UNIVERSITY OF LONDON THESIS

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THE MOLECULAR PATHOLOGY OF PAGET'S DISEASE OF THE VULVA AND THE BREAST

Patricia Elizabeth Ellis

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University College London,
University of London

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

Paget's disease of the vulva and breast are rare diseases. Unlike Paget's disease of the breast, where the consensus is that the great majority of cases are associated with an in-situ or invasive ductal carcinoma, only approximately 10-30% of cases of Paget's disease of the vulva have an invasive adenocarcinoma present. It is believed that the Paget cells in Paget's disease of the breast are derived from the underlying in-situ or invasive breast carcinoma, and these cells migrate up through the ducts onto the nipple epidermis. The histogenesis underlying vulval Paget's disease is unclear. Paget's disease of the vulva may become secondarily invasive even in the absence of an underlying malignancy.

The aim of the study was to identify abnormalities present in Paget's diseases of the vulva and the breast and to investigate whether there are any differences in molecular markers in the Paget cells of those cases with invasive disease compared to those cases without invasive disease. Such an analysis may identify some of the molecular pathways underlying both vulval and breast Paget's disease, as well as generating potential markers for clinical prognosis.

Archival paraffin wax-embedded sections of Paget's disease of the vulva or breast were used. Immunohistochemical analysis was performed to analyse markers involved in the cell cycle (p53, pRb, cyclin D1, Ki67), in angiogenesis (e.g. VEGF, PD-ECGF/TP, MVD) and cell adhesion molecules (e.g. plakoglobin, E-cadherin, β-catenin). Sections that were immunopositive for the tumour marker p53 were microdissected, the DNA extracted, amplified and sequenced. In situ hybridisation was also used to determine the presence of mRNA of the adhesion molecules in the

tissue sections. A national register for Paget's disease of the vulva was established requesting that clinicians who have patients with Paget's disease of the vulva should enrol them.

Results suggest E-cadherin, plakoglobin and pRb and p53 may have a role to play in the pathogenesis of Paget's disease of the vulva and pRb, plakoglobin, VEGF and PD-ECGF/TP in Paget's disease of the breast. Wide local excison was the preferred treatment option for patients registered in the PDV database. The information from the Paget's register and the results obtained from the thesis may increase the understanding of Paget's disease of the vulva and breast.

ACKNOWLEDGEMENTS

I would like to thank Dr Christopher Perrett, Non-Clinical Senior Lecture in Medical Sciences, for supervising me, and his discussions and comments concerning my work. Similarly, I am indebted to Professor Allan MacLean, whose own interest in Paget's disease stimulated my enthusiasm, and who gave me the opportunity to embark on this project.

When starting the study only three cases of Paget's disease of the vulva and 11 cases of Paget's disease of the breast were found at the Royal Free Hospital. Through the generosity of pathologists throughout the country the number of cases of Paget's disease of the vulva increased to 72 and Paget's disease of the breast to 37 cases. I would like to thank sincerely all the pathologists who kindly provided specimens.

My special thanks to Dr Kerstin Rolfe and Anne Christine Wong Te Fong for helping to score the sections, to Dr Julie Crow for histological analysis of biopsy specimens, and to colleagues at the Royal Free Hospital who helped me throughout the project. I would also like to thank Colin Hughes for his assistance.

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DECLARATION

The experimental work and results presented in this thesis except where accordingly acknowledged, are entirely my own.

None of the work contained within this thesis has been submitted previously either by my self or by any other unknown, for examination within the University of London or other awarding body.

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ABBREVIATIONS

AEA	Apocrine endothelial antigen
AJ	Adherens junctions
APC	Adenomatous polyposis coli
APES	Aminopropyltriethoxysilane
CA	Carcinoma
CEA	Carcinoembryonic antigen
CDK	Cyclin dependent kinases
DCIS	Ductal carcinoma in situ
DIG	Digoxigenin
DPEC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonuclease triphosphates
ECM	Extracellular matrix
EMA	Epithelial membrane antigen
EMPD	Extramammary Paget's disease
GCDFP	Gross cystic disease fluid protein
GSK	Glycogen synthetase kinase
H&E	Haematoxylin & eosin
HPV	Human papilloma virus
HRP	Horse Radish peroxidase
IHC	Immunohistochemistry
INV	Invasive
ISH	In situ Hybridisation
KDa	Kilo dalton
LCM	Laser capture microdissection
LEF/TCF	Lymphoid enhancer factor/T cell factor
MPD	Mammary Paget's disease
mRNA	Messenger ribonucleic acid
MVD	Microvessel density
NBT/BCIP	Nitroblue tetrazolium chloride/ bromo-chloro-idoly-phosophate
PBS	Phosphate bovine saline
PCR	Polymerase chain reaction
PDB	Paget's disease of the breast
PD-ECGF/TP	Platelet-derived endothelial growth factor/Thymidine phosphorylase
PDV	Paget's disease of the vulva
PPAR	Peroxisome proliferator-activated receptor
pRb	Retinoblastoma protein
RNA	Ribonucleic acid
SBC	Streptavidin biotin complex
SCC	Squamous cell carcinoma
SSC	Sodium chloride sodium citrate
TBS	Tris buffered Saline
TSG	Tumour suppressor gene
PBS	Phosphate buffered saline
VEGF	Vascular endothelial growth factor
VIN	Vascular intraepithelial neoplasia
vWF	Von willebrand factor

FULL PUBLICATIONS ARISING FROM THE THESIS

Ellis P.E., Wong Te Fong L.F., Rolfe K.J., Crow J.C., Reid W.M.N., Davidson T., MacLean A.B Perrett C.W (2002). The role of p53 and Ki67 in Paget's disease of the vulva and breast. Gynecol. Oncol. 86: 150-156

Ellis P.E., Wong Te Fong L.F., Rolfe K.J., Crow J.C., Reid W.M.N., Davidson T., MacLean A.B Perrett C.W (2002). The role of vascular endothelial growth factor-A (VEGF-A) and Platelet-derived endothelial cell growth factor/ thymidine phosphorylase (PD-ECGF/TP) in Paget's disease of the vulva and breast. Anticancer Res.22: 857-861.

A.B MacLean, M., Makwana, **Ellis P**, F.Cunningham (2004).The management of Paget's disease of the vulva. Journal of Obstetrics & Gynaecology. 24, 2 124-128 2004.

In addition, this work has resulted in five abstracts, four oral presentations including one international presentation and five poster presentations.

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CHAPTER 1 GENERAL INTRODUCTION

1.1 Who was Sir James Paget?

Sir James Paget was born in Great Yarmouth in 1814. After gaining his medical qualification at St. Bartholomew's Hospital, he was retained as a curator of the museum at the Royal College of Surgeons. In 1847 he became Assistant Surgeon at St. Bartholomew's Hospital and in 1858 was appointed as Surgeon to Queen Victoria. It was during his years at St. Bartholomew's Hospital that he described two different diseases which both bear his name today: Paget's disease of the breast and Paget's disease of the bone. In 1874 he published a report entitled "On Disease of the Mammary Areola preceding Cancer of the Mammary Gland". In this, he describes cases of Paget's disease of the nipple (Paget, 1874). This lesion is characterised by the presence of large cells, with abundant, pale cytoplasm and large nuclei with prominent nucleoli, in the surface epithelium of the nipple. These cells are generally referred to as Paget cells, although Treves et al. (1954) questioned this practice since Paget did not actually describe the histological features or the cellular morphology of the lesion. Similar conditions involving Paget cells located outside the mammary gland have been termed extramammary Paget's disease, the most common locations being the vulva and perianal region. Crocker in 1889 described the first case of extramammary Paget's disease. He reported lesions on the scrotum and penis, with clinical features similar to those described by Paget (Olson et al., 1991). Other affected sites include the axilla, eyelids, groin, buttock, thigh and the external ear canal (Whorton et. al. 1955; Fligiel et al., 1975; Chanda et al., 1985; Watanabe et al., 1993). Paget's disease of the bone is a completely different and unrelated condition, which Sir James Paget described in 1876 and which is characterised by abnormal remodelling and deformity of the bones. It has distinguishing clinical and radiological

appearances and there has been evidence to suggest that a virus may be involved in its aetiology.

1.2 Paget's disease of the vulva (PDV)

Paget's disease of the vulva is a rare intraepithelial in situ carcinoma that comprises approximately 1% of all vulval neoplasms (Curtin et al., 1990). It was W. Dubreuilh, Professor of Dermatology at Bordeaux University, who described the first case of Paget's disease in the vulva. In his paper he describes a 51-year old woman who presented with a 3-year history of an itchy, painful pimple on the clitoris which had been gradually increasing in size (Dubreuilh, 1901). On inspection there was a red indurated eroded lesion on the labium majorum. Within this were areas of white islands of hyperkeratosis. The red scaly lesions with hyperkeratosis are classical features of PDV. Figure 1.1 demonstrate clinical presentation of PDV.

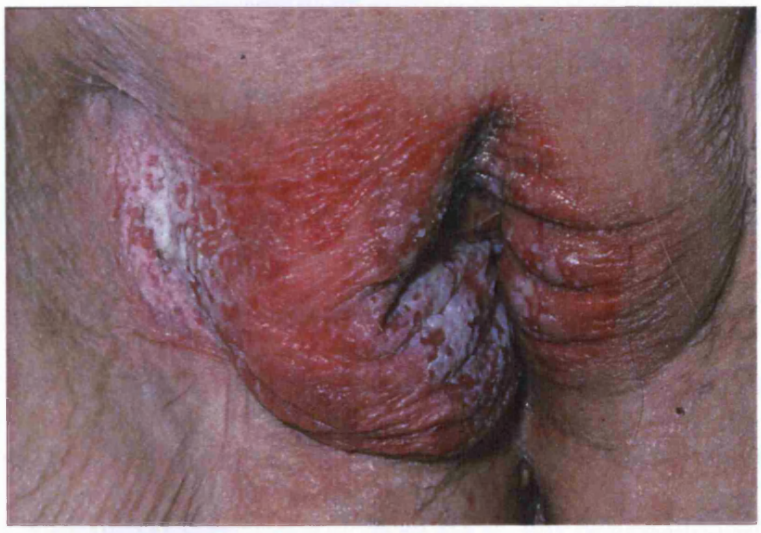

Figure1.1 Photograph of PDV (x100)

1.3 Development of the vulva

1.3.1 Development of the external genitalia

The transverse septum divides the primitive cloaca into an anterior urogenital portion and a posterior rectal portion. The urogenital portion of the cloacal membrane breaks down shortly after division is complete forming the urogenital sinus which develops into three portions. There is an external phallic part, a deeper narrow pelvic part between it and the region of the Müllerian tubercle, and a vesicourethral part connected superiorly to the allantois. Externally in this region the genital tubercle forms a conical projection around the anterior part of the cloacal membrane. Two pairs of swellings - a medial pair (the genital folds) and a lateral pair (genital swellings) - are then formed by the proliferation of mesoderm around the end of the urogenital sinus. Development up to this time (10 weeks'gestation) is the same in the male and the female. Differentiation then occurs. The genital tubercle enlarges only slightly and becomes the clitoris. The genital folds become the labia minora and the genital swellings enlarge to become the labia majora. The bladder and urethra form from the vesicourethral portion of the urogenital sinus and the vestibule from the pelvic and phallic portions.

1.3.2 Vulval skin

The epidermis of the vulval skin and its appendages, hair, sebaceous and sweat glands, are developed from the ectoderm. The dermis is developed from the mesoderm. The epidermis is established at about the 8^{th} day of gestation when the ectoderm differentiates within the developing embryo. Three cell types invade the developing epidermis during the first six months of intra-uterine life. Melanocytes, derived from the neural crest, and Langerhans cells, derived from the mesoderm, are

present at the end of the 3rd month while Merkel cells, the origin of which is uncertain, are present by the 6th month. Hair follicles begin to form during the 3rd month of gestation and sebaceous glands by the 4th month; differentiation of the primordial cells into sebum producing cells also proceeds rapidly. Eccrine sweat glands appear during the 3rd month of pre-natal life and apocrine glands develop after 6 months.

The intrinsic components of the dermis originate from the mesoderm during the 2nd month of embryonic life. The major cellular component is the fibroblast which synthesises and secretes the amorphous and structural connective tissue matrix to support the epidermal appendages and other component tissues.

1.4 Anatomy of the vulva

The vulva lies mainly within the urogenital triangle. The urogenital triangle constitutes the anterior section of the perineum; the posterior anal triangle being the posterior section. The perineum is derived from the body wall ectoderm, the hindgut endoderm and the intervening mesoderm. It forms part of the pelvic outlet caudal to the pelvic diaphragm.

The vulva consists of the mons pubis, the labia majora and minora, the vestibule of the vagina, the hymen, the greater vestibule glands of Bartholin, the clitoris with its prepuce and frenulum, the bulbs of the vestibule and the external urethral orifice (Figure 1.2.).

1.4.1 Mons pubis

Overlying the symphysis pubis, the mons pubis becomes recognisable at puberty with the deposition of subcutaneous fat and the appearance of pubic hair.

1.4.2 Labia majora

The labia majora originate from the mons pubis anteriorly and merge with the perineal body posteriorly. On inspection they consist of two cutaneous folds which form the lateral boundaries of the pudendal cleft. After puberty the deposition of subcutaneous fat within the labia majora produces a greater degree of prominence and the presence of pigmentation and hair on their lateral surfaces establish them as well defined structures. The medial surfaces of the labia majora are hairless and contain numerous sebaceous glands which may be in contact with each other or separated by the protrusion of the labia minora.

1.4.3 Labia minora

The labia minora are two thin folds of hairless skin, devoid of subcutaneous fat, which are situated between the labia majora on either side of the vaginal and urethral openings. The labia minora are separated from the labia majora by interlabial furrows. Anteriorly, the labia minora divide into lateral and medial parts. The lateral parts unite anteriorly to the clitoris, in a fold of skin overhanging the glans to form the prepuce of the clitoris. The medial parts unite on the under surface of the clitoris to form its frenulum. Posteriorly, the labia minora fuse to form a transverse fold, the frenulum of the labia, or fourchette, behind the vaginal opening. Being devoid of

adipose tissue the labia minora are composed mainly of elastic fibres and blood vessels and possess a rich innervation.

1.4.4 The vestibule

The cleft between the labia minora is termed the vestibule of the vagina and it extends from the clitoris to the fourchette. The urethra and the openings of the vagina, the ducts of Bartholin's glands and the minor vestibular glands are all found within the vestibule. The junction of the vestibule with the vagina is identified by the presence of the hymen or its remnants.

1.4.5 Bartholin's glands

Deeply situated within the posterior parts of the labia majora are the Bartholin's glands. The glandular secretion is clear, mucoid and alkaline and is increased during sexual arousal. The main duct of each Bartholin's gland opens at the lateral margin of the vagina just behind the mid-point and superficial to the hymen ring. The glands and their ducts can be a site of infection or cyst formation.

1.4.6 Minor vestibular glands

These glands are tubular structures; their acini are lined with columnar epithelium and their ducts with transitional squamous epithelium. They are found mainly in the posterior part of the vulva. The opening of the ducts can be seen with the naked eye.

1.4.7 The clitoris

The clitoris is situated in the midline at the apex of the vulval cleft. It is covered with stratified squamous epithelium that is thinly keratinised. No sebaceous, apocrine or sweat glands are present. It consists of erectile tissue and is the homologue of the penis. The bulbs of the vestibule, also composed of erectile tissue, participate in the formation of the clitoris. The external urethral orifice lies between the vagina and the clitoris.

1.4.8 Blood supply of the vulva

The arterial blood supply of the perineum is from branches of the internal iliac and femoral arteries. The superficial external pudendal artery, a branch of the femoral artery, pierces the deep fascia of the thigh anteriorly, to overlie the round ligament of the uterus. It runs medially to supply the mons pubis and labia of the vulva. The deep external pudendal artery, also a branch of the femoral artery, pierces the deep fascia of the thigh medially to enter the labia of the vulva. The venous drainage of the perineum is similarly arranged and eventually reaches the femoral and internal iliac veins.

1.4.9 Lymphatic drainage of the vulva

The regional lymph nodes of the perineum are situated in the groin at the base of the femoral triangle. These superficial lymph nodes subsequently drain to deep nodes in the pelvis and ultimately to para-aortic nodes on the posterior abdominal wall.

1.4.10 Nerve supply to the vulva

Innervation to the perineum is by the ilioinguinal nerve (L1) and the genital branch (L2) of the genito-femoral nerve (L1, 2). These supply the anterior part of the perineum. The lateral aspect of the perineum is supplied by the perineal branch (S1) of the posterior cutaneous nerve of the thigh (S1, 2, 3). The remainder of the cutaneous innervations is supplied by the pudendal nerve (S2, 3, 4) and the perineal branch of the 4th sacral nerve.

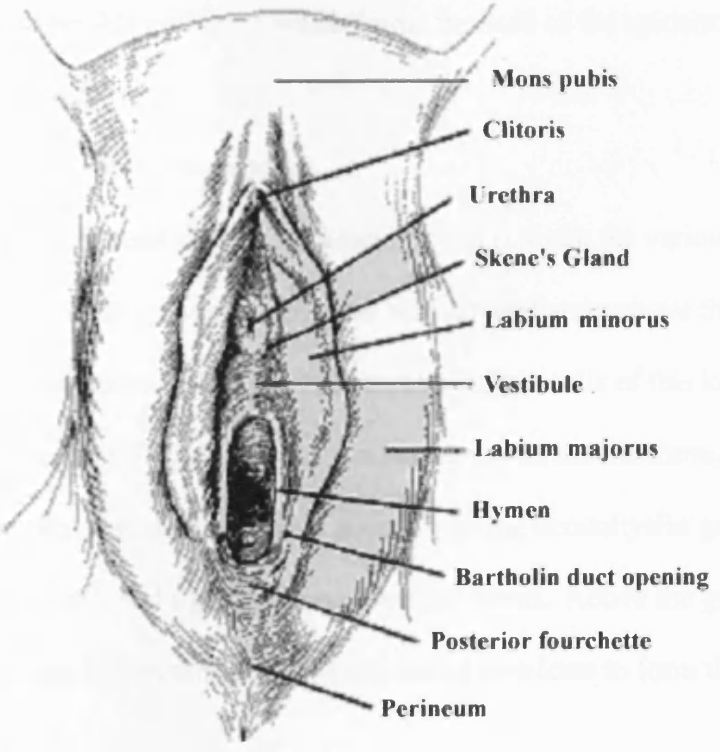

Figure 1.2 Diagram of the vulva.

1.5 Histology of the vulva

1.5.1 Epidermis

A stratified squamous epithelium, the epidermis varies in thickness in different regions of the body. In stained vertical sections its lower border, at the dermal-epidermal junction, presents an undulating appearance due to the epidermal or rete ridges.

Histologically, the epidermis of the vulva is described in four layers.

1. A basal layer, or stratum germinativum, the lower border of which rests on the basal lamina.
2. A spinous or prickle cell layer which forms the bulk of the epidermis.
3. A granular layer.
4. A horny layer or stratum corneum.

There is progressive differentiation of the keratinocytes through the various layers to form the tough, protective, outer surface of the skin. Immediately above the basal layer are the larger polygonal cells of the spinous layer. The cells of this layer are less basophilic than those of the basal layer. As the keratinocytes ascend through the epidermis they become flatter and broader, deeply staining keratohyalin granules appear in their cytoplasm and a granular layer is established. Above the granular layer an abrupt change occurs with the cells becoming anucleate to form the keratin of the horny layer.

1.5.2 Dermis

The dermis is divided into papillary and reticular layers. The papillary dermis projects upwards between the rete ridges and is composed of fine collagen fibres and elastic fibres which support the vascular and lymphatic channels as well as the nerve terminals. The reticular dermis lies below the papillary dermis and is composed of coarse collagen fibres lying parallel with the epidermal surface. The vascular and lymphatic plexuses which drain the papillary dermis lie within the reticular dermis, which also contains the nerve fibres associated with the papillary nerve terminals.

1.6 Clinical presentation of PDV

PDV is an intraepithelial lesion which usually appears in post-menopausal Caucasian women with a mean age of 64 years. Often the presenting symptoms are pruritus and soreness and it is not uncommon for the symptoms to have been present for some time before the patient first presents clinically. The condition appears as erythematous scaly patches with islands of hyperkeratosis, most commonly on the labia majora (Figure 1.1), but it can also involve the perineum and the perianal region. The diagnosis is made by biopsy and there is a distinctive histological appearance with pathognomonic 'Paget' cells. On the basis of histological examination, Wilkinson and Brown (2002) proposed a classification for PDV:

1a. Intraepithelial neoplasia.
1b. Intraepithelial neoplasia and stromal invasion.
1c. Underlying adenocarcinoma of skin appendage or a subcutaneous vulval gland.
2a. Secondary to anal or rectal adenocarcinoma.
2b. Secondary to urothelial neoplasia.
2c. Secondary to adenocarcinoma of other site.

1.7 Histopathology of PDV

Paget cells can be distinguished by their large vesicular nuclei often located centrally (Figure 1.3). Pronounced nuclear atypia and pleomorphism are usually present. Mitotic figures may be frequent. The basophilic or amphophilic finely granular cytoplasm is abundant and vacuolated, and often contains mucin. The cells tend to form nests or aggregates within the epidermis, most commonly in the basal layers. Two types of Paget cells have been documented (Goldblum and Hart, 1997). Classic type (type A) cells are characterised by vesicular nuclei with prominent nucleoli and abundant clear or amphophilic cytoplasm (Figure 1.3i). The signet ring type (type B) is characterised by an eccentrically displaced nucleus due to large cytoplasmic mucin droplets (Figure 1.3ii). Such cells may also be present in the sweat gland ducts, which have led some to suggest that the Paget cells are derived from a local apocrine gland neoplasm (Parker et al., 2000). In the literature very few studies (Goldblum and Hart, 1997; Helwig et al., 1963; Lee et al. 1977) have documented the different types of Paget cells present in their study. In the majority of cases type A Paget cells predominated. There was no correlation between type A and type B Paget cells and PDV with and without invasive disease in these studies.

In the current study, the majority of Paget cells were classic type A. There was no difference in the staining pattern of the antigens between the two subtypes of Paget cells.

The Paget cells contain neutral and acid mucopolysaccharides and carcino-embryonic antigen (CEA) has been identified (Olson et al, 1991). Other markers have also been identified in Paget cells which will be described later in section 1.13.

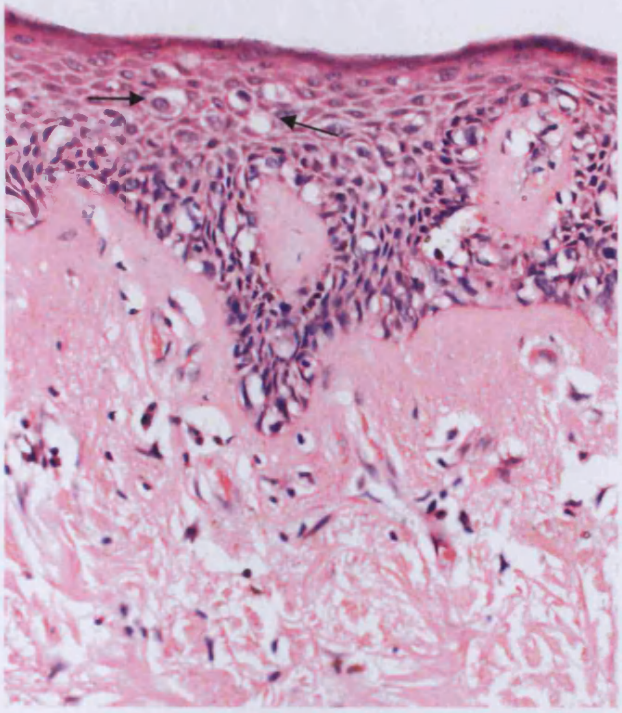

Figure 1.3 H&E staining of PDV (x200).

Arrows point to Paget cells.

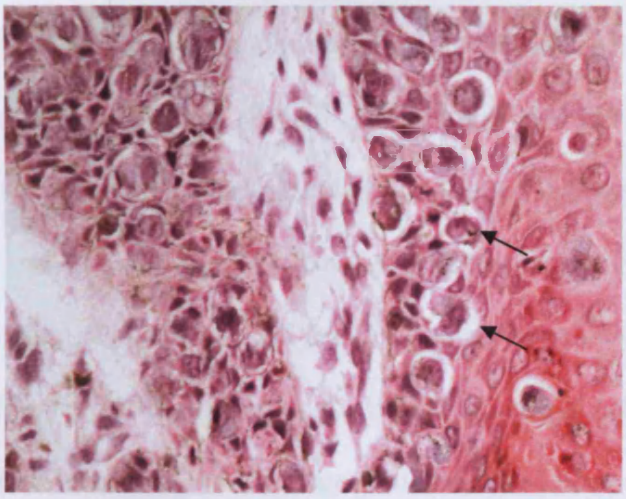

Figure 1.3i Photomicrograph of type A Paget cells (x400).

Arrows point to Paget cells.

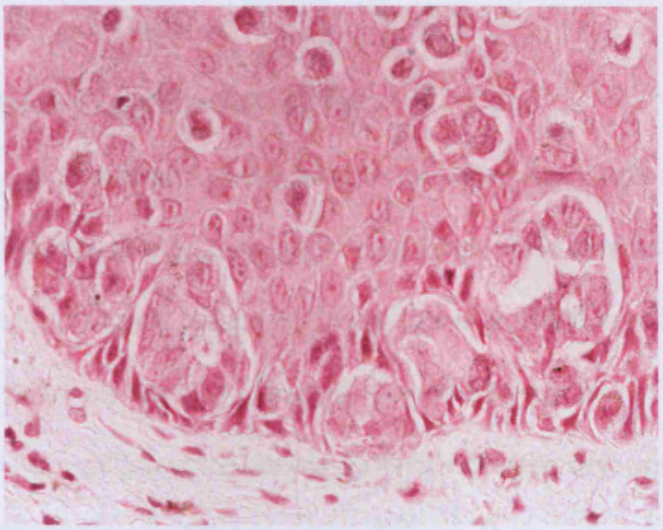

Figure 1.3ii Photomicrograph of type B Paget cells in the basal layer of the epidermis (x400).

1.8 Electron microscopy of Paget cells

There are few ultrastructural studies on PDV (Demopoulous, 1971; Lagios et al., 1984; Ordonez et al., 1984, 1987). Electron microscopy has shown that the large Paget cells contain numerous cytoplasmic organelles, including free ribosomes, smooth and rough endoplasmic reticulum, lysosomes, numerous enlarged mitochondria and Golgi membranes (Sagami, 1963; Sagebiel, 1969). The plasma membrane shows microvilli and desmosomal attachments, but fewer than those seen between keratinocytes. The keratinocyes close to the Paget cells have fewer desmosomes than usual. No desmosomal attachments are seen between Paget cells and melanocytes (Sagami, 1963; Lagios et al., 1984). Abnormal cells with characteristics of both keratinocytes and Paget cells have also been demonstrated (Sagami, 1963; Sagebiel, 1969; Lagios et al., 1984). These apparently intermediate cells have been designated pre-Paget cells (Lagios et al, 1984) and such observations support the concept of an in situ origin of Paget cells.

1.9 Differential histological diagnosis of PDV

'Pagetoid spread', is a term used to describe any condition where cells are distributed singly and in small groups throughout an epithelial layer. This term has been derived from the distinctive histological pattern of the epidermal infiltration of Paget cells. A variety of intraepithelial lesions may assume a 'Pagetoid appearance'. These include superficially spreading malignant melanoma, Bowen's disease, mycosis fungoides, Langerhans cell histiocytosis and Spitz naevus. Toker cells are normal epidermal cells that resemble Paget cells, found in 10% of normal nipples. Diagnostic problems arise, particularly if the biopsy is taken from degenerating areas, when only a few atypical cells are present, and when the cells contain melanin pigment.

Morphologically, these situations raise the possibility of a melanocytic lesion and dysplastic squamous changes. In most cases, with the help of immunostains and careful morphological examination, the correct diagnosis can be made.

1.10 Histogenesis of PDV

Many studies have sought to address the histogenesis of extramammary Paget's disease with various theories having been put forward. A tendency for Paget cells to be located within apocrine gland-bearing areas has led some authors to believe that their origins are from apocrine ducts (Mazoujian et al., 1984; Nagle et al, 1985; Olson et al, 1991). Boehm and Morris (1971) identified them as being of apocrine type on the evidence of their blue staining reaction with colloid iron. The presence of apocrine epithelial antigen (Kariniemi et al., 1984) and gross cystic disease fluid protein (Mazoujian et al., 1984) concurred with this hypothesis. These suggestions do

not explain the presence of Paget cells in areas without apocrine glands and others have found no evidence of apocrine differentiation (Tsukada et al., 1975) but have suggested that the cells are of eccrine sweat gland type (Webb and Beswick, 1983). Some have concluded that Paget cells represent an aberrant differentiation from pluripotential epidermal stem cells (Medennica and Sahihi, 1972; Frederich and Wilkinson, 1982; Guarner et al., 1989; Urabe et al., 1990).

The migration of Paget cells from an underlying carcinoma has been widely accepted as the origin of mammary Paget's disease. This led Koss et al. (1968) to suggest that extramammary Paget's disease was similar and that PDV was always associated with an underlying carcinoma. However, several studies have consistently shown that this is not so in the majority of cases. Boehm and Morris (1972) reviewed 100 cases of PDV reported in the literature and found that only 28 had been described with an underlying carcinoma. Chanda (1985) reviewed details on 197 published cases of extramammary Paget's disease of which 150 were women and 47 were men. Twenty-four percent had an underlying cutaneous or adnexal carcinoma and 29% of the cases were said to be associated with an associated internal malignancy. What is not clear from the review is whether the underlying cutaneous carcinoma was an invasion of Paget cells from the overlying epithelial disease or vice versa i.e an invasion of the epidermis from the underlying tumour, or was a separate carcinoma derived from a different clone of cells, thus unrelated to the intraepithelial Paget's disease. Defegu et al. (1986) found the overall incidence of associated malignancy for extramammary Paget's disease was 15% with vulval cancer constituting 33% of these. One theory that has been put forward is that extramammary Paget's disease arises as a primary intraepidermal neoplasm in most cases, with the Paget cells either

originating from the intraepidermal cells of the apocrine gland ducts or from pluripotent keratinocyte stem cells (Lloyd et al., 2000). Others have suggested that the Paget cells migrate upwards along the ducts from an adnexal carcinoma (Hastrup et al., 1988). Invasive disease can develop from this in situ disease (Goldblum and Hart, 1997) and this may have been a confounding issue in the consideration of the association with an underlying malignancy.

1.11 Management of PDV

Excluding invasion of PDV or a deeper underlying malignancy is the first essential step in the management and treatment of Paget's disease of the vulva. The need to exclude invasive disease warrants excision of the lesion. Previously, radical vulvectomy was the preferred treatment but others have recommended more conservative surgery. A simple vulvectomy at a depth of at least 5mm of subcutaneous tissue has been recommended as the optimal treatment for intraepithelial Paget's disease by Feuer et al. (1990). Bergen et al. (1989) performed skinning vulvectomy with split-thickness skin grafts to treat some of their patients with intraepithelial Paget's disease of the vulva. Others have advocated a wide local excision with sufficient depth of underlying dermis to detect invasion (MacLean, 2000).

Histologically, the extent of the disease is often greater than the clinically apparent lesion with residual disease present after excision. Obtaining frozen sections at the time of surgery has been advocated to ensure adequate surgical clearance (Stacy et al., 1986). Gunn and Gallager (1980) demonstrated that even this may not be

successful owing to the irregularity and multifocal nature of the disease and they noted that local recurrence is frequent unless a wide local excision is performed.

Some have advocated other methods of treatment. Burrows et al. (1995) treated their five patients who had extramammary Paget's disease (two cases involving the vulva) with radiotherapy. Ewing et al., (1991) treated six cases with CO_2 laser and others have used chemotherapy (Yamazaki et al., 1999; Voigt et al., 1992; Watanabe et al.2002). Parker et al. (2000) compared and evaluated the initial treatments of 76 patients in a retrospective study. They found that patients who had a wide local excision had a better prognosis than patients treated with simple vulvectomy, radical vulvectomy, radiotherapy or chemotherapy. Chapter 6 of this thesis will discuss this further.

1.12 Prognosis of PDV

In general the prognosis of PDV depends on whether there is dermal invasion or an underlying adnexal adenocarcinoma present. Similarly, disease location and type of treatment are also important prognostic factors. In his review, Chanda (1985) demonstrated a 46% higher mortality rate in those patients who had PDV with an associated underlying cutaneous adnexal adenocarcinoma compared to those without a carcinoma. In their retrospective study of 76 patients with Paget's disease of the vulva, Parker et al. (2000) also found that patients with Paget's disease and underlying adenocarcinoma (n=13), patients with a coexisting cancer (n=8), and those with invasive Paget's disease (n=9) had a poorer prognosis than those with purely intraepithelial Paget's disease (n=46). They also suggested that the location of the Paget's disease is important for the prognosis; those patients with clitoral

involvement had a worse outcome compared to those with lesions located at other sites in the vulva. Approximately a quarter to over half of all patients with PDV will have recurrence of the disease (Fanning et al., 1998; Zollo et al., 2000).

1.13 Literature review

One of the aims of this study was to investigate the expression of various molecular markers in Paget's disease of the vulva and to identify any differences in the expression of these markers between those with pure intraepithelial Paget's disease and those cases with invasive adenocarcinoma. Such an analysis might identify potential markers of clinical prognosis. The paucity of literature regarding the molecular pathology and the expression of molecular markers in PDV is in part due to the rarity of this disease. Most studies that have been performed have involved small numbers of cases. A summary of various studies performed in the literature is shown in Table 1.1.

Table 1.1 Review of expression of antigen markers in Extramammary Paget's disease

Authors	Study	Results
Kariniemi et. al. (1984)	Expression of CEA and AEA in 7 cases of MPD and 12 cases of EMPD	All cases of EMPD +ve for CEA and AEA
Kariniemi et al. (1985)	Cytokeratin expression in Paget cells	7 cases of EMPD positive for PKK_1 and RGE_{53}
Olso et al. (1991)	Immunohistochemical features of Paget's disease of the vulva with and without adenocarcinoma	No significant difference in the expression of GCDFP-15 ,EMA, CEA, B72.3, S-100 and oestrogen receptor between the two groups.
Diaz de Leon et. al.(2000)	Oestrogen, progesterone and androgen expression in 28 cases of EMPD.	Androgen receptor expression in 15/28 cases of EMPD (23 vulval cases) Oestrogen and progesterone not expressed
Brummer et al. (2004)	Her-2/neu expression in Paget disease of the vulva and the female breast	8 of 10 cases of PDV
Alo et al., (2005)	Fatty acid synthase expression in Paget's disease of the vulva	20 cases. Increased expression with increased aggressiveness

1. 14 Paget's disease of the vulva database

PDV is an uncommon disease and individual clinical experience is therefore limited. In 1997 the British Society for the Study of Vulval Disease (BSSVD) established a national register asking all clinicians who had patients with PDV to enrol them. This register has been publicised via the Royal College of Obstetricians and

Gynaecologists, the British Association of Dermatologists and the British Gynaecological Cancer Society. Members of the Department of Obstetrics and Gynaecology at the Royal Free and University College Medical School (Hampstead Campus) have continued to contact clinicians and collect information on cases throughout the country. Registration and follow-up forms have been devised and sent to clinicians so that information on the management and clinical outcome on cases of PDV can be gained; this will increase our understanding of this rare but important disease. This is discussed further in chapter 6 of this thesis.

1.15 Paget's disease of the breast (PDB)

Described in 1874 by Sir James Paget, less than 4% of all breast carcinomas are associated with PDB (Ashikari et al.,1970; Ascensao et al.,1985; Chaudary et al., 1986; Osther et al., 1990). The lesion is characterised by the presence of large, pale, intraepidermal neoplastic (Paget) cells within the nipple epithelium. As stated earlier, Sir James Paget did not actually describe the histological features or cellular morphology of the lesion. He did not regard the nipple lesion *per se* as neoplastic, but stated that 'a superficial disease induces in the structures beneath it, in the course of many months, such degeneracy as makes them apt to become the seats of cancer' (Paget, 1874). This view was no longer tenable after the first microscopic studies clearly defined the Paget cells as neoplastic. One of the earliest clinical descriptions of PDB has been attributed to John of Ardene (Graham, 1939) who recorded the development of a sore in the nipple of a priest in 1307, within two years the cancerous change extended into the underlying breast and was associated with changes in the overlying skin. In 1841, Velpeau described the dermatitis-like changes of the nipple and areola that preceded cancer of the breast in women.

However, it was Sir James Paget who has been credited with recognition of the lesion as a clinical entity. Jacobaeus (1904) provided a histological description of three cases of Paget's disease and advanced the concept that Paget's disease reflects an epidermotropic extension of an underlying adenocarcinoma into the surface epithelium. His findings were later supported by Muir (1927, 1929) and Ingis (1936).

1.16 The breast

Mammary glands are modified sweat glands with the specialised function of providing nutrients for the newborn infant. Located on the anterior chest wall, they serve as target organs for a variety of hormones (McManus and Welsch, 1984). These hormones have an active or a passive role in the physiology of mammary glands. Hormones that actively influence breast physiology are prolactin, oestrogen and progesterone. Oestrogen promotes the growth and development of the duct system, and progesterone stimulates lobular development. For oestrogen and progesterone to exert their effect, prolactin must be present (Reyniak, 1979). Oestrogen initiates mammary epithelial stem cell division, but prolactin, insulin, and growth hormone are necessary for the process to continue. A variety of other hormones, including human placental lactogen and thyroxine, influence DNA synthesis in the ductal epithelial cells (McManus and Welch, 1984). The various hormones act in concert to promote morphological and functional development of the breast through the establishment of the duct system and production of milk. These dynamic processes of mammogenesis and galactopoiesis commence during fetal life, continue throughout puberty and the reproductive years, and end following menopause.

1.17 Development of the breast

1.17.1 Fetal mammary development

The primitive milk streak (milk line) appears in the human embryo during the 5th week of gestation (Hamilton et al., 1968) as two ventral bands of slightly thickened ectoderm. The pair of milk lines extends from the axilla to the groin. Shortly after its formation, most of the band regresses except for a small segment that remains in the thoracic-pectoral region where it forms the mammary ridge. By the 7th to 8th week of gestation, the mammary anlagen have been established as a thickening of the milk line. This is followed by the development of the nipple. The smooth muscle of the nipple and areola form through differentiation of the mesenchymal cells in the chest wall between the 12th and 16th weeks of gestation. Sex differences become apparent toward the end of the 1st trimester under the influence of oestrogen and testosterone. During the 3rd trimester placental hormones initiate canalisation of the epithelial strips; this process continues from the 20th to the 32nd week of gestation. The development of the mammary lobules between the 32nd and 40th weeks results in an increase in the mammary tissue. Meanwhile, the pigmented nipple-areola complex develops. At birth, the mammary ducts open into a small shallow depression, referred to as the mammary pit. Shortly thereafter, evagination of the mammary pit results in the formation of protuberant nipples (Tavassoli, 1999).

1.17.2 Postnatal stage

A transient mammary hyperplasia is observed in some newborn infants as a result of transplacental transfer of the maternal hormones. A colostrum-like fluid may appear in the nipple of newborn males or females up to a week postpartum. The hyperplastic

breast tissue characterised by ducts distended with secretory material involutes by the 3rd to 4th postpartum week. During childhood, some branching of the primary duct system occurs, but significant changes do not become apparent until puberty. The changes taking place during puberty and adolescence result from a surge of hormonal activity by the pituitary gland and ovaries. The ovarian follicles secrete oestrogenic hormones, which stimulate further growth and branching of the duct system simultaneously with proliferation of the periductal stroma. Along with progesterone, the oestrogen secretion results in the development of the lobular units. The adult breast assumes its final protuberant appearance with pigmented nipple and areola about 3-4 years following the initial surge of the hormonal activity.

1.17.3 Nipple-areola complex

The nipple, rich in sensory nerve endings, is located in the centre of the complex surrounded by the areola and elevated above it over the 4th intercostal space. The initial development of the primitive nipples, which appear as a narrow crest of ectodermal cells, precedes that of the mammary gland (Hughes, 1950). The nipple nodule is composed of bundles of smooth muscle and elastic tissue traversed by lactiferous ducts, which have their orifice at the tip of the nipple. Stratified squamous epithelium covers the nipple and areola. Clear cells may be present in the surface epithelium. These cells lack intracytoplasmic mucin, have normal nuclei, fail to react to CEA antibodies and should not be mistaken for Paget's cells. Toker, (1970) described a glandular type of clear cells in12% of nipples in autopsy cases. These "Toker cells" have abundant clear cytoplasm, large nuclei and easily identifiable nucleoli. Sebaceous and apocrine glands are present in the nipple and areola, but hair follicles are found only in the areola. The areola surface is punctuated by rounded

elevations known as 'tubercles of Montgomery' which comprise the openings of the ducts of large sebaceous glands known as 'glands of Montgomery'.

1.17.4. Structure of the adult duct system

The breast consists of 15-20 segments or lobes (Cowie, 1974). Each lobe is drained by a collecting duct, but the segments are ill-defined and not identified on either gross or microscopic inspection. The lactiferous (collecting) ducts converge on the nipple, with the majority opening at the nipple. Between the ages of 13 and 15 years, the ducts elongate and the lobules are formed by lateral and dichotomous branching (Monaghan, 1990). The different parts of the duct system are given specific designations to help communicate the site of a lesion. Also referred to as the terminal duct-lobular unit, a small portion of the terminal duct together with the acini constitutes the lobule. Lobules are only fully developed and functional during lactation.

