity as measured by Ki67 labelling index (LI) significantly correlated to DCIS and IDC grades and it is shown for the first time that of all the

markers studied, was the best discriminant between normal, DCIS, IDC (LN negative) and IDC (LN positive or metastatic). Ki67 correlated positively to p53 and cerbB-2, and negatively to ER and bcl2.

Hoshi et al (58) have studied Ki67 in ADH versus DCIS and they showed that the MIB1 labeling index of ADH was very low (0 – 2.3%) and was the best parameter to distinguish it from DCIS ($p < 0.01$). Therefore, they suggested that the lower level of proliferative activity is one of the most distinctive features of ADH.

ER Status in Breast Carcinoma:

Oestrogen receptor (ER) expression has been intensively investigated in invasive breast cancers by both radioligand binding assays and immunohistochemistry and expression is reported to be present in about 60% of cases overall, but data on incidence of ER positivity in DCIS is controversial. In this study, overall 70% of breast carcinoma cases were ER positive with 72% of DCIS, 66% of LN negative IDC, 73% of LN positive IDC and 80% of LN metastases being positive. ER expression correlated negatively to increasing grades of DCIS and IDC. However, there was no significant difference in ER expression between DCIS and IDC. Normal tissues were significantly more positive than DCIS and IDC (LN negative) but not more than metastatic cases. ER correlated positively to bcl2 as discussed already, and negatively to Ki67, p53 and cerbB-2. ER positivity is known to correlate with low histological grade of IDC. In 70% of positive cases, ER is functional, which has implications for therapy and prognosis (59).

There have been some studies looking at hormone receptors in DCIS. Burr et al (60) included only 38 cases of pure DCIS in their study and found a correlation between large cell size and absence of ER. They found ER expression in 91% of non-comedo and 57% of comedo DCIS. Giri et al (61) studied 48 cases of pure DCIS using three different antibodies and showed ER positivity to be 42-45% overall. They found that less than one fifth of comedo carcinomas expressed ER, whereas more than half the cribriform, solid and micropapillary categories showed ER expression. Poller et al (62) looked at 151 cases of pure DCIS and found that 32% expressed ER and that ER expression was significantly associated with small cell size, higher S phase fraction and absence of cerbB2 expression. The association between high S-phase fraction and ER status in Poller et al's (62) study is in contrast to the present study, where ER expression is negatively associated with Ki67

labelling index as shown in table 8.21. Similar to the present study, Bobrow et al (11) found a correlation between PR expression and low proliferation rate. They showed PR positivity in 45% of 105 cases.

Two studies have looked at both ER and PR together in DCIS cases. Pallis et al (63) using a quantitative immunoassay on 57 cases of in situ carcinomas, some of which were lobular in type, showed that 54% were ER positive and 30% were PR positive. Wilbur and Barrows (64) showed ER and PR expression to be 75% and 65% respectively in lobular and ductal in situ cases studied with immunohistochemistry. It has been shown that there is a significant reduction in ER positivity from the periphery of the tumours towards the centre (65), which was a phenomenon inconsistently observed in this study. This is thought to be a biological phenomenon within the tumour and not due to delays in fixation at the centre of excised tumours. In fact, in one study (66), it was shown that none of the fixation or processing regimes had any deleterious effect on the sensitivity of the ER assessment performed by IHC.

Some researchers (67) have quantified immunohistochemically stained hormone receptor expression by computer-assisted image analysis and found good correlation between semi-quantitative and quantitative methods. Nevertheless, there remains the need for quality assurance (68) for immunohistochemical assays for the determination of hormone receptor status.

One recent study (69) has reported on the incidence of androgen receptor in DCIS, and its relation to the expression of ER and PR in DCIS. The study was done by Selim et al and showed positive AR expression by IHC in 19/57 (33%) of cases. AR expression did not correlate to degree of differentiation of DCIS unlike ER and PR, neither did it correlate to ER or PR expression. There was a suggestion that AR expression may be related to apocrine differentiation of DCIS.

P53 Expression in Breast Carcinoma:

In this study, p53 expression was seen in 24% of breast carcinoma cases with 22% of DCIS, 21% of LN negative IDC, 40% of LN positive IDC and 47% of LN metastases being positive. P53 expression correlated positively to increasing grades of DCIS and IDC, although it did not significantly differ between the two. However, it was increased in metastatic cases as compared to DCIS and IDC, although this difference was not

statistically significant. P53 correlated negatively to ER as mentioned above, and positively to Ki67 and cerbB-2.

Walker et al (70), in a study of 10 cases of DCIS, showed p53 expression in 5 cases (50%), 4 of which were comedo in type. Poller et al (71) have shown p53 expression in 25% of DCIS cases overall, similar to the present results. They noted a significant association between p53 and cell size but in contrast to the present results, they found no significant association between p53 protein expression and cerbB-2 expression or ER expression. However, Bobrow et al (11) found positive correlations between p53 and cerbB-2, as well as between p53 and KiS1 and negative correlations between p53 and PR as well as between cerbB-2 and PR, in DCIS, which is similar to findings in this study.

The expression of p53 protein, detected by IHC, has been shown to be associated with high histologic grade of IDC and poor outcome (72). In a study of 297 LN negative IDC by Ferrero et al, p53 status determined by immunoluminometric assay correlated positively to high histopathological grade and negatively to positive ER status (73), a finding similar to that of our study. Goel et al (74) have also found positive correlation between p53 and PCNA expression (determined immunohistochemically) and histopathological grade of 35 IDC cases.

CerbB-2 Expression in Breast Carcinoma:

CerbB-2 expression was (initially with microwaving for antigen retrieval) found in 46% of breast carcinoma cases, with 48% of DCIS, 42% of LN negative IDC, 47% of LN positive IDC and 53% of LN metastases being positive. CerbB-2 expression correlated positively to increasing grades of DCIS and IDC, but was not significantly different between these two, except in HNG as compared to GIII, where it was significantly lower in the latter. . The finding of 48% of DCIS cases positive for cerbB2 is in accordance with the literature (75) but 42% positivity in case of IDC is slightly higher than reported studies (15-30%). This finding could be explained partly by the small number of cases (n=38), especially of grade I IDC (n=6) as these are more often cerbB-2 negative; and partly due to improved antigen retrieval by the microwave method employed. When cases were repeated without antigen retrieval, the positivity was observed to be 30% in IDC and nil in normal breast tissue. Thus there is a need to standardise protocol across various laboratories in order to get consistent results.

CerbB-2 expression in DCIS has been studied by several groups (75;76) and it is well established that although it is expressed in only 15-30% of IDC, in DCIS it is expressed in 40-70% of cases predominantly in comedo-type and is associated with large cell size (75).

An inverse relationship between cerbB-2 expression and hormone receptor status has been reported in IDC and DCIS (62) and the present results have confirmed this relationship.

Molecular Marker Expression in the Pathogenesis of Breast Carcinoma:

Although the markers studied have previously been looked at in breast carcinoma, but their role in the pathogenesis of breast carcinoma is not entirely clear. Therefore, in the present study, these markers were studied in various different groups of breast carcinoma simultaneously, in order to better clarify their role. Normal breast tissue showed significantly lower expression of Ki67, p53 and cerbB-2 (poor prognostic markers) when compared against DCIS and IDC. Ki67 and p53 were also lower in normal breast tissue as compared to LN positive IDC and LN metastases. In contrast, bcl2 and ER (good prognostic markers) and bax (unknown value as a prognostic marker) did not significantly differ between normal breast tissue and other groups. When normal breast tissue was compared against individual grades of DCIS and IDC, Ki67 was the only marker significantly higher in well differentiated grades of DCIS and IDC. However, in addition to higher Ki67 values, p53 and cerB-2 expression was also higher and ER expression was lower in poorly differentiated grades of DCIS and IDC when compared to normal breast tissue.

Comparing DCIS with invasive lesions of the same differentiation grade, it was found that_proliferation was elevated in the invasive lesions. Altered expression of the other proteins was in general only slightly different in the invasive lesions compared with DCIS, except cerbB-2, which was significantly higher in HNG DCIS compared to GIII IDC. The number of proteins with altered expression per lesion was highest in poorly differentiated lesions and was comparable between DCIS and IDC of the same differentiation grade.