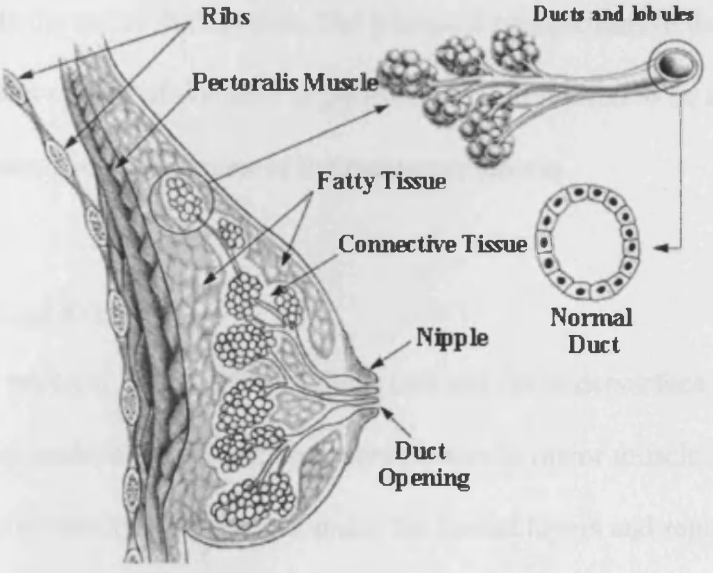

Figure 1.4 Diagram of the breast.

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1.18. Anatomy of the breast

1.18.1 Microscopic anatomy

The duct and lobule system constitutes the functional component of the breast although the fibroadipose tissue surrounding the duct system accounts for the major bulk of the organ. Except for a small segment of the collecting ducts at the nipple orifice where squamous epithelium lines the duct, the entire duct system is lined by two cell layers: the inner epithelial cell layer along the luminal surface and a surrounding interrupted layer of myoepithelial cells. The long axis of the inner epithelial cells is perpendicular to that of the myoepithelial cells. These cell layers are surrounded by a basal lamina. A stem cell population is dispersed throughout the duct system with the highest concentration in the lobules. Proliferation of these cells results in the expansion of the duct system during gestation and lactation and possibly during pathological proliferative and neoplastic processes. The intralobular stroma is loose and more cellular than the interlobular stroma. A thin rim of similarly loose stroma surrounds the entire duct system, but it is most conspicuous in the lobules where the compact cluster of ductules is present. This is believed to be a specialised, more hormone-sensitive component of the mammary stroma.

1.18.2 Anatomic relations

The superficial pectoral fascia envelops the breast and the undersurface of the breast lies over the deep pectoral fascia, which covers pectoralis major muscle. Fibrous bands (Cooper's accessory ligaments) connect the fascial layers and represent the natural means of supporting the breast.

1.18.3 Blood supply of the breast

The internal thoracic (mammary), the posterior intercostals as well as the highest thoracic, lateral thoracic, and pectoral branches of the thoracoacromial (multiple branches of the axillary artery) arteries provide the major blood supply to the breast. The posterior intercostal arteries and several other branches of the axillary artery, including the highest thoracic and pectoral branches of the thoracoacromial, also contribute to the blood supply. The anterior perforating branches of the internal mammary artery supply 60% of the breast (the medial and central parts), whereas the upper outer quadrant is supplied by the lateral thoracic artery. However, variations in the blood supply and patterns of circulation are common.

The three groups of veins that drain the breast are the intercostal veins, the axillary veins and the internal (thoracic) mammary vein perforators. Tumour embolisation through the venous channels plays a major supplementary role in the spread of breast carcinoma to the lungs, brain, liver, bone and other distant tissues.

1.18.4 Lymphatic drainage from the breast

75-97% of lymph from the breast flows to the axillary nodes and 3-25% to the internal mammary nodes (Hultorn et al., 1955; Hultorn et al 1974). A very limited drainage passes to the posterior intercostal lymph nodes located close to the articulation of the ribs and vertebrae. The lymphatic drainage serves as the major route for dissemination of mammary carcinoma. Based on a correlation of the location of carcinomas and the distribution of lymph node metastases, it appears that a preferential flow via certain pathways exists depending on where the primary

carcinoma is located in the breast. In general, tumours located in the upper outer quadrant metastasise mainly to the axillary nodes whereas only those located in the central or medial region of the breast may metastasise to the internal mammary chain. Only tumours in the upper outer and upper central part of the breast show metastases to Rotter's nodes, which are located between the pectoralis major and pectoralis minor muscles, and the axillary nodes.

1.18.5. Nerve supply to the breast

The overlying skin of the breast is supplied by the cutaneous branch of the intercostal nerves T4 -T6. Sympathetic fibres supply the blood vessels and glands, but the control of lactation is hormonal.

1.19. Clinical Presentation of PDB

PDB presents as a well demarcated, slightly indurated erythematous scaling or crusted area on the nipple or areola. It is rare before the 4^{th} decade and is most frequent in the 5^{th} and 6^{th} decades (Colcock et al.,1954; Rissanen et al.,1969; Paone et al.,1981). The early changes may be minimal, perhaps a small, crusted and intermittently moist area on the nipple (Figure 1.5.i) giving a brownish stain on clothing, or producing an itching, pricking or burning sensations. The lesion always appears first on the nipple, subsequently spreading to the areola and seldom involving the surrounding skin. Less often, a serous or blood-stained discharge from the nipple is the presenting symptom. This is often prominent in later the stages, when ulceration and erosion of the nipple tend to occur (Figure 1.5.ii). Nipple retraction may be seen, but this is not characteristic, and is most often caused by adhesions to an underlying tumour. Studies have shown three different clinical patterns at

inspection and palpation: 1) nipple changes only, 2) nipple changes and an underlying palpable tumour in the breast and 3) breast tumour only (subclinical Paget's disease of the nipple) when Paget's disease of the nipple is an incidental histological finding in a mastectomy specimen (Ashikari et al., 1970; Kister et al., 1970; Chaudary et al., 1986). In most series about half the patients with clinical Paget's disease of the nipple had a palpable breast tumour, and in the other half the clinical features were confined to the nipple (Ashikari et al., 1970; Kister et al., 1970; Paone et al., 1981; Ascensao et al., 1985; Johnson et al., 1987). Paget's disease of the breast is generally unilateral, but bilateral lesions have been reported (Anderson, 1979). Paget's disease is seen in both the female and male breast (Archibald, 1922; Crichlow and Czernobilsky, 1969; Fine, 1986; Desai et al., 1996). It has also been reported in ectopic breast tissue (Kao et al., 1986) and in supernumerary nipples (Martin et al., 1994). The observed incidence of subclinical Paget's disease of the nipple depends on the thoroughness of histological examination of mastectomy specimens, and consequently the figures vary greatly in different series. In most studies Paget's disease of the nipple was present in about one-fifth of breasts with palpable tumour (Rissanen et al., 1969; Ashikari et al., 1970; Kister et al., 1970; Ascensao et al., 1985). In such cases the associated breast tumour was not necessarily immediately adjacent to the nipple (Kister et al., 1970; Bussolati et al., 1975). The commonest presentation is Paget's disease associated with a non-invasive intraductal carcinoma. This may be limited to the ducts just beneath the nipple, or it may be more extensive in its distribution within the breast. Cases of PDB associated with ductal carcinoma in-situ (DCIS) alone usually have no palpable tumour within the breast, and the presence of a palpable mass in the context of Paget's disease should raise the suspicion of invasive carcinoma (Ashikari et al., 1970; Jones et al.,

1985; Chaudary et al., 1986). Cases of Paget's disease with no palpable mass account for 50% of all presentations. A palpable tumour is almost always present in the invasive carcinoma component of Paget's disease and, in 1/2 -2/3 of patients at this stage, axillary lymph node metastases are identified at the time of presentation (Ashikari et al., 1970; Ascenao et al., 1985; Chaudary et al., 1986; Eusebio et al., 1992). Among the histological subtypes of intraductal carcinoma, Paget's disease appears to be most commonly associated with the solid/comedo form (Stockdale et al., 1989; Eusebio and Deckers, 1992). This subtype is considered to be the most biologically aggressive, with a higher nuclear grade and greater number of mitoses (Martin et al., 1994).

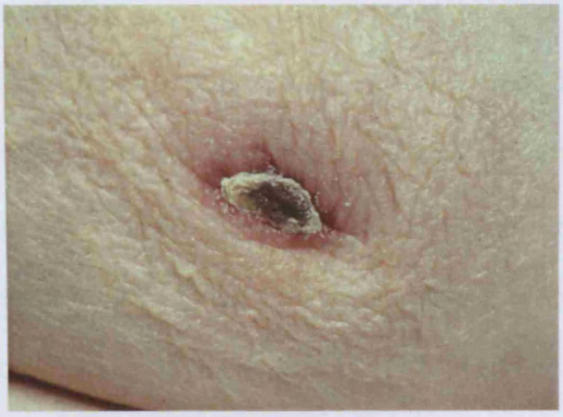

Figure 1.5.i. Early changes in PDB (x100).

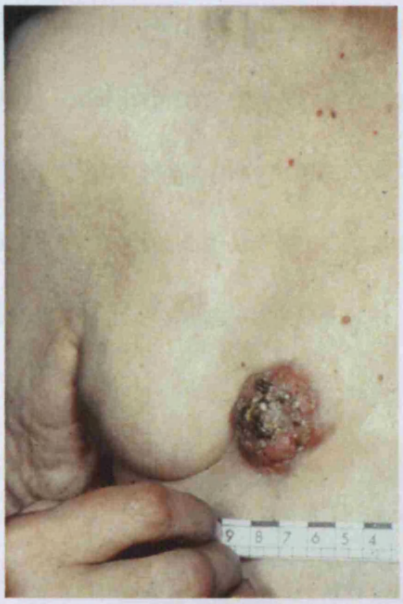

Figure 1.5.ii Ulceration and erosion of the nipple in PDB (x100).

1.20. Histopathology of PDB

The epidermis is thickened with papillomatosis, enlargement of the interpapillary ridges and hyperkeratosis or parakeratosis on the surface. Within the epidermis, the Paget cells are dispersed between the prickle cells (keratinocytes). They vary in number and, when profuse, the Malpighian layers may be disrupted. There is usually a chronic inflammatory reaction in the upper dermis. In the later stages the epidermis may be atrophic or eroded. The Paget cell's cytoplasm is usually periodic acid-Schiff (PAS)-positive and diastase resistant (Crawley, 1957) which indicates the presence of neutral polysaccharides and supports the glandular origin of the cells. They are disposed singly among the keratinocytes or in clusters which, if basal in situation, may mimic a malignant melanoma. The Paget cells may also extend down the ducts of appendages. An underlying breast carcinoma is not always seen on biopsy, as it may be more deeply situated. Careful examination usually reveals an intraductal carcinoma within the lactiferous ducts, usually situated distally from the nipple, but sometimes in the terminal ducts, and often appearing to spread between the two

layers of epithelial and myoepithelial cells of the duct. The carcinoma in-situ cells accumulate within and distend the ducts and spread in both directions. A number of ducts are usually involved. At a later stage the carcinoma becomes invasive and behaves like any other breast carcinoma, e.g. infiltrating ductal carcinoma of the breast. Figure 1.6 demonstrates H&E staining of PDB.

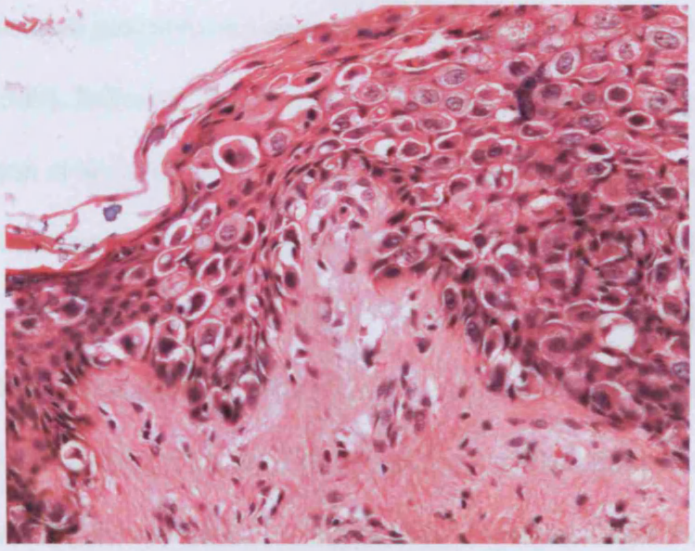

Figure 1.6 H&E stain of PDB (x200).

1.21. Histogenesis

Over the years, a variety of explanations have been offered about the nature of PDB cells. Orr and Paris (1962) suggested that PDB cells represented altered melanocytes. Derived in part from the presence of melanin pigment seen within some Paget cells, this viewpoint has been refuted since the cells fail to show a positive reaction to S-100 protein or the melanoma marker HMB45, but do show a positive reaction to low molecular-weight cytokeratin subclasses. Although the Paget cells in the nipple and the underlying carcinoma cells have been found to express similar markers in many

studies, a few cases have shown discordance, e.g. between the expression of CEA and gross cystic disease fluid protein (GCDFP)-15 at the two sites (Cohen et al., 1993). Furthermore, at the ultrastructural level, Paget cells of the breast show microvilli along the plasma membrane, intracellular and extracellular lumen formation, and desmosomal attachment to adjacent keratinocytes and other Paget cells (Sagebiel et al., 1969; Satiani et al., 1977; Jahn et al.1995), whereas well developed desmosomal junctions are almost never identified in melanocytic cells (Sagebiel et al.,1969). It is now accepted that PDB cells are capable of phagocytosing melanin (Culberson et al.,1956; Peison et al., 1985). Two other theories that have been put forward to account for the origin of PDB are : 1) the epidermotropic theory, which postulates that Paget cells are ductal carcinoma cells that have migrated along the basal membrane of the underlying ducts to the epidermis of the nipple (Lloyd et al., 2000) and 2) the concept of in situ transformation, which regards the Paget cells as malignant keratinocytes appearing in situ and thus considers Paget's disease of the nipple to be an independent in situ carcinoma (Willis, 1960; Paone et al.,1981; Lagios et al., 1984). This transformation theory is favoured for extramammary Paget's disease (Sitakalin et al., 1985) but could also account for the cases of PDB in which no underlying carcinoma can be identified in the mastectomy specimen, despite extensive sampling. The presence of cells with morphological features intermediate between Paget cells and keratinocytes, as well as the occasional "dyskeratotic" appearance of Paget cells as they migrate into the stratum corneum, support this possibility. Sagami (1963) and Sagebiel (1969) independently provided substantial evidence in support of an in situ transformation in the development of PDB. Both demonstrated desmosomal junctions between Paget cells and adjacent keratinocytes. Sagebiel's description of keratinocytes that exhibit a paucity of desmosomes and

separation of tonofibrils by a clear peripheral zone of cytoplasm adjacent to Paget cells may reflect early stages of transformation of keratinocytes to Paget cells at the ultrastructural level. On the other hand, Muir (1927, 1929) presented strong histological evidence for an intraepithelial extension of cancer cells (Paget cells) from an underlying intraductal carcinoma in the upper extremity of a lactiferous duct to the subpapillary epidermis, forming the background for the epidermotropic theory (Ingis, 1946, 1952; Toker, 1961, 1967; Ordonez et al., 1987; Osther et al., 1990). Most immunohistochemical studies which have revealed that the Paget cells express antigens closely resembling cells of mammary origin, have tended to favour the epidermotropic theory, while the theory of in situ transformation has found support mainly in ultrastructural studies. However, it is possible that both theories may be correct and there may be more than one mechanism involved. Further studies are needed to solve this enigma.

1.22. Immunohistochemical investigations of PDB

Immunofluorescence and immunoperoxidase techniques have demonstrated positive staining of PDB cells for casein, CEA, cytokeratin 7, epithelial membrane antigens, milk fat globules and lectins (Bussolati and Pich, 1975; Kariniemi et al., 1984, 1985; Kirkham et al., 1985; Mariani-Constantini et al., 1985; Cohen et al.,1993 (Table 1.2).

These results suggest that the Paget cells express antigens typical of glandular epithelia, which fit in well with the epidermotropic theory. Only a few investigators, however, compared the Paget lesions with the underlying tumour (Tani and Skoog, 1988; Cohen et al., 1993). This relationship is essential as a basis for a discussion of histogenesis. The epidermotropic theory, moreover, takes no account of the

observations of some cases of PDB without an underlying carcinoma (Muir, 1939; Ashikari et al., 1984; Lagios et al., 1984). Although the immunohistochemical data strongly suggest a mammary origin of the Paget cells, a possible alternative explanation of these data, based on the in situ transformation theory, is that the basal epidermal cells of the nipple, in response to unknown stimuli, acquire properties typical of apocrine and mammary ducts. This alternative could explain the findings of casein-containing basally located epidermal cells lacking the characteristic morphology of Paget cells (Bussolati and Pich, 1975). These cells may have been pre-Paget cells. Such a concept was supported by observations on the replication of Paget cells (Pierard-Franchimont and Pierard, 1984), showing proliferation of the cells to be topographically controlled within the epidermis.

Table 1.2 Review of expression of antigen markers in mammary Paget's disease

Authors	Study	Results
Kariniemi et. al. (1984)	Expression of CEA and AEA in 7 cases of MPD	5/7 of MPD +ve for CEA.and 6/7 of MPD +ve for AEA.
Kariniemi et al. (1985)	Cytokeratin expression in Paget cells	Six cases of MPD positive for PKK_I and RGE_{53}
Cohen et al.(1993)	Mammary Paget's disease and associated carcinoma. An immunohistochemical study	18/20+ve for cytokeratin (MAK-6), 6/20 +ve for keratin, 20/20 +ve for epithelial membrane antigens, 9/20+ve for GCDFP-15, 7/20 +ve for CEA, 7/20+ve for S100 protein, 1/20+ve for κ-casein and α-lactalbumin, 1/19+ve for oestrogen receptor, 1/25+ve for progesterone receptor
Brummer et al. (2004)	Her-2/neu expression in Paget disease of the vulva and the female breast	5 of 5 of PDB positive

1.23. Classification of PDB

On the basis of histological examination, clinical PDB may be classified into four stages (Lagios et al., 1984): Stage 0 - PDB confined to the epidermis, without an underlying lesion; Stage 1- PDB associated with DCIS limited to the ducts just beneath the nipple; Stage 2 - PDB accompanied by more extensive DCIS; Stage 3 - PDB accompanied by invasive ductal carcinoma. The existence of stage 0 is still debatable but there are reports of Paget's disease of the breast without an underlying carcinoma (Muir et al., 1939; Ashikari et al., 1970; Haagensen, 1970; my own personal observations described in Chapter 2, Page 84). In stages 1 and 2 there is usually no palpable tumour. These two stages comprise up to 50% of all cases (Colcock and Sommers, 1954; Culberson and Horn, 1956; Maier et al., 1969; Ashikari et al., 1970; Kister and Haagensen, 1970; Page et al., 1982; Ascensao et al., 1985). A palpable tumour is always present in stage 3, and in 1/2- 2/3 of patients with this stage there are axillary lymph node metastases (Maier et al., 1969; Ashikari et al., 1970; Kister and Haagensen, 1970; Paone and Barker, 1981; Ascensao et al.,1985).

1.24. Management of PDB

Until recently, mastectomy had been considered standard therapy for PDB regardless of the presence or absence of a palpable underlying mass (Mendez-Fernandez et al., 1980; Lichter and Lippman, 1981; Anelli et al., 1995). In a small number of cases, efforts have focused on a more limited excision of the nipple-areola complex, with or without radiation, and more limited surgical procedures (Plowman et al., 1986; Peterse et al., 1988; Menzies et al., 1989; Stockdale et al., 1989; Bulens et al., 1990; El-Sharkawi and Waters, 1992; Eusebio and Deckers, 1992; Anelli et al., 1995;

Pierce et al., 1996; Banerjee et al., 1997). The presence of a clinically palpable mass has been used as a discriminating factor in the selection of a surgical approach. For women presenting with a palpable lesion, treatment generally consists of some form of mastectomy (Colcock et al., 1954; Maier et al., 1969; Rissanen and Holsti, 1969). Reported experience with breast-conserving management of Paget's disease of the breast is very limited. The method of wide local excision of the nipple-areola complex followed by axillary node dissection in cases with small or micro invasive carcinoma, with or without radiation therapy, or with radiation therapy alone, has been used in a few patients (Rissanen and Holsti, 1969; Bulens et al., 1990; El-Sharkawi and Waters, 1992; Anelli et al., 1995; Pierce et al., 1996). The subjects for conservative therapy have been women without a palpable mass, those with lesions confined to the nipple without an underlying breast carcinoma, those with only a minimal intraductal carcinoma in the lactiferous ducts and all those reluctant to have mastectomy. It is difficult to verify that an underlying carcinoma is absent without a mastectomy, but if this could be accomplished with clinical and mammographic studies, conservative treatment would be an option for women with limited lesions. The relatively good results that have been obtained in the small number of reported cases of conservative surgery (Lagios et al., 1984; du Toit et al., 1988; Lichter and Lippman, 1988) are encouraging, but follow-up periods in most studies are short and the precise extent of the in situ carcinoma, the size of invasive carcinoma (if any), and the status of the surgical margins are either not well defined or not well documented. Local recurrence rates in PDB have varied from 0%-40% (Paone and Barker, 1981; Bulens et al., 1990; El-Sharkawi and Waters, 1992).

A combination of wide local excision of the lesion within the nipple-areola complex along with radiation therapy may be preferable so as to minimise chances of local recurrence.

1.25. Prognosis of PDB

The prognosis of the disease depends on whether there is an underlying carcinoma, the tumour size, and the status of the axillary lymph nodes. Patients with a palpable mass at presentation have a worse prognosis than those without a mass. Ashikari et al. (1970) reported that patients without a palpable mass and negative axillary nodes had a 5-year survival rate of 97%, which dropped to 95% at 10 years. Axillary lymph node metastases were found in 13% of women without an underlying palpable mass. The overall survival rate for those without an underlying palpable mass was 92% at five years and 87% at 10 years. In contrast, the 5-year survival rate for the 113 patients with a palpable mass was 40% at five years and 38% at 10 years. For patients with a palpable mass but negative nodes, the 5-year survival rate was 73%. This study clearly demonstrates the influence of tumour size and axillary node metastases on prognosis and survival; unfortunately, it is unclear whether the survival rates are actuarial or absolute. Salvadori et al. (1976) provided actuarial survival rates for 41 patients who presented without a palpable mass. The 5-year survival rate for these patients was 92%, dropping to 82% at 10 years with a median survival time of 16.4 years. Of these, 34 had a histological assessment of the lymph nodes and none had evidence of nodal involvement. Of 50 women who had a palpable mass, the survival rate was 38% at five years dropping to 22% at 10 years. Forty-eight of these patients had histological assessment of their lymph nodes and, of these, 42 (84%) had positive nodes. Among men, the 5-year survival rates are consistently worse (Lancer and

Moschella, 1982; Haagensen, 1986) at around 20% (Lancer and Moschella, 1982). This more aggressive behaviour has been attributed to the more advanced stage of the disease at presentation. Understandably, there are no reports of conservative treatment among men.

1.26. Hypothesis

PDB is frequently, though not exclusively, associated with an underlying ductal tumour. It is currently believed that such breast Paget cells are derived from this tumour and that they migrate up through the ducts into the nipple epidermis. In PDV, however, only about 10-30% of cases are associated with an invasive adenocarcinoma which may pre-exist or develop secondarily to the Paget's disease. This disease is thought to occur as a primary intraepidermal neoplasm which may then undergo subsequent steps of malignant progression with invasion. There are currently no markers to suggest which cases will progress to invasive disease.

Paget's disease of the breast and vulva may therefore have a different molecular pathology, which is reflected in their different clinical presentation. In addition, such differences in molecular pathology may reveal specific markers valuable in identifying those cases of PDV which could progress to malignancy.

The hypothesis which will be examined in this thesis will attempt to answer the following questions:

1. Is there a difference in the molecular pathology between PDV and PDB?
2. Are there differences in the molecular pathology when PDV is associated with or without an underlying invasive adenocarcinoma?

3. Are there biological molecular markers that can determine the progression of intraepidermal PDV to invasive PDV?
4. If there are differences in the molecular pathology between intraepidermal and invasive PDV are these reflected in different clinicopathological presentation?

1.27. Aims of the project

Specifically, the following areas of molecular carcinogenesis have been chosen to examine the hypothesis, since abnormalities in such pathways are very important in the malignant progression of several different tumour types, including vulval and breast carcinoma:

- cell proliferation, cell cycle control and oncogene expression
- angiogenic factors
- cell adhesion factors

In addition, a database was constructed (for the first time in the UK), to correlate clinicopathological parameters of PDV with invasion and prognosis, and to consider treatment options including surgical vs. non-surgical.

CHAPTER 2

ONCOPPROTEIN, TUMOUR SUPPRESSOR PROTEIN AND CELL CYCLE PROTEIN EXPRESSION IN PAGET'S DISEASE OF THE VULVA AND THE BREAST

Poster presentation:

Can Ki67 and p53 play a role in the Paget's disease of the breast? **Ellis P.**, Wong Te Fong L.F., Rolfe K.J. MacLean A.B Perrett C.W T Davidson. British Association of Surgical Oncology, Royal College of Surgeons, London, 2000

Oral presentations:

Tumour marker expression in Paget's disease of the vulva. British Society for the Study of vulval diseases. **Ellis P.**, Wong Te Fong L.F., Rolfe K. MacLean A.B., Perrett C.W. Manchester, September 2000

p53 and Ki67 expression in Paget's disease of the vulva with or without invasive disease. **Ellis P.**, Wong Te Fong L.F., Rolfe K.J. MacLean A.B Perrett C.W. British Congress of Obstetrics and Gynaecology, Birmingham, 2001

Abstracts:

Can p53 and Ki67 be useful in the clinical management of Paget's disease? **Ellis P.**, Wong Te Fong L.F., Rolfe K.J. MacLean A.B Perrett C.W T Davidson. European Journal of Surgical Oncology 26, 870 2000

Tumour marker expression in Paget's disease of the vulva. **Ellis P.**, Wong Te Fong L.F.,Rolfe K.MacLean A.B., Perrett C.W Journal of Obstetrics and Gynaecology 20 556, 2000

p53 and Ki67 expression in Paget's disease of the vulva with or without invasive disease. **Ellis P.**, Wong Te Fong L.F., Rolfe K.J. MacLean A.B Perrett C.W Proc. Br. Congress Obstet. Gynaecol. 156

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2.1 INTRODUCTION

Several studies have shown that there is an important association between genetic changes in oncogenes and tumour suppressor genes (TSGs) and the development of most human cancers. Some of the aberrant genes, and their respective proteins, have been suggested to be useful as markers of malignancy. In this chapter the expression of the oncoprotein c-erbB-2(*HER2/Neu*), the tumour suppressor proteins p53 and pRb($p105^{RB1}$,RB1) and the cell cycle- related proteins cyclin D1 (*CCND1/PRAD1/Bcl-1*) and Ki67 will be examined to determine whether they can be used as prognostic markers in PDV and PDB and their role in malignant progression of these diseases.

2.1.1 Cancer Progression

Transformation of a normal cell to the neoplastic state involves the derangement of its genetic programme controlling growth and differentiation. Willis (1952) described cancer progression as "growth of which exceeds, and is uncoordinated, with that of normal tissue and persists in the same excessive manner after cessation of the stimuli which evoke change"

As with other diseases, both genetic and environmental factors are implicated in the aetiology of human cancers. Cancers are heterogeneous conditions. Malignant cells require independence from mitogenic signals, they may no longer require cellular adhesion, or cell type-specific cytokines and may proliferate faster and lose the ability to undergo apoptosis. The process of carcinogenesis is believed to be a stepwise accumulation of genetic abnormalities. The exact nature of the abnormalities for each tumour is not well understood; however, early cytogenetic studies showed that many human solid tumours exhibited a reproducible loss of

genetic material at defined chromosomal loci. These losses are specific for a given tumour type and may differ between tumours. The absence of functional genes at these loci seems to be related to tumour development and, moreover, both alleles were sometimes lost (by deletion or gross chromosomal rearrangement). Hanahan and Weinberg (2000) have described six hallmarks essential for carcinogenesis (Figure 2.1).

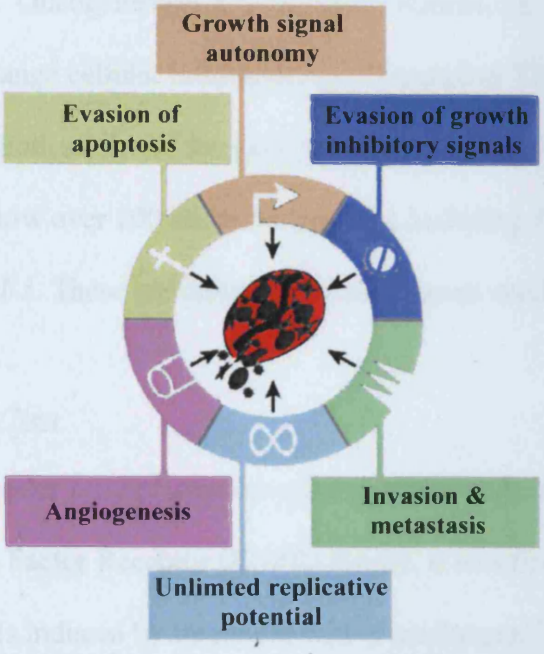

Figure 2.1 The six hallmarks of cancer.

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2.1.2 Oncogenes

Oncogenes, derived from the Greek word "oncos" meaning tumour, are mutated forms of proto-oncogenes, whose functions are to encourage and promote the normal growth and division of cells. Proto-oncogenes encode cellular proteins, e.g. growth

factors, growth factor receptors, intracellular signalling proteins, cell cycle-associated proteins and nuclear transcription factors, all of which relay signals to the cell's nucleus, stimulating growth. When proto- oncogenes change genetically to become carcinogenic oncogenes the result is excessive cell multiplication, as part of the stepwise progression to the development of cancer.

The activation of a proto-oncogene to express its oncogenic potential can occur in several ways: point mutation; chromosomal rearrangement; gene amplification, viral insertion and deletion. Oncogenes act in a dominant manner, i.e. only one mutant allele is required to change cellular behaviour, as compared to TSGs that act in a recessive manner, i.e. both alleles of the gene must be inactivated to change cellular behaviour. There are now over 100 known oncogenes including *HER2/ErbB-2/Neu* and *CCD1/PRAD1/Bcl-1*. These are discussed in subsequent sections.

2.1.2.i. *HER 2/ErbB-2/Neu*

The *HER2* oncogene codes for a plasma membrane protein, c-ErbB-2. It belongs to the Epidermal Growth Factor Receptor (EGFR) family. It was first described in rat neuroglioblastoma cells induced by treatment with a carcinogen (Schechter et al., 1985). *HER2* is 80% homologous to *EGFR* and 80% itself. The gene is located at 17q21- q22 (Fukushige et al., 1986). C-ErbB-2 is widely distributed. This transmembrane protein is a receptor-like tyrosine kinase which is phosphorylated on the intracellular tyrosine kinase domain by a ligand-receptor interaction. This phosphorylation leads to signal transduction by phosphorylating second messengers, inducing RAS-GTP. Studies have shown that over expression of c-ErbB-2 is associated with breast, stomach, ovarian and bladder cancers (Hupp et al., 1995; Wang & Prives 1995; Zauberman et al., 1995). Oncogenic transformation of the protein by truncation/ point mutation leads to phosphorylation and permanent

activation. Kunz et al., (1995) have described remission in 11% of patients with c-ErbB-2 over expressing metastatic breast cancer using a monoclonal anti- *HER2* antibody. Herceptin, a monoclonal antibody that binds the extracellular domain of c-ErbB-2 has been used for the treatment of metastatic breast cancer whose tumours overexpress c-ErbB-2. Herceptin functions through a combination of action including enhanced receptor degradation, inhibition of angiogenesis and recruitment of immune cells, resulting in antibody-dependent cellular cytotoxicity.

2.1.3. Tumour suppressor genes (TSGs)

That cancer may be genetically determined has been recognised for many years (Broca, 1866) and, consistent with this, it has also been clear that some types of cancers cluster in families however, inherited cancers only contribute to ~5% to all cancers. Through observations of the genetic epidemiology of the rare childhood cancer, retinoblastoma, Knudson suggested that a heterozygous child might inherit a (recessive) mutation of one allele of a tumour–predisposing gene at birth (an inherited first genetic "hit"). Such individuals will go on to develop cancer if the mutation is made homozygous by subsequent damage to the remaining intact allele (the second somatic genetic "hit"; Knudson, 1971). Knudson's proposal of a class of cellular genes that regulate cell growth by counteracting the action of growth promoting genes was novel. It was subsequently proposed that these latter genes, which are active at embryogenesis for proliferation ("transforming" oncogenes), are suppressed at differentiation "suppressor" genes (Comings, 1973). Therefore, Knudson named a proposed new class of gene as TSGs. Cancer-causing mutations in TSGs can formally be regarded as being recessive at the molecular level because both copies of the gene must be defective.

TSGs encode proteins that normally function to inhibit cell growth, cell division and to prevent cancer. Examples include the *BRCA1* gene, which is mutated in inherited breast and ovarian cancers, the retinoblastoma gene, *RB1* and *P53/TP53*, which is altered in approximately 50% of solid human sporadic cancers (Hollstein et. al., 1991) and in the Li- Fraumeni Syndrome, which predisposes patients to multiple primary cancers at an early age.

The interaction between oncogenes and TSG's can be clearly demonstrated in the molecular events that are implicated in colorectal cancer progression. (Fearon, 1997) Hereditary colorectal cancer develops by means of defined stages that go from lesions in the crypt of the colon through adenomas to manifest cancer. They are characterised by the accumulation of multiple mutations in tumour suppressor genes and oncogenes that affect the balance between cell proliferation and apoptosis.

2.1.4. The cell cycle

The major task of the cell cycle (Figure 2.2) is to ensure that DNA is replicated once during synthesis (S) phase and that identical chromosomal copies are distributed equally to two daughter cells during mitosis (M) phase (Heichman and Roberts, 1994). There are four main phases: G1, S, G2 and M. S is the DNA synthesis phase and M is the cell division phase (Figure 2.2). These two phases are separated by the gap phases G1 and G2. M phase can be divided into three further phases: prophase, metaphase and anaphase. G1, S, and G2 comprise the interphase. Cells can enter a non-proliferative phase from G1, called GO phase, resting phase or quiescence. Cells enter the cycle and execute DNA synthesis in response to external factors. During the first phase of the cycle, G1, cells are responsive to these mitogenic stimuli and are dependant on them in order to reach a critical restriction point, termed 'R', at the end

of G1 (Pardee,1989). Beyond R, cell cycle transition becomes autonomous. Transition through the cycle is regulated by the activities of cyclin dependent kinases and their inhibitors. A cyclin dependent kinase (CDK) is active as a serine/threonine kinase if it associates with a cyclin protein and becomes activated by it. This then results in the phosphorylation of the target proteins. This cyclin/CDK mechanism provides multiple levels of control. Cyclin proteins are only present during particular phases of the cell cycle. This is the result of the specific induction and elimination of such proteins at certain points. The development of human cancer represents the uncoordinated and unlimited growth of the cell. This is believed to be the result of a step wise accumulation of genetic abnormalities (Fearon, 1997). These genetic abnormalities are partly due to the aberrations of cell cycle regulators (Malkonen 1995)

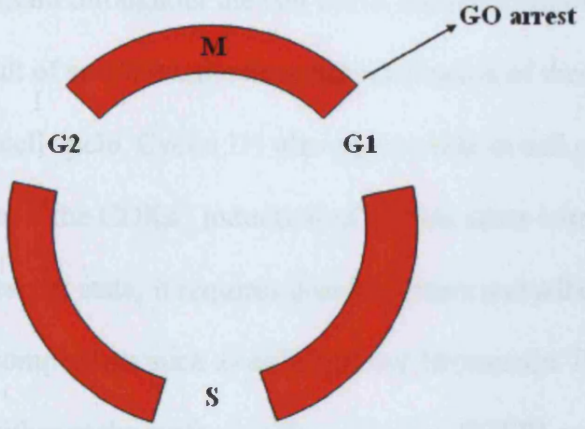

Figure 2.2 Diagram of the cell cycle.

2.1.5. *Cyclin D1*

The cyclins

The *cyclin D1* gene was isolated as the *PRAD1/ CCND1* oncogene located on chromosome 11q13, clonally rearranged and over-expressed in parathyroid adenomas

(Motokura et al, 1991); it is also identical with the *BCL1* oncogene which is translocated and over-expressed in a significant subset of B-cell neoplasms (Rosenberg et al., 1991; Withers et *al.*, 1991). Three different cyclin Ds have been identified (cyclins D1-D3); their distribution is cell type specific, with cyclin D1 being expressed in most epithelial and fibroblast cells and cyclin D3 in lymphoid cells (Sherr, 1995). The association of individual cyclins with their cognate cyclin dependent kinases (CDKs), together with additional regulation of CDKs by phosphorylation and by inhibitor proteins (CKIs), results in a regulated complex that drives the events of the cell cycle (Morgan,1995). The expression of CKIs may be induced by stimuli such as contact inhibition, extracellular anti-mitogenic factors like tumour growth factor β, and cell cycle checkpoints like the p53 DNA damage checkpoints (Saneverino et al., 2003). The cyclins D1-D3 functions as rate limiting controllers of G1 progression through the cell cycle. While levels of the CDKs remain relatively constant throughout the cell cycle, the presence of the cyclins varies; this is the result of specific induction and elimination of these proteins at various stages of the cell cycle. Cyclin D1 also plays a role in cell proliferation through the activation of the CDKs. Induction of cyclins starts when cells enter the cell cycle from a quiescent state; it requires growth factors and adherence of cells to extracellular matrix components such as collagen and fibronectin. Binding of growth factors to receptors, either at the surface of the cells (e.g. EGFR) or in the cytoplasm (in the case of steroids), triggers a cascade of events which links the ligand signal to binding the nucleus, resulting in the activation of transcription factors. Cyclin D1 protein associates with cdk4 or cdk6, and this complex is then positively regulated by cyclin activating kinase and cdc25 and negatively by CKIs. The final target of an activated cyclin D: cdk4 or cdk6 kinase complex is the pRb (Weinberger 1995).

Abnormal cyclin D1 expression has been found in several tumours including breast, vulval and prostate cancers. It has also been found to be amplified in breast cancers (Theillet et. al, 1990).

2.1.6. *RB1*

RB1 is located at 13q14 and codes for the retinoblastoma protein pRb ($p105^{RB1}$)
The main roles of pRb are to act as a signal transducer connecting the cell cycle clock with the transcriptional machinery. pRb exerts most of its effects in the first two-thirds of the G1 phase of the cell cycle. Through the preceding hours of G1, pRb is found in a hypophosphorylated form. The bulk of pRb prepared from cells during the last several hours of G1 is, in contrast, hyperphosphorylated. pRb maintains this hyperphosphorylated configuration throughout the remainder of the cell cycle, losing its multiple phosphate groups only upon emergence from mitosis. Several lines of evidence indicate that this phosphorylation causes the inactivation of the growth inhibitory functions of pRb. Firstly, oncoproteins made by three classes of DNA tumour viruses eliminate pRb function by binding and sequestering hypophosphorylated pRb, ignoring hyperphosphorylated forms. Secondly, the hypophosphorylated form binds and controls a number of other cellular proteins, e.g. E2F transcription factor; the hyperphosphorylated form appears to have lost the ability to interact with these proteins. Thirdly, conditions that cause pRb phosphorylation favour cell proliferation (Cobrinik et al., 1992). Cyclins of the D class (D1, D2 and D3) are most prominently implicated in the phosphorylation of pRb. These cyclins serve as regulators of the Cdk4 and Cdk6 kinases.
pRb binds to the transcription factor E2F to form a complex that blocks transcription in a way that is dependent on the cell cycle. Binding is disrupted by viral

oncoproteins (e.g. adenovirus E1A, simian virus T antigen, HPV16/18/E7) or by phosphorylation of pRb by Cdk4, and Cdk6 with Cyclin D1 complexes and CDK2 and Cyclin E (Weinberg, 1995). Hyperphosphorylated pRb dissociates from E2F allowing activation of S-phase genes. The balance between levels of active pRb and E2F proteins is important for regulation of cell-cycle progression. More than 20 other pRb-binding proteins have been identified, suggesting that pRb might play a variety of roles in the regulation of cell proliferation, in addition to its best understood role with E2F (Taya ,1997).

Loss of pRb immunostaining by deletion or mutations of the *RB1* gene has been demonstrated in a variety of tumours e.g., breast cancers (Sherbet et al., (2003), ovarian cancers (Gras et al., 2001), lymphomas (Rassidakis et al., 2004), and it has also been demonstrated to be an independent predictor of survival in bladder cancer (Agerbaek et al., 2003).

2.1.7. *P53/TP53*

The *P53/TP53* gene located at 17p13.1 codes for the p53 protein and was initially identified through its association with the papovavirus SV40 (Lane and Crawford, 1979; McCormick and Harlow, 1980). p53 is detectable at the plasma membrane during mitosis in normal and malignant cells and is distributed widely throughout the tissues.