Proliferation was found to be significantly higher in DCIS associated with IDC as compared to pure DCIS, and in LN negative IDC as compared to both pure DCIS and DCIS associated with IDC. Hence proliferation increased in the following order:

Pure DCIS ⟶ DCIS associated with IDC ⟶ LN negative IDC

However, none of the markers differed significantly between LN negative and LN positive IDC, suggesting that these markers are probably not involved in the critical step of invasion to metastasis. However, as 16% of cases were clinically node negative, the analysis was repeated after excluding them and again no significant differences were detected between LN +ive and LN –ive IDC. This could be attributable to a smaller number of histologically LN-ive cases. The only marker significantly reduced in metastatic LNs as compared to their primaries was bax.

In conclusion, proliferation seems to be the main differentiating factor in transition from normal breast to DCIS to IDC without lymph node involvement and bax is much reduced in LN metastases as compared to primaries. Other proteins (ER, cerbB-2 and p53) are mainly related to differentiation of DCIS and IDC but probably play a minor role in the transition from DCIS to an invasive breast lesion of the same differentiation grade. Well-differentiated in situ and invasive breast lesions share many of the aberrations in expression of these proteins, as do poorly differentiated in situ and invasive lesions. However, there are many differences between the well and poorly-differentiated lesions. This further supports the existence of different biological progression routes leading to breast cancer. None of the proteins studied seems to be involved in the development of metastasis.

The findings of the present study have been supported recently by a similar study done by Mommers et al (77), who have looked at alterations in the expression of proteins involved in proliferation and apoptosis in non-invasive and invasive ductal breast lesions. They performed IHC on 106 usual ductal hyperplasias (UDH), 61 DCIS lesions and 53 invasive ductal breast carcinomas. Increased proliferation (Ki67), overexpression of cyclin D1, cerbB-2, p21 and p53, and decreased expression of bcl-2 and p27 could already be found in UDH. Significant differences between UDH and DCIS lesions were found for only one protein when UDH was compared with well-differentiated DCIS (p27), for three proteins when compared with intermediately differentiated DCIS (p21, cyclin D1, Ki-67), and for

all proteins when compared with poorly-differentiated DCIS. Similar to the present results, comparing DCIS with invasive lesions of the same differentiation grade, proliferation was elevated in the invasive lesions. They concluded that the biggest changes in expression of these proliferation and apoptosis related proteins occured during the transition from hyperplasia to DCIS; they probably played a minor role in the transition from DCIS to an invasive breast lesion of the same differentiation grade.

Similar results have also been found by Warnberg et al (78) in a recent study. They studied p53, cerbB-2, Ki67, ER, PR and bcl2 in pure DCIS, small invasive lesions and mixed lesions with both invasive and in situ components and showed that all these markers correlated with grade rather than invasiveness. Therefore they concluded that the step between in situ and invasive cancer seems to occur independently of the grade. However, metastatic lymph nodes were not included the above mentioned two studies.

NUCLEAR GRADING OF DCIS BY IMAGE ANALYSIS:

Evaluation of the nuclear grade is the least satisfactory of the three elements of histological grading system in IDC due to lack of interobserver reproducibility and therefore contributes to differences in allocating overall grade (79). However, determination of nuclear grade is important both in DCIS and IDC as it has been found to predict recurrence in the case of DCIS (80) and is an important component of the grading system for IDC (81). The best way in which differences in nuclear features can be assessed with accuracy is by the use of morphometry or image analysis, which are expensive and time-consuming processes, not always practical in a routine laboratory.

In this study, by employing image analysis, it was shown that DCIS grading by mean nuclear area measurement correlated well with the visual grading by light microscopy and thus provides a quantitative, objective and reproducible means of classifying DCIS into three grades. This information was not available from published literature at the time.

It is known that mean nuclear area in DCIS is slightly higher than that in invasive breast cancer (82). It has also been shown that DCIS has a higher mean nuclear area as compared to benign lesions and ADH (58;83-85). One recent study by Tan et al (86) has reported on a significant correlation between nuclear grade assessed histologically and nuclear area similar to the present study. They reported nuclear area to be 44μm in LNG, 47 μm in ING and 72 μm in HNG.

P53 MUTATION:

P53 mutations could not be detected in this study in spite of successful amplification of *P53* exons 5 – 8 by PCR. This information would have been useful to confirm a *P53* mutation in cases immunopositive for p53 and furthermore to characterize incidence and type of mutation in different DCIS grades as published literature lacks enough information in this respect. If a mutant allele has been cloned, then the sequence can be determined easily using synthetic oligonucleotide primers, basing the sequence of these primers on the known sequence of the wild-type gene. If the mutant allele has not already been cloned, or where the mutation cannot be predicted (as in the case of *P53*), the quickest method is to amplify the region of interest by PCR or RT-PCR and then to sequence the PCR reaction product. In either case, the wild type sequence needs to be known for comparison with the mutants, and as the basis for primer design.

Mutations in *P53* are scattered throughout the *P53* coding sequence but tend to be clustered in exons 5-8. Studies have been published in which each individual exon has been amplified and subsequently sequenced but this is an extremely labour-intensive approach and is not suitable for analysis of a large number of specimens. However, samples can be pre-screened for mutations in specific exons using a modification of the PCR. This technique is called single-strand conformation polymorphism (SSCP) analysis and can detect nucleotide substitutions, insertions and deletions in PCR-amplified DNA fragments. SSCP relies on the sequence specific migration of single-stranded DNA in non-denaturing polyacrylamide gels. PCR products are radioactively labelled during amplification, run out on non-denaturing polyacrylamide gels alongside controls of wild-type DNA amplified with the same primers, and visualization is by autoradiography. In this way, the individual DNA strands of the amplified product are observed and size differences between wild-type DNA and sample DNA are an indication of polymorphism. The exons identified to contain mutations by SSCP are then sequenced.

Southern blotting and hybridization to detect RFLPs can detect large-scale DNA rearrangements, insertions and repeat expansions, but point mutations generally cannot. Other molecular techniques such as SSCP and DDGE can help to locate a point mutation, but only DNA sequencing can determine the molecular nature of the mutation precisely.

P53 mutation is a common event in sporadic breast cancer being found in 15-50% of invasive carcinomas. Although many studies have been published about *P53* alterations in breast cancer, there are limited data on the molecular biological detection of *P53* mutations in in situ lesions and the implications for breast carcinogenesis are unclear. P53 immunoreactivity correlates well with mutations of the corresponding gene, although this correlation is not absolute, since non-sense mutations and deletions can result in the absence of positive p53 immunostaining.

In one study (87), *P53* mutations were studied in 30 breast cancers using PCR and manual sequencing methods and 5/30 (17%) cases had *P53* mutations. All mutations were detected in ductal carcinomas NOS type. All the 4 lobular and 2 mucinous carcinomas showed wild-type *P53* sequence only. In another study (88), *P53* mutations in exons 5-8 were detected by DGGE and confirmed by sequencing in 14/82 (17%) of breast carcinomas.

Done et al (89) showed that a *P53* mutation present in invasive breast cancer was found in all surrounding areas of ductal carcinoma in situ (DCIS) but not in areas of hyperplasia or normal breast epithelium. In another study, Done et al (57) studied patients with DCIS, but without invasive breast cancer, to determine the spectrum of DCIS types that can harbour a *P53* mutation. Formalin-fixed, paraffin wax-embedded tissues from 94 patients with DCIS were stained immunohistochemically with an anti-p53 antibody. Positively stained tissue areas were analyzed for the presence of *P53* mutations by SSCP and direct sequencing. DCIS from 10 of 94 (11%) patients were found to contain *P53* missense mutations. All 10 were of a solid or a comedo histologic pattern and were either intermediate or high nuclear grade. The frequency of *P53* missense mutations was statistically significantly different among the three overall histologic grade categories, 0/49 (0%) of low-grade DCIS, 1/23 (4%) of intermediate-grade DCIS, and 9/22 (41%) of high-grade DCIS had a *P53* mutation. This finding that *P53* mutations can occur before the development of invasive breast cancer, particularly in DCIS of high histologic grade, has potentially important implications for prevention and treatment.

Ho et al (90) evaluated a series of microdissected, pure DCIS lesions comprising a spectrum of morphologic subtypes (comedo, micropapillary, papillary, cribriform, and solid) and their corresponding normal breast tissue for genetic aberrations in *HER-2/neu (cerbB-2)* and *P53*. *HER-2/neu* amplification was determined by differential polymerase

chain reaction, and *P53* mutations were identified by SSCP analysis. *HER-2/neu* amplification was identified in 12 of 30 DCIS samples (40%), and *P53* mutations were identified in 6 of 30 DCIS samples (20%). The genetic alterations were not present in any of the normal breast tissue samples. *HER-2/neu* amplification occurred predominantly in the comedo subtype (69% vs. 18% of the noncomedo subtype; $p = 0.008$) and in lesions of high nuclear grade (63% vs. 14% of low grade; $p = 0.01$). These workers did not find any difference in the frequency of *P53* mutations among the subtypes or between low grade and high grade lesions neither did they observe any correlation between the presence of the two genetic alterations. They concluded that the presence of *HER-2/neu* amplification, but not *P53* mutations, correlates with histologic subtype and nuclear grade. The relatively frequent occurrence of *HER-2/neu* amplification and *P53* mutations in DCIS tissue and their absence in normal breast tissue suggests that these genetic aberrations are important early events in breast duct carcinogenesis.

Lisboa et al (91) studied tissue samples from 83 patients with different stages of breast cancer and from 13 patients with benign breast lesions screening for *P53* gene mutations by polymerase chain reaction (PCR) followed by temperature-gradient gel electrophoresis (TGGE). P53 protein accumulation was analysed by immunohistochemistry (IHC). Additionally, 23 pairs of primary tumours and corresponding lymph nodes were examined. *P53* gene aberrations were found in 55.7% of the infiltrating carcinomas, in 31.5% of the ductal carcinomas in situ (DCIS) and in one atypical ductal hyperplasia. A positive correlation was seen with high-grade tumours and with comedo DCIS. Concordance between TGGE and IHC was seen in only 63% of the cases analysed. Among 8 pairs of primary tumours and their corresponding lymph node metastases showing *P53* mutation, only 3 harboured identical *P53* mutations in the same exon, while in 5 cases with mutant *P53* in the primaries, no mutation was seen in the lymph node. They concluded that *P53* mutations are frequent in breast tumours associated with unfavourable prognosis, including high-grade or the comedo-type and that *P53* gene alterations occur early in breast carcinogenesis, as mutations were detected not only in in situ carcinomas but also in atypical ductal hyperplasia.

Munn et al (92) studied 36 cases of pre-invasive breast lesions, including 35 cases of ductal carcinoma in situ (DCIS) for mutation of *P53* and allelic imbalance on 17p13. *P53* mutations and allelic imbalance on 17p were identified in cases of 'pure' DCIS as well as

those with associated invasive carcinoma and, furthermore, were identified in well-differentiated lesions as well as poorly differentiated ones.

O'Malley et al (93) performed p53 immunostaining using p53 antibody DO7 and direct sequencing following PCR amplification of exons 5,6 and 7 in 27 noncomedo and 12 comedo DCIS (a total of 39 DCIS). Four comedo DCIS cases that were p53 immunopositive were further screened for *P53* mutations by PCR/SSCP in exons 8 and 9 of the *P53* gene. Immunostaining with DO7 was positive in 4 of 12 comedo DCIS lesions (33%) while all noncomedo lesions were negative. Direct sequencing of PCR products confirmed wild-type DNA in exons 5 and 6 in all noninvasive papillary carcinomas, 3 noncomedo DCIS lesions, and 4 p53 antibody-positive comedo DCIS lesions. In these latter 4 cases, wild-type DNA sequences were preserved in exon 7 for all cases. A single case (1/4 or 25%) had a conformational shift in exon 8 within the four cases screened in exons 8 and 9. Direct DNA sequencing of this exon revealed a G to A point mutation resulting in an arginine-to-histidine substitution at codon 273 of the protein. They concluded that mutant p53 protein accumulation in pre-invasive lesions is limited to comedo (high grade) DCIS and that p53 positivity by immunohistochemistry does not correlate in all cases with specific *P53* mutations in exons 5 to 9, the most highly conserved regions of this gene.

Most of the afore-mentioned studies have used SSCP or TGGE for detection of p53 mutation followed in some cases by direct sequencing employing manual technique. Some of the studies (Done et al, O'Malley et al) found *P53* mutation confined to comedo-type whereas others disagree (Ho et al, Munn et al) and therefore there remains the need for clarification of relation of P53 mutations to histological grade of DCIS.

In this study, an attempt was made to detect p53 mutations in DCIS by employing semi-automated sequencer. Unfortunately, the samples could not be sequenced, the probable reason being that they had a secondary structure which meant that the sequencing primers could not work. The PCR was repeated by MWG Biotech to confirm that PCR worked, which was reported that it did. However, they were unable to sequence it. It is possible that cloning of the PCR product in a specific vector could help to sequence these samples.

FTIR AND FT-RAMAN AS POTENTIAL DIAGNOSTIC TECHNIQUES IN BREAST CARCINOMA:

Fourier Transform Infrared (FTIR) absorption spectra are well known for their sensitivity to composition and three-dimensional structure of biomolecules. The biochemical changes in the sub-cellular levels developing in abnormal cells, including a majority of cancers, manifest themselves in different optical signatures, which can be detected by IR spectroscopy. It is known that FTIR spectra of human tissues have specificity which can be used to discriminate between various disease states. This technique was employed to investigate whether morphological changes in DCIS and IDC grades translate into compositional differences.

The results obtained in this preliminary study have shown that it is possible to identify differences between the normal and the cancerous tissue, and may also be possible to differentiate between the different nuclear grades of DCIS and IDC. Although the results reported here are from the tissues obtained from paraffin wax-embedded sections, it is possible to obtain information on fresh tissues as well. It would be useful to compare the spectral data both from fresh and embedded tissues because comparable results in both these type of cases would mean that prospective studies could be carried out.

The differences in the intensity and positioning of peaks in the spectra are attributed to the compositional changes between the different tumours. The variation in intensity of these peaks is due to the variation in the amount of lipids, proteins and DNA contents present in the breast carcinoma. As the disease progresses, changes take place in the chromosomal structure and there is increase in the DNA content. These variations in the chromosomal structure and increase in the lipids/acylglyceride, proteins and collagens are well observed in the infrared spectra, which collectively help in classification of the grades.

The FTIR spectrum of normal breast tissue showed well defined peaks in the region of 3500 to 650 cm^{-1}. Spectra of normal and breast carcinoma cases showed well defined and prominent spectral bands at 3500-2700 cm^{-1} and 1700-900 cm^{-1} with differences in intensity and positioning of the peaks, which helped to differentiate between the healthy and diseased tissue. Dukor et al (94) employed exactly the same technique (fourier transform infrared coupled with ATR – Microspectroscopy) used in this study and

reported on the analysis of cells: benign, atypical hyperplasia and malignant tissues. Their study demonstrated that benign vs. malignant cells were discriminated with 100% accuracy, benign vs. atypical hyperplasia were discriminated with 100% accuracy and malignant vs. atypical hyperplasia were discriminated with an accuracy of 90% and higher.

Weng et al (95) have done preliminary investigation showing that FTIR spectroscopy in conjunction with fibre-optics can reflect differences between normal and malignant tissues and they stated that it may be possible to determine the applicability of the technique to cancer diagnosis. Grading of NHLs has been looked at with FTIR (96) with significant differences in spectra between different grades. Infrared microspectroscopy has been applied in combination with bivariate statistics for the detection of cell changes in oral/oropharyngeal squamous cell (97), lung (98) and prostate carcinoma (99) with a view to providing a potential diagnostic tool.

These findings reported in the literature encouraged the use of both FTIR and FT-Raman spectroscopic techniques for discriminating between the different nuclear grades of DCIS and IDC. Spectral area of 3500 – 2700 cm^{-1} and 1250-960 cm^{-1} showed significant spectroscopic differences between IDC grades. Spectral bands in the region of 1700 – 900 cm^{-1} showed significant spectroscopic differences between DCIS grades indicating HNG is lipid/acylglyceride rich, LNG is protein rich and ING contains both lipids/acylglyceride and protein.

A number of other investigators have reported spectroscopic differences between the normal and cancerous tissues (100-106). However, only one study reported by Jackson et al (107) has looked at IDC grades with FTIR and none has yet been reported in literature on the analysis of DCIS grades. Findings in the present study agree with the results reported by Jackson et al (107), who have also shown that higher nuclear grade tissues are rich in lipids and acylglyceride, intermediate nuclear grade contain both lipids/acylglyceride and proteins and low nuclear grade tissues are more protein rich. However, the FTIR technique used in the present study was more refined with the use of an ATR (attenuated total reflectance) objective equipped with a diamond crystal, which allows viewing of the specimen as well. It is also important to note that in this study it was the first time diamond crystal ATR was used for cancer tissue analysis. All the previous studies reported in literature have been carried out by employing zinc solenoid

and germanium crystals. Use of diamond ATR further enhances this technique by allowing the viewing of the sample area more clearly as compared to other crystals.