Similar to the paradigm of the retinoblastoma gene, (*RB1*) the nature of *P53* was recognised through the phenotype produced by the loss of function. A connection between the p53 protein and cell death, while consistent with the recessive (tumour suppressor) phenotype was directly demonstrated in complementing studies examining the effects of either adding or removing *P53* from the armamentarium of

the cell. Yonish-Rouach et. al.1991; Lowe et al., 1993 published results regarding loss and gain of p53 function, and provided strong evidence that *P53* is a regulator of apoptosis. While the relationship between *P53* and apoptosis had been recognised, numerous other reports demonstrated an important role for *P53* in regulating cell cycle dynamics. The wild type (wt) *P53* gene acts as a TSG negatively regulating cell growth and division. P53, a DNA binding transcription factor, also functions as a checkpoint regulator.

Loss of wt p53 function results in the loss of the G1 checkpoint and hence shortening of the time available for DNA repair before replication. The end result is a demonstrable genomic instability, with chromosomal aberrations accumulating at an increased rate. Such a loss of DNA fidelity is a potential mechanism for the increased plasticity of cancer cells and could thereby endow p53 loss with prognostic significance. Accordingly, p53 has been named the "guardian of the genome". Somatic mutations at the *P53* locus, usually point mutations which inactivate its TSG activity are the most common genetic change in human cancers and occur in 50%, including cancer of the breast, colon, pancreas, and bladder. (Dong. et al, 2003; Khaled et al. 2003; Tachibana et al. 2004).

Several studies have examined whether p53 aberrations may have a prognostic value in human malignancy. Abnormalities in p53 have been observed in as many as 50% of human cancers and can occur through both gene deletions and a variety of gene mutational events (Hollstein et al.1994; Malkin et al., 2001). An abundance of evidence suggests that p53 may play a significant role in both tumourigenesis and in anticancer treatment response. In animals with tumours lacking p53, loss of p53 disrupts an important apoptosis pathway, which results in refractoriness of these tumours to a wide assortment of standard antineoplastic therapies, including radiation

and chemotherapy (Lowe et al, 1993, 1994). Through its disruption of an apoptosis pathway, the loss of p53 function may confer a tumour with far reaching resistance to radiation therapy and a broad spectrum of anticancer drugs (Fisher, 1994). Correspondingly, the ability of reintroduced p53 to restore apoptotic death to p53 deficient cells further confirms p53 as a potentially important regulator of treatment response.

Accumulation of p53 protein can be detected immunohistochemically. The basis of the commonly used immunohistochemical stain is that p53 protein levels are regulated through protein degradation events. Normally, p53 protein is virtually undetectable by antibody-mediated staining. However, mutant (mt) p53 usually resists protein degradation for an increased length of time, leading to the appearance of immunohistochemical (IHC) p53 overexpression. There is a strong correlation between IHC p53 positivity and p53 mutations (mt p53) in some carcinomas (Bodmer et al., 1992; Maestro et al., 1992). However, p53 positivity does not always imply there is a mutation present in the gene as other factors can cause an increase in the half-life of the protein, e.g. a regulatory defect or stabilisation by other proteins such as Mdm-2.

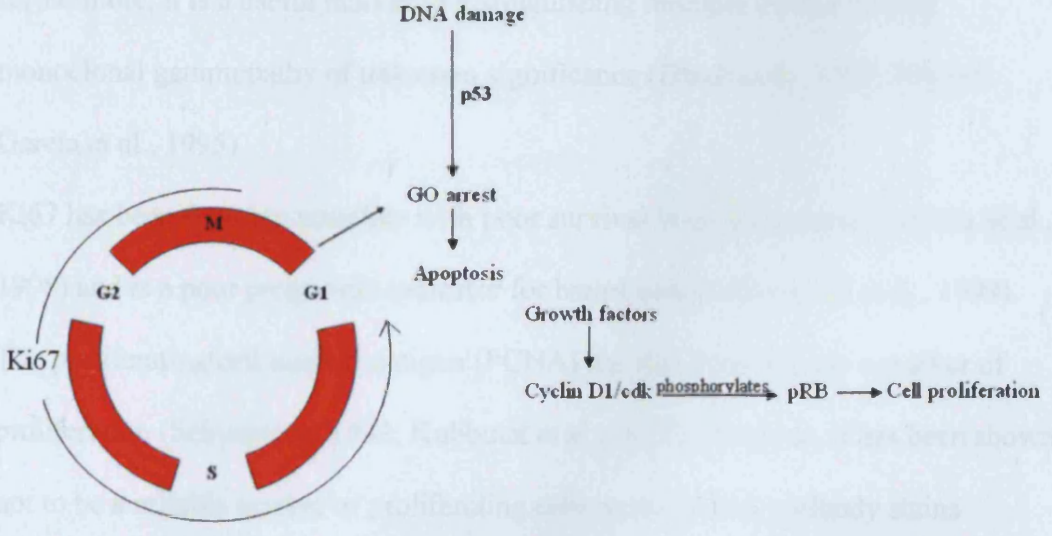

Figure 2.3 Some molecular events of the cell cycle.

2.1.8. Ki67

The gene encoding Ki67 is located at 10q25. Characterisation of the Ki67 antibody revealed that it was reactive with a nuclear structure present exclusively in proliferating cells. A detailed cell cycle analysis revealed that the antigen was present in the nuclei of cells in G1, S and G2 phases of the cell cycle as well as in M. Quiescent or resting cells in the GO phase did not express Ki67 (Gerdes et al., 1984). The cellular distribution of the Ki67 protein is not constant but is subject to dramatic changes during the cell cycle.

Because the Ki67 antigen is present in all proliferating cells (normal and tumour cells), it soon became evident that its presence is an excellent marker to determine the growth fraction of a given cell population (Thomas and Gerdes, 2000). For this reason, antibodies against the Ki67 protein have been increasingly used as diagnostic tools in different types of neoplasms. Ki67 expression has been shown in certain diseases, e.g. Barrett's oesophagus, to indicate increased proliferation in an early stage of neoplastic progression (Barrett et al., 1999). In the case of multiple myeloma it can be shown that Ki67 correlates with the course of the disease and, furthermore, it is a useful marker in distinguishing multiple myeloma from monoclonal gammopathy of unknown significance (Drach et al., 1992; Miguel-Garcia et al., 1995).

Ki67 has been found to correlate with poor survival in ovarian cancer (Anttila et al., 1998) and is a poor prognostic indicator for breast cancer (Giovanni et al., 1999). The proliferating cell nuclear antigen (PCNA) has also been used as a marker of proliferation (Schwarting, 1993; Kubbutat et al., 1994). However, it has been shown not to be a reliable marker of proliferating cells as the PCNA antibody stains

quiescent cells, and PCNA not only has a role in DNA replication but also has a role in DNA repair (Thomas and Gerdes, 2000).

2.1.9. The Immunohistochemistry Technique

The process of staining tissue with antibodies on sections is referred to as immunohistochemistry (IHC); it is an essential adjunct of modern diagnostic pathology. Immunohistochemistry has several advantages in that there is preservation of tissue architecture and cell morphology, and also there is direct visualisation of cell localisation. In the majority of cases, pathological tissue samples are fixed in formaldehyde and embedded in paraffin wax for examination of microscopic morphology. A major limitation of routinely processed tissues for IHC is that many potentially interesting antigens are altered during tissue fixation and processing. As an alternative, sections of snap-frozen tissues can be used to detect most of these antigens. Nevertheless, paraffin wax-sections offer well preserved tissue architecture and cytomorphology superior to that obtained in frozen sections and thus allow more accurate antigen localisation. Also, due to the rarity of PDV and PDB, obtaining tissue for analysis was very difficult and required a multicentre approach involving many hospitals to gain a sufficient numbers of cases. Paraffin wax-embedded samples therefore represented an invaluable resource of human tissues in a number of different hospitals accessible for retrospective studies going back two decades. Thus, for this research, the use of paraffin wax- sections was the preferred choice for IHC analysis.

2.1.9.i. Theory of fixation

Formaldehyde is the most commonly used fixative in histopathology. The commercially available solution contains 35-40% gas by weight; ("Formalin") which reacts with basic amino acids to form methylene bridges. Two buffers were used in this study. At the Royal Free, 10% formol saline was used. 10% neutral buffered formaldehyde was used at the Royal London Hospital.

The use of neutral buffered formalin and formol saline almost completely stops the formation of formalin pigment which occurs with non-buffered acid formaldehyde solutions, and also fixes tissues more rapidly.

2.1.9.ii Antibodies

Antibodies belong to a group of proteins called immunoglobulins (Igs) .They comprise five major classes; immunoglobulin G (IgG), IgA, IgM, IgD and IgE. Each immunoglobulin is composed of two identical heavy (H) chains and two identical light (L) chains. The H chains differ in antigenic and structural properties and determine the class and subclass of the molecule. The classes have been designated gamma (IgG), alpha (IgA), mu (IgM), delta (IgD) and epsilon IgE. Of the five classes, IgG and IgM are the most frequently utilised antibodies in IHC.

2.1.9.iii. Polyclonal antibodies

The antibodies available to detect tissue antigens can be either polyclonal antisera or monoclonal antibodies. Polyclonal antibodies are produced by different cells and, in consequence, are immunochemically dissimilar; they react with various epitopes on the antigen against which they are raised. By far the most frequently used animal

used for the production of polyclonal antibodies is the rabbit. The popularity of rabbits for the production of polyclonal antibodies is attributable primarily to the rabbit's easy maintenance. Rabbits offer other advantages also: human antibodies to rabbit serum proteins are rare, whereas those to ruminants, such as goat, are more common.

2.1.9.iv. Monoclonal antibodies

Monoclonal antibodies are produced from hybridoma clones of plasma cells and myeloma cells. Antibodies from a given clone are immunohistochemically identical and react with a specific epitope on the antigen against which they are raised. Mice are currently used almost exclusively for the production of monoclonal antibodies. There are numerous advantages of monoclonal antibodies in IHC over their polyclonal counterparts; these include high homogeneity, absence of non-specific antibody binding, ease of characterisation and lack of batch-to-batch variability. However, some pitfalls in the use of monoclonal antibodies should also be noted. Firstly, the methods for the screening of useful clones and for quality control must be identical to the methods for which the monoclonal antibodies were raised. Often, monoclonal antibodies are characterised using frozen tissue, for example, when the intended research is on formalin-fixed specimens. Secondly, the targeted epitope must survive fixation. In some cases an antigen may be shown to survive formalin fixation (by use of polyclonal antibodies) but the particular epitope with which the monoclonal antibody interacts does not. Similarly, reactivity of an epitope after optimal fixation does not necessarily assure survival under suboptimal fixation conditions. Thirdly, the targeted epitope must be unique to a given antigen. One of the greatest benefits that comes with the use of monoclonal antibodies, namely

specificity, will be lost if the antibody is directed against an epitope shared by two or more antigens. The cross reactivity of polyclonal antisera can be absorbed out, but the cross-reactivity of a monoclonal antibody cannot.

2.1.9.v. Antigen retrieval for immunohistochemical reactions

Alterations to antigens in paraffin wax-embedded tissues are related to a variety of changes in the three- dimensional structure of proteins resulting from cross-linking by formaldehyde and, to a lesser extent, from heating and dehydration during paraffin wax-embedding. As a consequence, epitopes may be destroyed, denatured or masked, which may diminish or abrogate their detection.

2.1.9.vi Heat-induced epitope retrieval (HIER)

The development of HIER procedures has been the critical breakthrough in paraffin section immunohistochemistry (Shi et al., 1991; Cattoretti et al., 1993; Norton et al., 1994). HIER methods substantially increase the sensitivity of reactions of antibodies directed to paraffin-resistant antigens, especially those located in the nucleus. Moreover, some antibodies that have never before reacted in paraffin sections show specific staining following HIER pre-treatment.

There are several variations of HIER. Many laboratories have attempted to improve the original method by altering the buffer solutions as well as the source and mode of heating. Currently, the most popular HIER technologies use pressure cookers, microwave ovens or autoclaves as the heat source and low-molarity buffers with acid or alkaline pH (Cattoretti et al., 1993; Bankflavi et al.1994; Beckstead et al., 1994; Norton et al., 1994; Miller et al., 1995). The exact mechanism by which HIER works is unknown. It is thought to reverse the masking effects of formaldehyde fixation and

routine tissue processing. Hydrolytic-proteolytic cleavage of formaldehyde-related crosslinks, unfolding of inner epitopes, as well as the extraction of calcium ions from co-ordination complexes with proteins are among the hypothesised mechanisms (Morgan et al., 1994; Taylor et al., 1996; Morgan et al., 1997).

Experimental data suggest that the pH and molarity of the retrieval buffers are among the most important factors for efficacy (Shi et al., 1991; Cattoretti et al., 1993; Morgan et al., 1994; Shi et al., 1995; Taylor et al., 1996; Morgan et al., 1997). Citrate buffer (sodium citrate-citric acid) at pH 6.0 is a very popular retrieval medium and has been used at molarities between 0.01 and 0.1 M (Shi et al., 1991; Cattoretti et al., 1993). Successful protocols can be established by varying key conditions in the antigen retrieval process. The use of different temperatures can markedly enhance the retrieval of the antigen. The process is optimised by trial and error of different temperature regimes.

2.1.9.vii Protease digestion

Protease induced epitope retrieval (PIER) was introduced by Huang (1975) and it was the first method to counteract the antigen masking effects of formalin fixation. It was the most commonly used antigen retrieval method before the advent of heat based methods. A wide variety of enzymes has been used for this purpose including proteinase K, trypsin, DNAse and pepsin. The beneficial effects of the PIER mechanism is related to cleavage of the molecular cross-linking by the proteolytic enzyme, allowing the epitope to return to its normal configuration , enabling more effective binding of the antibody. However, the cleavage is non-specific and some antigens might be negatively affected by this treatment (Battifora et al., 1986).

2.1.9.viii. Antigen detection

The use of enzymes linked to Ig to identify specific substances emerged with the work by Nakane and Pierce, who labelled an Ig with the peroxidase enzyme rather than with a fluorescent compound (Nakane and Pierce, 1966). The labelled antibody binds the antigen and the attached enzyme catalyses the oxidative reaction. This direct-labelled technique was the forerunner of numerous other methods that bring enzymes and antibodies together to allow the enzyme action to identify the location of the antigen through the antibody intermediary. Along with the direct-labelled methods, the indirect-labelled methods were developed, providing amplification and universality. By using a labelled secondary antibody, any number of primary antibodies can be used (to which the secondary antibody binds; Farr et al., 1981). This allows great freedom in selecting antigens to study and antibodies with which to study them. All that is needed is secondary "anti-antibody" reagent with an enzyme attached. The important aspect of this technique is the increased amplification of the signal obtained.

2.1.9.ix Enzymes used for antigen detection

Two enzymes frequently used for antigen detection are alkaline phosphatase and horseradish peroxidase. The peroxidase enzyme has been used over many years in IHC assays. Peroxidase has an oxidative function when used in conjunction with a source of oxygen. The peroxidase enzyme found in the horseradish plant has been widely used since it is easily obtained and it is antigenically different from all mammalian forms of the enzyme. The oxidative function of this enzyme allows for the use of chromogens which, when oxidised, not only change colour but precipitate in such a manner as to render permanent detection.

In the experiments described in this work horseradish peroxidase was used as part of the streptavidin-biotin complex method.

2.1.9.x The streptavidin-biotin complex (SBC) method

The streptavidin-biotin complex method (Figure 2.4) involves, at the core, the vitamin biotin and the protein streptavidin, which bind together irreversibly (10^{-19} M dissociation constant). By establishing a biotin link, through streptavidin, between the horseradish peroxidase enzyme and a secondary antibody reagent, enzyme localisation can be achieved at the site of primary antibody interaction with the specimen. The streptavidin and the biotinylated peroxidase are mixed together at the appropriate concentrations at RT for 30 min. to form the streptavidin-biotin complex. Originally the the glycoprotein avidin was used to conjugate with biotin, however, avidin may bind non-specifically to negatively charged structures such as the nucleus, and secondly, as it is a glycoprotein, it can react with molecules such as lectins. Because of these disadvantages avidin was replaced with streptavidin.

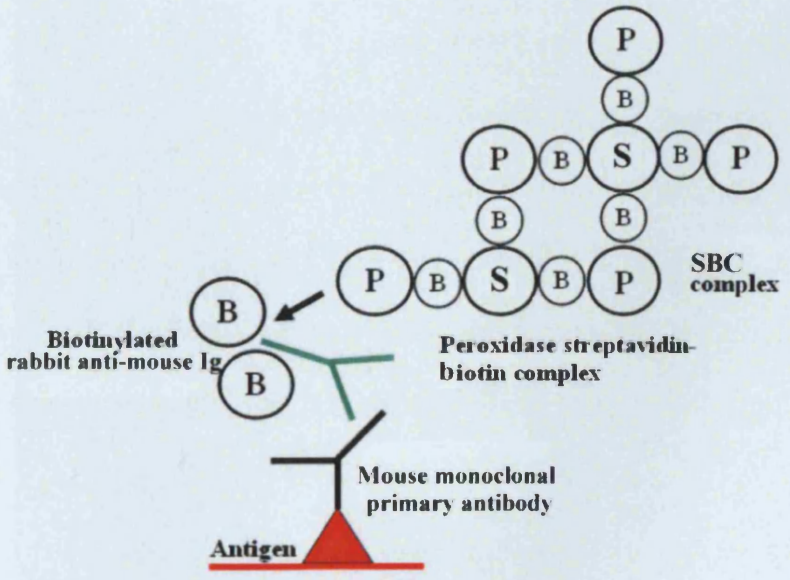

Figure 2.4 Streptavidin-biotin complex immunolabelling method.

2.1.9.xi Chromogenic substrates

In addition to many enzyme systems available there are, with each, a series of chromogenic substrate solutions that can be used to create different colours to identify the location of the antigen. For the peroxidase system, there are numerous oxidisable compounds that precipitate as a permanent colour. The most common, and the one which was used in this work, was 3, 3-diaminobenzidine tetrahydrochloride (DAB). This compound precipitates to a brown colour when in solution with peroxidase and hydrogen peroxide. The signal can be intensified with the use of imidazole. This material is also insoluble in alcohol and xylene, and therefore the tissue may be routinely dehydrated and cleared without loss of chromogen

2.2. METHODS

2.2.1. Sample collection and preparation

LREC approval was obtained for the use of archival vulval and breast tissue.

Tissue specimens

Seventy-two cases of PDV and 38 cases of PDB were retrieved from the Histopathology Department at the Royal Free Hospital and from collaborators as listed in the Acknowledgements. The archival cases had all been formalin fixed and paraffin wax- embedded (see Appendix I). These cases were diagnosed and treated between 1984 and 2000. Ten cases of PDV were associated with invasive adenocarcinoma. Of the 38 cases of PDB, six had an underlying invasive carcinoma; 14 had DCIS alone and six had both DCIS and invasive carcinoma. Twelve cases of PDB had no DCIS or underlying carcinoma despite exhaustive sampling by the referring hospital pathologist, and review by Dr Julie Crow (JCC), Consultant Histopathologist at the Royal Free Hospital. Eleven cases of underlying breast ductal carcinoma were also available for the study. A pathologist from each referring histopathology department originally diagnosed all cases. JCC reviewed all of the haematoxylin and eosin (H&E) stained slides of the cases to confirm the original diagnosis. The number of PDV and PDB cases available for IHC for some markers were fewer than 72 and 38 respectively due to the limited amount of archival tissue. Wax-embedded sections of Paget's tissue, 5μm thick, were cut using a microtome and mounted on 3-aminopropyltriethoxysilane (APES; see Appendix I)-coated glass slides to aid adherence. Slides were stored at 4^0C if there were not used immediately

as there has been reports of loss of antigen when left at room temperature (Prioleau and Schnitt, 1995; Jacobs et al., 1996).

2.2.2. Optimisation of the immunohistochemical protocol

Immunohistochemistry was optimised to ensure reduction of non-specific background staining, using suitable positive controls for each respective antibody. Optimising of antibody dilutions was performed for all primary antibodies, starting with the recommended manufacturer's dilution and then using a range either side. Incubation time was also optimised for all antibodies, along with incubation temperature, allowing for specific staining with the lowest concentration of antibody but still providing intense specific staining. Various antigen retrieval steps e.g none, microwaving, pressure cooking and protease digestion were also optimised using various concentrations and incubation times.

A known positive control was used in each staining procedure to ensure optimal quality of reagents and methods, and to ensure that the staining was successful. The same control specimen was used for each run for a given antigen to assess the intensity of the stain. Negative controls (same specimen as positive control) were also included in each staining run. In the negative controls the primary antibody was omitted and replaced by phosphate buffered saline (PBS) in one reaction, and the secondary antibody replaced by PBS in another.

2.2.3. Immunohistochemical staining of PDV and PDB sections with p53, Ki67, Cyclin D1, pRb and c-erBb2 antibodies

Immunohistochemical staining was performed using the streptavidin-biotin preroxidase method. Tissue sections were deparaffinised in xylene (3x5 min) and then rehydrated in different percentages of ethanol up to distilled water (3x5 min).

3% hydrogen peroxide (10min) was placed on the sections to block endogeneous peroxidase. They were then washed in Tris-buffered saline (TBS) or PBS for 2x5 min. Optimum antigen retrieval was performed by microwaving the slides in warmed 10mM citrate (see Appendix I) buffer for 10 min and leaving to cool to room temperature for 30 min. The slides were then washed in TBS (Ki67) or PBS (2x5 minutes). Non- specific binding was blocked by incubating the slides with either normal rabbit serum (NRS), normal goat serum (NGS) or normal horse serum (Table 2.2). The sections were then incubated with the primary antibody. Washes were with either TBS or PBS depending on the antibody. All incubations took place in a humidity chamber. Table 2.1 describes the clone, working dilution, method of antigen retrieval, incubation time and positive controls used. This was followed by the incubation with the secondary antibody. Incubation times were 45 min. Table 2.2 describes method of non specific binding and secondary antibodies used for each antigen. Following incubation with their respective primary and secondary antibodies, all sections were then incubated with streptavidin-biotin-horseradish peroxidase complex (Dako), diluted 1:200 in TBS, for 30 min.

Antibody binding was visualised with a solution containing the chromogen DAB for 8-12min and then terminated with tap water. The sections were then counterstained with Mayer's haematoxylin. Finally, the slides were dehydrated in methanol, cleared in xylene to allow the refractive index to be 1. They were then mounted in Depex and a coverslip added. They were then viewed under a light microscope at various magnifications. For each case of PDV and PDB there was a separate H &E stain. When assessing the immunostains of each antigen, the H& E section was viewed at the same time to identify the Paget cells. The results were analysed by four

independent observers [JCC, myself (PEE), Dr Kerstin Rolfe (KJR) and Ann ChristineWong Te Fong (ACWTF)].

Sections were scored positive for p53, Cyclin D1 and c-erBb2 if >10% of the Paget cell nuclei were stained brown. This value was chosen to indicate a firm cut-off point in the antigens immunohistochemical staining above potential background levels. Furthermore, such p53-positive levels enabled mutational analysis to be performed on dissected specimens (Chapter 3). The full scoring system is indicated in Tables 2.3 and 2.4. Staining intensity was also noted for all markers. Ki67 staining was expressed as a percentage of positive Paget cell nuclei to the nearest 5% (Ki67 staining index). The total staining index for all samples in each clinical group was then divided by the number of samples. The result obtained was the 'mean Ki67 staining index' for each group.

When performing double marker expression, two markers were assessed on two sections of the same case, each stained with a separate antibody, and not using two different stains on the same section.

Due to the limited amount of specimens, sections were stained on two separate occasions. They were also scored three times by different individuals to ensure reproducibility. There was <5% variation between sections and observers.

Due to the lack of archival material of PDV only cases of PDB were examined for the presence of the c-erbB-2 antigen.

2.2.4 Statistical analysis

The Fisher's exact test was applied for p53, pRb, and cyclin D1 and c-erbB-2 analysis (since samples were either positive or negative for these proteins). It was also used to assess double marker expression in PDV. The Wilcoxon Rank-Sum test

applied for Ki67 analysis as it was a continuous variable. A p value <0.05 was considered significant.

Table 2.1 Antibodies used for IHC

Antigen	Source	Clone	Working dilution*	Antigen retrieval	Incubation time and temp.	Positive control
P53	Dako M7001	DO7	1:100	Microwave	1h R.T	Ovarian carcinoma
Ki67	Immunotech. IM0505	MIB-1	1:25	Microwave	1½h R.T	Tonsils
Cyclin D1	Novocastra NCL-CYCLIN D1	DCS-6	1:20	Microwave	Overnight R.T	Breast carcinoma
PRb	Novocastra NCL-RB	1F8	1:20	Microwave	Overnight R.T	Breast carcinoma
c-erbB-2	Dako	Polyclonal	1:100	Microwave	1½h R.T	Breast carcinoma

* In PBS-BSA, :0.1%

Table 2.2 Non specific binding and secondary antibody used

Primary antibody	Solution used to block non-specific binding	Secondary antibody
P53	NRS 1:10 PBS-BSA 10min	Biotinylated rabbit anti-mouse serum 1:200 in 0.1% PBS-BSA
Ki67	NGS 1: 10 PBS 10min	Biotinylated Goat anti-rabbit/mouse 1:200 in 0.1% PBS-BSA
Cyclin D1	NRS 1:5 PBS-BSA 10min	Biotinylated rabbit anti-mouse 1:400 in 10% NHS
PRb	NGS 1:100 PBS-BSA 10min	Biotinylated goat anti-rabbit mouse 1:100 in10% NHS
c-erbB-2	Normal horse serum 1:100 TBS 20min	Biotinylated horse anti-rabbit/mouse in 10% NHS

NHS –normal human serum

Table 2.3 Scoring of primary antibodies used in IHC. Staining was nuclear and counted as positive.

Antigen	% Paget Cells staining	Score
Cyclin D1, p53	≤10% ≥10%	Normal Abnormal
PRb	< 1% ≥ 1%	Abnormal Normal
Ki67	Staining scored to the nearest 5% (Ki67 staining index) and the mean taken for each clinical group	
c-erbB-2	≥10% of Paget cells expressing c-erbB-2 and membranous staining of the Paget cells was considered to be positive.	

Table 2.4 Scoring system for p53, cyclin D1 and c-erbB-2

% of Paget cell nuclei stained	Score
≤ 10%	0
11-24%	1
25-49%	2
50-74%	3
≥ 75%	4

2.3 RESULTS

2.3.1. p53 and Ki67 expression in PDV and PDB

p53

Fifteen cases (29%) of PDV expressed p53 with scores ranging from 1-4, as identified by Table 2.4. Four of these cases were associated with an invasive adenocarcinoma of which three had a score of 2 and the remaining one had a score of

4. In PDB, five cases (13%) were positive for p53 with a score range of 1-4. Of the five cases two had an associated DCIS with scores of 1 and 2; two cases which had both DCIS and invasive carcinoma had scores of 3 and 4, and the remaining case which had no underlying DCIS or invasive carcinoma, had a score of 3. In all cases, the intensity of the staining was strong. These results are summarised in Table 2.5. There was no significant difference between those cases of PDV with or without invasive disease or underlying carcinoma. Similarly, there was no significant difference in the expression of p53 between cases of PDB associated with DCIS, invasive carcinoma, both DCIS and invasive carcinoma and those without. Figures 2.5 and 2.7 demonstrate p53 expression in PDV and PDB. In both cases the intensity of the staining was strong.

Ki67

The mean Ki67 staining index for PDV was 17% and for PDB it was 11%. The mean Ki67 staining index for the vulval cases without invasion was 16% and for those with invasion it was 19% (Table 2.5). The four cases with invasion that were positive had proliferation rates of 5, 5, 50 and 75% respectively and for those cases without invasion, the proliferation rates were between 5 and 40%.There was no statistical difference in the Ki67 staining index between PDV cases with and without invasive disease in PDV. Similiarly, there was no difference between cases of PDB with DCIS, with invasive carcinoma, both DCIS and invasive carcinoma and without. Figures 2.6 and 2.8 demonstrate Ki67 expression in PDV and PDB respecctively. In both cases the intensity of the staining was strong.

The expression of both p53 and Ki67 was examined in PDV to assess whether there was any correlation between the markers (Tables 2.6). There appeared to be no correlation in the expression of p53 and Ki67.

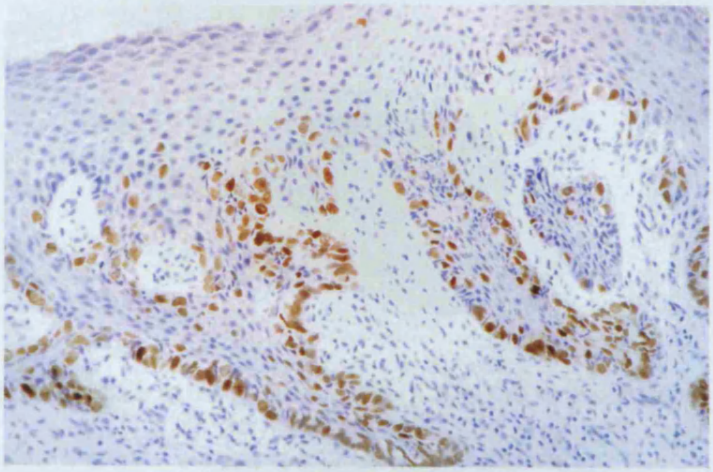

Figure 2.5 p53 expression in PDV (x 40) with a score of 4.

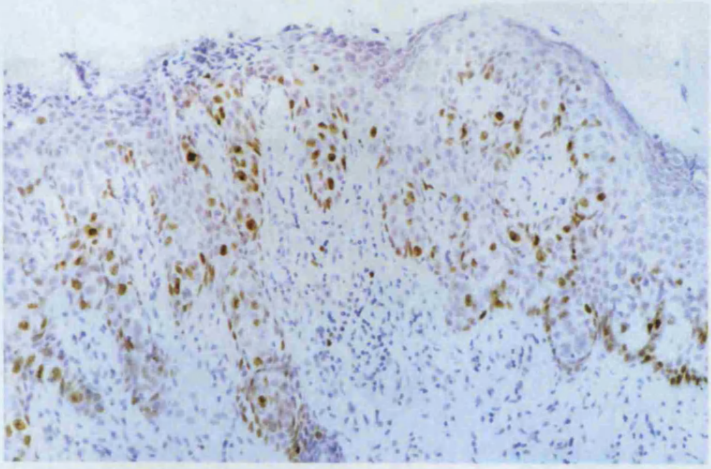

Figure 2.6 Ki67 expression in PDV (x 40) with a Ki67 staining index of 40%.

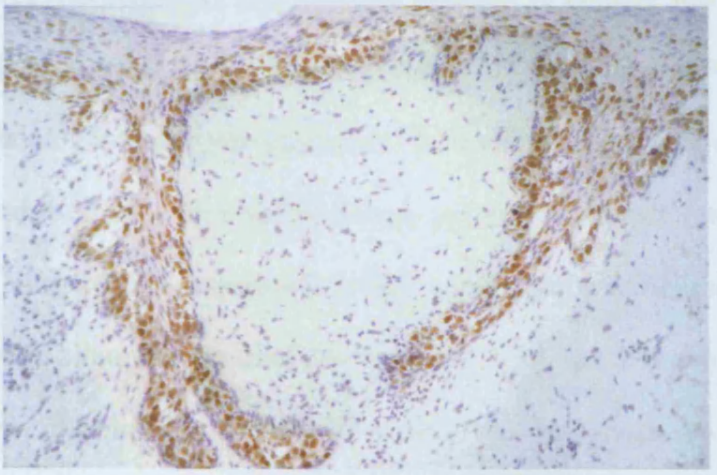

Figure 2.7 p53 expression in PDB (x 40) with a score of 4.

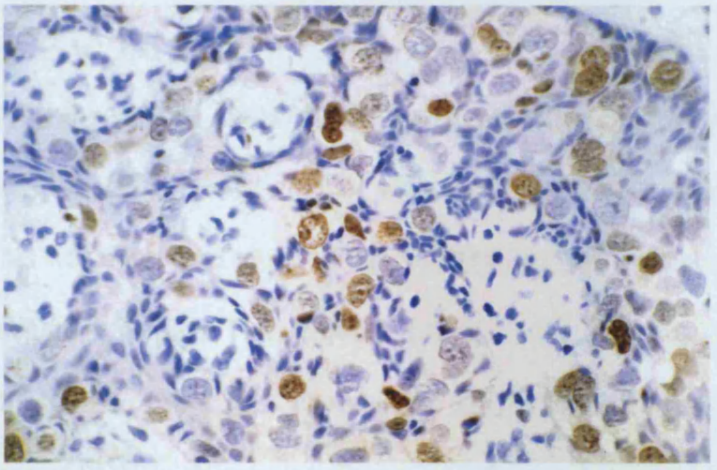

Figure 2.8 Ki67 expression in PDB (x100) with a Ki67 staining index of 40%.

2.3.2. Cyclin D1 and pRb expression in PDV and PDB

Cyclin D1

Forty-one cases (59%) of PDV stained positive for cyclin D1. Thirty-six of these (61%) of PDV without invasive disease expressed this protein. The range of score was between 1 and 4. The intensity of staining was mild to moderate. This was compared to 5 /10 (50%) cases of invasive PDV which were positive for cyclin D1.

The range of scores was between 1 and 3 and the intensity of staining was also mild to moderate. Figures 2.9 and 2.10 demonstrate cyclin D1 expression in PDV with a score of 3. The staining intensity was moderate. There was no significant difference between those cases of PDV with invasive disease and those cases without.
In PDB, three of 34 cases (8%) expressed cyclin D1 with a score of between 1 and 4. Two cases had DCIS alone and the third case had both DCIS and invasive disease. Again, the intensity of staining was also mild to moderate. The results are summarised in Table 2.5. Figure 2.11 demonstrate Cyclin D1 expression in PDB.

pRb

Thirty-two of seventy-two (44%) cases of PDV had abnormal expression of pRb. Twenty-five of sixty two (40%) cases of PDV without invasive disease were negative for the protein. This was compared to seven cases (70%) of PDV with invasive disease that was negative for pRb. The expression of both pRb and cyclin D1 was assessed in each PDV case (Table 2.2). There was a significant difference $p= 0.048$ between those cases of PDV with invasive disease and those cases without invasive disease expressing both proteins (Table 2.6). Twenty four of thirty-six PDB cases (67%) did not express the protein pRb. These included nine cases of DCIS, six cases with invasive disease and one case with DCIS and invasive disease. The intensity of staining in all cases was mild to moderate. In most cases epithelial cells adjacent to the stained Paget cells were also positive for the pRb. The results are summarised in Table 2.5. Figures 2.12 and 2.13 demonstrate pRb expression in PDV and PDB.

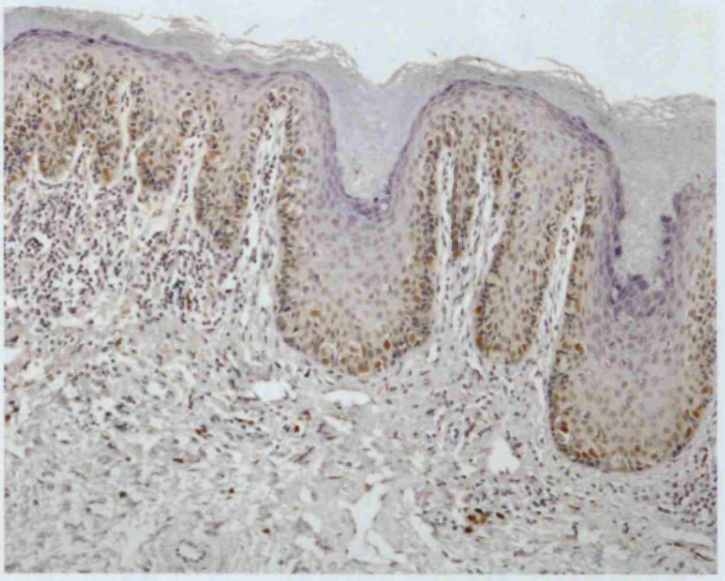

Figure 2.9. Cyclin D1 expression in PDV (x 50) with a score of 4.

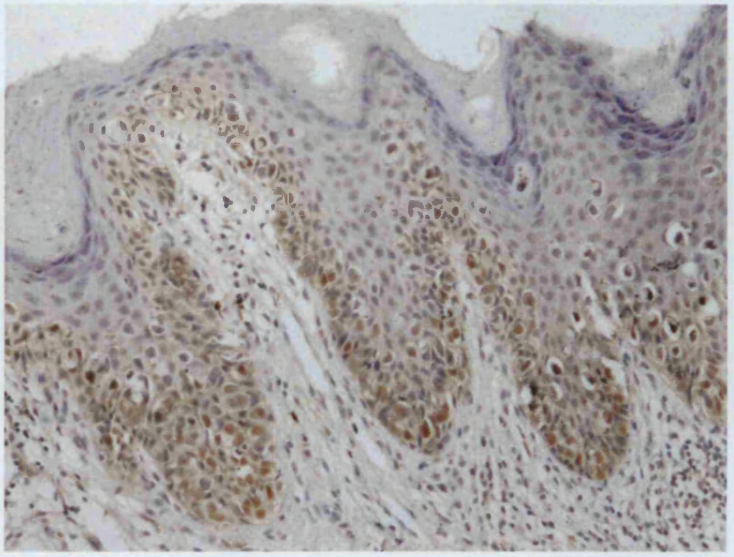

Figure 2.10 Cyclin D1 expression in PDV (x100) with a score of 4.

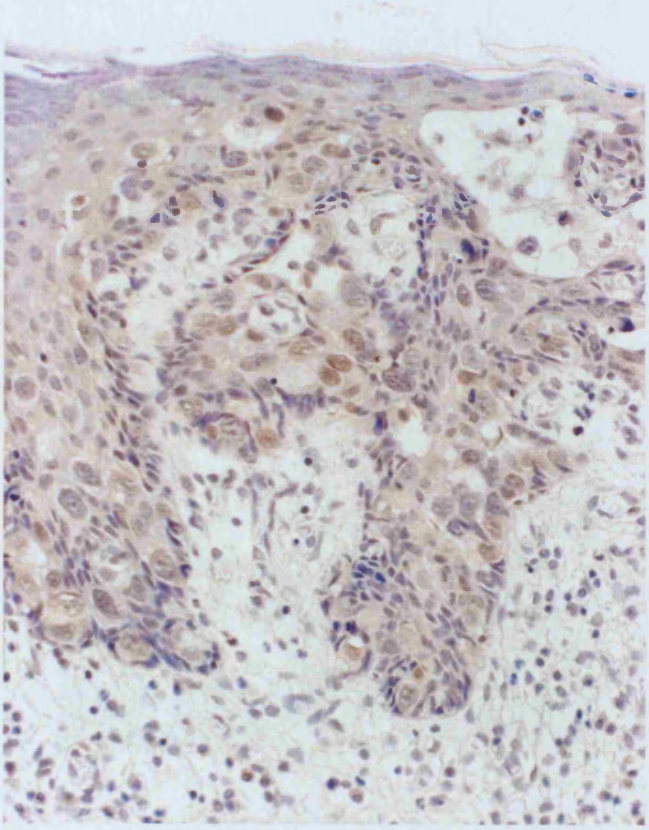

Figure 2.11 Cyclin D1 expression in PDB (x 200) with a score of 2.

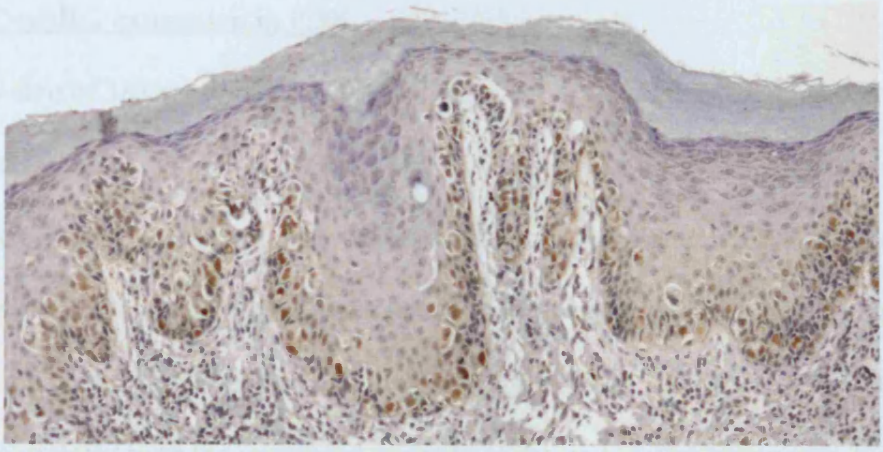

Figure 2.12 pRb expression in PDV(x100).

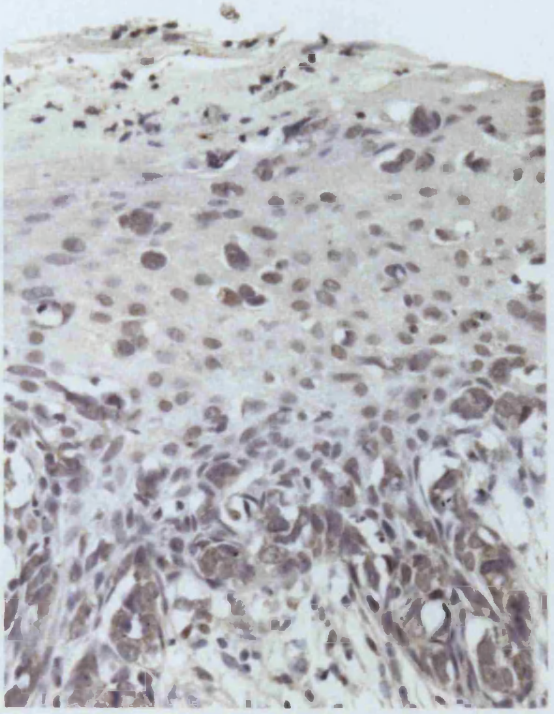

Figure 2.13 pRb expression in PDB (x 200) with a score of 3.