It may be possible to relate the spectral variation of breast tumour grades to normal variations in histology. However, this raises the question of whether it is possible to detect the much less pronounced changes in the spectrum expected as a consequence of the progression of the disease? Many spectral changes expected to accompany disease progression may be masked by variations in acylglyceride and/or collagen content. For example, progression to high grade tumour is accompanied by the appearance of nuclear abnormalities (e.g., altered chromosomal structure, increased DNA content). The infrared absorption bands most useful in studies of DNA arise from the stretching vibrations of phosphodiester groups in the region of 1082 – 1244cm^{-1}. Since this region of the spectrum is dominated by absorptions from both collagen and acylglyceride, the changes in cellular DNA that accompany disease progression may be masked by the large alterations in collagen and acylglyceride contents that are possible between different tumour types. Similarly, changes in protein expression accompanying the disease process are also expected to manifest changes in the amide I absorption that may also be masked by differences in collagen content. In addition, the changes in cell membrane properties would be evident by the C – H absorption bands indicating differences in acylglyceride contents.

Andrus et al (108) reported on 39 freeze-dried tissue samples from 17 lymphoid tumours (nine malignant non-Hodgkin's lymphomas) using FTIR spectroscopy. They reported on the cellular RNA/DNA ratio of overlapping absorbances, if present, due to collagen or glycogen. Absorbance attributable to collagen increased with lymphoma grade and was greater in benign inflammatory tumours than in low-grade lymphomas. They claimed that the trend observed in their study may form the basis of a universal cancer-grading parameter to assist with cancer treatment decisions and may also be useful in the analysis of cellular growth perturbation induced by drugs or other therapies. They also claimed that their findings may potentially be applied to cell clusters and discrete areas of tumor tissue sections using the FTIR microscope, allowing correlation with morphology and a high degree of spatial resolution.

Ramesh et al (109) reported on the IR absorption spectra of monolayers of cultured normal and H-ras transfected mouse fibroblasts, using a microscopic Fourier transform IR

(micro-FTIR) technique. They observed an increase in the RNA/DNA ratio for H-ras transfected fibroblasts, which correlates with the increased transcriptional activity expected for the cancerous cells and concluded that the variation in absorbance between normal and ras transfected fibroblasts may be due to changes in the cell dimensions.

Schultz et al (97) employed FTIR spectroscopy to observe the changes in tissue biochemistry induced by well-differentiated and poorly differentiated oral/oropharyngeal squamous cell carcinoma. The biopsies analyzed were each proven SCC positive and compared with tissue taken from the contralateral normal site. Individual infrared spectra, recorded from specific tissue areas, were correlated with histopathological structures normally found in the oral mucosa. Infrared mapping of these areas allowed the generation of biochemical images of molecular structures such as lipids, sugars, and proteins. The visualization of DNA and tissue structures containing keratin (well expressed in all epithelia) revealed distinct differences between normal and SCC-positive biopsies. Bivariate histogram analysis of cell components (e.g., DNA and keratin) indicated that cancer cells produced relatively homogeneous and clearly abnormal cell biochemistry, whereas differentiated epithelial cells presented a very heterogeneous distribution of cellular components. They concluded that using these features, tissue containing abnormal or cancer cells can easily be distinguished from normal epithelial structures. The abnormal keratin distribution in poorly differentiated SCC and in keratin pearls (present only in well-differentiated SCC) offered insight into the process of malignant tissue transformation in squamous epithelium.

Sukuta et al (110) reported on the Fourier transform infrared fiberoptic evanescent wave (FTIR-FEW) spectra, in the middle infrared (MIR) region, of human normal skin tissue and cancer tumors by identifying protein peptide bond and lipid carbonyl vibrations. Most importantly, the study demonstrated that the FTIR-FEW technique has potential as a clinical diagnostic tool.

Further studies have also been reported on the FTIR analysis of normal and neoplastic colon (111), breast tissue (107;112;113), skin (114;115) and bladder (116) in terms of such differences in DNA hydrogen bonding interactions and membrane and protein structural properties. Some questions have been raised but many answers have been achieved in these studies.

Given the number of potential confounding variables associated with cancer cytology, a multivariate statistical or neural network analysis would appear to be necessary before the implementation of FTIR technology in clinical laboratories. To address some of the question highlighted in the reported studies, it will be highly recommendable to create a comprehensive spectral database of cancerous tissue and standardisation of the classification of breast cancer tissues especially in consultation with the pathologists.

A standardised method of analysis using FTIR spectroscopy could only be achieved by consensus of pathologists and spectroscopists. A combination of both would further enhance the diagnosis by removing some of the inter-observer variation of individual pathologists. Although the technique is quite specialized, with a comprehensive computer database, minimal training and expertise will be required.

It is important to note that FTIR spectroscopy can merely act as an alternative, but will be an excellent complementary tool to histopathological discrimination among tumours of different grades, and it can assess variations in many variables simultaneously. In the case of the spectra, these variables may include the absolute intensity of one or more absorption bands, the position of one or more absorptions, the relative intensities of two absorptions, the width of one or more absorptions, the relative width of two absorptions and so on.

FTIR-microspectroscopy used in this study was applied to the tissue samples mounted on a glass slide and spectra obtained in reflectance mode. It may also be possible to mount samples on infrared transparent windows, such as calcium fluoride, potasium bromide, sodium chloride and obtain spectra in the transmission mode. The spectra obtained in the transmission mode would provide more detailed and precise information as compared to that obtained in reflectance mode. Samples mounted on any of these windows can be stored after analysis for further investigations, as this is a non-destructive technique and samples are not altered in any way by exposure to the infrared beam. The FTIR spectroscopic technique may be extremely valuable in the further study of archived samples in later years, as the sections stored on glass slides or in paraffin wax-embedded blocks can easily be analysed by FTIR. FTIR microspectroscopy combined with ATR sampling may also be a valuable clinical evaluation system for regular monitoring of the effects of therapy (chemo- and radio-therapy) and in predicting the type and progression of disease.

FTIR chemical imaging employed in this study was used for the first time for analysis of cancerous tissues, which illustrated the immense potential of this technique to analyse a large number of samples within a short period of time. Only recently, a couple of studies have been reported on the analysis of cancerous and normal tissues employing FTIR imaging techniques and coupling it with artificial neural network.

An interesting study by Zhang et al (117) reported on the classification of Fourier transform infrared microscopic imaging data of human breast cells by cluster analysis and artificial neural networks. They employed cluster analysis and artificial neural networks (ANNs) to the automated assessment of disease state in Fourier transform infrared microscopic imaging measurements of normal and carcinomatous immortalized human breast cell lines. Cell pixels were subsequently classified into carcinoma and normal categories through the use of a feed-forward ANN computed with the Broyden-Fletcher-Goldfarb-Shanno training algorithm. Inputs to the ANN consisted of principal component scores computed from Fourier filtered absorbance data. A grid search optimization procedure was used to identify the optimal network architecture and filter frequency response. Data from three images corresponding to normal cells, carcinoma cells, and a mixture of normal and carcinoma cells were used to build and test the classification methodology. They claimed the development of a successful classifier, although differences in the spectral backgrounds between the three images were observed to complicate the classification problem. They claimed the robustness of the final classifier through the use of a rejection threshold procedure to prevent classification of outlying pixels.

Sheng et al. (118) reported on the FTIR microspectroscopic analysis of colorectal carcinoma and found substantial differences in the spectral bands of cancer and non-cancerous cells. A shift of phosphodiester groups was noted in the cancerous cells as compared to the non-cancerous one, with a sensitivity of 90.91%.

FT-Raman is a technique which provides complementary information to FTIR. It also showed differences between normal and breast carcinoma tissue, between IDC grades and between DCIS grades. Most of the studies (95) on Raman analysis of cancerous tissue reported to date have utilized the intensity of the 1660 and 1442cm^{-1} band to distinguish between the grades and the type of cancerous tissue, similar to the findings in this study.

Frank et al (119) reported on the Raman spectroscopy of normal and diseased human breast tissues. In their study Raman spectra of histologically normal human breast biopsy samples were compared to those exhibiting infiltrating ductal carcinoma (IDC) or fibrocystic change. They reported that sample to sample and patient to patient variation for normal specimens were less than 5% for the ratios of major Raman bands. The Raman spectra The spectrum of infiltrating ductal carcinoma samples is similar to that of human collagen. Differences between benign (fibrocystic) and malignant (IDC) lesions were smaller than those between normal and IDC specimens, but were still reproducible.

Similar findings were observed in this study, the increase in intensity of the C – H peaks suggesting the change in the lipids, proteins and DNA contents. The absolute intensity of each of the peaks in 3500 to 2700cm^{-1} and 1660 to 1450cm^{-1} region varies with increasing grade (I, II and III), indicating varying concentrations of fatty acyl chains. Results obtained on IDC different nuclear grades showed an increase in intensity and shifting of peaks in the area of 1660 to 600cm^{-1} (carbonyl stretching and amide-bending vibrations in the DNA) confirm the changes in the chemical structure of breast tissue and this change in intensity provides useful information in differentiating between the IDC grades, The results were reproducible and variation in spectral resolution was less than ± 0.1cm^{-1}. The sensitivity and reproducibility of the technique has also been investigated by a number of investigators (120-126) and reported that the resultant sensitivity of cancer prediction is very good. One of the author reported as high as 91%, with 97% specificity and an error margin of $p<0.0001$.

Raman studies on DCIS grades have not been reported in literature to date. The results obtained in this study suggest that the higher nuclear grade spectrum is rich in acylglyceride and the low nuclear grade spectrum is rich in protein contents. The intensity of the absorption bands at 2958, 2920 and 2851cm^{-1} was highest in HNG progressing to the lowest relative intensity in LNG, which indicated that the tissue section giving rise to the spectrum from HNG is lipid/acylglyceride rich and the one giving rise to the spectrum from LNG is protein rich while the one giving rise to the ING spectrum contains significant amounts of both lipids/acylglyceride and protein. These findings nicely compliment the FTIR studies.

Raman spectroscopy is not very sensitive to hydroxyl bands (water contents), but this limitation of the technique can be utilised as an advantage, as fresh tissue, which is high

in water content, can be easily analysed by Raman without getting interference from water content.

Currently a number of clinical diagnostic techniques, such as imaging by CT and MRI scans are widely used. As both these are visual tools and do not add to the chemical structural confirmation of the cancer tissue, it is extremely difficult to diagnose type and grade of cancer without taking biopsies and conducting histopathological evaluations. There is a need to develop a clinical diagnostic technique that can offer chemical analysis of the tissues helping the clinicians not only in detecting the cancer but, also potentially determining its type and further classification of nuclear grades in vivo. The potential of Raman spectroscopy to do this opens the possibility of using this technique with a minimally invasive needle probe for diagnosing lesions, which are detected mammographically. At present attempts are being made to develop such a probe to analyse the tissues in vivo, which is in its final stages of development.

The detection of in-situ and early malignancy is important because of the improved survival rate associated with early treatment of cancer. As mentioned earlier, Raman spectroscopy is sensitive to the changes in molecular composition and molecular conformation that occur in tissue during carcinogenesis, and recent developments in fibre-optic probe technology enable its application as an in vivo technique (127). Raman spectroscopy may become a useful adjunct to pathological diagnosis allowing directed or guided biopsies. Manoharan et al (100) have reported on developing optical methods based on near infrared Raman spectroscopy and fluorescence photon migration for diagnosis and localization of breast cancer. They have demonstrated the ability of Raman spectroscopy to classify accurately normal, benign and malignant breast tissues, which may prove an important step in developing Raman spectroscopic needle probes as a tool for improving the accuracy of needle biopsy. Raman spectroscopy is considered to have the potential for diagnosis of arterial disease, and cancer of gynaecological tissues, soft tissues, breast, colon, bladder and brain (101;128).

Mammography is the only diagnostic tool at present by which breast cancer can be detected in its pre-invasive (in situ) stage although MRI is showing some promise. Around 95% of cases of DCIS are now detected by mammography in their preclinical, asymptomatic phase (129). Detection is based on the presence of mammographically significant microcalcifications that are associated with most of these lesions. Well

differentiated DCIS is more likely to be missed mammographically than poorly differentiated DCIS. In one series alone (3), 7/59 (12%) of DCIS were mammographically occult and six out of these were well differentiated DCIS. The main disadvantage of mammography is that it usually underestimates the size of DCIS as compared to histological examination, especially in the case of standard mammographic views (oblique and cranio-caudal). If magnification views are used then the discrepancy in mammographic and histological size assessment of DCIS is less than 2 cm in 80-85% of the cases (3). Furthermore, mammogram does not reliably distinguish between DCIS grades.

SUMMARY AND CONCLUSIONS:

This study has looked at DCIS grades in relation to each other and in relation to IDC grades by employing various techniques. The immunohistochemistry suggested that DCIS grades are distinct in terms of molecular marker expression although LNG and ING seem to be more similar. The IDC grades were also distinct. DCIS grades correspond to respective IDC grades and the main differentiating factor seems to be proliferation in this study.

However, the immunohistochemical analysis in this study has highlighted many of the problems of small 'molecular marker' studies including the use of a clinically heterogeneous group of tumours, the reliability of the information obtained by the antibody staining, need for standardisation of staining and interpretation, small number of cases available for subgroup analyses and lack of strong association of many markers with prognosis resulting in limited validity of such data. This is now understandable in the light of recent data on molecular profiling of breast cancer (130) which helps to categorise patients into high risk and low risk groups in order to decide on better use of adjuvant chemotherapy, especially in node-negative patients. It has been recognised that there are at least 70 different gene alterations in breast cancer and hence the limited value of small marker studies, for instance 6 in this study is a rather small number.

Breast cancer 'gene expression profiles' have been identified through DNA-microarray technology and seem to be better predictors of clinical outcome in young women (less than 55 years old) with stage I or II breast cancer, when compared to the currently used clinical-pathological criteria. Remarkably, these molecular data suggest that the

prognostic profile of breast cancer does not depend on lymph node status and that it is possible to identify a group of node-positive breast cancer patients with an unexpectedly good prognosis. Prospective and independent confirmation is needed, but these data are fascinating and carry the hope that chemotherapy decision-making in breast cancer will be greatly facilitated in the future (130).

The image analysis in this study although provided a means of objectively classifying DCIS grades, but the problem of selection bias remains as the study is retrospective, therefore, there remains a need for prospective validation before it can be recommended for routine use. Furthermore, with current technology, its rather time consuming and new technological advances in software development may render it more practical to use.

The p53 mutation analysis was disappointing and needs to be repeated in future. It highlights the fact that in spite of repeated attempts, sometime a technique may not work due to complex interplay of various factors involved.

The spectral database developed in this study on the other hand suggests an enormous potential to offer pre-operative diagnosis of DCIS, IDC, their grades and extent as well as the nodal status in order to offer better management to patients resulting in better outcome. It may be possible in future to assess the extent of DCIS and IDC in vivo and to assess nodal status preoperatively to help in better management and outcome.

There are, I believe, the following reasons for distinct FTIR spectra for DCIS and IDC grades:

1) Minimal heterogeneity the spectra were taken only from nuclei of each case. Surrounding stroma was not included as it is likely that areas of stroma, collagen, calcification, necrosis, mucin, milk etc will have their own distinct spectra. Therefore, rare subtypes of DCIS like apocrine, clear cell, papillary, signet ring were not included. All IDC were not otherwise specified. The purpose of the study was to establish a database of nuclei of DCIS and IDC first as no such database exists. Once that is done, then in future, variations in normal histology can be looked at and compared against the baseline. This fact has been highlighted by Jackson et al who in the study of IDC noted that if consistent points are not compared with each other, tissue heterogeneity can pose considerable problem.

Therefore, the results of FTIR study basically mean that type and content of DNA is different in the three DCIS and IDC grades.

2) Selection bias in this retrospective study there is a clear selection bias as LNG cases that were selected had morphologically small nuclei and HNG had big nuclei, this was confirmed on automated image analysis. In addition, the lower grades were ER and BCL2 positive and higher grades were HER-2, p53 positive and had a very high proliferative activity on immunohistochemical analysis. Therefore, basically it is a feasibility and reproducibility study demonstrating "proof of principle" that is morphological difference in nuclei due to increased DNA content and increased proliferation proteins is reflected in chemical structural analysis. This can be particularly useful in proteomics research. The selection bias was introduced on purpose as mentioned above to establish baseline databases.

3) Non-employment of statistics; no attempt was made to analyse the spectra statistically because the statistical analysis involving spectra are quite complex and as yet there is no consensus in the literature which one to use. Furthermore, it was considered important validating the findings prospectively on unselected cases before trying to establish parameters like sensitivity, specificity, positive predictive value and negative predictive value of the technique.