2.3.3. C-erbB-2 expression in PDB

Twenty-five of 38 cases (66%) of PDB were positive for c-erbB-2 expression with a score between 2-4. The 25 cases included nine DCIS, two cases with DCIS and invasive carcinoma and two cases of invasive carcinoma alone. In all cases the staining was in the plasma membrane and the intensity was strong. In 11 cases the underlying ductal carcinoma was available for assessment. In all cases the PDB results correlated with the epidermal sections of the underlying carcinoma. Similar scores were obtained. Figures 2.14 and 2.15 demonstrate c-erbB-2 expression in PDB. The expression of c-erbB-2 in combination with other markers was also assessed in each PDB case (Table 2.7). No correlation was found between the different proteins.

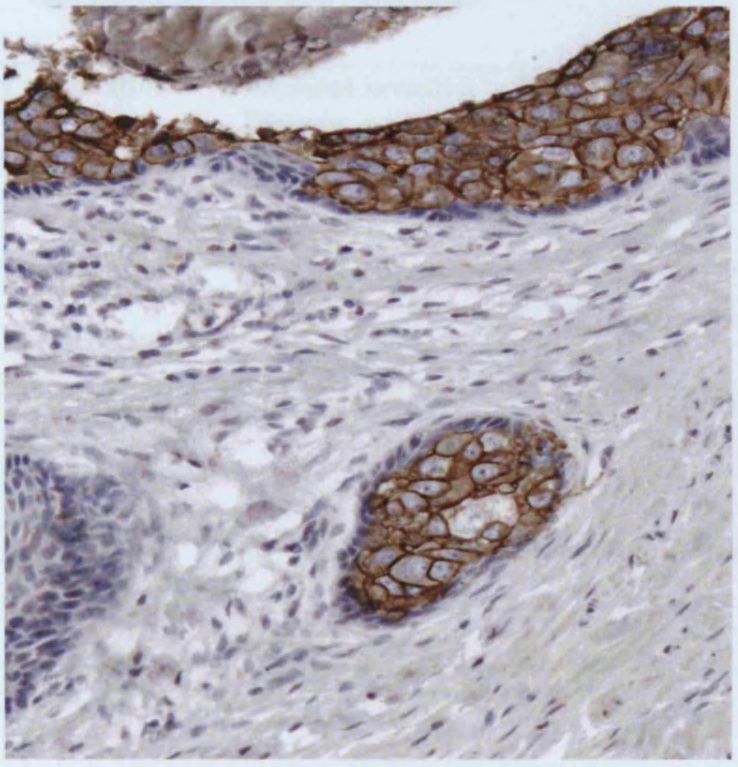

Figure 2.14 C-erbB-2 expression in PDB(x 200) with a score of 4.

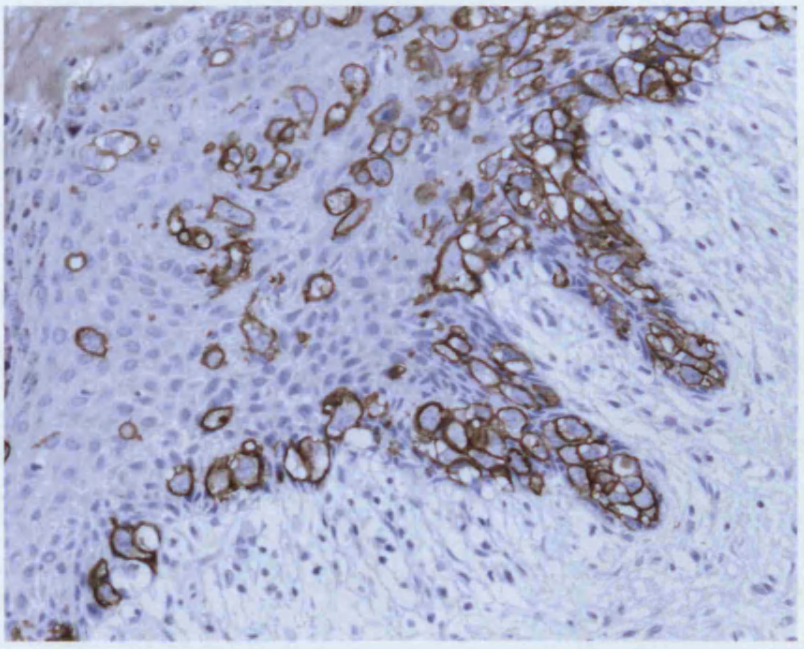

Figure 2.15. C-erbB-2 expression in PDB(x100) with a score of 4.

Table 2.5 Immunoreactivity for p53, Ki67, cyclin D1, pRb and c-erbB-2 expression in PDV and PDB cases

Antibody	Location	Without invasive Disease	With invasive disease	All cases
p53	Vulva	26% (11/42)	40% (4/10) p=0.62	29% (15/52)
	Breast			13% (5/37)
Ki67	Vulva	*16±22%	*19±28%	17 ±23%
	Breast			11±19%
Cyclin D1	Vulva	61% (36/59)	50%(5/10) p=0.75	59% (41/69)
	Breast			8% (3/34)
pRb	Vulva	40% (25/62)	70% (7/10) p=0.16	44% (32/72)
	Breast			67% (24/36)
c-erbB-2	Breast			66% (25/38)

+DCIS occurs only in PDB.

* mean ±SD

Table 2.6 Double marker expression in PDV

Proteins	Non invasive PDV	Invasive PDV
p53 + Ki67 +	8/52(15%)	2/10(20%)
P53- Ki67 +	11/52(21%)	2/10(20%)
Cyclin D1+ pRb+	21/62(34%)	0/10(0%)
Cyclin D+ pRb-	16/62(26%)	5/10(50%)
Both negative	10/62(16%)	2/10(20%)
Cyclin D1- pRb +	8/62(13%)	2/10(20%)

+ positive - negative

Table 2.7 Double marker expression in PDB

Proteins	PDB only (n=11)	PDB+DCIS (n=15)	PDB+DCIS+invasive (carcinoma n=3)	PDB+invasive carcinoma (n=8)
C-erbB-2 + +Ki67	1/11(9%)	1/15(6%)	1/3(33%)	1/8(12%)
C-erbB-2 + Ki67-	2/11(18%)	1/15(6%)	0/3(0%)	0/8 (0%)
C-erbB-2 +pRb+	2/11(18%)	2/15(13%)	1/3(33%)	1/8(12%)
C-erbB-2 +pRb-	7/11(64%)	6/15(40%)	0/3(0%)	3/8(37%)
Both negative	1/11(9%)	2/15(13%)	2/3(67%)	3/8(37%)
C-erbB-2 – pRb+	0/11(0%)	1/15(6%)	3/3(100%)	1/8(12%)

2.4. DISCUSSION

2.4.1 p53 and Ki67 expression in PDV

p53

Reports of the immunohistochemical detection of p53 in PDV have shown positivity ranging from 0-80% (Table 2.8A). A study conducted by Takata et al.(1997) demonstrated p53 expression in three of their 14 cases of extramammary intraepithelial Paget cases, which included two cases of PDV, neither of which were positive. In the three cases that were positive, <5% of the cells were immunopositive for p53. Others (Scheistroen et al., 1997) noted only 12% of their PDV cases to be positive for p53. The converse was found in the report by Crawford et al., (1999). Their study demonstrated p53 positivity in 16 of 20 cases (80%) of PDV cases. The variable results demonstrated could be due to the diversity of different antibodies used and different methodologies in the immunohistochemistry . Examples include the use of p53 clone Bp53-12, (Kobe) in the Nakamura study, NCL-CM1 p53 clone (Novacastra) and DO1 p53 clone (Scandinavian Diagnostic Services) in the

Scheistroen study; the incubation of the p53 antibody overnight at 4°C in the Takata study and no apparent antigen retrieval in the Kanitakis study. Similarly, the lack of a standardised immunohistochemical criterion to classify a section as positive may have played a part. The use of paraffin wax -embedded archival tissue to detect the p53 protein also has its limitations. Alteration of the p53 protein during tissue fixation processing and the limited choice of antibodies available for use on paraffin-embedded archival tissue may all contribute to the diverse results seen in the literature. In this current study a section was scored positive only if >10% of Paget cells had positive nuclear staining. Although Crawford et al.,(1999) used the same p53 antibody (clone DO7, Dako) no details were given with respect to incubation times, and antibody concentrations or what criteria were used to classify a section as p53 positive.

p53 protein expression has also been examined in other epithelial skin lesions (Ro et al., 1993). It was not detected in benign skin disease such as seborrhoeic keratoses or keratoacanthomas, but 56% of squamous cell carcinomas and 42% of basal cell carcinomas were positive for p53. Dysplastic skin lesions were also positive (27%). The authors suggested that p53 may be involved in the progression towards invasive malignancy in human squamous skin lesions. Our group has shown that the p53 protein is over expressed in 69% (47/68) cases of vulval squamous cell carcinoma. Rolfe et al., (2003; unpublished data) and we have also demonstrated p53 over expression in vulval intraepithelial neoplasia and lichen sclerosus adjacent to vulval cancer, but p53 was not expressed in adjacent normal vulva to the vulval squamous cell carcinoma. Furthermore, it was not expressed in histologically normal vulval biopsies (Rolfe et al., unpublished data).

In this study, it has been demonstrated that 29% of PDV cases over expressed p53. p53 may therefore have a role to play in PDV progression. In addition, in some cases, p53 over expression may be associated with progression of PDV towards invasive carcinoma, since more samples were p53 positive in PDV cases associated with an invasive carcinoma compared to PDV cases without invasive disease, although this did not reach statistical significance.

Table 2.8A p53 expression in extramammary Paget's disease

Study	No positive for p53	No of PDV
Kanitakis et al (1993)	0/5 (0%)	0/2 (0%)
Takata et al (1997)	3/14 (21%)	0/2 (0%)
Wieneke et al (1994)	6/12 (50%)	0/3 (0%)
Scheistroen et al (1997)	4/34 (12%)	4/34 (12%)
Nakamura et al (1995)	11/28 (39%)	2/10 (20%)
Crawford et al (1999)	16/20 (80%)	16/20 (80%)
Zhang et al., (2003)	4/14 (28%)	4/14 (28%)

Ki67

There have been no previous studies that have examined Ki67 expression in PDV. However, several studies have sought to investigate whether Ki67 could be used as a marker of premalignant progression in CIN (Kruse et al., 2004; 2005) and Lichen Sclerosus (Rolfe et al., 2001). These studies have demonstrated an increase in Ki67 expression as the dysplasia increases. It has also been shown to correlate with the degree of dysplasia in Barretts oesophagus (Hong et al. 1995) and in ulcerative colitis

(Andersen et al., 1998). Hendricks et al. (1994) demonstrated that the pattern of Ki67 immunostaining might have prognostic significance in vulval squamous cell carcinoma. In addition to common prognostic factors, patients with a localised Ki67 labelling pattern tended to live longer than those with a diffuse pattern. Ki67 has also been found to correlate with tumour grade and poor survival in vulval squamous cell carcinoma. Cases with negative or weak Ki67 expression were associated with a better prognosis than those where Ki67 overexpression was high. The mean staining index for Ki67 in normal vulva has been shown to be 3.4% (Rolfe et al. in ppn.). The mean staining index in the cases in this study of PDV with and without invasive disease was 19% and 16% respectively. It is unlikely, on the evidence so far, that Ki67 can therefore be used as a prognostic marker in PDV.

Very few studies have examined the correlation between p53 positivity and Ki67 expression in premalignant vulval lesions. Rolfe et al., (2001) found increased expression of p53 and Ki67 in areas of squamous hyperplasia and VIN which correlated with subsequent development of invasive squamous cell carcinoma of the vulva. In the current study I found no correlation in p53 and Ki67 expression (Table 2.6) in PDV without invasive disease as compared to those cases with invasive disease.

2.4.2. pRb and cyclin D1 expression in PDV

pRb

As discussed earlier, the loss or inactivation of many tumour suppressor genes may be an important aetiological or prognostic factor in cancer. Absent or decreased pRb expression has been demonstrated in lung cancer (Habour et al., 1988), glioblastomas (Venter et al., 1991) and breast carcinomas (Lee et al., 1988). Inactivation of the pRb

gene by the E7 protein of HPV 16/18 has been demonstrated, and has been associated with the development of cervical cancers (Howley et al., 1991). In other studies, pRb expression has been the same in normal cervical epithelium, CIN and cervical carcinoma (Parker et al., 1997; Sano et al., 1998; Skomedal et al., 1999). pRb inactivation has been shown to be involved in the progression of vulval cancer, and premalignant VIN and lichen sclerosus (Rolfe et al., 2001). The study also demonstrated that loss of pRb expression was associated with poor tumour grade. Similarly, Chan et al., (1998) also demonstrated that inactivation of pRb in SCC of the vulva increases from stage I to stage IV disease suggesting a role for this protein in SCC.

There have been no studies analysing the expression of pRb in Paget cells. In this study 70% (7/10) of invasive PDV did not express the protein as compared to 40% (25/62) of the cases of PDV without invasive disease. The difference may suggest that pRb inactivation is involved in the malignant progression of PDV. Those cases of PDV without invasive disease that expressed abnormal pRb may require closer clinical surveillance. It is not yet known if such inactivation of pRb is loss or if there is a mutation present. Long term clinical follow up of these patients may lead to the early identification of disease progression and therefore improve the prognosis.

Cyclin D1

Cyclin D1 has been implicated in a number of cancers and over expression of the cyclin D1 gene has been reported in bladder (Proctor et al.1991), head and neck (Schuuring et al., 1992), and lung cancers (Berenson et al., 1990).

Rolfe et al., (2001) demonstrated that abnormal expression of cyclin D1 in SCC of the vulva was statistically significant as compared to VIN, lichen sclerosus and

adjacent normal epithelium. (the group also found that abnormal expression of cyclin D1 was associated with a greater depth of invasion). There have been no studies analysing cyclin D1 expression in Paget cells of the vulva. It was found in this work that 61% (36/59) of cases of PDV over expressed the cyclin D1 protein and 50 % (5/10) of the cases of PDV with invasive disease. It is not known whether this over expression of the cyclin D1 protein is due to amplification of the gene or loss of gene regulation. This could suggest a role for cyclin D1 in the pathogenesis of PDV but not malignant progression.

pRb regulates cyclin D1 expression and both are involved in the regulation of the G1 phase of the cell cycle. Several studies have sought to address the nature and significance of the interaction between cyclin D1 and pRb (Bates et al., 1994; Nielsen et al., 1997; Kourakalis et al., 2006) . It has been shown that mutation of *RB1* leads to the absence of cyclin D1 function (Bates et al.,1994). Jiang et al. (1993) also demonstrated the occurrence of cyclin D1 amplification/over expression and the presence of *RB1* aberrations in a distinct subset of oesophageal tumours. In this study, 16 PDV cases without invasive disease (26%) and five cases of PDV cases with invasive disease (50%) were positive for cyclin D1 and negative for pRb (Table 2.6). The abnormal expression of cyclin D1 and pRb in these cases, in particular the PDV cases with invasive disease may represent significant events in the disease process of PDV. There was a significant difference ($p=0.048$) in the combined positive expression of cyclin D1 and pRb in PDV without invasive disease as compared with those cases with invasive disease (Table 2.6).

2.4.3. p53 and Ki67 expression in PDB

p53

There have been very few studies (Table 2.8B) examining p53 expression in PDB. Kanitakis et al. (1993) reported that four of 12 cases (33%) of PDB over expressed the protein and in the study by Nakamura et al. (1995) three of six cases (50%) were positive for p53. p53 expression has been well documented in breast carcinomas themselves (Thor et al., 1992; Allred et al., 1993; Silvestrini et al., 1993; Jacquemier et al., 1994; Rosen et al., 1995). The range of p53 positivity has been shown to vary between the different histological types of breast tumour. In one study (Thor et al., 1992) 68% of medullary carcinomas expressed p53 as compared to 9% of lobular and 23% of ductal carcinomas. It was not expressed in certain low grade carcinomas (tubular or papillary). PDB is usually associated with ductal carcinoma.

Five of the PDB cases (13%) in this current study over expressed p53. p53 protein changes may therefore only have a role to play in a minority of PDB cases.

Table 2.8B p53 expression in Paget's disease of the breast

Study	No positive for p53
Urano et al., (1992)	0/2 (0%)
Kanitakis et al., (1993)	4/12 (33%)
Nakamura et al., (1995)	3/6 (50%)
Fu et al., (2001)	6 /14 (43%)

Ki67

Several studies (Walker et al., 1988; Bouzubar et al., 1989; Isola et al., 1990; Ioachim et al., 1996 Fu et al., 2001) have demonstrated that a high Ki67 score is associated with high histological grade in breast tumours. In this current study there was no correlation between Ki67 score and histological grade of breast tumour associated with PDB. In addition, the proliferation rate in PDB itself was generally low.

2.4.4. pRb and Cyclin D1 expression in PDB

pRb

From the literature there appears to be no study examining the expression of pRb in PDB. Several authors have implicated that pRb has a role to play in the pathogenesis of breast cancer. (Lee et al., 1988; T' Ang et al., 1988). Bieche and Lidereau (2000) quantified retinoblastoma mRNA by reverse transcriptase- polymerase chain reaction in 129 primary breast tumours. From their results they suggested that the *RB1* gene promoted breast tumour aggressiveness and rapid tumour cell proliferation. This current study demonstrated similar findings; sixty seven percent of the PDB cases did not express the pRb protein which may suggest a role in the pathogenesis of PDB.

Cyclin D1

Eight percent of PDB cases in this study over expressed cyclin D1. The only other study examining cyclin D1 expression in PDB found a much higher figure (Fui et al., 2001). They found all 14 cases (100%) of their PDB over expressed cyclin D1. The discrepancy between this and the study by Fui et al. may be due to differences in the methodology e.g. a cocktail of 2 antibodies (clone P2D11F11, Novocastra and clone DCS-6, Dako) were used.

Gredts and Ingram (2000) examined cyclin D1 expression in 81 invasive breast carcinomas. They found that 28/81 (35%) of their cases expressed the protein. Bartkova et al., (1994) found that half of the 170 primary breast carcinomas over expressed cyclin D1. Over expression of cyclin D1 protein or mRNA has been demonstrated in high grade DCIS, which shows more frequent recurrences than low grade tumours (Weinstat-Saslow et al., 1995; Simpson et al., 1997) However, Van Diest et al.,(1997) reported that over expression of cyclin D1 is not by itself an indicator of prognosis. The variations in these results could be as a result of the fact that over expression of the cyclin D1 protein is linked to the oestrogen receptor (Hui et al., 1996) and is up-requlated by activated oestrogen receptor. Overexpression of cyclin D1 protein in breast cancer may result in"normal" stimulation by oestradiol.

2.4.5. C-erbB-2 expression in PDB

It has been shown that the c-erbB-2 protein is over expressed in 20 % of all breast cancers and 25% of invasive ductal cell carcinomas (De Potter and Schelfhout 1995); however, it appears not to be expressed in lobular carcinomas (Portern et al, 1999). It has also been shown to be expressed in PDB. Lammie et al., (1989) found that 41/45 (91%) of their PDB cases over expressed the c-erbB-2 protein. Similarly, Fu et al., (2001) found a high percentage of their cases (93%) expressing the protein and Brummer et al (2004) demonstrated all five cases of PDB over expressed c-erbB-2 protein.

The c-erbB-2 protein act as an intermediary between the chemotactic factor secreted by epidermal keratinocytes. This chemotactic factor attracts the Paget cells to spread into the epidermis and acts via the c-erbB-2 protein. In this study it was found that 66% of PDB cases over expressed the c-erbB-2 protein. Similarly, those underlying ductal carcinomas that were available for assessment had similar scores to the PDB

section. There was no significant difference in those cases of DCIS and invasive carcinomas compared to those that did not express the protein. This study suggests c-erbB-2 protein may be important in the pathogenesis of PDB.

There have been reports that c-erbB-2 overexpression may be involved in cell cyle regulation through p27, D cyclins, pRb and c-myc (Loden et al., 2003). In this study no correlation was found between the cell cycle proteins and c-erbB-2 overexpression in PDB.

This is the largest immunohistochemical study on cases of PDV and PDB. These lesions are very uncommon and, even after collecting cases from multiple centres, it was difficult at times to obtain numbers to allow for statistical significance. A PDV register for PDV in the UK has now been established (chapter 6) to gain access to more cases. However, with current data, p53, Ki67 and c-erbB-2 immunohistochemistry does not provide information on prognosis or management. pRb may be involved in the pathogenesis of PDV and PDB, and cyclin D1 in the pathogenesis of PDV. An even larger series of well documented cases is required if a firm conclusion is to be made regarding the role of these cell cycle markers in the pathogenesis and prognosis of Paget's disease of the vulva and of the breast. The collaboration with societies such as the British Society for the Study of Vulval Disease or the International Society for the Study of Vulval Disease may be able to provide more cases that are required.

CHAPTER 3

MUTATION ANALYSIS OF THE *P53* GENE IN PAGET'S DISEASE OF THE VULVA AND THE BREAST

3.1. INTRODUCTION

3.1.1. *P53* gene

The *P53* gene has been described as the most commonly mutated gene in human cancers (Hollstein et al., 1999). Located on the short arm of chromosome 17p 13.1., it encodes a nuclear phosphoprotein involved in cell proliferation and apoptosis and is classified as a TSG. Loss of its function by mutation, deletion or binding of proteins (e.g. Mdm2 or E6 from HPV) has been implicated in a number of cancers (Barker et., 1990; Sliutz et al., 1997) and has been shown to be useful as a prognostic parameter in some cancers (Hietanen et. al., 1995).

Mutations in the *P53* gene were first identified in colon and lung cancer in 1989 (Barker et al., 1989; Takahasi et al., 1989). It was thought at the time that mutations in the *P53* gene were predominantly localised in exons 5-8. However further analysis of the entire coding region has shown a number of mutations in exons 4, 9 and 10 (Hasimoto et al., 1999; Skaug et al., 2000).

Immunohistochemical staining and DNA sequencing have been used to assess abnormalities in the *P53* gene. Most abnormalities are point mutations leading to the synthesis of a stable mutated protein that accumulates in the nucleus of the tumour cells which can be detected by IHC (Dowell et al.,1994). The correlation between p53 accumulation and p53 mutation is approximately 80% as frameshift mutations do not lead to p53 accumulation and therefore will not be detected by IHC analysis.

Our study analysed exons 5-8 of the *P53* gene for mutations because of the greater chance of finding a mutation within this region. Furthermore, exons outside this region have a significant greater number of frameshift and nonsense mutations.

3.1.2. Laser Capture Microdissection (LCM)

Microdissection is a long established method to obtain purified cell populations for analysis of genetic alterations at the DNA level from both fresh and fixed tissues and cell preparations. Microdissection originally involved manual and/or micromanipulator guidance of a needle to scrape off an area of interest (e.g. Paget cells) of a thin tissue section (Going et al., 1996). This method is highly operator-dependent, tedious and can be inaccurate, owing to the uncertainty of the population of cells obtained.

The first technological advance in microdissection was Shibata's procedure of selective ultraviolet radiation fractionation, which relied on negative selection: ablation of the unwanted areas of tissue on the slide (Shibata et al., 1992). A pigment painted over the areas of interest provides a protective shield from ultraviolet radiation, which destroys the unprotected tissue and improves the purity of subsequent manual microdissection of the selected areas. Micromanipulators have improved the accuracy and reliability of manual dissection which, however, remains an intrinsically slow process for procuring pure cell populations from tissues. LCM was designed to diminish the uncertainties that remain with other manual, ablation and mechanical dissection techniques. Operator contact with the tissue during the LCM is almost negligible and targeting and procurement is simple, reliable and rapid. Multiple similar targets can be easily accumulated onto the same polymer transfer film. Individual single laser shots can be used to procure specific cells or cell clusters. In addition, multiple shots can be combined in order to procure complicated tissue structures. The morphology of all procured microsamples during the microtransfer is retained so that further inspection and archiving of images is easily accomplished. LCM was first described by Becker in 1996. It was originally conceived and first

developed as a prototype research tool at the National Institute of Child Health and Human Development and the National Cancer Institute of the National Institutes of Health. It has since been commercialised and used in many laboratories.

3.1.2.i. Principles of LCM

LCM provides a method for the selective adherence of visually targeted cells and tissue fragments to a thermoplastic membrane activated by a low energy infrared laser pulse. The system consists of an inverted microscope, a solid state near infrared laser diode, a laser control unit, a joy-stick controlled microscope stage (Figure 3.1) with a vacuum chuck for slide immobilisation, a camera, and a colour monitor. The thermoplastic membrane used for the transfer of selected cells has a diameter of approximately 6mm and is mounted on an optically clear cap, which fits on standard 0.5ml microcentrifuge tubes for further tissue processing (Figure 3.2). The cap is suspended on a mechanical transport arm and placed on the desired area of the dehydrated tissue section under standard pressure. After visual selection of the desired cells guided by a positioning beam, laser activation leads to focal melting of the ethylene vinyl acetate membrane. The melted polymer expands into the section and fills the extremely small hollow spaces present in the tissue. The polymer resolidifies within milliseconds and forms a composite with the tissue. The adherence of the tissue to the activated membrane exceeds the adhesion to the glass slide and allows selective removal of the desired cells. Laser impulses, usually between 0.5 and 5 milliseconds in duration, can be repeated multiple times across the cap surface, which allow the rapid isolation of large numbers of cells. The selected tissue fragments are harvested by simple lifting of the cap, which is then transferred to a microcentrifuge tube containing the buffer solutions required for the isolation of the

molecules of interest (eg. DNA, proteins). The morphology of the transferred cells is preserved and can be readily visualised under the microscope.

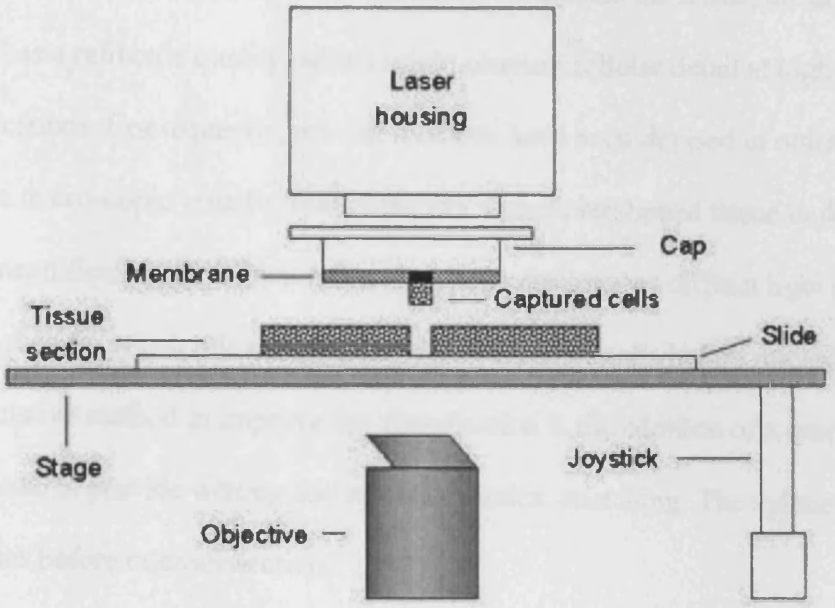

Figure 3.1 Diagram of the LCM tissue transfer process.
Ref. cgap-mf.nih.gov.

An advantage of LCM is its speed and precision. Depending on the chosen laser spot size, the architecture features of the tissue and the desired precison of the microdissection, thousands of cells can be collected within a few minutes. The morphology of both the captured cells as well as the residual tissue is well preserved. The isolated cells are attached firmly to the cap, minimising the danger of tissue loss. In contrast, most other microdissection techniques require the removal of isolated cells with the help of a needle tip or using a microcapillary step, requiring skill and practice. Because LCM is very fast and does not destroy adjacent tissues, several tissue components can be sampled sequentially from the same slide, such as normal

and neoplastic cells. A disadvantage of LCM is that the microdissected tissue section is not coverslipped. Coverslipping would prevent physical access to the tissue surface, which is a requirement of any current microdissection. Without a coverslip, and the index matching between the mounting media and the tissue, the dry tissue section has a refractile quality, which might obscure cellular detail at high magnifications. Consequently, several methods have been devised in order to optimise microscopic visualisation of the dry, non-coverslipped tissue under high-power magnification. A diffusion filter has been designed to diffract light passing through the cap, which allows the clear visualisation of cells before microdissection. An alternative method to improve the visualisation is the addition of a drop of xylene to the tissue to provide wetting and refractive index -matching. The xylene evaporates before microdissection.

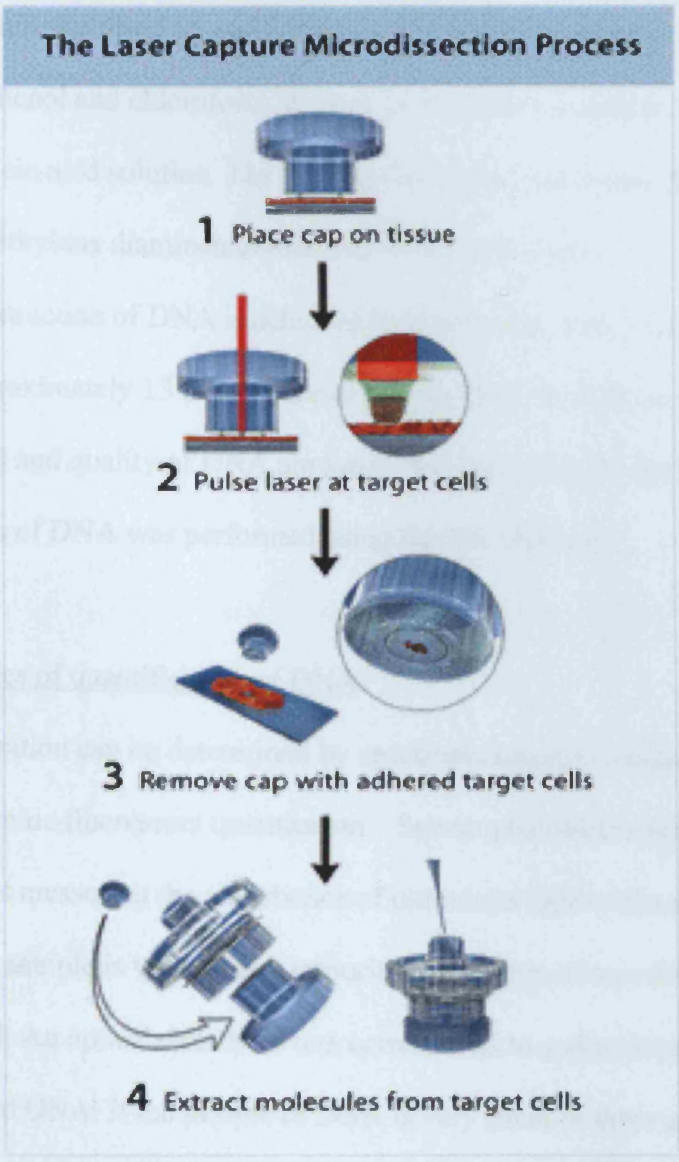

Figure 3.2 Laser capture microdissection process.
Ref. www.mcg.edu.

3.1.3 DNA Extraction

Techniques for the extraction of DNA from fresh tissue, cell or cultured cells have been devised. Similarly, they can be applied to archival material such as paraffin wax-embedded tissue. These procedures involve incubating the tissue with proteinase K (Jackson et al., 1992), a proteolytic enzyme, for times ranging from minutes up to 24hl or more. After extraction, the proteinase K is inactivated by heat treatment at

95°C for 10 min, followed by purification of the DNA by a number of extraction steps using phenol and chloroform. Phenol: choloroform is used to remove proteins from the nucleic acid solution. The DNA is then resuspended in a buffer such as Tris-EDTA (ethylene diaminetetra-acetate) or in sterile water.

More rapid extraction of DNA is achieved by boiling the tissue in sterile distilled water for approximately 15 min. This can provide DNA of sufficiently good quality although yield and quality of DNA are lower than that using the traditional method. Quantification of DNA was performed using the Dot Blot test.

3.1.4. Principles of quantification of DNA

DNA concentration can be determined by spectrophotometric measurement or Ethidium Bromide fluorescent quantitation. Spectrophotometric determination, which involves measuring the absorbance of ultraviolet light of the sample, is ideally used when the sample is without any impurities. Readings of wavelengths at ~ 260nm should be used. An optical density of one corresponds to approximately 50μg/ml of double stranded DNA. If the sample of DNA is very small or there are significant impurities then ethidum bromide can be used. The fluorescence emitted by the ethidium bromide is proportional to the total mass of DNA; the quantity of DNA can therefore be estimated using a series of standards.

3.1.5. Polymerase Chain Reaction (PCR)

Originally devised by Kary Mullis (1986) the polymerase chain reaction (PCR) is an in vitro technique that allows the amplification of a specific DNA region that lies between two regions of known DNA sequence. The technique is based on repeated cycles of high temperature template denaturation, oligonucleotide primer annealing

and polymerase -mediated primer extension. The simplicity of the reaction, as well as its speed and sensitivity, has made it an important tool in a wide variety of applications, including the diagnosis and characterisation of infectious and genetic diseases (including cancer), forensic analysis, environmental microbiology and evolutionary studies.

3.1.6. Basic principle of the PCR

PCR utilises two primers (short, single-stranded DNAs) complementary to the opposite strands of the DNA sequence to be amplified. The extension of each primer creates a DNA strand including the sequence complementary to the opposite primer. After heat-mediated denaturation of the template DNA, the primers bind to their respective sequences (anneal) on the template DNA and a DNA polymerase synthesises a complementary strand. It is a cyclical process: denaturation of the DNA to become single stranded, annealing of the primers and extension by utilising nucleotides. This results in exponential amplification of the specific sequence with each cycle doubling the amount of specific DNA sequence being amplified. However, there will come a point whereby additional cycles do not lead to any further increase in the DNA yield. This results from exhaustion of the reagents.

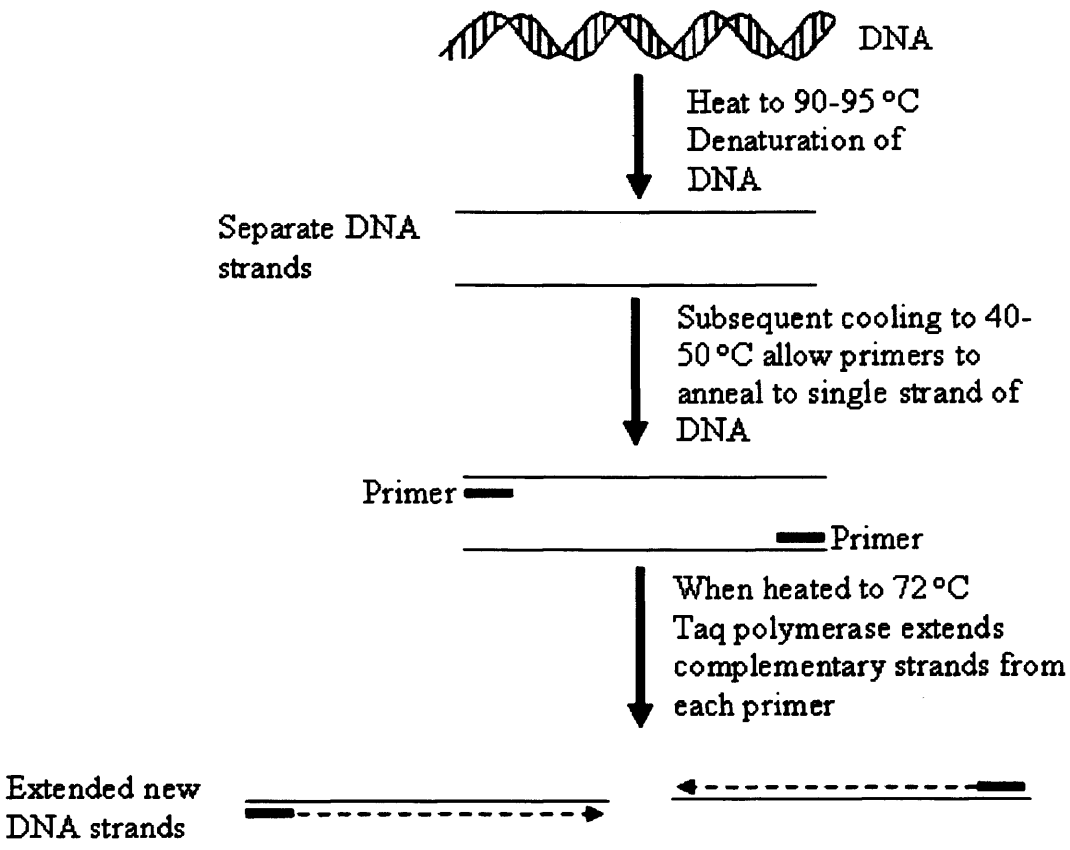

Figure 3.3 Diagram of a PCR cycle.

3.1.7. Primers

Successful specific amplification of a desired target sequence in the PCR is dependent upon design and optimal use of a primer pair. The consensus of opinion (Innis et al., 1990; Kocher et al; 1991; Old et al., 1994) has provided a set of rules for the design of PCR primers:

1. Primers should range from 15-30 bases in length.
2. Base composition should be 50-60% guanine and cytosine.
3. Long runs with more than three or four of the same base should be avoided.
4. Primers should not have a secondary structure (e.g. hairpin loops).
5. Ideally, primers should not contain sequences that are complementary to each

other. This will avoid the annealing of such primers.

6. Palindromic sequences should be avoided.
7. Primer melting temperatures (Tms) between 55-80 °C are preferred.

The annealing temperature used in the PCR is dependent on both the length and composition of the primers. Ideally, it should be between 1 °C and 5 °C lower than the lowest Tm value. (Warford et al.,1988).

3.1.8. DNA polymerase

A DNA polymerase enzyme is required to synthesise the new strands of DNA, thus joining the two strands of the target DNA as templates and the two oligonucleotide primers as starting points for new strand synthesis. The enzyme *Taq* is the most frequently used DNA polymerase and is isolated from the bacterium *Thermus aquaticus*, which works optimally at 72 °C and over a pH range of 7.0-7.5, adding ~ 100 nucleotides/s to the primer under these conditions. It is a 94kDa protein with a half-life of 40 min at 95 °C. It is heat stable and therefore allows the withstanding of denaturation cycles. However, it has a lack of 3'→5' exonuclease activity, this can lead to misincorporation of nucleotides.

3.1.9. Deoxyribonucleoside triphosphates (dNTPs)

Deoxyribonucleoside triphosphates (deoxyribonucleotides) are essential for the synthesis of new DNA. These are heat resistant and have a half-life of more than 40 cycles of PCR (Innis et al., 1988). Usually, each dNTP concentration should be between 50 μM and 200 μM. This concentration is sufficient to synthesise 6.5μg and

25μg of DNA respectively. Deoxyribonucleotides provide energy and nucleosides for the synthesis of the new strands of DNA.

3.1.10. Reaction buffer

The most often used is a 10mM Tris-Hcl buffer with a pH range of 8.5-9.0 at 25 °C. It also usually contains 50mM kcl. Detergents such as Tween 20, Triton X-100 and/or extra protein, e.g bovine serum albumin (BSA) or gelatin may help to prevent the precipitation of the hydrophobic *Taq*.

3.1.11. $MgCl_2$

Taq polymerase is an Mg^{2+} dependent enzyme. In addition, free Mg^{2+} may affect primer annealing, strand dissociation, product specificity and fidelity. The concentration of Mg^{2+} infuences the efficiency of primer to template annealing. It is important therefore to optimise the Mg^{2+}concentration in the reaction. In the current study 1.5mM of Mg^{2+} was optimal for PCR amplification of exons 5-8 of *P53*.

3.1.12. Principles of electrophoresis

Electrophoresis is a technique used to separate, identify and purify DNA fragments. The location of DNA can be determined with the fluorescent intercalating dye ethidium bromide by direct examination on a UV transilluminator and the DNA directly recovered from the gel if required. The gel itself is composed of either agarose or polyacrylamide, each of which have attributes suitable to a particular task. Agarose is a polysaccharide extracted from seaweed. Agarose gels have a large range of separation, but relatively low resolving power. Fragments of DNA from about 200 to 50,000bp can be separated using standard electrophoretic techniques. Agarose gels

are used to check the progression of restriction enzyme digestion, to determine the yield and purity of DNA, to identify PCR products and to size fractionate DNA molecules, which then can be eluted from the gel. It is easy to prepare and is non toxic.

Polyacrylamide is a cross-linked polymer of acrylamide. It is more difficult to prepare; oxygen inhibits the polymerisation process and also acrylamide is a potent neurotoxin. The polyacrylamide gels have a small range of separation, but very high resolving power. In the case of DNA, polyacrylamide is used for separating fragments of less than about 500bp. Non denaturing polyacrylamide gel is used for the separation and purification of fragments of double-stranded DNA, whereas denaturing polyacrylamide gel is used for the separation of single -stranded DNA. In contrast to agarose, polyacrylamide gels are used for separating and characterising mixtures of proteins.

3.1.13. Principles of sequencing

DNA sequencing is the process of determining the nucleotide order of a given DNA fragment. In most cases DNA sequencing is performed using the chain terminator method developed by Frederick Sanger (Sanger et al., 1977).

3.1.13.i. Sanger sequencing

This method uses sequence-specific termination of a DNA synthesis reaction using modified nucleotide substrates. Extension is initiated at a specific site on the template DNA using a short oliognucleotide primer complementary to the template at that region. The oligonucleotide primer is extended using a DNA polymerase. Included with the primer and DNA polymerase are the four deoxynucleotide bases, along with

a low concentration of a chain terminating nucleotide most commonly a di-deoxynucleotide. Limited incorporation of the chain terminating nucleotide by the DNA polymerase results in a series of related DNA fragments that are terminated only at positions where that particular nucleotide is used. The fragments are then size-separated by electrophoresis.