It seems that the initial part of the study employing immunohistochemical detection of molecular markers did not suggest LNG and ING DCIS as distinct entities, but spectroscopy did. This may be due to the reason that in this study immunohistochemistry looked at only 6 molecular markers which may not have been significantly different between the 2 groups, whereas spectra in each case were a collective reflection of all the proteins present in a particular case; and there can be a lot of proteins at one point from where a spectrum is obtained. Therefore, spectroscopy probably provides a more accurate reflection of a particular point's chemical structure.

CHAPTER 10: FUTURE WORK:

A considerable amount of study has been carried out on breast carcinoma as evidenced by the huge number of publications on this topic. However, particularly in the area of DCIS there is a need to do more research work. In this study, classification of DCIS according to nuclear grades appears to be clinically valid as indirectly evidenced by the statistically significant correlation of high nuclear grade with markers of poor prognosis (Ki67, p53 and cerbB-2). In contrast, low nuclear grades correlated with markers of favourable prognosis (ER, Bcl2). It would be useful to determine the prognostic significance of these molecular markers prospectively in DCIS studied as there are only a few studies evaluating this (1-3). In one study, Ringberg et al (4) have evaluated the prognostic value of ER, PR, cerbB-2, bcl2, p53, DNA non-diploidy and Ki67 in a study of 187 cases of DCIS. It is likely that the combination of molecular markers studied will prove to be of prognostic significance in DCIS in a manner similar to that in IDC and that it helps augment the information provided by the histological classification alone. In this study, bax was also studied in DCIS and its prognostic value for this stage of the disease still needs to be evaluated. After appropriate follow-up, this could be done on these cases but in order to do this, the patients would now need to be contacted in person and their informed consent obtained.

Other molecular markers may also be of importance in DCIS and pathogenesis of breast carcinoma, for instance TGFβ, cyclins, pS2, cadherins, VEGF (5-10) and a better clarification of their role in prognostication of DCIS is desirable.

In addition to a possible role in prognosis of DCIS, the molecular markers studied may also be useful for treatment prediction in this stage as well as in invasive disease. For example, ER determination may guide the use of adjuvant anti-oestrogen treatment. A beneficial effect of adjuvant tamoxifen for patients with DCIS operated upon with a lumpectomy and postoperative RT has been reported both with regard to ipsilateral and contralateral recurrences (11). Therapies based on monoclonal antibodies directed against cerbB-2 may also be a treatment alternative in the future, since a high proportion of DCIS overexpresses cerbB-2. It would also be desirable to find markers for sensitivity or resistance to RT in DCIS, as in IDC, *p53* mutations have been suggested to predict sensitivity to RT (12).

It would be desirable to include ADH as part of the spectrum of the disease and compare molecular marker expression in normal, and UDH as well as ADH, DCIS and IDC to better understand the pathogenesis of breast carcinoma. There is some evidence from the work of Hoshi et al (13) that proliferative activity is a good discriminant between ADH and DCIS and it was found to be a good discriminant between normal, DCIS and IDC in this study as well. It is known that cerbB-2 appears earlier in breast carcinogenesis, at the level of atypical hyperplasia, whereas p53 overexpression is confined to carcinomas (14). However, in one study of 44 normal and benign breast tissues (15), Kandel et al demonstrated *P53* gene mutations (all missense) in 10/44 (23%) of cases employing PCR-SSCP analysis for exons (4-10), the significance of which is yet unclear. In this study, p53 positivity was not seen in normal tissues. Therefore, a study of normal tissues, perhaps obtained as a part of reduction mammoplasty or biopsied for other reasons but subsequently demonstrated to be normal, is desirable for clarification; as the normal tissues in this study came from cancerous breasts (surrounds) rather than from completely normal breasts.

In this study, the main differentiating factor between normal tissues, DCIS and IDC as well as between the comparable subgroups of DCIS and IDC was proliferative activity, whereas the principal difference between IDC LN positive and its metastatic lymph nodes was bax protein expression. It is quite possible that during early stages of breast carcinogenesis, when DCIS develops from normal tissue and IDC, the increase in proliferation of epithelial cells is the predominant process, while the development of metastasis probably requires marked decrease in apoptosis in addition. To confirm this, a larger number of metastatic cases would need to be studied, looking at bax expression at molecular level, for instance to do RT – PCR for bax mRNA detection. This would aid in understanding the development of metastasis with a view to prevent it and/or treat it.

Topoisomerase IIα is another proliferation marker, which can be looked at in DCIS and IDC. It is expressed in G_1, S and G_2 phases of the cell cycle. In a multivariate analysis of prognostic factors in 863 breast cancers, topoisomerase IIα expression was found to have the greatest prognostic value with regards to overall survival (OS), second only to nodal status (16)

For the cases studied by PCR amplification, repeat PCR followed by cloning and subsequent DNA sequencing needs to be done.

The results of cases studied by FTIR and FT-Raman are quite interesting. This study was carried out on paraffin-wax embedded sections, it would be extremely useful to analyse fresh tissue by both IR and Raman spectroscopic techniques. Furthermore, as the fibre optic probe for in vivo use is in its final stages of development, it would be possible to employ both IR and Raman techniques to investigate patients in future. As the development of the new FTIR chemical imaging system has provided detailed information at a very short interval of time, this will allow building up a spectral data base of cancerous tissues at a very rapid pace, which is a missing link at present, as limited amount of such information are available in literature. In addition there is a need to develop a statistical model for both IR and Raman spectroscopic studies on biological tissues.

The successful demonstration that IR and Raman spectroscopy of in vitro samples can distinguish normal from abnormal samples suggests the possibility that the same techniques may prove very useful in an in vivo analysis.

An extremely important aspect of the future study shall be to carry out a joint investigation with spectroscopists and pathologists, which should address the following issues in detail:

- What is there, i.e., the detection with confidence
- How far along is the disease process
- Assurance of precisely and accurately distinguishing between the diseased and healthy tissue
- Grading of cancerous tissue both by spectroscopic and pathological means.
- Predicting an accurate statistical model

With the blending of ideas from diverse fields, a new set of perspectives may be arrived which will ultimately improve the health of individuals.

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APPENDIX I:

PROTOCOLS FOR VARIOUS STEPS IN IMMUNOCYTOCHEMISTRY:

Preparation of paraffin-wax sections for immunocytochemistry (ICC) (1):

(a) Sections were cut at a thickness (3 to 4 μm).
(b) Floating of sections was done on 20% alcohol followed by immersion in a water bath at 45°C.
(c) Sections were picked on APES coated slides.
(d) The slides were immediately placed to dry at 37°C in an oven for 48 hours.

Preparation of 3-aminopropyl-triethoxysilane (APES) glass coated slides (1;2):

This was carried out to avoid section loss during microwave pre-treatment. Coated slides can be stored at room temperature indefinitely.

(a) Slides were degreased in hot detergent solution, rinsed in distilled water then alcohol (74OP) and air dried for 10 minutes.
(b) Slides were immersed in a freshly prepared 2% solution of APES in acetone, five dips approximately (5 seconds).
(c) Acetone was allowed to drain away.
(d) This was followed by washing in distilled water twice.
(e) Slides were stored air dried at 40°C overnight.
(f) Slides were stored at room temperature in a dust free environment.
Note 300ml. of APES solution is sufficient for 200 slides

Blocking Endogenous Peroxidase Activity (1;2):

Endogenous labels can produce a reaction product indistinguishable from specific immunostaining, hence need to be blocked prior to staining.

(a) Slides were transfered to a humidity chamber at room temperature and the sections were covered with freshly prepared 0.5% H_2O_2 in water for 10 to 15 minutes.
(b) The solution contains 0.2ml (200μl) H_2O_2 (30% w/vol.) and 11.8 ml of distilled water.

This is enough for 20 slides. 30 ml is needed for one full rack and for two racks, 60 ml is required (59ml of water + 1ml of H_2O_2).

Antigen Retrieval by Microwave Method (1-3):

Sections must be mounted on APES slides for this method to avoid section loss. The mechanism of action of microwave oven heating for antigen retrieval is thought to be due to alteration of cross-linking by formaldehyde.

(a) Plastic jars were filled with 0.01M citric acid buffer (pH 6.0) and the sections were submerged with tissue facing to the bottom of the coplin jar.
(b) Two other plastic coplin jars were filled with cold water in the same manner. This helps to stop some evaporation by absorbing some of the heat.
(c) Coplin jars were evenly spaced around the outside of the rotating plate.
(d) Microwave was set at 700 watts for 5minute intervals, topping up solutions at the end of each interval. Slides must be covered throughout the microwave heating process.
(e) Coplin jars were left to cool in running tap water before placing slides in appropriate buffer for immunocytochemistry technique.

TBS buffer for immunocytochemistry (1;2)

Composition:-

1-	Sodium Chloride	=	80g
2-	Tris (Tris-hydroxymethylamine)	=	6.6g
3-	Hydrochloric acid 1M	=	44ml
4-	Double distilled water	=	10 litres

(a) Reagents were mixed with 2 litres of distilled water on a magnetic stirrer with a magnetic flea.
(b) When components were dissolved, the solution was transferred to a plastic 10 litre container.
(c) Added 8 litres of water and mixed thoroughly.
(d) An aliquot was taken and pH adjusted to 7.6 (±0.05) using buffer solution.

Developing Sections with Freshly Prepared 3,3diaminobenzidine (DAB) (1;2):

(a) Buffer solution was prepared by adding Tris = 121.4mg to 12.6ml of double distilled water and the pH was adjusted to 7.3 to 7.7 by adding 0.1M hydrochloric acid (HCl). 7.6ml of HCl was normally sufficient to obtain the required pH range.

(b) 2ml of buffer solution was added to 12 mg of 3, 3 – diaminobenzidine tetrachloride (DAB)(Sigma) stored at -20°C to the weighing crucible (boat) inside the microbalance and then remaining buffer was added to DAB in a coplin jar. This minimises the risk of spillage. The solution was stored in the dark at room temperature.

(c) Just prior to use, 8µl of 30% H_2O_2 (stored at 4°C) was added to the solution and this was applied directly to the slides.

(d) The slides were left for 2 to 10 minutes at room temperature and then inspected microscopically. The reaction with TBS was stopped, if adequate staining developed. At this stage either freshly made up substrate was reapplied if the reaction was weak, or 0.1M imidazole (Sigma) was added at a concentration of 20µl/20ml DAB solution.

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APPENDIX II:

Recommended IHC scoring method for cerbB-2 staining

Score	CerbB-2 Expression	Staining Pattern
0	Negative	No staining is observed, or membrane staining is less than 10% of tumour cells
1	Negative	A faint membrane staining in more than 10% tumour cells
2	Borderline	Weak to moderate complete membrane staining in more than 10% tumour cells
3	Positive	Strong complete membrane staining in more than 10% of tumour cells

Quick Score for Assessment of ER Immunostaining As Recommended by the National Coordinating Group for Breast Screening Pathology:

Score for proportion staining	Score for staining intensity
0 = no nuclear staining	0 = no staining
1 = < 1% nuclear staining	1 = weak staining
2 = 1 – 10 % nuclear staining	2= moderate staining
3 = 10 – 33 % nuclear staining	3 = strong staining
4 = 33 – 66 % nuclear staining	
5 = 66 – 100 % nuclear staining	

Adding the two scores together gives a maximum score of 8 (range from 0 – 8).

Experience suggests that if this scoring system is used the appropriate cut off values for treatment of advanced disease are as follows:

- A score of 0 indicates that endocrine treatment will not work
- A score of 2 – 3 indicates a small (20%) chance of response to endocrine therapy
- A score of 4 – 6 indicates an even (50%) chance of response
- A score of 7 – 8 indicates a good (75%) chance of response

Reference:

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APPENDIX III:

Research Publications:

1. S. Rehman, J. Crow and P. A. Revell

   Bax Protein Expression in DCIS of the Breast in Relation to Invasive Ductal Carcinoma and Other Molecular Markers, Pathology Oncology Research, vol. 6, no. 4, 256-263 (2000).

2. S. Rehman, J. Wilson, E. Miseo and I. Rehman (Presentation)

   Analysis of Cancerous Tissues Using Fourier Transform Infrared Rapid Scan Imaging, Pittcon exhibition and conference, USA (2003)

   [This is one of the major conferences and exhibitions in the world. The paper was extremely well received and was sponsored by Digilab,].

3. S. Rehman and P. A. Revell

   Fourier Transform Infrared Spectroscopic Analysis of Breast Cancer Tissues; Identifying Differences between Normal, Invasive IDC and DCIS

   (Submitted) Interfaces, Journal of the Royal Society (2004)

Papers in Progress:

4. **S. Rehman and P. A. Revell**
   Fourier Transform Raman Spectroscopic Analysis of Breast Cancer Tissues; Identifying Differences between Normal, Invasive IDC and DCIS (nearly completed and will be submitted in Clinical Investigations)

5. **S. Rehman and P. A. Revell**
   Fourier Transform Infrared chemical imaging of cancerous tissues (in preparation)

GLOSSARY:

Allele: one of the possible different forms of a gene at a given locus.

Amplification: increase in the number of copies of a specific DNA molecule.

Anneal: the hybridization of a single-stranded DNA molecule to another single-stranded DNA molecule of complementary sequence.

Aneuploid: any chromosomal number that is not the exact multiple of the normal haploid number.

Autoradiography: the process of exposing X-ray film to a radioactive source to generate an image of the radioactive components of that source. The developed film showing the image is called an autoradiogram or autoradiograph.

Autosome: any chromosome that is not a sex chromosome or mitochondrial chromosome, there are 22 pairs of autosomes in humans.

Bacteriophage: a bacterial virus. These are modified and used as vectors for DNA cloning.

Base pair (bp): a pair of complementary nucleotides in double-stranded DNA.

Centromere: the point at which two chromatids of a chromosome are joined and where the spindle fibres become attached during mitosis and meiosis.

Chromatid: one of the two strands that make up the chromosome, held together by the centromere.

Chromatin: stainable material of interphase nucleus consisting of nucleic acid and associated histone protein packed into nucleosomes.

Chromosomal aberration: an abnormality in the number or structure of a chromosome.

Chromosome: a structure of DNA and associated proteins that contains the hereditary information of the cell in the form of a linear array of genes.

Clone: cells having the same genetic constitution and derived from a single cell by repeated mitosis.

Codon: three adjacent nucleotides in a nucleic acid capable of encoding a single amino acid.

Co-efficient of variation: the ratio of the standard deviation to the mean.

Complementary DNA (cDNA): DNA synthesized from an mRNA template by the enzyme reverse transcriptase. It does not contain introns.

Complementary sequence: sequence which will hybridize with a specified sequence.

Cosmid: a modified plasmid containing sequences from bacteriophage that allow the insertion of large DNA fragments. Cosmids are circular double-stranded molecules typically 45-48 kb long, including the vector sequences.

Cowden disease (CD): It is also called multiple hamartoma syndrome and is a cancer associated genodermatosis with an autosomal dominant pattern of inheritance. It was named after the first patient described in 1963 by Lloyd and Dennis. The main clinical features are hamartomas of the skin, breast, thyroid, oral mucosa and intestinal epithelium. The gene responsible has been localized to chromosome 10q22-23 by linkage analysis and is probably a tumour suppressor gene.

Deletion: loss of a part of a chromosome.

Denature: to separate the two complementary strands of double-stranded DNA, usually done either by treatment with alkali or by use of elevated temperature.

Deoxyribonucleic acid (DNA): nucleic acid comprising four different nucleosides (deoxyadenosine, deoxycytidine, deoxyguanosine and deoxythymidine) linked by phosphate groups.

2', 3'-dideoxynucleotide (ddNTP): a 2'-deoxyribonucleotide analogue which does not have a hydroxyl group at the 3'position.

Diploid: a cell having two chromosome sets, or an individual with two chromosome sets in each of its cells. Humans are diploid.

DNA cloning: the process of isolating a particular DNA fragment of interest and its insertion into a vector, so that it can be cloned and prepared in large quantities independent of other sequences.

DNA polymerase: the enzyme that replicates/synthesizes DNA.

DNA probe: a piece of single-stranded DNA which can be labelled with a radioactive isotope or a fluorescent signal and used to detect DNA fragments whose sequence is complementary to the sequence of the probe, by hybridization.

DNAse: an endonuclease with preference for DNA.

DNA sequence analysis: determination of the nucleotide sequence of a length of DNA molecule. Typically, this is performed by cloning the DNA molecule of interest, so that enough can be prepared to allow the sequence to be determined, usually by the Sanger dideoxy chain termination or Maxam Gilbert chain degradation techniques. The resulting reactions are then run on a large sequencing gel, capable of resolving single nucleotide differences in chain length. Recently PCR based methods have obviated the need to clone the DNA under some conditions and automated DNA sequencing has become widely available.