3.1.13..ii. Automated commercial sequencing

A new technology has emerged to replace the Sanger Method. Automated commercial sequencing is similar to the chain terminator sequencing except that a flouresecent dye is attached to the di-deoxynucleotides (ddNTP's). The reactions are performed in a single tube containing all four ddNTP's, each labelled with a different colour dye (Russell, 2002). These dyes fluoresce at different wavelengths, which are read via a machine (Metzenberg). As in the Sanger's method, the DNA is separated on a gel, but they are all run on the same lane as opposed to four different ones. It has been developed so that more DNA can be sequenced in a shorter period of time Automated commercial sequencing was used in this study.

The aim of this chapter was to identify and confirm any mutations in exons 5 to 8 of the *P53* gene in immunohistochemically positive sections of PDV and PDB. Such confirmation would be important in clinical practice in finding improvements in the treatment of PDV and PDB and understanding the molecular mechanisms underlying these diseases. If a common mutation is found, *P53* genechips could be analysed. Also, clonality could be performed to provide further information on the origin of the underlying invasive carcinoma.

3.2. METHODS

3.2.1. Sample preparation

Six immunohistochemical samples, three cases of PDV (including two associated with invasive carcinoma) and three cases of PDB (one case associated with invasive breast carcinoma, one case with DCIS only and the remaining case not associated with either) were selected according to the percentage (25-100%) of cells immunohistochemically positive for the p53 antibody . The stained slides were immersed in xylene solution to remove the coverslip. Prior to LCM the sections were rehydrated in graded ethanol rinses (70%, 90% and 100% for 3 min each) and then immersed in xylene for 5 min. The final dehydration and xylene steps are crucial for successful LCM. Any moisture present in the sample will impede transfer efficacy. The slides were then air dryed ready for LCM.

3.2.2. LCM

A CapSure LCM cap (Arcturus, California) was placed onto an area where the Paget cells were stained positive for the p53 protein. To capture the cells, the infrared laser was pulsed. The laser activates the transfer film on the CapSure LCM cap which then expands down into contact with the tissue. The Paget cells adhered to the CapSure LCM cap. The CapSure LCM cap with the Paget cells attached to the film surface was removed, the surrounding tissue remaining intact. This was then inserted into a microcentrifuge tube ready for the addition of the extraction buffer.

3.2.3. DNA extraction

50µl of the extraction buffer (5mm Tris- Hcl [pH 8.5], 0.5mM EDTA, 0.5mg/ µl proteinase K [Sigma-Aldrich], 0.5%v/v Tween 20) was placed into the sterile microcentrifuge Eppendorf tube containing the microdissected Paget cell's DNA. The tubes were placed in a thermomixer overnight at 37 °C. The solution was then centrifuged at 3500rpm for 10 min and the supernatant removed, placed in a 0.5ml sterile Eppendorf and stored at 4 °C. Prior to PCR, proteinase K was inactivated at 95 °C for 10minutes.

Quantification of the DNA was performed using standard DNA concentrations (MW markers) diluted to varying concentrations with ethidium bromide (based on ethidium bromide fluorescence). The concentration of the extracted DNA was then estimated on a transilluminator.

3.2.4. PCR

4 µl of DNA solution was added to 46 µl of PCR mix giving a final overall composition (50 µl): 200µM dNTPs, 75mM Tris-HCL (pH8.8, RT) 1.5mM $Mgcl_2$, 0.01%v/v Tween 20, 20mM $(NH_4)_2 SO_4$. PCR cycling conditions, primer amounts and primer concentrations are described in Table 3.1.

Table 3.1 PCR conditions and primers

Loci	Primers	Primer concentration	PCR conditons
p53 exon 5	U-GGAATTCTGTTCACTT GTGCCCTGACTTTCAAC D-GCAACCAGCCCTGT CGTCTCTCCA	14pmoles	Denature-94°C for 5 min. initial cycle; 94°C for 1min for 35 cycles. Anneal- 60°C for 1 min. x 35cycles; Extend- 72°C for 1min. x 35 cycles. 72°C for 10 min. for final cycle. Enzyme:*Taq* (2.5units, Advanced Biotechnologies)
p53 exon 6	U-CCTGCTTGCCACAGG TCT D-CCGGAAATGTGATGA GAGGT	15 pmoles	Denature-94°C for 5 min. initial cycle; 94°C for 1min for 35 cycles. Anneal- 60°C for 1 min. x 35cycles; Extend- 72°C for 1min. x 35 cycles. 72°C for 10 min. for final cycle. Enzyme: *Taq* (2.5units, Advanced Biotechnologies)
p53 exon 7	U-CCTGCTTGCCACAGG TCT D-CCGGAAATGTGATGA GAGGT	17 pmoles	Denature-94°C for 5 min. initial cycle; 94°C for 1min for 35 cycles Anneal- 60°C for 1 min. x35cycles; Extend- 72°C for 1min. x 35 cycles. 72°C for 10 min. for final cycle. Enzyme: *Taq* (2.5units, Advanced Biotechnologies)
p53 exon 8	U-AGGTAGGACCTG ATTTCCTTACTGCC D-GGAATTCTGAGGCAT AACTGCACCCTTGGTCT	17 pmoles	Denature-94°C for 5 min. initial cycle; 94°C for 1min for 35 cycles Anneal- 60°C for 1 min. x 35cycles; Extend- 72°C for 1min. x 35 cycles. 72°C for 10 min. for final cycle. Enzyme:*Taq* (2.5units, Advanced Biotechnologies)

3.2.5. Polyacrylamide gel analysis

10% non-denaturing polyacrylamide gel electrophoresis in a vertical chamber was used (Bio Rad). Running buffer used was 1xTBE. The gel was run at 200v for 30min, 8V/cm. Once completed the PCR product was stained in EtBr (0.5μg/ml in 0.5xTBE) and viewed in the transilluminator. Standard MW markers were used for size comparison and were also loaded onto the gel alongside bromophenol blue, which was used as the tracking dye. PCR was repeated twice to ensure reproducibility of the results.

3.2.6. Purification of PCR product

DNA was purified from the polyacrylamide gel using Qiagen PCR purification kit. Five volumes of PBS buffer to one volume of the PCR mix were added, mixed, and placed in a mini centrifuge column. This was then centrifuged for one min. The flow-through was discarded and 0.75ml of the PE (wash) buffer was added to the tube to wash the remaining mix. This mix was then centrifuged for one min. The flow-through was again removed and the mix was again centrifuged for a further min. to ensure all the residual ethanol from the PE buffer was removed. The mix was then placed in a sterile centrifuge tube along with 30µl of elution buffer. This mixture was then centrifuged for 1 min.

3.2.7 Sequencing

Automated commercial sequencing was performed on the PCR product at the Divison of Genomic Medicine, University of Sheffield. Sequencing of both DNA strands was visualised using an automated sequencer (ABI-Prism). The sequences were analysed using Sequence navigator software (ABI).

3.3. RESULTS

3.3.1. Photomicrographs

Figure 3.4 p53 expression in PDV (x 200) with a score of 4.

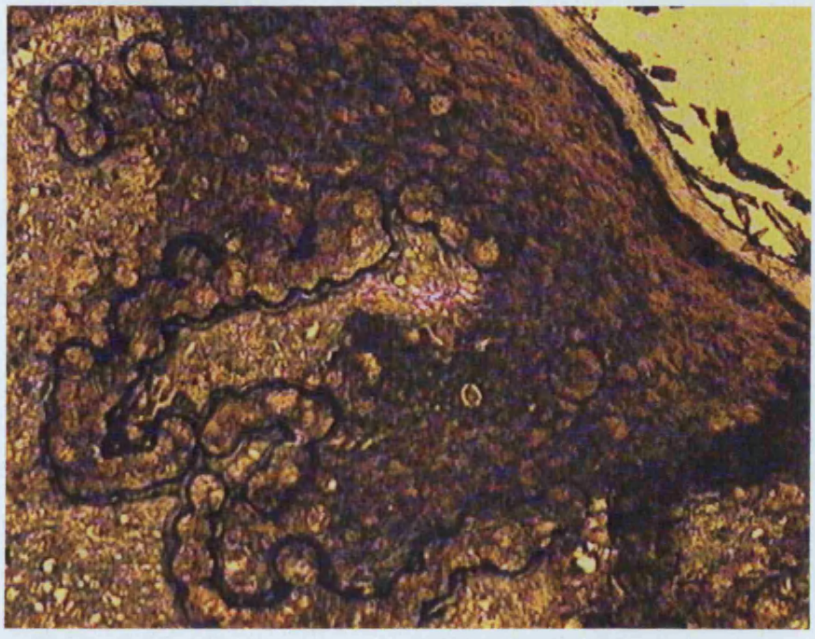

Figure 3.5 Photomicrograph of section of PDV following pulse laser of Paget cells (x 200).

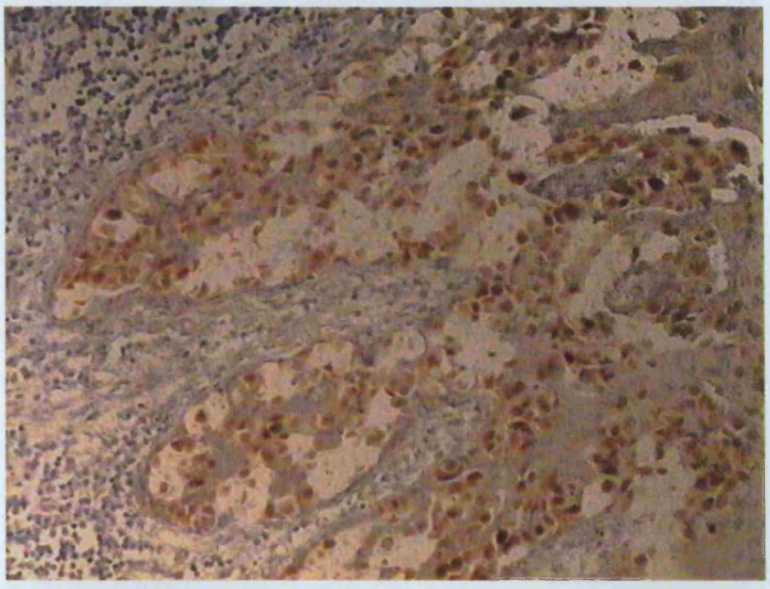

Figure 3.6i p53 expression in PDB (x 400) with a score of 4.

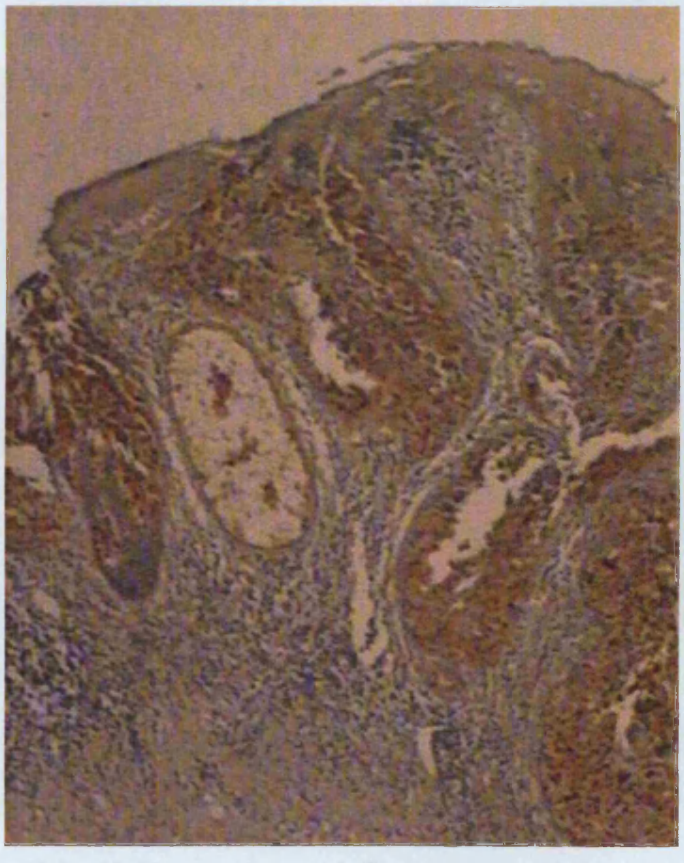

Figure 3.6ii p53 expression in PDB (x 100) with a score of 4.

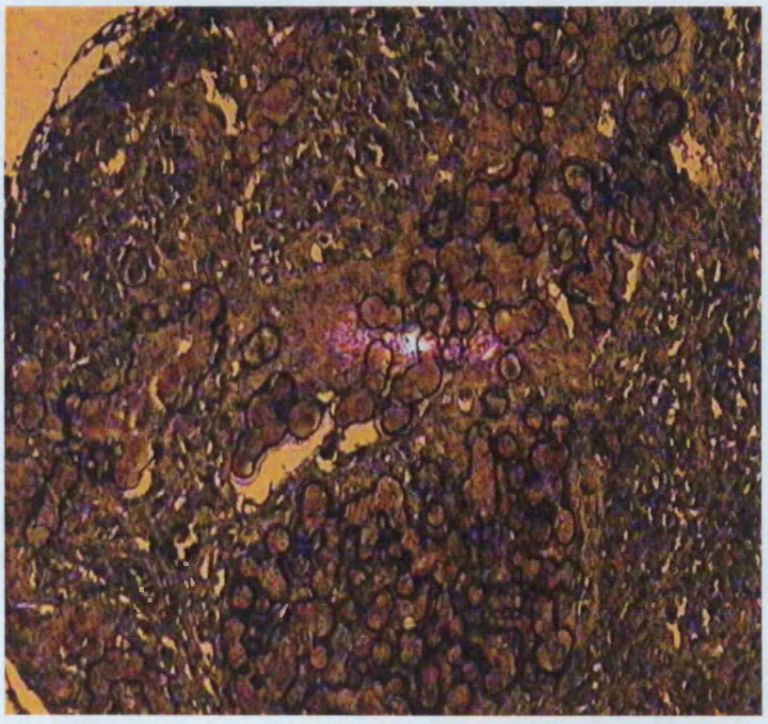

Figure 3.7 Photomicrograph of section of PDB following pulse laser of Paget cells (x 200).

Figure 3.8 p53 Photomicrograph demonstrating the captured PDB cells (x 400).

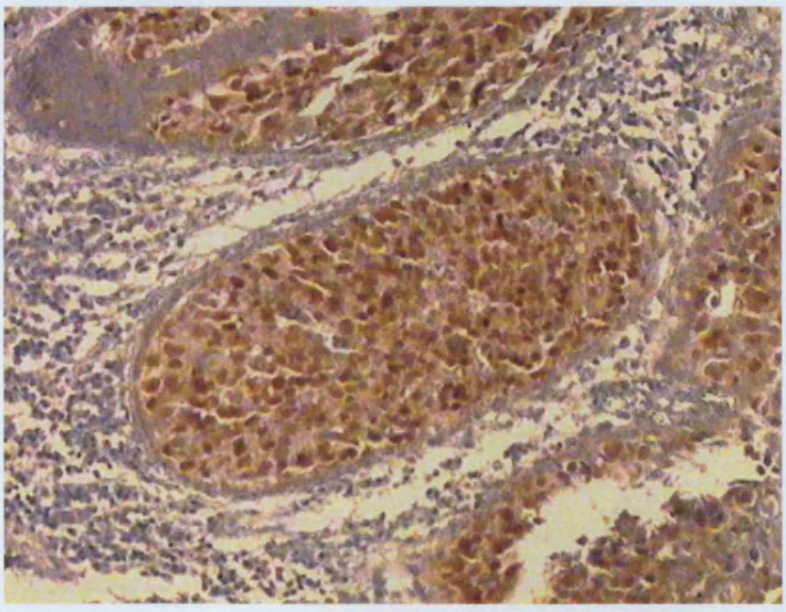

Figure 3.9 p53 expression in PDB (x 400) with a score of 4.

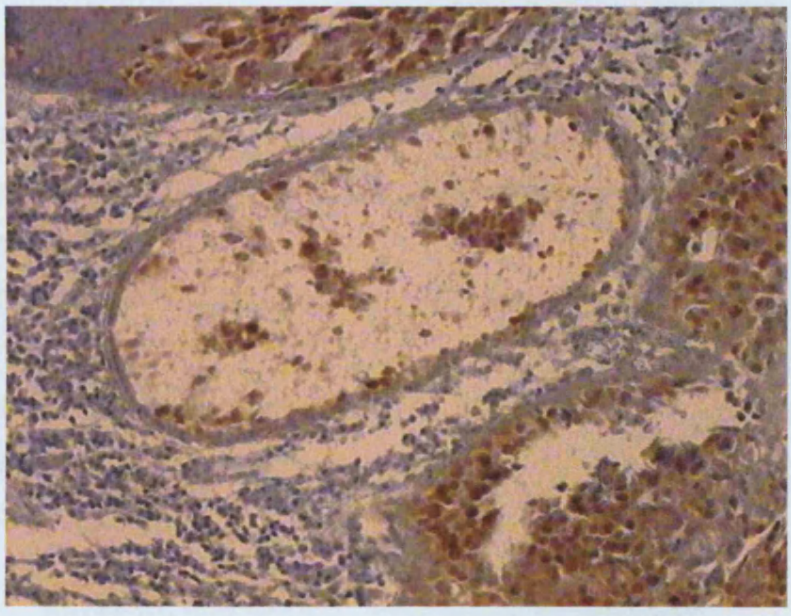

Figure 3.10 Section of PDB after removal of Paget cells (x 400) following LCM.

Figure 3.11 Photograph of cap demonstrating adherent Paget cells (x 400).

3.3.2. Mutation analysis of exons 5-8 of the *P53* gene in PDV and PDB

```
1     CCTCTTCCTA    CAGTACTCCC    CTGCCCTCAA    CAAGATGTTT
      TGCCAACTGG
51    CCAAGACCTG    CCCTGTGCAG    CTGTGGGTTG    ATTCCACACC
      CCCGCCCGGC
101   ACCCGCGTCC    GCGCCATGGC    CATCTACAAG    CAGTCACAGC
      ACATGACGGA
151   GGTTGTGAGG    CGCTGCCCCC    ACCATGAGCG    CTGCTCAGAT
      AGCGATGGTG
201   AGCAG
```

Figure 3.12 Sequence (5'-3') of genes in exon 5 of the *P53* gene in PDB associated with infiltrating carcinoma.

```
1    CCCTCCTCAG    CATCTTATCC    GAGTGGAAGG    AAATTTGCGT
     GTGGAGTATT
51   TGGATGACAG    AAACACTTTT    CGACATAGTG    TGGTGGTGCC
     CTATGAGCCG
101  CCTGAGGTCT    GGTTTGCAAC    TGGGGTCTCT    GGGAGGAGG
```

Figure 3.13 Sequence (5'-3') of genes in exon 6 of the *P53* gene in PDV associated with invasive disease.

```
1    TTATCTCCTA    GGTTGGCTCT    GACTGTACCA    CCATCCACTA
     CAACTACATG
51   TGTAACAGTT    CCTGCATGGG    CGGCATGAAC    CGGAGGCCCA
     TCCTCACCAT
101  CATCACACTG    GAAGACTCCA    GGTCAGGAGC    CACTTGCCAC
     CCTGCACACT
151  GGCCTGCTGT    GCCCCAGCCT    CTGCTTGCCG    CTGACCCCTG
     GGCCCACCTC
201  TTACCGAT
```

Figure 3.14 Sequence (5'-3') of genes in exon 7 of the *P53* gene in PDV.

```
1    TCTCTTTTCC    TATCCTGAGT    AGTGGTAATC    TACTGGGACG
     GAACAGCTTT
51   GAGGTGCGTG    TTTGTGCCTG    TCCTGGGAGA    GACCGGCGCA
     CAGAGGAAGA
101  GAATCTCCGC    AAGAAAGGGG    AGCCTCACCA    CGAGCTGCCC
     CCAGGGAGCA
151  CTAAGCGAGG    TAAGCAAGCA    GGACAAGAAG    CGGTGGAGGA
     GACCAA
```

Figure 3.15 Sequence of gene (5'-3') in exon 8 of the *P53* gene in PDB associated with DCIS.

Table 3.2 Analysis of exons 5-8 of the *P53* gene in PDV and PDB

Sample	Diagnosis	p53 IHC positivity	P53 sequencing
Case no 42	PDV without invasive disease	>75%	wt
Case no 28	PDV with invasive disease	>40%	wt
Case no 30	PDV with invasive disease	>75%	wt
Case no 130	PDB with DCIS	>25%	wt
Case no 92	PDB with DCIS and invasive caricnoma	>50%	wt
Case no 131	PDB without DCIS or invasive disease	>50%	wt

There appeared to be no mutations in exons 5-8 of the *P53* gene in any of 3 cases of PDV or the 3 cases of PDB.

3.4. DISCUSSION

3.4.1. LCM vs other microdissection techniques in cancer studies

The microdissection of cells in premalignant lesions is a requirement for identifying molecular genetic-changes occurring during the process of tumouriogenesis. LCM has the advantage over other common microdissection techniques such as manual tissue dissection, laser microbeam microdissection (LMM) and laser pressure catapulting (LPC) in that it is a very rapid method, versatile and very precise. Manual dissection has the disadvantage of not being suitable for small lesions (<50cells) and the high risk of contamination, and LPC has the danger of destroying the actual cells that is required. (Walch et al. 2000). LMM can be more time consuming and requires greater skills than LCM however, the smaller laser beam diameter and the high precison for 1-10 cells makes LMM more suited for microdissection of tissue at single cell level.

Because of its speed and it does not destroy adjacent tissues, LCM can be used to sample both normal and neoplastic cells together on the same slide and thousands of cells can be collected within a few minutes. Combining IHC, LCM and sequencing of the *P53* gene Ren et al. (1997), identified mutations in the *P53* gene within synchronous dysplasia, carcinoma in situ and invasive sqamous carcinoma of the skin. p53 mutations were also detected in the adjacent normal keratinocytes. LCM was also used to evaluate p53 mutations in premalignant oesophageal lesions and oesophageal adenocarcinoma (Djaalivand et al., 2004).

LCM was used in this study because of ease and rapidity of the procurement of Paget cells and also it allowed the preservation of the morphology of the transferred Paget cells, which could be visualised readily under the microscope

3.4.2. Loss of *P53* function in tumours

Many studies have shown that there is an important association between activation of oncogenes and /or inactivation of tumour suppressor genes and the development of most human cancers. Some identified genes and their respective proteins have been suggested to be useful as prognostic markers for particular cancers (Duffy et al.,1993). Mutation and loss of heterozygosity (LOH) of the *P53* gene has been reported to be the most common cancer -related genetic change (Hollstein et. al 1991). As well as loss of function in certain cancers, the function of the p53 protein may also be abrogated through binding to viral oncoproteins, and Mdm2. HPV E6 has been shown to bind the *P53* gene product and degrade it. (Werness et al.1990). Mutational analysis of the *P53* gene has been well documented in vulva and breast cancer. *P53* expression has been shown to be a useful prognostic marker in vulvar carcinoma, VIN and squamous cell hyperplasia (Hantschmann et al., 2005) and Chulvis et al., (2004) have demonstrated mutations in the *P53* gene in two of their

recurrent /progressive cases of VIN. Very few studies have sought to examine mutations in the *P53* gene in PDV. Takata et al. (1997) examined the LOH at the loci 17p13.1 where the *P53* gene is located, in 14 cases of extramammary Paget's disease, two of which were cases of PDV, and direct sequencing of exons 5-8 of the *P53* gene in 8 cases. No LOH or mutations were found in any of the cases. This would be in keeping with our results although it does not correlate with the immunohistochemical results in the current study which suggested that p53 protein expression may be involved in disease progression in PDV.

In breast cancer the *P53* gene has been extensively studied and several studies have demonstrated mutations in the *P53* gene and its role as prognostic marker in breast cancer patients (Berns et al., 1998; Falette et. Al.,1998). Wt p53 expression has also been demonstrated in primary breast cancers (Nenutil et al., 2005; Marchetti et al., 2003). As discussed in chapter 2, very few studies have examined p53 expression in PDB (Table 2.8B Page 106) and so far no study have performed mutational analysis of the *P53* gene in PDB. No mutation was found in any of the three cases of PDB, which included a case of DCIS, and DCIS with invasive carcinoma.

3.4.3 Expression of wt p53:

Sequencing results in the current study suggest that wt p53 is being expressed as there were no mutations found at exons 5-8 of the *P53* gene. Overexpression of the p53 protein could be due to the fact that the mutation is in fact located outside of the exon 5-8 region. Only exons 5-8 were sequenced in the six cases of PDV and PDB in the current study. However, mutational analysis of the whole *P53* genome have shown that it is uncommon for mutations to lie outside the region of exons 5-8. Different cellular and environmental conditions may cause the accumulation of wt

p53. DNA damage from cytotoxic drugs, smoking and viruses are known to be inducers of p53 and hence cause the accumulation of the p53 protein.

PDV and PDB may habour molecular changes that could inactivate the p53 pathway and hence the accumulation of wild type p53.

3.4.4. Further work:

More samples need to be analysed to assess *P53* mutations in PDV and PDB. Applying LCM to this work would be valuable in assessing clonality of PDV and PDB. This would allow us to determine the origin of the Paget cells in those cases of PDV with invasive/associated adenocarcinoma. DNA chip analysis could be a method used to assess the *P53* gene as it combines a good sensitivity and a high throughput (Ahrendt et al., 1999).

CHAPTER 4

MARKERS OF ANGIOGENESIS IN PAGET'S DISEASE OF THE VULVA AND THE BREAST

Poster Presentations:

VEGF-A and disease progression in Paget's disease of the vulva and breast. **Ellis P.**, Wong Te Fong L.F., Rolfe K.J. MacLean A.B Perrett C.W 181st meeting of the Pathological Society Nottingham July 2000.

PDECGF/TP expression in Paget's disease of the vulva. Blair Bell Society Meeting. **Ellis P.**, Wong Te Fong L.F., Rolfe K.J. MacLean A.B Perrett C.W London Oct 2000. .

Oral presentation:

An examination of the role of VEGF-A and PD-ECGF/TP in Paget's disease of the vulva and breast. **Ellis P.**, Wong Te Fong L.F., Rolfe K.J. MacLean A.B Perrett C.W. International institute of Anticancer Research. Athens Greece June 2001

Abstract:
Vascular Endothelial Growth Factor (VEGF) and disease progression in Paget's disease of the vulva and breast. **Ellis P.**, Wong Te Fong L.F., Rolfe K.J. MacLean A.B Perrett C.W. Journal of Pathology 192ss:P23A 2000

Full publication:

Ellis P.E., Wong Te Fong L.F., Rolfe K.J., Crow J.C., Reid W.M.N., Davidson T., MacLean A.B Perrett C.W (2002). The role of vascular endothelial growth factor-A (VEGF-A) and Platelet-derived endothelial cell growth factor/ thymidine phosphorylase (PD-ECGF/TP) in Paget's disease of the vulva and breast. Anticancer Res.22: 857-861.

4.1. INTRODUCTION

4.1.1. Angiogenesis

Angiogenesisis is the formation of new capillary blood vessels from pre-existing microvessels. Vasculogenesis is the process whereby the early vascular plexus forms from the mesoderm by the differentation of angioblasts (vascular endothelial cells that have not yet formed a lumen), which subsequently generate primitive blood vessels. Mesoderm-inducing factors of the fibroblast growth factor family are crucial in inducing paraxial and lateral plate mesoderm to form angioblasts and haematopoietic cells. Angiogenesis precedes and sustains tissue growth and therefore plays a critical role in normal tissue physiological processes such as reproduction, wound healing or bone remodelling (Philips et al., 1990; Brown et al.,1992 ; Clarke et al.,1996). Angiogenesis involves the migration of endothelial cells from the parent vessel towards a chemo-attractant. As well as the ability to promote endothelial cell migration, angiogenic factors must also degrade the matrix through which the cells move. Endothelial cell invasion, as part of the angiogenic process, involves secretion of urokinase-type plasminogen activator and its inhibitor (PAI-1). Fine maintenance of the proteolytic balance seems to be crucial for the correct development of new blood vessels. Having migrated, the endothelial cells must proliferate, lay down their own basement membrane and form a lumen, which is the basis of the new capillary. This sequence of events-cell migration, matrix degradation and proliferation is the basis of angiogenesis. Many different factors have been identified which promote the proliferation, migration and morphogenesis of new blood vessels, both in embryonic development and in diseases such as cancer. The most extensively studied is vascular endothelial growth factor (VEGF).

4.1.1.i. Tumour Angiogenesis

Angiogenesis is an important process in tumour growth and metastasis (Figure 4.1) and allows tumours to facilitate their expansion beyond a certain volume (Folkman, 1990, 1995). In theory, the development of tumours is associated with two phases of tumour growth (Brem et al., 1977; Adcock et al.,1982; Burke et al., 1987; Folkman et al., 1987;). First is the "pre-vascular phase", which is seen with various intraepithelial neoplasias, can persist for many years, and is not capable of metastasis (Weidner et al., 1991). In contrast, the "vascular phase" is characterised by rapid tumour growth and the potential for metastasis (Weidner et al., 1991). The vascular phase, otherwise termed the "angiogenic phase', occurs when a tumour possesses the innate ability to potentiate its own growth. Oncogenesis of this type, whereby the extent of neovascularisation is directly correlated with metastasis, has been demonstrated in several cancers e.g. breast carcinoma (Weidner et al., 1991) and in cutaneous melanoma (Brem et al., 1977; Adcock et al., 1982; Burke et al., 1987). In situ carcinomas may exist for a long time without neovascularisation and, as a consequence, they remain limited to a small ~ few mm^3. Some tumour cells then switch to the angiogenic phenotype and recruit new capillary blood vessels that support the growth of both the angiogenic and non-angiogenic cells (Folkman et al.,1989; Kandel et al., 1991; Weidner et al., 1991; Folkman, 1994) . Expansion of the tumour mass is made possible, not only because of perfusion of blood throughout the tumour, but also because of the paracrine stimulation of tumour cells by numerous growth factors and matrix proteins that are produced by the new capillary endothelium (Nicosia et al., 1986; Hamada et al., 1992; Rak et al., 1994). The switch to the angiogenic phenotype itself depends on a net balance of positive angiogenic factors released by the tumour (Sinevad et al.,1989; Bouck et al., 1990; Dameron et

al., 1994). The positive factors include α fibroblast growth factor (FGF) (Maciag et al., 1984), βFGF (Shing et al.,1984), VEGF (Ferrara et al., 1989), angiogenin (Fett et al.,1985) and angiopoietin -1. (Folkman and Shing, 1992). They can be exported from tumour cells, mobilised from the extracellular matrix, or released by macrophages attracted to the tumour. Although this increased production of positive angiogenic factors (Table 4.1) is necessary, it is not sufficient for the angiogenic phenotype. Negative regulators of angiogenesis must also be decreased. Of several naturally occurring negative regulators of angiogenesis thrombospondin-1 was the first for which it was demonstrated that the inhibitor is produced constitutively by normal cells but down-regulated during tumourigenesis. Thus, by the time tumour cells have become angiogenic, they are producing only 4-6% of the thrombospondin originally generated by their normal precursor cells (Folkman and Shing, 1992).

Table 4.1 Some endogenous regulators of angiogenesis

Stimulators of angiogenesis	**Inhibitors of angiogenesis**
VEGF	Thrombospondin-1
PDECGF/TP	Vascular endothelial growth factor inhibitor
Angiopoietin -1 and Angiopoietin-2	Angiostatin
Insulin –like growth factor	Endostatin
Fibroblast growth factor	Vasostatin
Epidermal growth factor	Fragment of platelet factor 4
Placental growth factor	Derivative of prolactin
Tumour necrosis factor	Interleukin- 1,12
Matrix metalloproteinase	Restin
Ephrin family	Placental proliferin-related protein
Transforming growth factor	Anti-thrombin III
Granulocyte macrophage colony stimulating factor	Meth-1and Meth-2
Interleukin-8	Interferon α and Interferon β

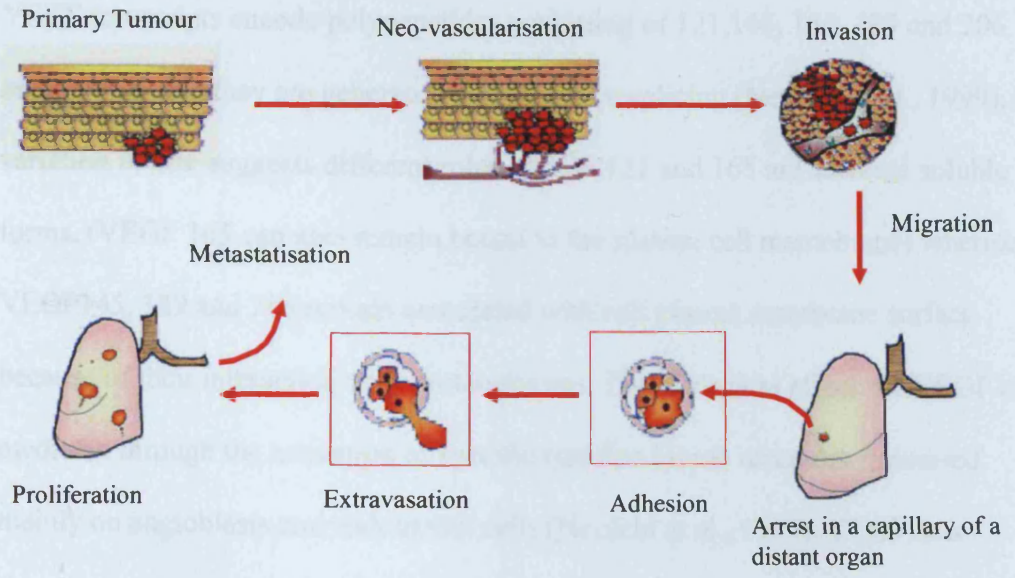

Figure 4.1 An example of tumour angiogenesis.

Ref www.itb.cnr.it

4.1.2. VEGF

Vascular endothelial growth factor (VEGF), a heparin binding glycoprotein, was originally identified in the media conditioned by normal bovine pituitary folliculo-stellate cells ; Ferrara and Henzel ,1989) and by a variety of transformed cell lines (Levy et al.,1989;Tisher et al., 1989; Conn et al.,1990) It is a multifunctional cytokine with potent angiogenic activity. VEGF stimulates angiogenesis through its action as an endothelial mitogen and its ability to increase vascular permeability. There are several members of the VEGF family (Table 4.2): VEGF, VEGF-B, VEGF-C (Andre et al.2000), VEGF-D (Olofson et al.,1998), VEGF-E, and the structurally-related placenta growth factors (PlGFs 1-3). VEGF, VEGF B, VEGF E and PlGF stimulate angiogenesis as well migration and proliferation of endothelial cells. VEGF C and VEGF D have been shown to stimulate lymphangiogenesis and

act as lymphangiogenic growth factors (McColl et al., 2004). The five isoforms of the VEGF transcripts encode polypepetides consisting of 121,145, 165, 189 and 206 amino acids and they are generated by alternative splicing (Neufeld et al., 1999). The variation in size suggests different roles. VEGF 121 and 165 are secreted soluble forms, (VEGF 165 can also remain bound to the plasma cell memebrane) whereas VEGF145, 189 and 206 remain associated with cell plasma membrane surface because of their interaction with proteoglycans. The biological effects of VEGF are mediated through the activation of specific tyrosine kinase receptors expressed mainly on angioblasts and endothelial cells (Neufeld et al., 1999). VEGF acts selectively on endothelial cells by binding to specific class III receptor tyrosine kinases. The receptor VEGFR-2 binds VEGF, VEGF-C and VEGF-D and promotes the proliferation and motility of endothelial cells. VEGF-R1 shows 10-fold lower kinase activity than VEGF-R2, and has been proposed to act as a negative regulator of VEGF-induced endothelial –cell proliferation (Hiratsuka et al., 1998). VEGF-R3, which in adults is exclusively expressed in lymph node endothelial cells, binds VEGF-C and VEGF-D (Neufeld et al., 1999). VEGF is distributed widely in different tissues. Several studies have demonstrated its over expression in a variety of tumours including those of the breast (Toi et al.,1994) and vulva (MacLean et al.,2000). Anti VEGF therapy has been used in clinical trials to inhibit angiogenesis and is showing promising results in cancer treatment therapies. Bevacizumab, a recombinant humanized monoclonal antibody to VEGF has been shown to induce apoptosis of breast cancer cells and is currently been used in combination with 5-FU for the first-line treatment of metastatic colorectal cancer (Wedham et al., 2006). Figure 4.2 demonstrate VEGF involvement in the angiogenesis signalling cascade.

Table 4.2 Roles of VEGF receptors and respective ligands

VEGF receptor	Ligands	Function
VEGFR-1	VEGF , VEGF-B, PlGF	Essential for vascular development, VEGF signalling, release of growth factors
VEGFR-2	VEGF, VEGF-C, VEGF-D VEGF-E	Essential for vascular development, proliferation, migration, angiogenesis
VEGFR-3	VEGF-C, VEGF-D	Proliferation, migration, role in vascular development, signals for lymphangiogenesis

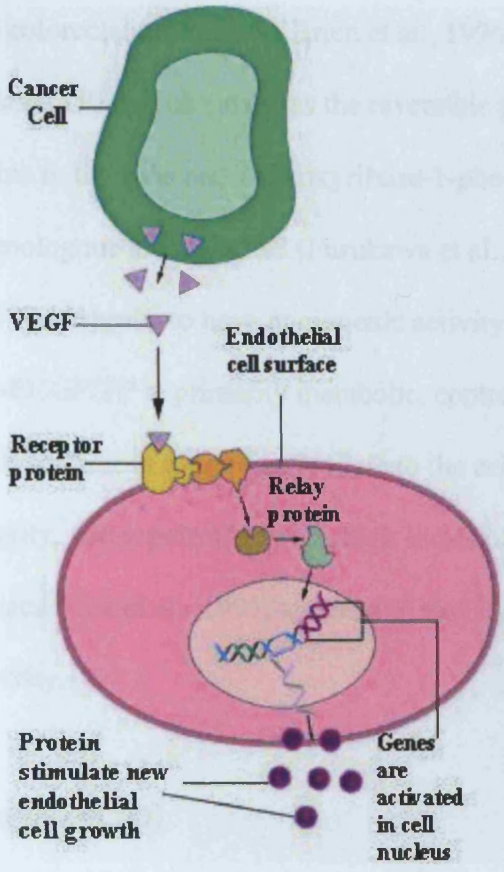

Figure 4.2 The Angiogenesis signalling cascade.

Ref: rex.nci.nih.gov/behind the news/uangio/13uangio/13uangio.htm

4.1.3. Platelet derived- endothelial growth factor/Thymidine phosphorylase

Originally isolated from platelets in 1987, platelet-derived endothelial growth factor (PD-ECGF) is a 47 kilo Dalton (kDa) protein that promotes cell growth and chemotaxis in endothelial cells in vitro and angiogenesis in vivo (Ishikawa et al., 1998). It is a non-glycosylated intracellular protein, with no heparin binding activity, found in various tissues such as placenta, lung and endometrium (Fox et al., 1995) as well as in certain cancers e.g vulva (Wong Te-Fong et al.,2000), breast (Yonenga et

al., 1998) bladder and colorectal tumours (O'Brien et al., 1996). The enzyme thymidine phosphorylase (TP), which catalyses the reversible phosphorylitic breakdown of thymidine to thymine and 2- deoxyribose-1-phosphate, has been identified as being homologous to PD-ECGF (Furukawa et al., 1992). The by-product, 2-deoxy-D-ribose, has been shown to have angiogenic activity (Haraguchi et al., 1994). The role of PD-ECGF/TP is primarily metabolic, controlling the thymidine levels in the cell. Accumulation of thymidine is toxic to the cells and causes errors in DNA replication, integrity, and repair. However, high levels of TP have been identified in macrophages (Fox et al., 1995) suggesting that it provides other functions such as angiogenic activity.

4.1.4. Microvessel density (MVD)

MVD is a measure of tumour angiogenesis. Several studies have demonstrated an association between increasing tumour MVD and prognosis in solid tumours (Weidner, 1995, 1997 Weidner and Folkman,1996). MVD has been shown to be a prognostic indicator in breast cancer (Tsutsui et al., 2003) and vulva cancer (Obermair et al.,1996), and is increased in cases of VIN 3 as compared to cases of VIN1 and VIN 2 (Saravanamuthu et al., 2003). It is considered the gold standard in estimating angiogenesis.

4.1.5. Endothelial cell markers

A variety of endothelial cell markers have been used to identify microvessels for the purpose of counting. The most commonly used include factor VIII related antigen (F8RA)/ von Willebrand factor (vWF), CD31/PECAM-1, and CD34 (Fina et al., 1990; Johnson et al., 1993). F8RA forms part of the vWF complex and plays a critical

role in the process of haemostasis (Franchini et al. 2006). CD31(PECAM-1) a platelet- endothelial cell adhesion molecule is a transmembrane glycoprotein involved in cell adhesion (DeLiser et al., 1994) and CD34 is a surface glycoprotein of unknown function (Krause et al., 1996). Tissue sections are stained with antibodies specific for the endothelial cell marker expressed by the vascular endothelial cells. Ideally the markers should be specific, only being expressed by endothelial cells and not other cell types, and occur on endothelial cells.

In our group three endothelial markers have been used, vWF, CD31 andCD34 to measure tumour vascularity. vWF was chosen as the endothelial cell marker to be used in this study due to its consistent staining and good contrast between microvessels and other components.

The objective of this study was to determine whether the expression of angiogenic growth factors VEGF, PD-ECGF/TP, and MVD differed between intra-epithelial PDV and those cases of Paget's disease associated with an invasive adenocarcinoma, and their expression in PDB, in order to investigate their role as potential prognostic marker and indicate some of the molecular mechanisms underlying PDV and PDB angiogenesis. I also investigated whether growth factors worked synergistically.

4.2. METHODS

4.2.1. Sample collection and preparation

Table 4.3 Number of PDV archival cases available for IHC

Marker	PDV without invasive disease	PDV with invasive disease
VEGF	44	10
PD-ECGF/TP	44	10
VWF	60	8

Table 4.4 Number of PDB archival cases available for IHC

Marker	PDB	PDB with DICS alone	PDB with DCIS and invasive carcinoma	PDB with invasive carcinoma
VEGF	38	14	6	6
PD-ECGF/TP	38	14	6	6
VWF	33	13	6	6

Preparation and collection was as per section 2.2.1, chapter 2, Pages 85.

4.2.2.Immunohistochemical staining with the VEGF-A, PD-ECGF/TP and vWF (F8RA) antibodies

Sections were deparaffinised in xylene and rehydrated in different percentages of ethanol up to distilled water for 10 min. 3% hydrogen peroxide was placed on the sections to block endogenous peroxidase for 10 min. They were then placed in distilled water for 10 min at 37°C.

Optimisation of the protocol was performed for all primary antibodies as per section 2.2.2, chapter 2, Pages 86. Optimisation of the antigen retrieval method was performed using 3 different methods for each protein; microwaving in sodium citrate buffer (pH 6.0) for 10 mins at 750 watts; protease digestion (bacterial protease Type 24, Sigma) using 12.5mg in 100ml of PBS at 37°C for 10mins, and finally using no antigen retrieval. Different protease digestion times (using different time intervals of 5, 7.5,10 and 15mins.) and protease concentrations (6, 10, 12.5, and 15mg) as well as different incubation times and temperatures were also tried. The optimal incubation time and temperature was identified when specific staining was achieved with the lowest concentration of antibody but still providing intense staining. The optimal protease concentration used was12.5mg and the digestion time was 10mins. The

endothelial cell markers were each assessed in terms of specificity and staining. CD31/PECAM-1 and CD34 cross reacted with other tissue components such as macrophages. vWF gave the most consistent staining pattern. Table 4.5 summarises the optimal conditions used for IHC.

Table 4.5 Antibodies and optimum conditions used for immunohistochemistry

Antigen	Source	Clone	Working dilution	Antigen retrieval	Positive Control	Negative control	Incubation Time	Incubation temp.
Human VEGF	R&D Systems Abingdon, UK	26503.11, IgG_2B	1:40	Protease digestion	Placenta	IgG_2B	overnight	4°C
Human PD-ECGF/TP	John Radcliffe Hospital, Oxford,UK	P-GF.44C	1:10	Microwave	Placenta	TBS	1 hr	RT
vWF	Dako,Ely, Cambs,UK	F8/86	1:40	Protease digestion	Placenta	PBS	1 hr	RT
CD34	Serotec, Oxfordshire, UK	QBEN D/10	1:300	Microwave	Placenta	PBS	1 hr	RT
CD31	Dako,Ely, Cambs,UK	JC/70A	1:50	Microwave	Placenta	PBS	1 hr	RT

Biotinylated rabbit anti-mouse serum (Dako, Cambs. UK) dilution 1:400 inTBS, PBS (vWF) was used as the secondary antibody in all cases.

4.2.3 MVD counting

Any brown staining endothelial cell or endothelial cell cluster that was clearly separate from adjacent microvessels, tumour cells, and other connective tissue elements was considered as a single countable microvessel. Large vessels with lumina greater than approximately seven red blood cells were excluded from the count. Blood vessels were detected by a method similar to Bosari et al. (1992).

Areas of highest neovascularisation, i.e containing the highest number of capillaries and small venules per area (" hot spots") were found by scanning the whole tissue section at low power (x 40 and x100) using a light microscope. Five fields in each section with the highest number of hot spots were selected. The highest vessel density

(HVD) of five fields at x 200 field (0.74mm^2 under the light microscope) and x 400 field (0.17mm^2 under the light microscope) was recorded and the average vessel density (AVD) was also recorded in these five fields at x200 and x400. This was repeated using the HVD and AVD of three fields. The area of HVD and AVD using five fields did not differ significantly from the values obtained using three fields and therefore the analysis was performed using three fields. Individual MVD were made at both x200 and x400 magnification within each hot spot. The MVD is confined to an area within 500μm of dermal tissue just beneath the basement membrane of the epidermis and expressed as HVD/AVD per mm^2.

Sections were stained on 3 separate occasions to ensure reproducibility. Results were analysed by three independent observers (PEE, ACWTF and JCC). In all cases there was <5% variation in results between sections and observers.

4.2.4. Scoring

4.2.4.i. VEGF quantification

The slides were viewed under a light microscope at a magnification of x100. The presence of VEGF was quantified by counting the number of positive (brown) focal areas representing VEGF per cm^2 of tissue section and scoring them accordingly. VEGF imunostaining was examined for in both the cytoplasm and nucleus of the cells. A section was scored positive if 10 or more focal areas were present in the dermis and epidermis combined. A focal area consisted of 10 or more stained cells. The number of focal areas in the epidermis and dermis were also recorded. The blood vessels of the placenta express VEGF and were used as the positive control. When scoring the sections for VEGF, the positive control is checked alongside the PDV or PDB section to determine whether VEGF is expressed in the section.

4.2.4.ii. PD-ECGF/TP quantification

A section was scored positive for PD-ECGF/TP if 10% or more Paget cells were stained brown at x100 magnification. Samples were assessed to the nearest 5% of cells showing positivity. Both nuclear and cytoplasmic positivity was counted. PD-ECGF/TP expression was noted in the epidermal and dermal cells. The intensity of staining was also recorded; 1= mild intensity; 2 =moderate intensity and 3=strong intensity.

4.2.5. Statistical analysis

Statistical analysis was performed using the Fisher exact test for PD-ECGF/TP and VEGF expression. The Mann-Whitney U test was used to compare MVD expression between invasive and non invasive cases of PDV and cases of PDB with DCIS and invasive carcinoma. The McNemar's chi squared test was used for analysing double marker expression of VEGF and PD-ECGF/TP. A p value of <0.05 was considered significant.

4.3. RESULTS

4.3.1. VEGF expression in PDV and PDB

None of the Paget cells present in PDV or PDB expressed VEGF. Positive focal areas of VEGF expression (Figure 4.3) were found within the epidermal and dermal cells in 14 cases of PDV. Of the fourteen cases 5 were PDV with invasive disease. VEGF expression (Figures 4.5 and 4.6) was also seen in the epidermal and dermal cells in 23 cases of PDB, seventeen of these cases were associated with an underlying tumour. VEGF expression was found to be significantly higher ($p=0.001$) in the breast epidermal and dermal cells compared to the vulval epidermal and dermal cells.

Table 4.6 and 4.7 summarises the results.

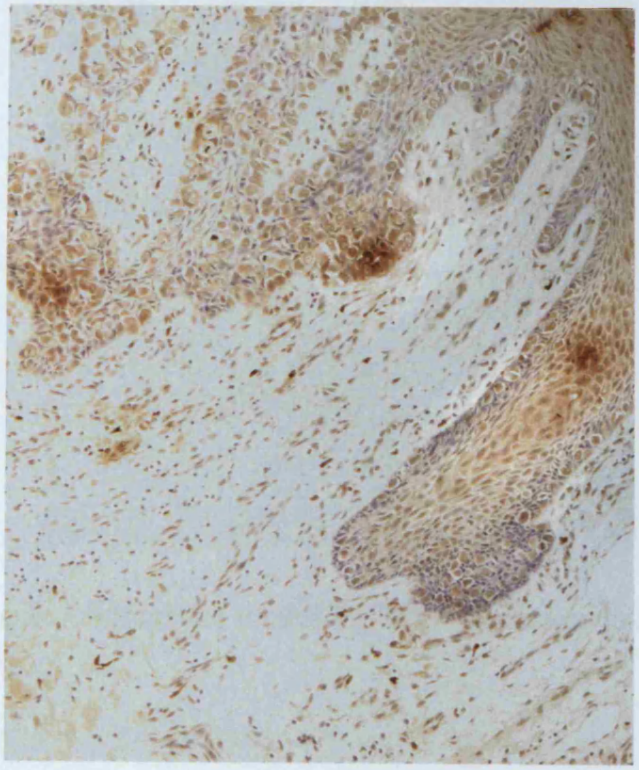

Figure 4.3 VEGF expression in PDV (x 200).

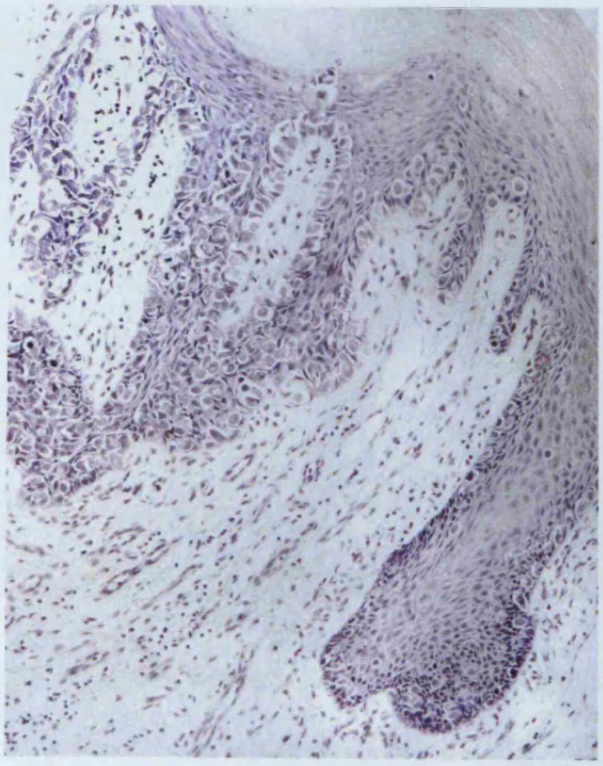

Figure 4.4 Negative control for VEGF expression in PDV (x 200).

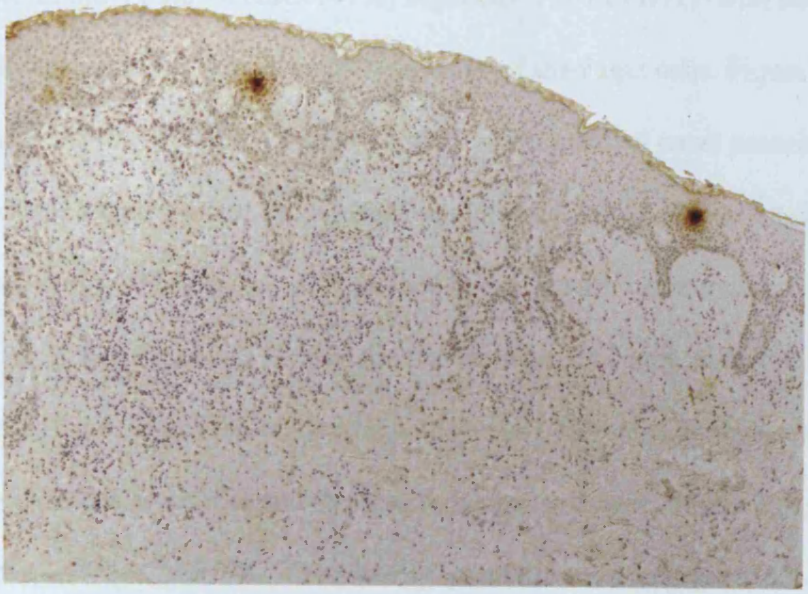

Figure 4.5 Two VEGF focal areas in PDB (x 50).

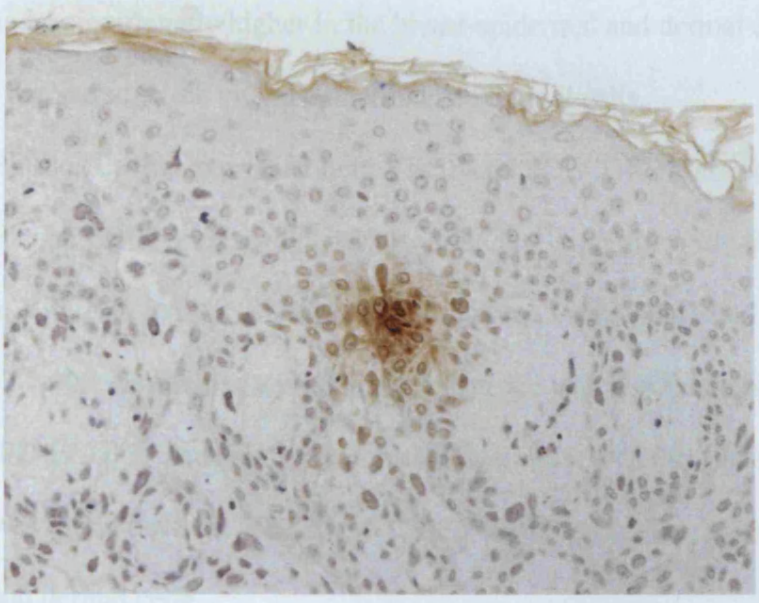

Figure 4.6 A VEGF focal area within stromal cells of the dermis in PDB (x 400).

4.3.2. PD-ECGF/TP expression in PDV and PDB.

Twenty-two of the 54 vulval cases (41%) expressed PD-ECGF/TP with both nuclear and cytoplasmic staining present in 50% or more of the Paget cells. Figure 4.7 demonstrates PD-ECGF/TP expression in PDV. Of the vulval cases associated with invasive adenocarcinoma four (40%) expressed PD-ECGF/TP. Table 4.8 demonstrate the results. Seventy-five percent of Paget cells were positive in one case, 50% of the cells positive in two cases and 20% in the remaining case. There was no difference in the intensity of staining of the epidermal and dermal cells between those cases of PDV without invasive disease and those with invasive disease. In PDB 21/38 (55%) cases expressed PD-ECGF/TP. The staining intensity was moderate and the range of positivity was 10-75% of cells. PD-ECGF/TP expression in the underlying breast DCIS and invasive carcinoma is demonstrated in Table 4.9. PD-ECGF/TP expression

was noted to be significantly higher in the breast epidermal and dermal cells ($p<0.0005$) compared to the vulval epidermal and dermal cells.

PDV and PDB cases that expressed both VEGF and PDECGF/TP are shown in Table 4.6. PD-ECGF/TP expression was also found to be significantly different compared to VEGF in the vulval Paget cells and in the vulval epidermal and dermal cells with $p < 0.001$. Similiarly, a significant difference was also found between VEGF and PD-ECGF/TP expression in breast Paget cells, $p < 0.001$. No significant difference was found between VEGF and PD-ECGF/TP expression in breast epidermal and dermal cells.

Table 4.6 VEGF and PD-ECGF/TP expression in PDV and PDB.

Location	VEGF expression	PDECGF/TP expression	VEGF/ PD-ECGF/TP double marker expression
Vulval Paget cells	0/54	22/54 (41%)	-
Vulval epidermal and dermal cells	14/ 54 (26%)	29/54 (54%)	12/14 (85%)
Breast Paget cells	0/38	21/38 (55%)	-
Breast epidermal and dermal cells	23/38 (61%)	36/38 (95%)	19/38 (50%)

Table 4.7 VEGF expression and associated underlying breast tumour

Cases	VEGF expression in epidermal/dermal cells and underlying tumour
PDB with DCIS	11/13 (85%)
PDB with DCIS and carcinoma	2/6 (33%)
PDB with carcinoma alone	4/6 (67%)

Table 4.8 Immunoreactivity for PD-ECGF/TP expression in relation to invasive carcinoma

Location	Without invasive adenoncarcinoma	With invasive adenocarcinoma
Vulva	18/44 (41%)	4/10 (40%)

Table 4.9 PD-ECGF/TP expression in breast Paget cells and underlying DCIS and invasive carcinoma

Cases	%Paget cells stained	Underlying tumour and % positive
Case 90	20%	Carcinoma 75%
Case 91	75%	Carcinoma 75%
Case 96	50%	DCIS/Carcinoma 10%
Case 98	0%	DCIS 0%
Case 99	50%	DCIS 75%
Case 102	0%	DCIS 50%
Case 103	75%	Carcinoma 75%
Case 113	40%	Carcinoma 10%
Case 116	5%	DCIS/Carcinoma 5%
Case 143	0%	Carcinoma 50%

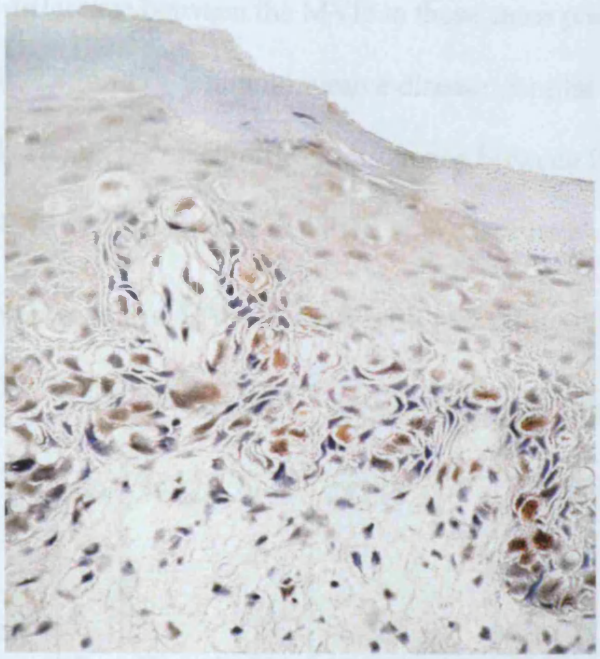

Figure 4.7 PD-ECGF/TP expression in PDV (x200).

4.3.3. Microvessel Density in PDV and PDB

There appeared to be no significant difference in the MVD in intraepidermal PDV as compared with PDV associated with invasive disease. Figures 4.8 and 4.9 demonstrate microvessels in PDV. MVD was also found to be similar in PDB associated with DCIS, invasive disease and PDB alone. Figure 4.10 demonstrates microvessels in PDB. The mean HVD and AVD values are summarised in Tables 4.10 and 4.11.

Correlation with VEGF and MVD, PD-ECGF/TP and MVD expression in intraepidermal PDV and PDV with invasive disease x200 field are shown in Tables 4.12A and 4.12B. There appeared to be a trend towards higher HVD and AVD in VEGF and PD-ECGF/TP positive cases of invasive PDV compared to those cases that did not express VEGF or PD-ECGF/TP. However, the numbers were too small to perform statistical analysis. Similar results were demonstrated at x 400 field. There

was no significant difference between the MVD in those cases positive or negative for VEGF or PD-ECGF/TP in PDV without invasive disease. Similar analysis was performed on PDB. There was no significant difference between PDB cases with DCIS, PDB with DCIS and carcinoma, and PDB with carcinoma.

Table 4.10 Mean values of the MVD in PDV with and without invasive disease

Cases	*n*	HVD(x200)	AVD(x200)	HVD(x400)	AVD(x400)
PDV without invasive disease	60	39 (22.7)	27 (19.3)	60.5 (35.3)	40.1 (22.3)
PDV with invasive disease	8	27.6 (26.3)	19.1 (17.97)	71.2 (55)	51.2 (31.8)

n, number of cases. The SDs of the mean are given in brackets.

Table 4.11 Mean values of the MVD in PDB

Cases	*n*	HVD(x200)	AVD(x200)	HVD(x400)	AVD(x400)
PDB with DCIS	13	34.1 (17.7)	23 (16.1)	68 (18.4)	50.9 (18.2)
PDB with DCIS/ invasive carcinoma	6	19.7 (13.5)	15.8 (12.0)	53 (27.1)	43.7 (22.3)
PDB with invasive carcinoma	6	31.1 (5.4)	22.8 (4.3)	114.7 (82.3)	95 (71.8)

n, number of cases. The SDs of the mean are given in brackets.

Table 4.12A. MVD in correlation to VEGF and PD-ECGF/TP in PDV cases without invasive disease

PDV without invasive disease	*n*	Mean HVD (x200)	Mean AVD (x200)
VEGF positive	19	44.6 (27.9)	32.4 (27.7)
VEGF negative	17	33.4 (22.7)	23.24 (16.9)
PD-ECGF/TP positive	25	41.2 (27.7)	29.3 (25.9)
PD-ECGF/TP negative	21	41.4 (19.9)	29.1 (13.9)

n, number of cases. The SDs of the mean are given in brackets.

Table 4.12B. MVD in correlation to VEGF and PD-ECGF/TP in PDV cases with invasive disease

PDV with invasive disease	*n*	Mean HVD (x 200)	Mean AVD (x 200)
VEGF positive	4	40.5 (27.2)	28.04 (16.9)
VEGF negative	3	4.1 (0)	2.2 (0.78)
PD-ECGF/TP positive	4	34.1 (32.9)	23.5 (21.67)
PD-ECGF/TP negative	2	16.9 (18.1)	11.5 (11.9)

n, number of cases. The SDs of the mean are given in brackets.

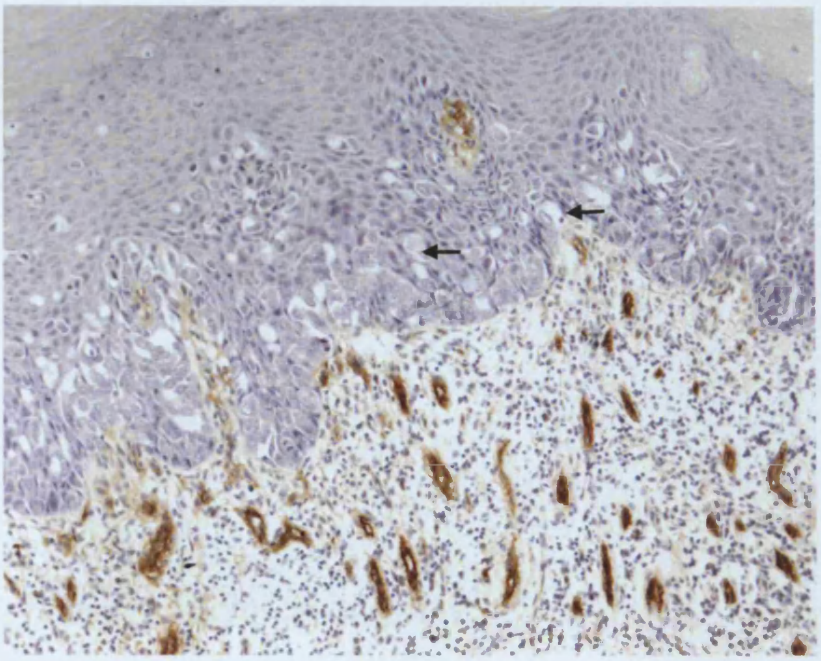

Figure 4.8 vWF expression demonstrating microvessels in PDV (x200).
Arrows = Paget cells.

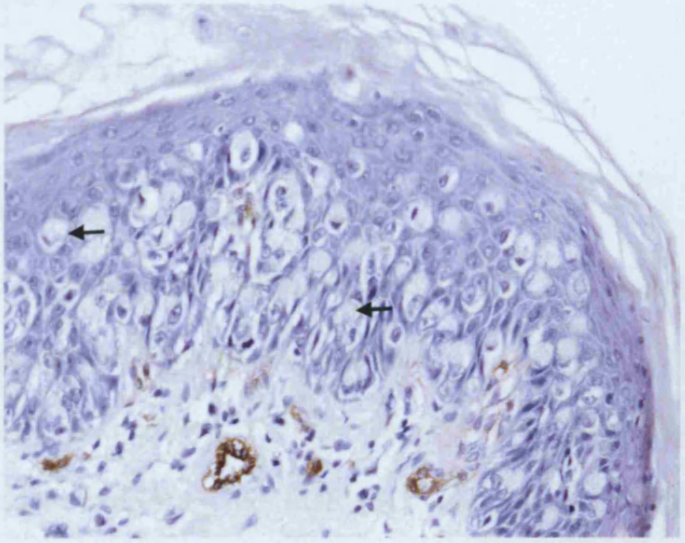

Figure 4.9 vWF expression demonstrating microvessels in PDV (x200).
Arrows = Paget cells.

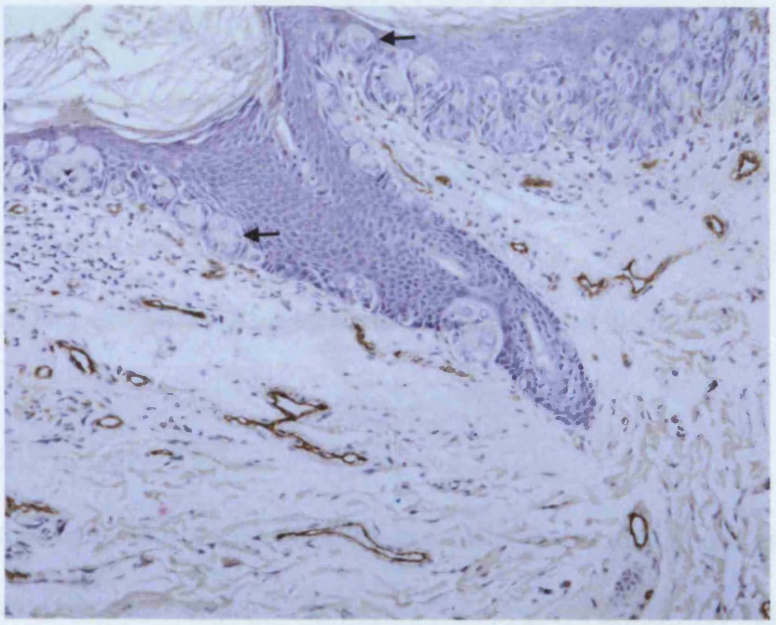

Figure 4.10 vWF expression demonstrating microvessels in PDB (x200).
Arrows = Paget cells.

4.4. DISCUSSION

4.4.1. VEGF expression in PDV

4.4.1.i VEGF in general pre-malignant lesions

It is becoming increasingly apparent that angiogenesis may be acquired early in tumour development and increased angiogenic activity has been reported in several pre-malignant lesions. Guidi et al. (1995) examined the association between angiogenesis and the expression of VEGF mRNA in cervical intraepithelial neoplasia and invasive cervical carcinoma by in situ hybridisation. From their results they demonstrated that VEGF expression and MVD were significantly increased in high-grade intraepithelial lesions and invasive cervical carcinoma compared to low-grade cervical lesions and benign squamous epithelium. Dobbs et al., (1997) demonstrating VEGF expression using immunohistochemistry found similar results; a significant

increase in VEGF and MVD expression from normal cervix through CIN I to CIN III to invasive squamous cell carcinoma. VEGF expression has also been found to be significantly upregulated in oesophageal metaplasia, dysplasia and adenocarcinoma of the oesophagus (Kitadai et al., 2004; Vallbohmer et al., 2006) and benign, premalignant and malignant prostate tissue (Kollermann et al., 2001)

4.4.1.ii VEGF in vulval lesions

Several studies have demonstrated VEGF expression in pre-malignant and malignant vulval disease (Obermair et al., 1996; Bancher-Todesca et al., 1997; MacLean et al. 2000). Bancher-Todesca et al., using immunohistochemistry, examined the expression of VEGF and microvessel density (MVD) in vulval intraepithelial neoplasia (VIN). They demonstrated that for both VEGF expression and MVD the differences between VIN I and VIN III and between VIN II and VIN III were statistically significant and the highest values were found in VIN III. In addition, it has been reported that patients with vulval tumours with a high MVD and moderate to strong VEGF expression had a poorer survival rate compared to those patients with low MVD or weak VEGF expression (Obermair et al., 1996). In contrast, Doldi et al. demonstrated that VEGF mRNA expression was high in VIN, with no difference between VIN I and VIN III, but low in vulval cancer. Work from our group (MacLean et al., 2000) has shown that VEGF is not expressed in normal vulval tissue but is expressed in 6% of VIN lesions and 92% of vulval cancers. Wong Te-Fong, et al (2000) has demonstrated similar results. Such data suggest that VEGF in both invasive and potentially invasive (VIN) lesions of the vulva may be involved in malignant progression and may be a valuable marker of vulval lesions which go on to become invasive.

To my knowledge there has been no previous study that has examined VEGF expression in Paget's disease of the vulva. VEGF was not expressed by Paget cells, even in those cases associated with invasive disease. However, VEGF expression was found in the epidermal and dermal cells in 26% cases of PDV, five of these were cases with invasive disease. The significance of this is not clear. As stated earlier, VEGF is not expressed in normal vulval lesions (MacLean et al., 2000). It is therefore possible that in some cases of PDV the presence of Paget cells stimulate epidermal and dermal cells to release VEGF and instigate angiogenesis.

4.4.2. PD-ECGF/TP expression in PDV

4.4.2.i PD-ECGF/TP in pre-malignant and malignant lesions

Very few studies have examined PD-ECGF/TP expression in precancerous lesions. Increased expression has been demonstrated in dysplastic oesophageal tissue as compared to normal oesophageal mucosa (Kitadai et al, 2004). It has also been examined in cervical intraepithelial neoplasia and invasive cervical carcinoma (Isaka et al., 2002) and vulva intraepithelial neoplasia (Wong-Te-Fong et al., 2000; MacLean et al., 2000) These studies demonstrate an increase in the expression or intensity of PD-ECGF/TP in invasive cervical carcinoma and vulvar cancer relative to the premalignant lesions and suggest that PD-ECGF/TP may have a prognostic value.

Because of the relative rarity of PDV, my numbers are small; however, 40% of the PDV cases with invasive disease expressed PD-ECGF/TP compared to 41% of cases without invasive disease. Epidermal and dermal cells were positive in 54% of cases. There was a signifcant difference in expression of PD-ECGF/TP and VEGF in the vulval Paget cells and the vulval epidermal and dermal cells. The data suggest that

PD-ECGF/TP maybe involved in PDV pathogenesis, but it is not a marker of malignant progression of PDV.

4.4.3. Double marker expression in PDV

One of the questions that remained to be answered was whether VEGF and PD-ECGF/TP acts synergistically. Wong-Te-Fong et al., (2000) and MacLean et al., (2000) have both assessed these angiogenic factors in VIN and vulval cancers. There appeared to be no relationship between VEGF and PD-ECGF/TP expression in VIN and vulva cancers. In the current study I found a good correlation between VEGF in the epidermal and dermal cells, and PD- ECGF/TP. Eighty five percent of cases coexpressed both markers in PDV. This suggests a possible synergistic effect but whether this effect plays a role in the angiogenesis and the malignant transformation of PDV remains to be elucidated.

4.4.4. MVD in PDV

The method of MVD has been used in many studies to investigate the role of angiogenesis in patients with cancer. The role of angiogenesis as determined by MVD has been examined in vulvar lichen scleorosus, VIN and vulvar cancer (Bancher-Todesca et al., 1997; Saravanamuthu et al., 2003) MVD is not a useful parameter in determining potential malignant progression in vulvar lichen sclerosis but could be valuable in VIN 3 in determing progression to invasive disease (Saravanamuthu et al., 2003). Other techniques have been used to measure tumour angiogenesis. The Chalkey method measures the relative area of vessel profile in a high-density region of the tumour as compared to MVD, which measures the density

of the vessels (Hansen et al., 2004). Hollingsworth et al. (1995) described a method using vascular volume. Determination of vessel density by vascular volume represents an average of the entire section rather than focusing on areas of most intense neovascularisation and therefore does not reflect the angiogenic activity of tumour cells or metastatic potential.

vWF was used as the endothelial cell marker of choice because of its consistent staining and it was less likely to react with other tissue components as compared to CD31 and CD34 endothelial cell markers. However, vWF does have its limitations; it is unable to distinguish between new and pre-exisitng blood vessels; the monoclonal antibody E-9 is the only endothelial marker capable of distinguishing between the two but can only be used on frozen sections (Wang et al.,1994). vWF can also be identified in lymphatic endothelial cells and platelets (Mietten et al., 1994). From the results obtained in my study, MVD appears not to be a useful parameter in determining which cases of PDV will develop invasive disease. There did appear to be a trend towards higher MVD in VEGFand PD-ECGF/TP positive cases in PDV with invasive disease as compared to those cases that did not express VEGF or PD-ECGF/TP.

4.4.5 VEGF expression in PDB

4.4.5.i VEGF in preinvasive breast disease

Angiogenesis in preinvasive lesions may differ from that seen in invasive tumours. The increase in vessels is seen in the normal stroma around or beneath the in situ tumour rather than between clusters of tumour cells, as in invasive disease. Therefore, in in situ tumours, angiogenic factors produced by inflammatory and

stromal cells may play a greater role than those derived from the tumour cell themselves (Rice et al., 2002)

Up-regulation of VEGF protein and mRNA expression has been reported in DCIS and other preinvasive lesions of the breast. (Yoshiji et al., 1996; Guidi et al.,1997 Heffelfinger et al., 1999; Viacara et al., 2004). VEGF mRNA expression by tumour cells in DCIS was greater than that observed in adjacent benign ductal or lobular epithelial cells in 96% of the cases (Guidi et al., 1997). In another study, VEGF protein expression was increased in carcinoma in situ relative to atypical hyperplasia of the breast (Heffelfinger et al., 1999). However, they did not find any difference in VEGF expression between carcinoma in situ and invasive disease. We found that VEGF was not expressed in the Paget cells of PDB but was found to be expressed in the dermal and epidermal cells in 61 % of PDB cases. The presence of Paget cells in some cases of PDB may stimulate epidermal and dermal cells to release VEGF and promote angiogenesis. However, other angiogenic factors may be involved.

4.4.5.ii VEGF in invasive breast disease

VEGF expression is well documented in invasive breast cancer (Lawerence et al., 1995; Locopo et al., 1998; Rajesh et al., 2004; Choi et al., 2005). It has been suggested that VEGF is an independent prognostic indicator for invasive breast cancer; increased expression correlated with poorer prognosis in node positive and node negative patients (Gasparini et al., 1997). There was no correlation between grade of underlying tumour and VEGF expression. In the current study VEGF expression was highest in the epidermal and dermal cells associated with PDB with

DCIS (85%; Table 4.7) followed by PDB with carcinoma alone (67%) and then PDB with both DCIS and carcinoma (33%).

4.4.6. PD-ECGF/TP expression in PDB

PD-ECGF/TP expression has been shown to be increased in breast carcinomas as compared to benign breast disorders (Yonenga et al., 1998). In Toi et al., (1995) study, 39% (39/100) of their invasive ductal carcinoma cases were positive for PD-ECGF/TP expression. Nagaoka et al., (1998) examined PD-ECGF/TP expression in 117 invasive breast carcinomas. They assessed PD-ECGF/TP in the cancer and stromal cells separately. Ffity two percent of cases were classified as PD-ECGF/TP positive in cancer cells and 37.6% of cases were classified as positive for PD-ECGF/TP in stromal cells. They demonstrated that PD-ECGF/TP expression in stromal cells correlated with both tumour size and microvessel count. They also found that relapse free survival and overall survival were significantly worse in patients with PD-ECGF/TP –positive stromal cells than in patients with negative cells. They concluded that PD-ECGF/TP expression in stromal cells correlated with tumour angiogenesis and can be used to predict the prognosis of patients with invasive breast cancer. In my study, Paget cells in 55% of PDB cases expressed PD-ECGF/TP and in 95% of cases it was positive in the epidermal and dermal cells. There was concordance in the expression of PD-ECGF/TP in the underlying tumour and Paget cells in 80% of the cases. These results suggest that PD-ECGF/TP may play an important role in the promotion of angiogenesis in PDB, and it may be that the PD-ECGF/TP produced from the epidermal and dermal cells may play a greater role in the angiogenic process than the PD-ECGF/TP derived form the Paget cells themselves.

4.4.7. Double marker expression in PDB

Toi et al., (1995) suggested that both VEGF and PD-ECGF/TP may be involved in the neovascularisation of human breast cancers. They found a significant correlation in the expression between the two markers, and were frequently coexpressed in the highly vascularised breast tumours with a high microvessel count. Sixty three percent of the tumours expressed both markers. In the current study, epidermal and dermal cells and Paget cells coexpressed VEGF and PDECGF/TP in 50% of PDB cases.

However, there were no differences in the expression of the markers and the type of tumour. These results suggest there is an unlikely to be a synergistic effect from the coexpression of these markers, and it plays no role in the disease progression in PDB.

4.4.8. MVD in PDB

Several studies have shown that MVD is a prognostic factor for several solid tumours (Bosari et al. 1992; Horak et al. 1992; Macchiarini et al. 1992.; Weidner et al., 1993) Weidner and co workers demonstrated that intratumour MVD is an independent factor for breast carcinoma (Weidner et al., 1992). They found a correlation between MVD and overall and relapse free survival in patients with early-stage breast carcinoma. Hall et al. (1992) were unable to find a relationship between MVD and breast metastasis. Another study, demonstrated no correlation between VEGF and MVD in 34 node negative breast carcinoma. (Rajesh et al., 2004). Similarly, Viacara et al., 2004) did not find any correlation between VEGF expression and MVD in their preinvasive lesion of the breast. In my study there appeared to be no correlation between MVD and VEGF or PD-ECGF/TP in PDB associated with PDB with DCIS or DCIS+ carcinoma. In conclusion my data suggest that VEGF and PD-ECGF/TP

may have a role to play in the angiogenesis of PDV and PDB but not their malignant progression. In Paget's disease of the breast, the angiogenic process may continue even after migration of Paget cells from the underlying tumour and that angiogenic factors released from the epidermal and dermal cells may have an important role in the angiogenesis of PDV and PDB. MVD does not appear to be a useful parameter in determining disease progression in PDV or PDB.

CHAPTER 5: CELL ADHESION MOLECULE EXPRESSION IN PAGET'S DISEASE OF THE VULVA ANDTHE BREAST

5.1. INTRODUCTION

5.1.1. Cell Adhesion

The evolution of specific mechanisms for cell adhesion was one of the crucial steps in the development of metazoan organisms. During embryonic development adhesive interactions play a major role in triggering a variety of morphogenetic processes including cell motility, growth, differentiation and survival. Such interactions may involve either direct cell-cell contact or adhesion to extracellular matrix (ECM) networks. These processes involve two critical and distinct stages, namely a binding event mediated through cell-surface receptors, which specifically and directly interact with the external ligand, and a morphogenetic event, which depends on the activity of force-generating cytoskeletal systems within the cells. Studies have shown that these transmembrane structural linkages are also involved in signal transduction (Bershadsky et. al.1995; Gumbiner, 1996). Malignant transformation is characterised by disruption of cytoskeletal organisation and altered adhesion dependent responses. The growth of many cancer cells is 'anchorage independent', and such cells have often lost the negative regulation of cell proliferation conferred by excessive cell-cell adhesion (i.e. have lost the 'contact inhibition of growth'; Ben-Ze'ev,1997). The reduction or lack of cell-cell adhesion molecules have been demonstrated in various carcinomas and have been implicated in the invasion and metastatic process (Shiozaki et al., 1991). Abnormal expression of cell-cell adhesion molecules has been reported in various skin carcinomas and has been implicated in the process of invasion and metastasis (Tada, 1996). Loss of expression of these molecules in PDV and PDB may determine which cases may have appeared in the epidermis secondary to an underlying adenocarcinoma, or which go on to develop invasive disease.

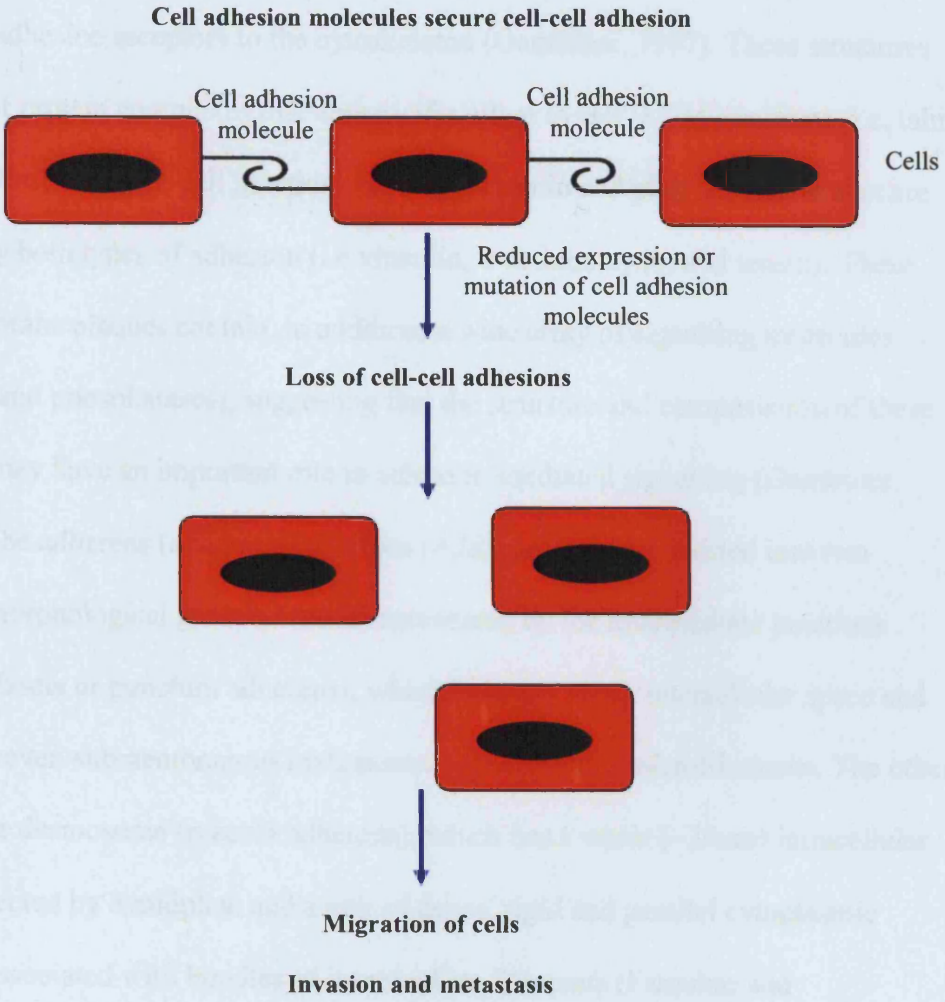

Figure 5.1 Schematic diagram of cell-cell adhesion.