Electrophoresis: a technique for separating molecules based on their differential mobility in an electric field.

Endonuclease: an enzyme that cleaves nucleic acids at positions within the chain. Some of these act on both RNA and DNA.

Exon: a segment of a gene that is represented in the final spliced mRNA product.

Exonuclease: an enzyme that degrades DNA from the end of the molecule.

Fidelity: the accuracy with which a polymerase synthesizes the strand complementary to the template DNA.

Fluorochrome (fluorophore): a dye which absorbs light at one wavelength and then emits light at another (longer) wavelength. These characteristic wavelengths are the absorption and emission spectra respectively.

Frameshift: when the normal protein coding information changes from one frame to another. This may be due to a mutation or a sequencing error, or may be part of a regulatory mechanism.

Gene: the fundamental physical and functional unit of heredity. Genes are parts of DNA molecules and many of them encode proteins.

Genetic marker: a genetically controlled phenotypic feature used in inheritance studies.

Genome: the total amount of genetic material in the cell.

Genotype: the genetic constitution of an individual.

Haploid: a cell having one chromosome set, or an individual with one chromosome set in each of its cells.

Hapten: a small molecule which can be detected by its high-affinity binding to a detector molecule, for example biotin can be detected by use of avidin, streptavidin or a specific antibody.

Heterogenous: composed of varied cell types.

Heterozygote: an individual possessing two different alleles at the corresponding loci on a pair of homologous chromosomes.

Homogenous: consisting of or composed of similar elements or ingredients, of a uniform quality throughout.

Homozygote: an individual possessing identical alleles at the corresponding loci on a pair of homologous chromosomes.

Hybridization: the process of complementary base pairing between two single strands of nucleic acid.

Hypothetical model of breast carcinogenesis[1]**:** columnar cell change > columnar cell hyperplasia > columnar cell hyperplasia with atypia > ADH > low grade DCIS > tubular and grade I carcinoma

[1] **Schnitt SJ and *Vincent-Salomon A,*** Columnar Cell Lesions of the Breast, *Advances in Anatomic Pathology* 2003; 10(3):113-124.

Intron: a segment of a gene not represented in the final mRNA product because it is removed through splicing together of exons on either side of it.

Kappa: a measure of the degree of non-random agreement between observers or measurements of the same categorical variable.

Kilobase pair (kb): a measure of the length of a nucleic acid molecule. One kb is 1000 base pairs (bp) of nucleic acid.

Karyotype: the number, size and shape of the chromosomes in a cell.

Label: a detectable entity covalently linked to a nucleic acid. Typical labels include radioisotopes, fluorochromes and haptens.

Ligase: an enzyme that joins the ends of two nucleic acid molecules to form a single molecule.

Ligate: to covalently join two ends of nucleic acids.

Linkage: the co-segregation of two unrelated DNA sequences which are physically close together on the chromosome.

Linkage disequilibrium: the association of particular alleles at two linked loci more frequently than expected by chance.

Locus: the site of a gene on a chromosome.

Mitotic index (MI): the number of mitoses expressed as a percentage of the total number of the tumour cells.

Mosaic: individuals with two different cell lines in their constitution.

Multicentric DCIS: two or more areas of DCIS separated by an uninvolved area of at least 4 cm. Typically, however, DCIS is unicentric.

Multifocal DCIS: two or more foci of DCIS separated by an uninvolved portion of duct of any length less than 4 cm. In most cases it is less than 1 cm.

Mutation: change in DNA sequence resulting in a different sequence by addition, subtraction or substitution of one or more bases.

Neoplasm: see 'tumour' below.

Northern blot: the RNA equivalent of a Southern blot.

Nucleoside: a base covalently linked to a sugar (ribose or deoxyribose). A nucleoside is a nucleotide without the phosphate group.

Nucleosomes: the repeating structural units of chromatin, each consisting of approximately 200 base pairs of DNA wound around a protein core.

Nucleotide: the basic repeat unit of DNA and RNA molecules, comprising a nitrogeneous base covalently linked to a pentose sugar which is linked to a phosphate group.

Oligonucleotide: several nucleotides joined together to form a short, single-stranded DNA molecule.

Oligonucleotide primer: a short, synthetic DNA molecule of known sequence, typically 18-24 bases long, which anneals to the complementary sequence on the template DNA.

Oncogenes: genes which when altered in their structure or expression, contribute to the abnormal growth of cancer cells. The mechanism of alteration includes mutation, amplification and translocation.

Oncogenic: an agent capable of causing or inducing the formation and development of a neoplasm.

Penetrance: the proportion of individuals with a particular genotype who also have the corresponding phenotype.

Phenotype: the appearance of an individual, resulting from the effects of both environment and genes.

Plasmid: an extrachromosomal, circular DNA molecule found in some bacteria which can be modified and used as a vector for DNA cloning.

Ploidy: a term that describes the number of chromosome sets, namely 23=haploid (1 set), 46=diploid (2 sets).

Polymerase chain reaction (PCR): a technique for amplifying a specific segment of DNA.

Polymorphism: a variation in the sequence of DNA, so that two or more versions exist.

Positioned cloning: a method used to isolate gene/s whose protein product/s is/are not known, but whose existence can be inferred from the disease phenotype.

Primer: an oligonucleotide which anneals to its complementary sequence, forming a short double-stranded region. This is extended by a DNA polymerase. Primers are required for the chain termination method of DNA sequencing and for PCR.

Primer walking: a strategy for sequencing in which a new sequencing primer is designed based on the most distant reliable sequence obtained from the previous sequencing reaction. This primer is then used to sequence the next unknown section of the template. Thus the sequence is determined in a methodical, stepwise fashion.

Proofreading: the ability of a polymerase to excise incorrectly incorporated nucleotides and replace them with correct ones. Proofreading polymerases have a 3'-5'exonuclease activity with which they remove the mismatched base.

Proteinase K: an enzyme which degrades proteins.

Pulsed field gel electrophoresis (PFGE): a technique for separation of large fragments of DNA. It can be used for long range mapping of the genome to detect major deletions and rearrangements.

Recombinant: a DNA molecule generated by ligating heterologous DNA molecules.

Restriction endonuclease (restriction enzyme): an enzyme which recognizes a short, specific DNA sequence and cleaves both strands.

Restriction fragment length polymorphism (RFLP): DNA can be cut by restriction endonucleases to give a set of DNA fragments and the lengths of these fragments can be measured by electrophoresis. A polymorphism may lead to the production of fragments of

different sizes from different versions of the sequence. This length polymorphism is called RFLP.

Reverse transcriptase: an enzyme capable of synthesizing DNA from an RNA template (an RNA-dependent DNA polymerase).

Ribonucleic acid (RNA): nucleic acid comprising the nucleosides adenosine, cytidine, guanosine and uridine. The main difference from DNA is ribose sugar in the molecule instead of deoxyribose and uridine base instead of thymidine.

RNA polymerase: the enzyme that synthesizes RNA, based on a DNA template.

Southern blot: DNA that has been electrophoretically separated and immobilized on a solid support (nylon or nitrocellulose). Named after Ed Southern, who devised this technique.

Splicing: removal of the introns from an unprocessed RNA molecule.

Stop codons: codons which terminate translation. These are UAA, UGA and UAG.

Stringency: conditions that affect the specificity of hybridization between nucleic acid molecules. Low stringency conditions allow strands to form hybrids even when they are not perfectly complementary. Increasing the temperature and decreasing the ionic strength increases stringency.

Template: a nucleic acid molecule used by a polymerase to direct the synthesis of a new nucleic acid molecule of complementary sequence.

Transcription: the process by which an RNA molecule is synthesized from a DNA template in the presence of RNA polymerase.

Transformation: the introduction of a DNA molecule into an organism (e.g. a bacterium), so that the DNA molecule can be inherited by subsequent generations.

Translation: the process by which protein synthesis occurs from the information encoded in mRNA molecule.

Translocation: the transfer of an area of one chromosome to another non-homologous chromosome.

Tumour (tumor): an abnormal mass of tissue that results from excessive cell division that is uncontrolled and progressive, also called a neoplasm. Tumours perform no useful body function. They may be either benign (non-cancerous) or malignant (cancerous).

Tumour suppressor genes: genes which suppress cell cycle progression and promote apoptosis.

Vector: a small, circular DNA molecule used to clone DNA segments of interest by allowing their propagation in bacteria or other organisms such as yeast.

Western blot: the protein equivalent of a southern blot.

Wild – type: the normal allele of a gene.