5.1.2 Cellular Junctions

Historically, cellular junctions have been placed into two major categories on the basis of their morphological appearance: the occludens junctions, which display a sealing of the intracellular space, and regulate the permeability characteristics of the paracellular space between adjacent cells (Gumbiner, 1987), and the adherens junctions, which have a widened intracellular space and are associated with various forms of submembranous densities termed plaques (Farquhar and Palade 1963).

These submembrane plaques of cell-cell and cell-ECM junctions are contacts that link the adhesion receptors to the cytoskeleton (Gumbiner, 1997). These structures consist of protein complexes that are specific either to cell- ECM junctions (i.e. talin and paxillin) or to cell-cell junctions (α- and β-catenin and plakoglobin) or that are shared by both types of adhesion (i.e vinculin, α-actinin, zyxin and tensin). These submembrane plaques contain, in addition, a wide array of signalling molecules (kinases and phosphatases), suggesting that the structure and composistion of these plaques may have an important role in adhesion-mediated signalling (Gumbiner, 1996). The adherens (adhering) junctions (AJs) can again be divided into two distinct morphological groups. One is represented by the intermediate junctions (zonula, fascia or punctum adherens), which have a ~ 20nm intracellular space and loosely woven submembranous mats associated with actin microfilaments. The other type is the desmosome (macula adherens), which has a wider (~30nm) intracellular space bisected by a midplate and a pair of dense, rigid and parallel cytoplasmic plaques associated with bundles of intermediate filaments (Farquhar and Palade,1963; Drochmans et al., 1978; Cowin et al.,1985a).

5.1.3. Zonula Adherens Junctions

The zonula AJ's are required for both the establishment and maintenance of epithelial layers (Geiger et al., 1989). In addition, these junctions have been identified in several other cell types including cardiac myocytes and fibroblasts. Zonula AJ subserve several important functions including mediating intercellular adhesion, sensing the presence of neighbouring cells, and anchoring the actin cytoskeleton. Zonula AJs are multiprotein complexes that are assembled around cell adhesion molecules called cadherins. Cadherins are a multifunctional family of calcium-

dependent, transmembrane, glycoproteins which promote cell-cell adhesion (Takeichi, 1991). The cadherin extracellular domain mediates homophilic interactions between like cadherin molecules on neighbouring cells, while the intracellular domain interacts with several cytoplasmic proteins which include: a-catenin, ß-catenin, γ -catenin (plakoglobin) and the tyrosine kinase substrate $p120^{cas}$ (Ozawa,1990 ; McCrea, 1991; Knudsen, 1992). Cadherin-catenin interactions are required for complete cadherin activity to regulate the interaction between cadherins and the actin-based cytoskeleton (Ozawa,1990; McCrea, 1991; Takeichi,1991).

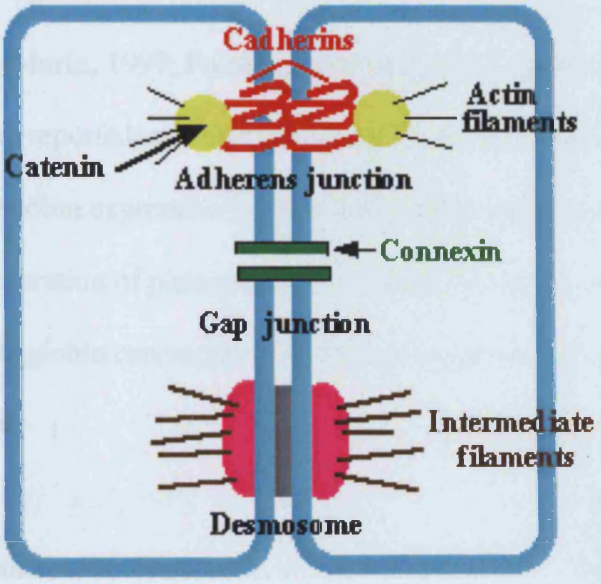

Figure 5.2 Cellular junctions.

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5.1.4. Plakoglobin

Plakoglobin, also known as γ –catenin, is an 83kDa protein that localises to the cytoplasmic face of both desmosomal and adherens junctions (Cowin et al., 1986). Plakoglobin was first demonstrated to associate with the desmosomal cadherins,

desmoglein and desmocolin, and was subsequently shown to associate with the classical cadherins E- and N- cadherin (Knudsen et al.,1994).

plakoglobin is highly homologous to β-catenin, and both possess dual roles in cell adhesion and in the Wnt /Wingless signalling pathways. Within AJs, plakoglobin links the cell adhesion molecule E-cadherin to the actin cytoskeleton via α-catenin. It also has an additional role to play in cell adhesion as an integral component of desmosomes (Cowin et al.,1996; Smith et al., 1998). Catenin-mediated cell adhesion and Wnt signalling both play multiple roles in various developmental processes and also in the adult organism. In contrast to frequent mutations in β-catenin in tumours of various types (Morin, 1999; Polakis, 1999) only one case of a plakoglobin mutation has been reported, and that was in gastric cancer (Caca et al.,1999). Moreover, plakoglobin expression is often lost during cancer progression (Aberle et al., 1995) and restoration of plakoglobin expression in several highly tumourigenic cells lacking plakoglobin can suppress their tumourigenicity (Simcha et al.,1996; Ben-Ze'ev,1997).

5.1.5. E-cadherin

E-cadherin is a member of the multifunctional family of calcium ion-dependent transmembrane glycoproteins with molecular masses ranging between 120-140 kDa. They promote and maintain cell adhesion in virtually all multicellular organisms. The cadherin superfamily comprises over fourty proteins which are, on average, 50-60% homologous. Cadherin expression is required for the assembly of cells into solid tissues and, importantly, cadherins are expressed in a tissue-specific fashion (Takeichi,1991). E-cadherin (also known as a 'classic' cadherin along with N- and P-cadherin) interacts in a homophilic manner, i.e. cadherins of the same type bind to

each other. Other members of the cadherin superfamily include the desmosomal cadherins (desmogleins and desmocollins), which are associated with the keratin filament network, and the proto-cadherins which are distantly related to the classical cadherins in their extracellular domains and differ completely in their cytoplasmic tail (Suzuki,1996). E- cadherin consists of an extracellular domain, a transmembrane segment, and a cytoplasmic domain. The most direct effect of cell-cell adhesion is on morphogenesis, i.e on the assembly of individual cells into highly ordered tissues and organs through cell-cell adhesion junctions (Gumbiner, 1996). These interactions between cells involve transmembrane cell adhesion receptors of the cadherin family (Takeichi, 1995).

Levels of E-cadherin, which is expressed in epithelial cells, are often reduced in many carcinomas, including carcinomas of the head and neck, oesophagus, skin, thyroid, lung, breast, stomach, liver, kidney, pancreas, colon , bladder, prostate and female genital tract (Vleminckx et al.,1991). E-cadherin downregulation also usually correlates with lower survival rates (Vleminckx et al.,1991). In vitro studies have demonstrated that in cultured cells restoration of E-cadherin levels by cDNA transfection leads to suppression of invasiveness and of the tumourigenic capability of cells (Frixen et al., 1991;Vleminckx et al. ,1991) and can decrease protease secretion by the tumour cells (Miyake et al., 1995). In addition to downmodulation of E-cadherin levels, studies have demonstrated that mutations/deletions in the E-cadherin gene can correlate with malignancy in tumour cells. For example, 50% of diffuse-type gastric carcinomas harbour a mutation that affects the calcium ion-binding site of E-cadherin (Becker et al., 1994) and deletions in the extracellular domain of E-cadherin were reported in infiltrative lobular breast carcinoma (Berx et al. 1995).

5.1.6. β- catenin

β-catenin is a 92kDa protein which shares 70% amino acid identity with both plakoglobin (γ-catenin) and the product of the Drosophila segment polarity gene armadillo (Peifer, 1990; McCrea, 1991; Takeichi,199). The armadillo protein is part of a multiprotein junctional complex and is a required component of the Drosophila wingless wnt signal transduction pathway (Peifer, 1990; McCrea, 1991). B-catenin, plakoglobin and $p120^{cas}$ are homologous but distinct proteins which contain between 10-13 copies of a 42-44 amino acid motif first identified in the armadillo protein and referred to as armadillo repeats. These armadillo repeats mediate the interaction between β-and γ-catenin and the cadherin cytoplasmic domain. In addition they mediate β-catenin's interaction with the tumour suppressor protein Adenomatous Polyposis Coli (APC; Rubinfeld, 1993; Peifer, 1994). Regulation of β-catenin levels by the Wnt pathway is essential for ensuring proper development and differentiation of many tissues. Wnt signalling elicits a very broad range of catenin-dependent and independent responses (Peifer and Polakis, 2000), including specification of cell fate at different stages of development (Wodarz and Nusse,1998), regulation of cell proliferation (Gat et al.,1998; Kolligs et al., Orford et al.,1999) and survival (Orford et al.,1999), cytoskeletal remodelling to define cell polarity (Peifer and Polakis, 2000) and cell motility (Wallingford et al., 2000).
In the absence of a Wnt signal, cytosolic β-catenin is rapidly eliminated by the ubiquitin –proteasome pathway (Aberle et al., 1997). This is initiated by the action of a multi-component destruction complex containing glycogen synthase kinase3β (GSK 3β), axin and APC protein; which promotes the phosphorylation of β-catenin at its N terminus.

Several studies have demonstrated elevated β- catenin levels in various cancers (Ben-Ze'ev, 1997; Ben-Ze'ev and Geiger, 1998; Morin 1999; Polakis, 1999). These increases in β- catenin levels result mostly from mutations in β- catenin itself that affect residues in the GSK 3β phosphorylation site critical for β-catenin degradation. In addition, mutations in key components of the degradation machinery, such as APC or axin, have also been detected in some tumours, e.g. colorectal carcinomas (Morin et al., 1997; Satol et al., 2000). The result of such mutations is an elevation in β-catenin content and activation of β-catenin lymphoid enhancer factor/T-cell factor (LEF/TCF)- dependent transcription. This could contribute to uncontrolled cell proliferation and tumour progression (Gumbiner, 1997; Korinek et al., 1997). Several target genes of the β- catenin -LEF/TCF-binding sites might provide the molecular basis for growth regulation by β- catenin signalling. Induction of the genes that encode c-Myc and cyclin D1, whose promoters contain LEF/TCF-binding sites, might provide the molecular basis for growth regulation by β-catenin signalling (Shtutma et al., 1999; Tetsu and McCormick, 1999), whereas the induction of matrilysin expression could promote cell invasion (Crawford et al., 1999). In addition, PPARδ, a transcription factor involved in colon cancer, is also a target for the β- catenin- LEF/TCF complex (He et al., 1999).

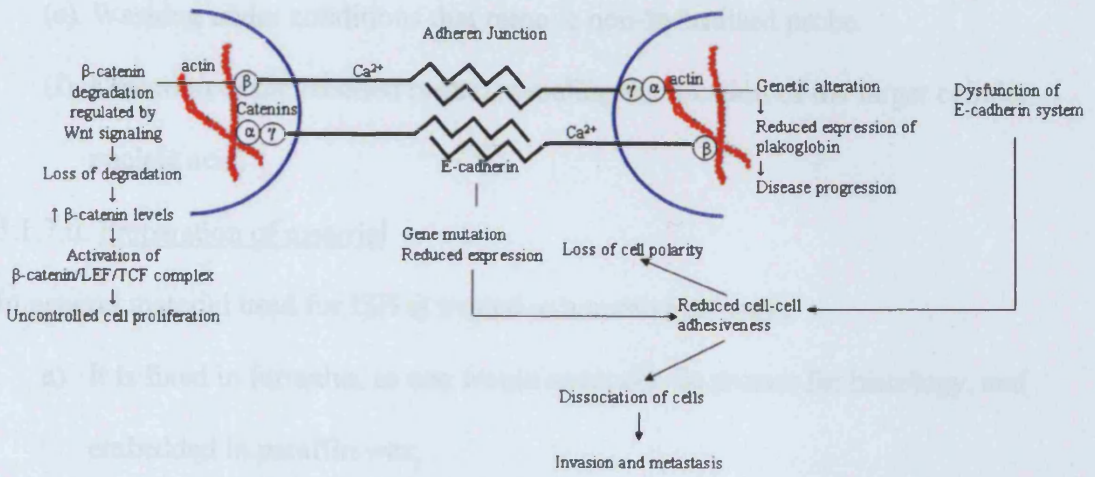

Figure 5.3 Mechanisms of inactivation of plakoglobin, E-cadherin and ß-catenin in cell mediated adhesion system in human cancers.

5.1.7. Principles of *In situ* hybridisation (ISH)

5.1.7.i. Introduction

ISH is a method of localising either mRNA within the cytoplasm or DNA within the chromosomes of the nucleus, whereas IHC is a method of detecting the presence of specific proteins in cells or tissues. The method uses hybridisation of the sequence of interest to a complementary strand of a nucleotide probe. ISH takes advantage of the specific annealing of complementary nucleic acid molecules, which can be DNA and /or RNA, through hydrogen bonds formed between bases attached to the sugar-phosphate backbone. Any nucleic acid sequence can therefore be specifically detected by use of a probe that is the 'antisense', complementary sequence. ISH involves the:

(a) Generation of a nucleic acid probe, labelled to enable subsequent detection.

(b) Preparation of chromosome spreads or fixation of tissues.

(c) Pre-treatment of tissues to increase accessibility of target nucleic acid.

(d) Hybridisation of labelled probe to chromosomes or tissues.

(e) Washing under conditions that remove non-hybridised probe.

(f) Detection of the labelled probe, revealing the location of the target cellular nucleic acid.

5.1.7.ii. Preparation of material

In general material used for ISH is treated in a number of ways:

a) It is fixed in formalin, as one would normally fix tissues for histology, and embedded in paraffin wax.

b) It is snap frozen, and embedded in a special support medium for cryosectioning. The sections are subsequently lightly and rapidly fixed in paraformaldehyde, just prior to processing for hybridisation.

c) Cells in suspension can be cytospun onto glass slides and fixed with ethanol or methanol

d) Preparation of metaphase chromosomal spreads, normally fixed with a mixture of methanol and acetic acid.

5.1.8. Choice of Probes

Probes can be as small as 20-40 bases or up to 1000 bases. Optimisation of conditions used is very important. The strength of the bonds between the probe and the target plays an important role. The strength decreases in the order RNA-RNA, DNA-RNA, DNA-DNA. This stability is in turn influenced by the temperature, pH, guanine (G) and cytosine (C) content of probe, concentration of Na^+ and the amount of formamide in the reaction mixture.

5.1.9. Probe types

A number of different types of nucleic acid probes can be prepared for use in ISH:

5.1.9.i. Oligonucleotide probes:

These are produced synthetically by an automated chemical process. The method utilises readily available deoxynucleotides, which are economical but requires the specific nucleotide sequence to be known. These probes are resistant to RNAase and are small, generally from 20-40 bases. This is ideal for ISH because their small size allows for easy penetration into the cells or tissue of interest. In addition, because they are synthetically designed, it is possible to make a series of probes that have the same GC content. Since G/C base pairs bond more strongly than A/U base pairs, differences in GC content would require different hybridisation conditions. Another advantage of the oligonucleotide probes is that they are single stranded therefore excluding the possibility of renaturation. The disadvantages are that because they are so small they cover less of the target than conventional DNA/RNA probes. One way of getting around this shortcoming is to prepare non-overlapping oligonucleotides to effectively hybridise to a larger area of the target.

5.1.9..ii. Single-stranded DNA probes

These have similar advantages to the oligonucleotide probes except they are much larger, in the 200-500 bp size range. They can be produced by reverse transcription of RNA or by amplified primer extension of a PCR-generated fragment in the presence of a single antisense primer. That is, having amplified

the sequence of interest, a subsequent round of PCR is carried out using the first PCR product as template, but only using the anti-sense primer, thus producing single-stranded DNA.

5.1.9.iii. Double-stranded DNA probes

These can be produced by the inclusion of the sequence of interest in a vector and then introduced into bacteria which are allowed to replicate. After lysing the vector DNA is then extracted and purified. The sequence of interest is excised with restriction enzymes. On the other hand, if the sequence is known then by designing the appropriate primers one can produce the relevant sequence very rapidly by PCR, potentially obtaining a very clean sample. Because the probe is double stranded, it means that denaturation or melting has to be carried out prior to hybridisation in order for one strand to hybridise with the mRNA or DNA of interest. Double- stranded DNA probes are generally less sensitive because of the tendency of the DNA strands to re-hybridise to each other. However, bacterial preparation has the advantage of obtaining large quantities of the probe and was the method preferred for this thesis.

5.1.9.iv. RNA probes or riboprobes

RNA probes have the advantage that RNA-RNA hybrids are very thermostable and are resistant to digestion by RNAases. This allows the possibility of post-hybridisation digestion with RNAase to remove non-hybridised RNA and therefore reduces the possibility of background staining.

There is one method of preparing RNA probes:

In vitro transcription of linearised plasmid DNA with RNA polymerase can be used to produce the RNA probes. In this thesis plasmid vectors containing RNA polymerase promoters from bacteriophages T7 and SP6 were used.

These probes are very difficult to prepare as they are very sensitive to RNAases and can easily be destroyed; therefore, scrupulous sterile techniques must be observed. However, an advantage is that antisense RNA can be synthesised as the probe and sense RNA synthesised as the negative control.

5.1.10. Choice of labelling

Traditionally, probes were radiolabelled with ^{32}P, ^{35}S or ^{3}H but more recently non-radioactive labels have been developed which, in general, are more versatile, have equal or greater sensitivity, produce quicker results and also, of importance, avoid the handling of hazardous materials. Radiolabelled probes are still the choice for many workers, however. ^{32}P and ^{35}S are the most commonly used radioisotopes because their high activity is necessary to detect transcripts present in low amounts.

Non-radioactive labels, such as biotin and digoxigenin, require an additional step for colour development, but other labels such as fluorescein and rhodamine can be linked directly to the nucleotide.

Digoxigenin (DIG) is a steroid isolated from the digitalis plant and, as blossoms and leaves are the only known source of digoxigenin, the anti-DIG antibodies are not likely to bind to other biological material. The digoxigenin is linked by a spacer arm containing 11 carbon atoms attached to the C-5 position of the uridine nucleotide and can be incorporated into the sequence in a number of ways. The advantage of this

probe is that it can be detected with antibodies conjugated to a number of different labels such as alkaline phosphatase, which results in a blue precipitate when the enzyme is incubated in the presence of the substrate NBT/BCIP (4- nitroblue tetrazolium chloride/ 5-bromo-4-chloro- 3-idolyl-phosphate) or becomes a fluorescent label when incubated with HNPP (2-hydroxy-3 napthoic acid- 2' phenylanilide phosphate) and fast Red. The anti–DIG antibodies can also be conjugated to other labels that require no development, such as fluorescein. DIG-labelled probes were used in this study as they are more sensitive than biotin and have comparable sensitivity to ^{35}S labelled probes.

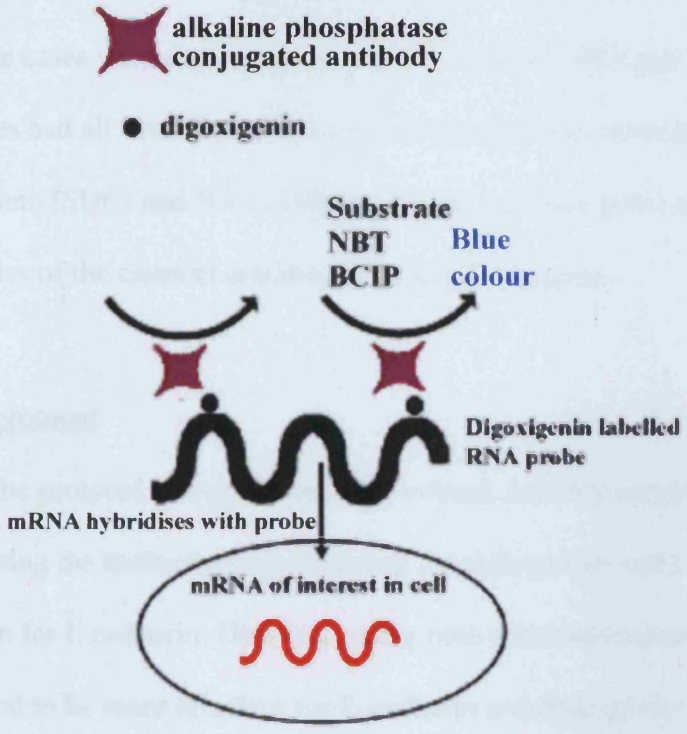

Figure 5.4 In situ hybridisation labelling process.

5.2 METHODS

5.2.1. Immunohistochemical staining with plakoglobin, E-cadherin and β-catenin antibodies

Sixty-three cases of PDV, including eight associated with invasive disease and 23 cases of PDB, which included 10 cases with DCIS only, 4 cases had both DCIS and invasive carcinoma, 5 cases had an underlying invasive carcinoma and 4 cases had no underlying DCIS or invasive carcinoma, were analysed for the expression of plakoglobin, E-cadherin and β-catenin. These cases were retrieved from the Histopathology Department at the Royal Free Hospital and collaborators as listed in the appendix. The cases were diagnosed and treated between 1984 and 2000.
The archival cases had all been formalin fixed and paraffin wax-embedded. Dr Salvador Diaz-Cano (SDC) and JCC (collaborating histopathologists) reviewed all H&E stained slides of the cases to confirm the original diagnosis.

Optimisation of protocol

Optimisation of the protocol was performed and refined. Initially antigen retrieval was performed using the method of microwaving for plakoglobin and β-catenin and protease digestion for E cadherin. However, using both microwaving and pressure cooking was found to be more effective for E-cadherin and plakoglobin. β -catenin was just as effective without antigen retrieval. Optimising of antibody dilutions was performed for all primary antibodies, starting with the recommended manufacture's dilution and then using a range either side. The sections from the control tissue were then assessed for intensity of staining with the presence and absence of non-specific staining. Incubation time was also optimised for all antibodies, along with incubation

temperature, allowing for specific staining with the lowest concentration of antibody but still providing intense specific staining.

Paraffin wax-embedded sections of Paget's tissue, 5μm thick, were cut and mounted on APES-coated glass slide. The sections were deparaffinised in xylene (2x5 min) and rehydrated in graded solutions of alcohol (100%, 90% and 70% for 3 min each). To block endogenous peroxidase sections were immersed in 3% H_2O_2 in methanol for 10 min. The sections were then left in running water for two min. Conditions for antigen retrieval, incubation times and the primary antibodies used are described in Table 5.1. Sections were incubated with the secondary antibody rabbit anti mouse IgG (1:50 dilution in PBS) for 30 min and washed in PBS (3x5 min) Addition of streptavidin HRP complex (Dako, Cambridge, UK) to the sections (1:500 dilution) for 30 min was followed by a further wash in PBS (1x3 min) . Antibody binding was visualised with a solution containing the chromogen 3,3'- diaminobenzidine (Sigma-Aldrich, Poole, UK). The sections were counterstained with Mayer's haematoxylin (Merck, Lutterworth, UK). Finally, the slides were rehydrated in graded alcohol rinses, cleared in xylene and mounted in DPX.

Normal vulval skin was used as the positive control for PDV cases and normal breast skin for PDB cases and were used in each staining procedure to ensure optimal quality of reagents and methods, and to ensure that the staining was successful. Apocrine and eccrine glands were used as internal controls. Due to the limited amount of specimens, sections were stained on two separate occasions. They were also scored by two individuals (SDC, PEE) to ensure reproducibility. There was <5% variation between sections and observers.

Scoring

The location of the pattern of staining of the protein was recorded as one of the following: membranous, diffuse cytoplasmic, paranuclear, and nuclear. The percentage of Paget cells expressing each protein had a score of <5%, <25%, <50% <75% and <100%. The intensity of the staining was also recorded as either nil, mild or strong.

Statistical analysis

Statistical analysis was performed using the Chi Square for trends and Fisher exact test when appropriate. A p value of <0.05 was considered significant.

Table 5.1 Conditions, antigen retrieval, incubation times and antibodies used for Immunhistochemistry

Antigen	Source	Clone	Working dilution*	Antigen retrieval	Incubation time	Positive controls
Plakoglobin	Zymed Laboratories Cat no. 13-8500	15	1:00	microwave pressure cooking	1½ h	vulval skin (PDV) breast skin (PDB)
E-cadherin	Zymed Laboratories Cat no.33-4000	36	1:250	microwave pressure cooking	1 ½ h	vulval skin (PDV) breast skin (PDB)
B-catenin	Zymed Laboratories Cat no. 138400	14	1:50	none	1 ½ h	vulval skin (PDV) breast skin (PDB)

* Diluted in PBS

5.2.2 *In-situ* hybridisation for plakoglobin, E-cadherin and β-catenin

To evaluate the mRNA for plakoglobin, E-cadherin and β-catenin selected cases of PDV and PDB were subjected to in situ hybridisation. DIG- labelled RNA probes, both sense and antisense, were prepared as described in detail in Appendix I.

All materials and solutions (except Tris based solutions) used for in situ hyubridisation were pretreated with 2% diethylpyrocarbonate (DEPC) to inactivate RNAses and autoclaved. Tris reacts with DEPC and therefore Tris-based solutions were made with water pretreated with DEPC and autoclaved.

Preparation of PDV and PDB tissue sections

Paraffin waxed-embedded 4μm sections were cut using a sterile blade and mounted on RNAase free APES-treated slides. Sections were dewaxed (xylene 2x5min) and rehydrated in graded ethanol rinses (100% for 10min, 70% for 5 min) and then in water containing 1% DEPC. The following procedures were performed to prepare the sections for pre-hybridisation. This preparation was done to make the RNA within the cell more accessible to the probe. For antigen retrieval two methods were tried.

Method 1: Sections were microwaved in pre-warmed 10% citrate buffer for 10 min and then left to cool in DEPC-treated water for 10 min. This was followed by incubating sections for 10 min in 0.25% acetic anhydride to acetylate the sections. The slides were then rinsed in PBS in DEPC water (2x5 min).

Method 2: Sections were incubated in 100 μl of Poteinase K solution at 37°C for 15min. Proteinase K was inactivated by submerging sections in 0.2% Glycine (in PBS) for 10min and rinsing twice in DEPC PBS. This was followed by incubating the sections with 0.25% acetic anhydride for 10min and then again rinsing twice in

DEPC PBS. This method was found to be less effective and therefore the first method was used.

Pre-hybridisation

Sections were dried partially to remove excess PBS and encircled using a wax pen to restrict pre-hybridisation solution to the area of the section. Following this, sections were incubated in the pre-hybridisation solution for 4 h at 42°C.

Hybridisation

The probe was heated to 100°C for 1 min to remove secondary structure and stored on ice. The pre-hybridisation solution was discarded and sections were then hybridised overnight with 40ng of RNA probe at 42°C in hybridisation solution. Sections were covered with Parafilm to prevent evaporation.

Post-hybridsation

The parafilm coverslip were removed and slides rinsed twice in 2xSSC (composition detailed in Appendix I) at room temperature (2x5min). The sections were washed with increasing stringency in SSC at different concentrations (2x SSC,1x SSC, 0.5xSSC and 0.1x SSC) with 50% formamide at 42°C for 30 min each time, at each concentration and then incubated for 30 min with blocking solution (prepared using 3µl of normal sheep serum and 20µl of Triton X-100 per ml of buffer1 solution). This was followed by incubating the sections with the anti-DIG alkaline phosphatase conjugated sheep Fab fragments (diluted 1:400 in buffer 1) containing 1% normal sheep serum to reduce non specific binding of the antibody, for 90 min at room temperature. The sections were washed in buffer 1 (2x5 min) and then in buffer 2 (1x5 min). To visualise antibody binding of the probes NBT /BCIP chromogen solution (Sigma) was added to the sections and left overnight at 4°C. To stop the

colour reaction sections were washed in buffer 3 (10mM Tris HCL, 0.5 molar EDTA and distilled water) for 30 min. The slides were air-dried and mounted using aqueous mount. Composistion of solutions are described in Appendix I page 244.

Stained sections were scored for the intensity of the mRNA signal of the intracellular junctions. Nuclear and cytoplasmic staining was also recorded. Sections were stained on two separate occasions and the results analysed by two independent observers (PEE and Dr Lucy Ghalia). There was <5% variation between sections and observers. The surrounding apocrine/eccrineglands and epidermal cells were used as positive controls. The sense probe of each section was used as the negative controls.

5.3 RESULTS

5.3.1 Immunhistochemistry results

5.3.1.i Evaluation of plakoglobin, E-cadherin and β- catenin expression in PDV

20 cases (34%) of PDV were grouped histologically in a nested pattern compared to 40 cases (68 %) that found the Paget cells to be lying in a single pattern. There appeared to be no significant differences in the expression of the adhesion molecules between the two groups (the Paget cells in two cases were found to have neither a single or growth pattern and had an infiltrative and solid growth pattern respectively, and in another case the Paget cells were arranged in both a single and nested pattern; these cases were not included in the final count). Four of the PDV cases with invasion had a nested pattern, two were arranged in a singular pattern, and two were neither but had an infiltrative pattern. The cases were further divided as to whether there was any lichenoid inflammation reaction surrounded the cells. Twenty-three cases (39%) had lichenoid inflammation reaction surrounding the Paget cells as compared to 36 cases (61%) that did not. Three of the PDV cases with invasive

disease had a lichenoid inflammatory reaction surrounding the Paget cells; five did not. Tables 5.2, 5.3, and 5.4 demonstrate the histological pattern and percentage score for each protein marker.

Table 5.2 Plakoglobin score and associated histological pattern in PDV

Histological pattern	<5%	<25%	<50%	<75%	<100%
Nested	7	4	3	4	2
Single	18	8	5	7	2
Nested + lichenoid	0	2	1	3	0
Single + lichenoid	5	4	3	2	0

Table 5.3 E-cadherin score and associated histological pattern in PDV

Histological pattern	<5%	<25%	<50%	<75%	<100%
Nested	0	3	2	7	8
Single	2	3	5	14	13
Nested + lichenoid	0	1	0	2	4
Single + lichenoid	1	2	1	7	4

Table 5.4 β-catenin score and associated histological pattern in PDV

Histological pattern	<5%	<25%	<50%	<75%	<100%
Nested	9	4	5	2	0
Single	12	18	6	2	2
Nested + lichenoid	3	2	1	1	0
Single + lichenoid	3	1	2	1	1

Plakoglobin

Thirty six (57%) of the 63 cases of PDV had a score of greater than 5% or more; in 4 cases (6%) >75% expressed plakoglobin; in 11 cases (17%) it was >50% of the Paget cells ; 8 cases had a score of >25% and 13 cases a score of >5% but < 25%. This was compared to 27 cases (43%) that had a score of <5%. In most cases the staining was membranous; 34 cases (58%) had membranous staining, 9 (15%) had diffuse cytoplasmic staining and 4 had nuclear staining (6%). In the 41% of cases the staining intensity was mild and in 37% it was strong.

Sixty percent (33/55) of the intraepidermal PDV cases had >5% of Paget cells expressing the Plakoglobin protein compared to 37% (3/8) of cases of PDV with invasive disease. Table 5.5 demonstrate the results. Figure 5.5a and Figure 5.5b demonstrate plakoglobin protein expression in non invasive PDV with a score of <5%. The staining was membranous and mild in intensity.

Table 5.5 Plakoglobin expression in PDV

Samples	<100%	<75%	<50%	<25%	<5%
PDV without invasive disease (n=55)	3	11	8	11	22
PDV with invasive disesase (n=8)	1	0	0	2	5

E-cadherin

Fifty eight of the 61 cases (95%) had a score of greater than 5% with 22 cases having a score of >75%. Only 3 cases had a score of <5%. Again the staining pattern was mostly membranous (58 cases out of 59), the remaining case was nuclear. The intensity of staining was mostly strong (49 cases).

Ninety-eight percent (53/54) of PDV cases had > 5% of Paget cells expressing the E-cadherin protein compared to 71% (5/7) of PDV with invasive disease. This result was significant, p value = 0.005. Table 5.6 demonstrate the results.

Figure 5.6 demonstrate E-cadherin protein expression in non invasive PDV with a score of <100%. The staining was membranous and cytoplasmic, and the intensity was strong. The Paget cells were in a nested pattern and lichenoid inflammatory reaction was present.

Table 5.6 E cadherin expression in PDV

Samples	<100%	<75%	<50%	<25%	<5%
PDV without invasive disease (n=54)	20	21	8	4	1
PDV with invasive disesase (n=7)	2	0	1	2	2

β catenin

β catenin expression in PDV was similar to plakoglobin expression with 41 (65%) cases had a score of between 100 and 5%. Twenty-two cases (37%) had a score of < 5%. As before, the staining pattern was mostly membranous (34 cases), 13 cases had nuclear staining, 11 cases had diffuse cytoplasmic staining pattern and 4 had paranuclear staining. The intensity of the staining was mild.

Sixty-nine percent (38/55) of PDV had >5% of Paget cells expressing β –catenin compared to 37% (3/8) of PDV cases with invasive disease. Table 5.7 demonstrate the results. Figure 5.7 show β –catenin protein expression in non invasive PDV with a score of <50%. The staining was membranous and the intensity was strong.

Table 5.7 β-catenin expression in PDV

Samples	<100%	<75%	<50%	<25%	<5%
PDV without invasive disease (n=55)	2	3	11	22	17
PDV with invasive disesase (n=8)	0	1	0	2	5

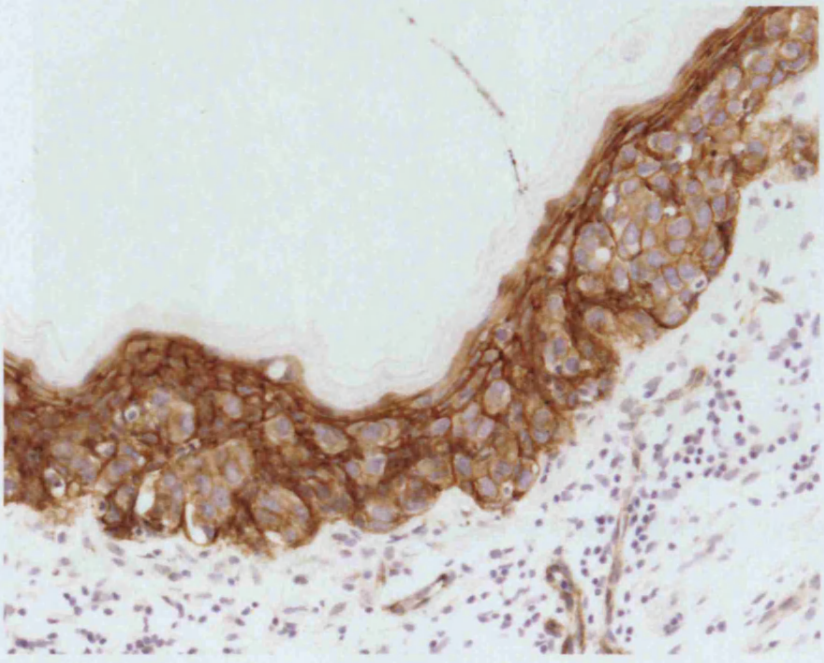

Figure 5.5a Plakoglobin protein expression in non invasive PDV (x100) with a score of < 5%.

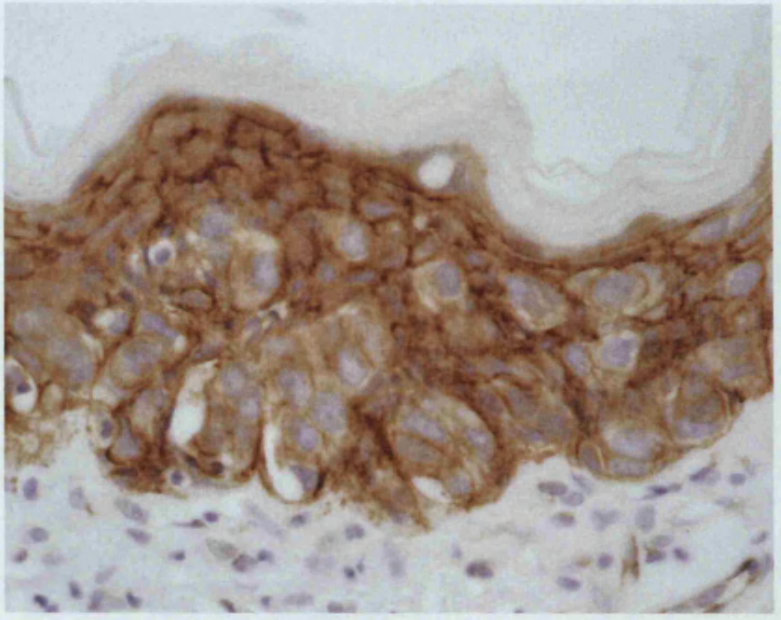

Figure 5.5b Plakoglobin expression in Paget cells in PDV (x400) with a score of < 5%.

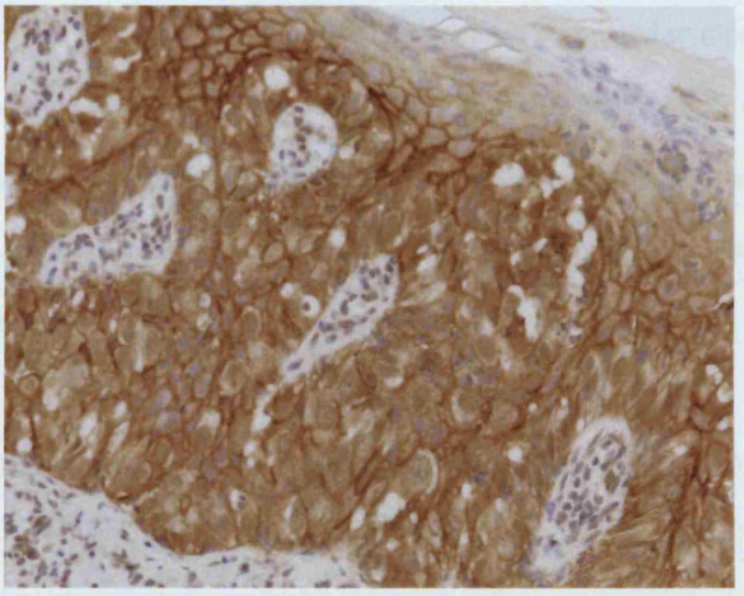

Figure 5.6 E cadherin protein expression in non invasive PDV (x200) with a score of <100%.

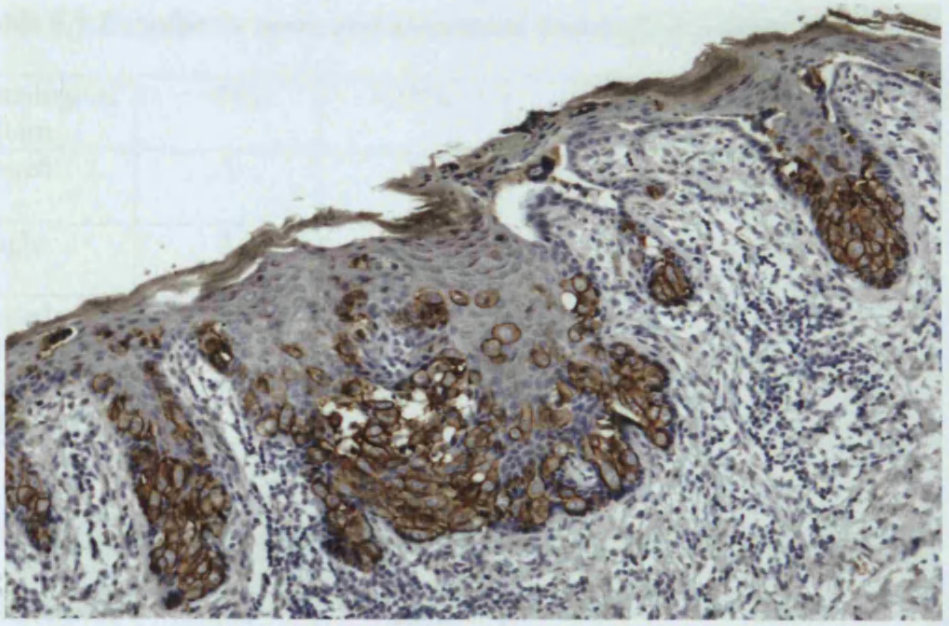

Figure 5.7 ß- catenin protein expression in non invasive PDV (x200) with a score of <50%.

5.3.1.ii. Evaluation of plakoglobin, E-cadherin and ß- catenin proteins in PDB

Twelve of the 24 (50%) PDB cases had a single pattern compared to nine PDB cases that shared a nested pattern. Three cases had an infiltrative pattern. Five of the 24 (21%) PDB cases had a lichenoid inflammatory reaction surrounding the Paget cells. Tables 5.8, 5.9 and 5.10 demonstrate the histological pattern with the percentage score for each marker.

Table 5.8 Plakoglobin score and associated histological pattern in PDB

Histological pattern	<5%	<25%	<50%	<75%	<100%
Nested	6	1	0	2	0
Single	10	2	0	0	0
Nested + lichenoid	1	0	0	0	0
Single + lichenoid	2	2	0	0	0

Table 5.9 E-cadherin score and associated histological pattern in PDB

Histological pattern	<5%	<25%	<50%	<75%	<100%
Nested	1	2	1	1	4
Single	3	1	0	4	4
Nested + lichenoid	0	0	0	1	0
Single + lichenoid	0	0	0	2	2

Table 5.10 β-catenin score and associated histological pattern in PDB

Histological pattern	<5%	<25%	<50%	<75%	<100%
Nested	4	1	1	2	0
Single	7	2	0	2	0
Nested + lichenoid	0	0	0	1	0
Single + lichenoid	1	1	0	1	0

Plakoglobin

Six of the twenty three cases (26%) had a score of >5% with 17 cases (74%) with a score of < 5%. Expression of the protein was found in the membranes in 78% of cases (two cases were nuclear and three cases were paranuclear). In most cases (70%) the intensity of the staining was strong. Table 5.11 demonstrate the results. Table 5.12 show plakoglobin protein expression in PDB with/without DCIS or underlying carcinoma. Figure 5.8 demonstrate plakoglobin protein expression in PDB with DCIS and invasive carcinoma with a score of <75%.

E-cadherin

E-cadherin expression in PDB was similar to its expression in PDV with 19 (83%) of the cases having a score greater than 5% (97% in PDV). Similarly, the protein expression was found mainly (78%) in the membranes and the intensity of the staining was strong in 70% of cases. Table 5.11 demonstrate the results. Table 5.13 show E-cadherin protein expression in PDB with/without DCIS or underlying carcinoma.

Figure 5.9 demonstrate E-cadherin protein expression in PDB with a score of <100%. The staining is membranous and the intensity is strong.

β-catenin

Eleven cases (52%) had a score of >5% and 10 cases (48%) had a score of <5%. In two cases the tissue was unsuitable for scoring. The staining was membranous in 11 cases (52%), 8 cases (38%) were nuclear and 2 cases (9%) were paranuclear. The intensity of the staining was mild in 57% of the cases and strong in 33%. Table 5.11 demonstrate the results. Table 5.14 shows β-catenin protein expression in PDB with/without DCIS or underlying carcinoma. Figure 5.10 demonstrate β- catenin protein expression in PDB only with a score of <100%. The staining is membraneous and the intensity is strong.

Table 5.11 Plakoglobin, E-cadherin and β- catenin protein expression in PDB

Protein	<100%	<75%	<50%	<25%	<5%
Plakoglobin n=23	1	3	0	2	17
E-cadherin n=23	9	6	1	3	4
β- catenin n=21	0	6	2	3	10

Table 5.12 Plakoglobin protein expression in PDB with/without DCIS or underlying carcinoma

Sample	<100%	<75%	<50%	<25%	<5%
PDB only n=4	0	0	0	0	4
PDB +DCIS n=10	1	0	0	2	7
PDB with DICS +invasive carcinoma n=4	0	2	0	0	2
PDB +invasive carcinoma n=5	0	1	0	0	4

Table 5.13 E- cadherin protein expression in PDB with/without DCIS or underlying carcinoma

Sample	<100%	<75%	<50%	<25%	<5%
PDB only n=4	1	1	0	1	1
PDB +DCIS n=10	3	2	1	2	2
PDB with DICS +invasive carcinoma n=4	2	2	0	0	0
PDB +invasive carcinoma n=5	3	1	0	0	1

Table 5.14 β-catenin protein expression in PDB with/without DCIS or underlying carcinoma

Sample	<100%	<75%	<50%	<25%	<5%
PDB only n=4	0	0	0	1	3
PDB +DCIS N=9	0	2	1	2	4
PDB with DICS +invasive carcinoma n=4	0	2	0	0	2
PDB +invasive carcinoma n=4	0	2	0	0	2

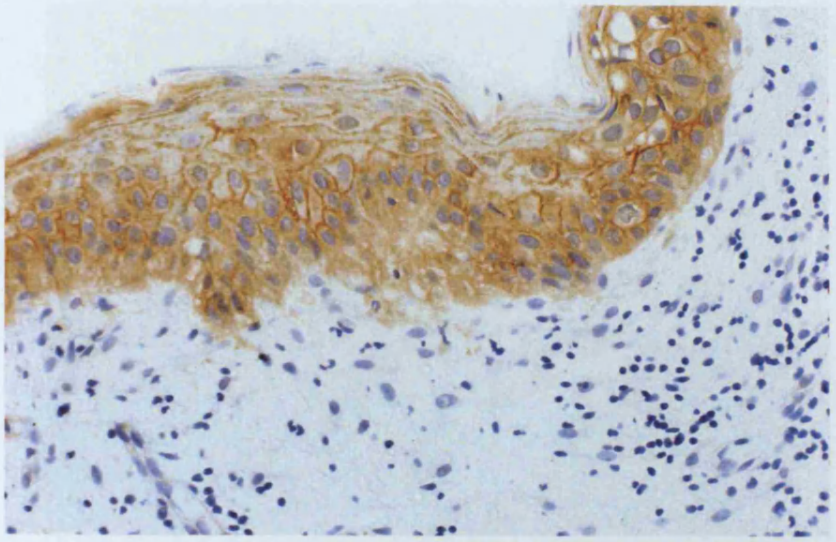

Figure 5.8 Plakoglobin expression in PDB with DCIS and invasive carcinoma (x200) with a score of <75%.

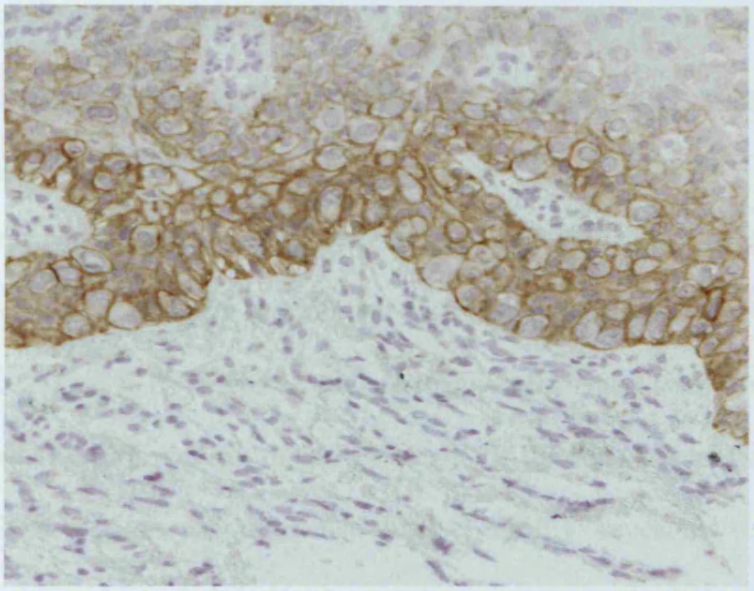

Figure 5.9 E-cadherin protein expression in PDB only (x200) with a score of <100%.

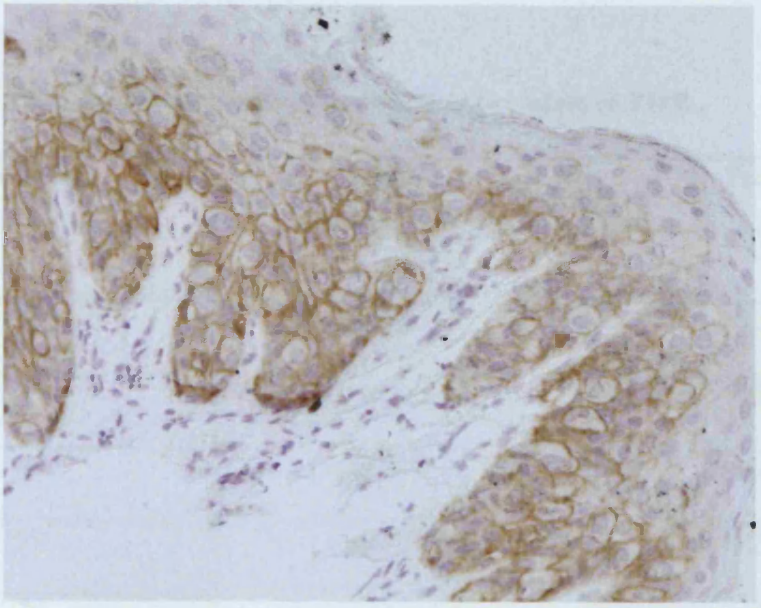

Figure 5.10 ß- catenin protein expression in PDB only (x200) with a score of <100%.

5.3.2. In situ Hybridisation results

5.3.2.i. Evaluation of Plakoglobin, E-cadherin and ß- catenin mRNAs in PDV

Plakoglobin

Ten PDV cases were analysed for mRNA but only eight including one case of invasive PDV were suitable for scoring. All cases had decreased mRNA signal in the Paget cells compared to the apocrine and epidermal cells. Apart from two cases where there was no difference in the mRNA signal in the antisense and sense slides, the mRNA signal was absent in the sense slides. Table 5.15 reveal the six cases available for comparison of plakoglobin protein expression and mRNA signal in PDV.

Table 5.15 Plakoglobin mRNA and protein expression in PDV

Case no.	mRNA	Protein
10	decreased	<5%
15	decreased	<5%
16	decreased	<50%
45	decreased	<100%
59	decreased	<25%
79	decreased	<5%

E-cadherin

Seven cases including one case of invasive PDV were suitable for scoring which revealed decreased mRNA signal in the Paget cells as compared to the surrounding apocrine glands and epidermal cells. Figures 5.11 reveal E-cadherin mRNA signal in Paget cells in PDV (antisense probe ;x200) and Figure 5.12 reveal the negative control. Table 5.16 demonstrate seven cases available for comparison of E-cadherin protein expression and mRNA signal in PDV.

Table 5.16 E-cadherin mRNA and protein expression in PDV

Case no.	mRNA	Protein
1	decreased	<100%
16	decreased	<100%
45	no decrease	<100%
52	decreased	<100%
59	decreased	<75%
72	decreased	<75%
82	decreased	<50%

β -catenin

Fifteen were cases were analysed but only nine were suitable for scoring. Eight cases revealed decreased mRNA signal in the Paget cells compared to the surrounding apocrine glands and epidermal cells. One case revealed no difference in the signal in the antisense and sense slides. Table 5.17 reveal the eight cases available for comparison of β-catenin protein expression and mRNA signal in PDV.

Table 5.17 β-catenin mRNA and protein expression in PDV

Case no.	mRNA	Protein
1	decreased	<5%
16	decreased	<5%
48	absent	<5%
54	absent	<5%
55	decreased	<25%
79	decreased	<25%
72	decreased	<50%
82	absent	<25%

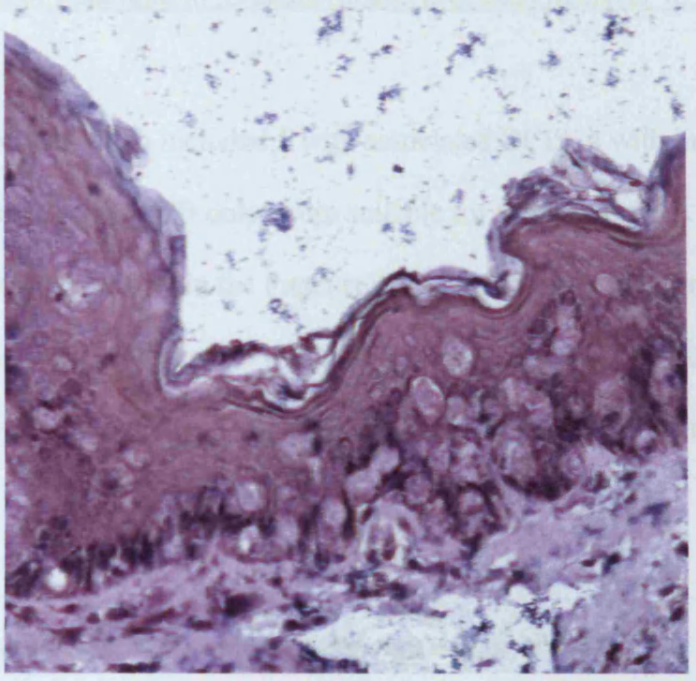

Figure 5.11 E-cadherin mRNA signal in Paget cells in PDV (antisense) (x200).

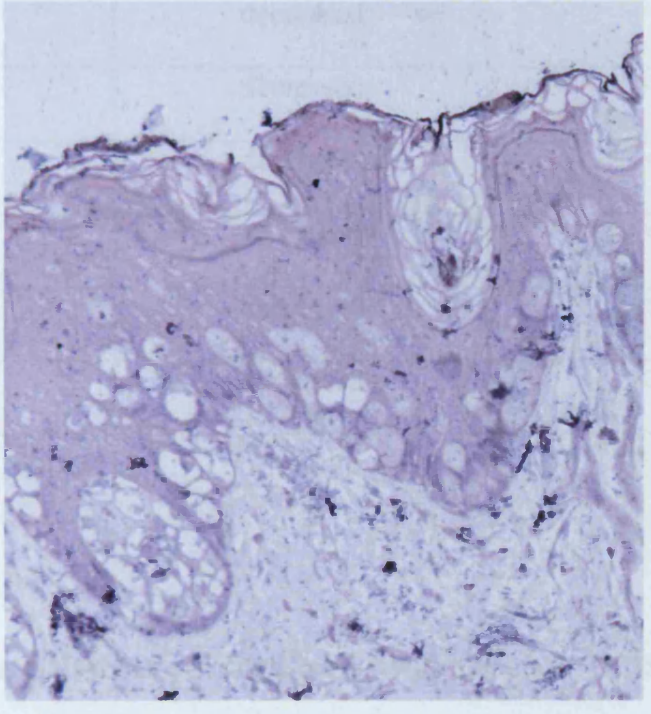

Figure 5.12. Negative control. E-cadherin mRNA signal in Paget cells in PDV (sense probe; x200).

5.3.2.ii Evaluation of Plakoglobin ,E-cadherin and β-catenin mRNAs in PDB

Plakoglobin

Eight of 11 PDB cases which included 5 with associated DCIS, 4 with invasive carcinoma and 2 cases with PDB only, were suitable for scoring. mRNA signal was noted to be decreased or absent in the Paget cells as compared to the surrounding apocrine glands and epidermal cells. Table 5.18 demonstrate the seven cases available for comparison between plakoglobin mRNA signal and protein expression in PDB.

Table 5.18 Plakoglobin mRNA and protein expression in PDB

Case no.	mRNA	Protein
100	decreased	<5%
103	decreased	<5%
105	decreased	<5%
120	decreased	<5%
122	decreased	<5%
124	decreased	<25%
136	decreased	<50%

E-cadherin

Nine of the fourteen PDB cases which included 4 with associated DCIS, 4 with invasive carcinoma, and one case of PDB only, analysed were suitable for scoring. In all cases mRNA signal was found to be reduced in the Paget cells as compared to the surrounding apocrine glands and epidermal cells. Table 5.19 demonstrate the five

cases available for comparison between E-cadherin protein expression and mRNA signal in PDB.

Table 5.19 E-cadherin mRNA and protein expression in PDB

Case no.	mRNA	Protein
89	decreased	<5%
98	decreased	<5%
100	decreased	<75%
103	decreased	<100%
124	decreased	<5%

β-catenin

Thirteen PDB cases were analysed which included 6 with associated DCIS, 4 with invasive carcinoma and 2 cases with PDB only. In the twelve that were suitable for scoring, mRNA signal was noted to be decreased in 10 cases as compared to the surrounding apocrine glands and epidermal cells. The signal was found to be similar in intensity in both the antisense and sense slides in two cases. Table 5.20 demonstrates the six cases available for comparison between β-catenin protein expression and mRNA signal in PDB.

Table 5.20 ß-catenin mRNA and protein expression in PDB

Case no.	mRNA	Protein
94	decreased	<5%
100	decreased	<75%
105	decreased	<5%
122	decreased	<5%
124	decreased	<5%
136	decreased	<5%

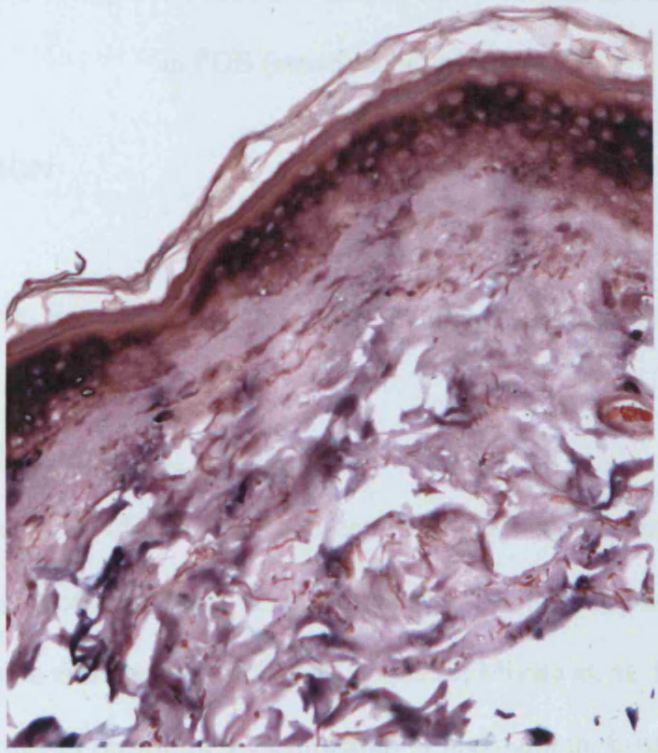

Figure 5.13 ß-catenin mRNA signal in Paget cells in PDB (antisense; x100).

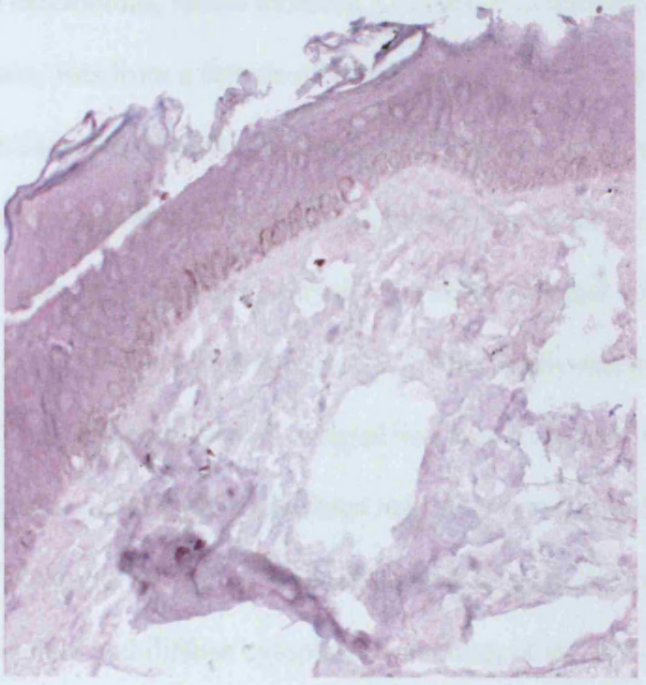

Figure 5.14 Negative control. ß-catenin mRNA signal in Paget cells in PDB (sense probe; x100).

5.4. DISCUSSION

5.4.1. Plakoglobin, E-cadherin and ß- catenin protein and mRNA expression in PDV

5.4.1.i Plakoglobin expression in PDV

This is the largest study of plakoglobin, E-cadherin and ß- catenin expression in PDV and PDB. The reduced expression of cell adhesion molecules has been implicated in promoting invasion and metastasis (Oka et. al., 1992; Miyata et. al, 1994). The paucity of information regarding the expression of plakoglobin, E-cadherin and ß-catenin in PDV is evident (Tada et.al 1996, 2000; Shirahama et al.,1996; Takata et al., 1999) and, as such, the role of these molecules have not been clearly established in PDV. To my knowledge, only one study has examined the expression of plakoglobin in PDV. Tada et al. (2000) examined the expression of desmoglein I and

plakoglobin in skin carcinomas, which included 11 cases of extramamary Paget's disease, only one case, was from a female of which the location was documented as the pudendum. This case was negative for plakoglobin. In the current study fewer Paget cells expressed plakoglobin in the intraepidermal compartment of the PDV cases with invasive disease when compared with the intraepidermal compartment of PDV cases without invasive disease, although this did not reach statistical significance ($p=0.27$) . Plakoglobin mRNA signal was also decreased as compared to the surrounding apocrine glands. These findings may suggest a possible dysfunction in the regulation of plakoglobin in PDV with invasive disease as compared to those without invasion. Nuclear and diffuse cytoplasmic staining of the plakoglobin protein was noted in 13 of the cases with only one case being associated with invasive disease. Plakoglobin has been reported to be involved in the Wnt signalling pathway (Karnovsky et al. 1997; Merriam et al. 1996), however this remains controversial. Whether extramembranous expression of the protein is significant in these cases remains uncertain.

5.4.1.ii. E-cadherin expression in PDV

Since loss of expression of E-cadherin has been suggested as one of the mechanisms contributing to the development of invasion and metastasis of cancer cells, its expression has been investigated in several cancers with a view to correlating lack of expression with invasive disease. Searching the literature only two studies have examined this role in PDV (Shirahama et al., 1996; Tada et al., 1996). Both studies three and eight cases of PDV respectively (Table 5.21) demonstrated decreased expression of the E-cadherin protein.

In the current study we found similar results. E-cadherin expression was significantly reduced ($p= 0.005$) in the intraepidermal compartment of the PDV cases with invasive disease when compared with the intraepidermal compartment of PDV cases without invasive disease. Loss of E-cadherin expression may lead to reduced cell adhesiveness and therefore detachment of Paget cells from the intraepidermal lesion with the result being invasive disease. There appeared to be no significant difference in the cellular localisation of the proteins

Loss of mRNA signal was also noted to decrease from surrounding apocrine glands and epidermal cells to in situ PDV, and to invasive PDV cases. However when comparing mRNA signal and protein expression the results do not correlate in some cases. One possible reason could be due to the mRNA degrading at a faster rate than the protein.

5.4.1.iii. β- catenin expression in PDV

The transcriptional activity of β- catenin and its degradation is regulated by the Wnt pathway. In normal epithelial cells, β- catenin is localised at the cell membrane. The unbound β- catenin is degraded by the ubiquitin-proteosome system which involves the kinase GSK-3 β. Stabilisation of the cytoplasmic β- catenin by the aberrant activation of the Wnt signalling leads to its accumulation, complexing with LEF/TCF transcription factors and transactivation of LEF/TCF target genes. Activation of these genes can lead to cell proliferation or inhibition of apoptosis. Nuclear accumulation of β- catenin can be the result of gene mutations (Sparks et al., 1997). In colon cancers, disruption of the Wnt signalling pathway by mutations of either the APC or the β-catenin gene plays a crucial part in the early stage of tumourigenesis (Morin et al., 1997). In the current study diffuse cytoplasmic and paranuclear staining of the β-

catenin protein was found in 11 and four cases of PDV respectively. Three of these cases were in invasive PDV. These results may suggest involvement of aberrant activation of the Wnt signalling pathway in some cases of PDV.

5.4.2. Plakoglobin, E-cadherin and β catenin protein and mRNA expression in PDB

5.4.2.i. Plakoglobin expression in PDB

Plakoglobin has not been examined in PDB. Very few studies have sought to examine the role of plakoglobin in breast carcinomas (Bukholm et al., 1995: Zschiesche et al., 1997). Loss of heterozygosity and reduced expression of plakoglobin associated with disease progression has been demonstrated in breast cancers (Sommers et al., 1994; Aberle et al., 1995). In the current study only 26% of the PDB cases had >5% of Paget cells expressing plakoglobin. This implicates a possible role for plakoglobin in the formation of PDB. There was good correlation between plakoglobin mRNA and protein expression.

5.4.2.ii. E-cadherin expression in PDB

The role of E-cadherin in breast cancers is debatable. Reduced expression of E-cadherin has been reported to be associated with a poor outcome (Oka et al.,1999; Charpin et al.,1997) where as other investigators have demonstrated no independent prognostic value for E-cadherin in breast carcinomas (Lipponen et. al., 1998; Soler et. al, 1999). To my knowledge only one study (Shirahama et al.,1996) has examined the expression of E-cadherin in PDB and both cases were negative fo the protein. Eighty-three percent of the PDB cases in my study had >5% of Paget cells expressing

E-cadherin. It is therefore unlikely that reduced E-cadherin mediated cell-cell adhesion is an important factor in PDB carcinogenesis. In two cases mRNA and protein expression did not correlate, which as previously discussed, could be due to mRNA degradation at a faster rate than the protein.

5.4.2.iii β- catenin expression in PDB

Few studies have sought to examine the role of β- catenin in breast carcinomas (Bukholm et al., 1995: Zschiesche et al., 1997) and no one has examined the role of β-catenin in PDB. Forty- eight percent of my PDB cases had nuclear and paranuclear staining of β-catenin protein. The extramembranous accumulation could be due to mutation in the β-catenin gene or due to lack of its degradation by the GSK3 β/ proteasome/ APC system as a result of abnormal disruption of the Wnt signalling pathway. Except in one case, there was good correlation between mRNA signal and protein expression.

The adhesive function of cadherins is dependent on its interaction with catenins. Some reports have revealed reduced expression of both cadherins and catenins in certain tumours (Muzio et al., 1999). In the current study there appeared to be no correlation between reduced expression of E-cadherin and β-catenin.

In summary reduced expression of E-cadherin may a have a role to play in the pathogenesis of invasive PDV, unlike PDB where in the majority of cases normal expression of the protein was demonstrated. Abnormal plakoglobin and wnt signalling pathway may be involved in the formation of some cases of PDV and PDB.

Table 5.21 review of studies on the expression of plakoglobin, E-cadherin and β-catenin in PDV and PDB

Authors	Title of study	Results
Shirahama et al. (1996)	E-cadherin and P-cadherin expression in tumour tissues and soluble E-cadherin levels in sera of patients with skin cancer	Five cases (3 cases of PDV and 2 cases of PDB) negative for E- and P-cadherin
Tada et al.(1996)	Expression of E-cadherin in skin Carcinomas.	Eight cases of PDV. Decreased expression in the invasive (4cases) compared to in situ (4 cases)
Takata et al. (1999)	ErbB-2 overexpression but no activation of β- catenin gene in EMPD	Positive membraneous β-catenin protein expression in all 6 female cases
Tada et al. (2000)	Expression of desmoglein I and plakoglobin in skin carcinomas	11 cases of EMPD only 1 case from female pudendum. Negative for plakoglobin

CHAPTER 6: PAGET'S DISEASE OF THE VULVA DATABASE

Poster presentation

A.B MacLean, **P E Ellis**, F Cunningham, M Makwana, C Perrett. Paget's disease of the vulva and associated carcinoma. Proc 17th Meet. Int. Soc. Study Vulvovaginal Dis. Salvador, Brazil 2003.

Oral presentation

Ellis P. Paget's disease of the vulva. 30th British Congress of Obstetrics and Gynaecology. Glasgow, 2004.

Publication

A.B MacLean, M., Makwana, **Ellis P**, F.Cunningham (2004).The management of Paget's disease of the vulva. Journal of Obstetrics & Gynaecology. 24, 2 124-128 2004.

6.1 PDV national register and database

6.1.1 Introduction

PDV is a rare disease. Few clinicians have seen cases of Paget's disease of the vulva, with less than 1% seeing five cases or more (Tidy et. al. 1996). At the Royal Free Hospital, only four cases of PDV were diagnosed among 1000 women seen in the vulval clinic (MacLean et al., 1998). The paucity of information regarding the treatment and management of this disease has led to a number of treatment options being offered to the patient. A survey in 1996 in the management of preinvasive vulval lesions by gynaecologist and dermatologists in the UK revealed that dermatologists referred 66% of their Paget cases to gynaecologists; gynaecologists managed more than 80% of their cases of Pagets themselves, but referred 10% to other gynaecologists and 5% was referred to dermatologists. Dermatologists used excision, cryotherapy, topical 5-fluorouracil and retinoids, while 73% of gynaecologists advocated wide local excision, vulvectomy or radical vulvectomy (MacLean et al., 2004). Because of the few numbers of PDV cases any one clinician may have, the information regarding management, recurrence rate and prognosis is limited. With this in mind, the British Society for the Study of Vulval Diseases set up a national register and database for PDV in 1997. Forms (Figure 6.1) were sent out to clinicians requesting that they would register their cases of PDV. Clinicians submitted the completed questionnaire to the University Department of Obstetrics & Gynaecology at the Royal Free and University College Medical School. These patients have come from various parts of the UK and as far a field as South Africa. So far 106 cases of PDV have been registered. Seventy-eight (74%) of the cases were

from gynaecologists, 17 (16%) from dermatologists, 6 (5%) from clinical oncologists, and 5 (5%) cases were from plastic surgeons and general practitioners. The age range of the women registered was between 47 and 98 years. Average age at diagnosis was 68 years old. Twenty five percent of the referrals were women aged 80 years or more. Where the interval between onset of symptoms and diagnosis was recorded, in 27 cases it was less than one year, for 20 cases, it was between one and five years and for 11 cases it was more than five years with the longest interval being 13 years. A biopsy was taken in most cases to confirm the diagnosis.

6.1. 2 Underlying cancer

Thirty of the 106 patients registered had an underlying cancer. Thirteen patients had an invasive vulval adenocarcinoma discovered at the time the Paget's lesion was excised. Nine patients were diagnosed with intraepithelial Paget's disease initially, but subsequently developed invasive adenocarcinoma. The initial age of diagnosis of PDV was between 56 and 80 years. One patient developed invasion within a year and therefore may have had pathology that was missed at initial surgery. Two of these patients have died of metastatic disease.

6.1.3 Other cancers

From our register there were 16 patients who had a history of an associated cancer: for six there was dermal invasion diagnosed at the time of primary biopsy or excision, for nine, progression from intraepithelial to invasive PDV occurred 1-19 years (mean 7 years) after the original diagnosis. In the 16 cases with associated cancers, in three the timing of the diagnosis of cancer is unknown, in five it preceded the diagnosis of Paget's, in four it was synchronous and in two it was subsequent to

the diagnosis of Paget's. Figure 6.2 demonstrate year of diagnosis and associated cancers. Table 6.1 list the anatomical site of the various associated cancers. Seven of these patients have died from their cancers. It was interesting to note that eight cases of PDV were associated with breast cancer. Chanda (1985) also found that the highest number of cases of internal malignancy at a particular site was the breast. There have been reports in the literature of an association between previous PDV and breast cancer. Friedrich et al. (1975) reported 14 cases of PDV and breast carcinoma, Farrell et al., (1999) also reported two patients with PDV associated with invasive adenocarcinoma and a history of breast cancer. There have been cases in the literature documenting an association between PDV and PDB (Boehm and Morris, 1971; Popiolek et al 1998). In the database there was only one case of PDV associated with PDB.

All patients with PDV should be screened for associated cancers by mammography, ultrasound scan of the pelvis plus vaginal speculum examination, cervical cytology and endometrial biopsy. If the lesion involves the anal skin examination for an ano-rectal or colon cancer could include occult bloods and endoscopy, and similarly urine cytology, cystoscopy and intravenous urography considered for urothelial cancers.

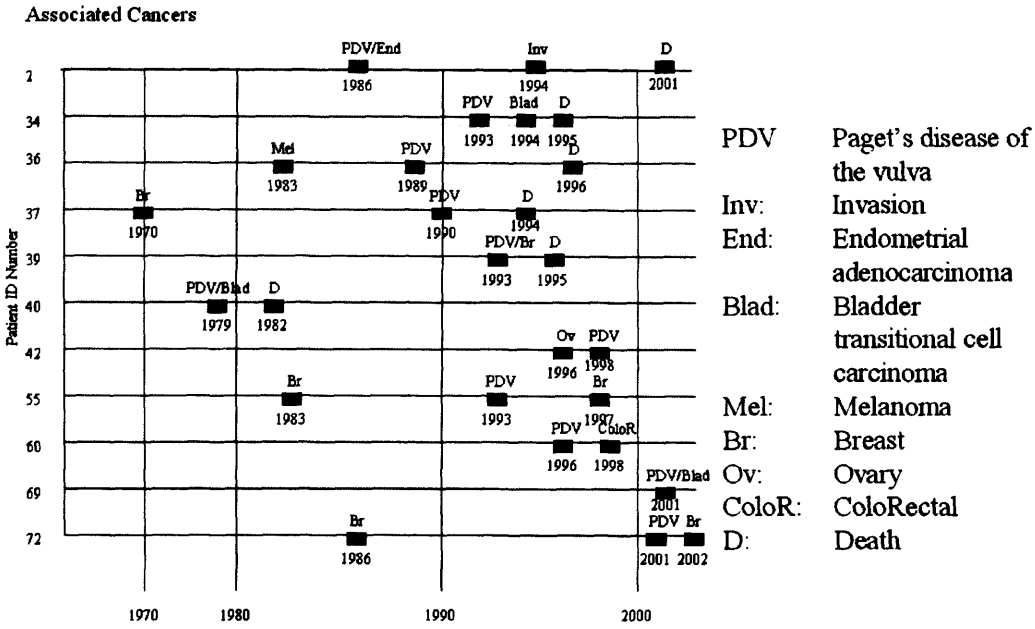

Figure 6.1 Time of diagnosis and associated cancers.

Table 6.1 Anatomical sites of associated cancers

Associated cancer	No of cases
Breast	8
Bladder	4
Ovary	1
Cervix	1
Endometrium	1
Colorectal	1

6.2 Management of PDV

6.2.1 Surgical treatment

The majority of patients (84 cases) in our database were treated by surgery and many had more than one procedure. Many of the women had co-morbidites such as diabetes and ischaemic heart disease which made anaesthesia and surgical management more difficult.

Previously, radical vulvectomy was the preferred treatment for PDV but others have recommended more conservative surgery (Feuer et al., 1990; MacLean et al., 2000). From the database we found that most clinicians preferred treating PDV by a wide local excision (Table 6.2), often with closure of the defect by the gynaecologist, but sometimes with a plastic and reconstructive surgeon using grafts, or pedicle flaps to close the defect. Other gynaecologists opted to treat their patients by a vulvectomy usually a simple vulvectomy, but sometimes radical, with the removal of inguinal-femoral nodes when there was an associated cancer recognised preoperatively. It is not possible from the information we have to comment on lymph node involvement.

6.2.2 Non surgical treatment

Several patients had no treatment, or topical corticosteroid cream only. These patients had either advanced or metastatic disease elsewhere or complex medical problems and were unsuitable for surgery. Immiquimod was given to 7 patients either as primary therapy after biopsy, or for persisting disease after surgery. In two of these patients there is no demonstrable disease, but in the others, although Paget's disease remains the symptoms have been controlled. As indicated (Table 6.2), some patients underwent lesion ablation with the carbon dioxide laser or photodynamic therapy, or

by radiotherapy. In most cases these treatments were used for recurrences after surgery and the numbers are too few to allow comment on efficacy. One patient developed recurrence following wide local excision and was treated with 42Gy over 10 fractions. The initial symptom relief was good, but her discomfort increased until repeat surgery became necessary.

Table 6.2 Management options from PDV database

Treatment	**No of cases**
Wide local excision	55
Vulvectomy	24
Surgery + radiotherapy	3
Surgery + photodynamic therapy	2
CO_2 laser	1
Immiquimod	7
Topical corticosteroids	2
No treatment	3

6.3 Follow-up of treated patients

As mentioned previously, the recurrence rate in PDV is high and over a half of all treatd patients will develop recurrent disease. Sometimes this will be symptomatic and demand further intervention, but symptoms of pruritis and irritation are not inevitable and observation may be appropriate. However, there is a small risk that apparently in-situ lesions can become invasive. Nine of the registered patients from the database showed such progression occurring 1-21yrs after a diagnosis of Paget's,

and with age at diagnosis of invasion ranging from 63-88 (mean 76.2yrs). Therefore, the patients need long term follow-up, at recommended intervals of 6 months if symptomatic and yearly if asymptomatic.

We have requested updates (Figure 6.3) on the cases of PDV to follow the outcome of these patients and the natural history of the disease. To date follow-up information has been received on 88 of the patients. Eleven women have been lost to follow-up because they have left the country or left the general practioners surgery or in some cases, no longer registered with the surgery and therefore presumed dead. There were 22 deaths with at least 14 of them dying of Paget's disease. Twelve died with widespread metastatic disease involving the lungs, liver and bone. Of the remaining patients the majority were alive but with evidence (clinical or on repeat biopsy) of persisting or recurring disease.

It is our hope that the information gained from the database will increase our understanding in the management and clinical outcome. We will continue to gather information on this rare but important disease.

PAGET'S DISEASE OF THE VULVA

REGISTRATION FORM

PATIENT'S NAME:_____

DATE OF BIRTH:_____

REFERRING CLINICIAN:_____

SPECIALTY: _____

ADDRESS: _____

_____

POSTCODE_____

Date of initial diagnosis (year):_____

How long were the symptoms present before diagnosis was made?_____

(Please **circle or tick** as appropriate responses)

Has a biopsy been taken for diagnosis? Yes / No

Could this tissue be made available for further research? Yes / No

SITE: Anterior
Posterior
Perianal
Both - Anterior and Posterior
Other: please specify _____

MANAGEMENT

Date – year only	Previous to diagnosis / date	Synchronous to diagnosis/ date	Subsequent to diagnosis / date
Only Biopsy (please circle)			
Topical application specify: Circle or add - Dermovate -Betnovate - 5 Fluorouracil - Synalar - Trimovate - Nerisone - Hydrocortisone - Daktacort -			
Wide Excision			
Vulvectomy			
Groin nodes			
Radiotherapy			
Photo DynamicTherapy			
Observation			
Other (specify)			

Is there an Associated Cancer ?
Please indicate which category it falls into and add the date if known

Type	Previous to diagnosis / date	Synchronous to diagnosis / date	Subsequent to diagnosis / date
Breast			
Genital Tract			
-Endometrium			
-Vulva			
-Ovary			
-Cervix			
-Vagina			
Colo-rectal			
Sarcoma			
Other (specify)			

Date of Diagnosis of Cancer_____

Current Status of Pagets – please circle

Persisting Disease symptomatic asymptomatic

New Disease symptomatic asymptomatic

No Apparent Disease _____

Was the Paget's **intraepithelial** or **invasive**? Please circle

Diagnosis of Invasion - within area of Pagets
at follow up - genital tract
- vulva
- colo-rectal
- elsewhere, please specify_____

Current Status of Patient Alive - and free of Paget's disease
Alive - with disease
Dead - date of death _____

- cause of death _____

Can I write for an update? Yes / No / NA

Please return to: Professor A B MacLean
University Dept of Obstetrics and Gynaecology
Royal Free and University College Medical School
Royal Free Campus, University of College London,
Rowland Hill Street, London NW3 2PF

On behalf of **The British Society for the Study of Vulval Disease**

Figure 6.2 Registration form.

For database use only
Patient ID Number _____ ***Registration Date*** _____ *Ref:pagets/pagets form2b/4'00*

FOLLOW-UP FORM

Date as Postmarked

Dear

We are updating our Paget's Register - we had originally thought we would do this annually but that now seems too frequent.

I enclose a form designed for computerising our data, but if you wish to photocopy correspondence and clinical notes on your patients, we will do the necessary form filling, or contact you for missing data. This exercise is producing some interesting UK-based information.

I appreciate your active input into this.

Yours sincerely

Professor A B MacLean
Head of Dept of Obstetrics and Gynaecology

Figure 6.3 follow-up form.

CHAPTER 7: CONCLUSION

The aim of the study was to identify any differences in the molecular pathology between PDV and PDB, and to investigate whether there were any differences in molecular markers in the Paget cells of those cases with invasive disease compared to those without invasive disease in PDV. The pathogenesis of PDV and PDB is unclear, thus the identification of biological factors involved in PDV and PDB carcinogenesis may be useful in clarifying the natural history of these diseases. Immunohistochemical, mutation and mRNA analysis was performed on molecular markers of cell cycle control and apoptosis (pRb, p53, and cyclin D1), cell proliferation (Ki67), molecular markers of oncogenesis (c-erbB-2), markers of angiogenesis (VEGF, PD-ECGF/TP and MVD). Proteins involved in cell adhesion (E-cadherin, plakoglobin and β-catenin were also analysed. All these molecular markers were chosen because they have been shown to be involved at different stages of the carcinogenic process and therefore could be involved in the development of PDV and PDB.

Sections that were immunopositive for the tumour marker p53 were then microdissected using LCM, the DNA extracted, amplified and sequenced to analyse for mutations in the exon 5-8 region of the *P53* gene. In situ hybridisation was also used to determine mRNA expression levels of the adhesion molecules in PDV and PDB.

This thesis attempted to answer the following questions:

1. Is there a difference in the molecular pathology between PDV and PDB?
2. Are there differences in the molecular pathology when PDV is associated with or without an underlying invasive adenocarcinoma?

3. Are there biological molecular markers that can determine the progression of intraepidermal PDV to invasive PDV?
4. If there are differences in the molecular pathology between intraepidermal and invasive PDV are these reflected in different clinicopathological presentation?

Is there a difference in the molecular pathology between PDV and PDB?

p53, cyclin D1 and pRb expression were generally higher in PDV compared to PDB. There was significant difference in the expression of cyclin D1 ($p < 0.001$) and pRb ($p = 0.03$) in PDV compared to PDB. The difference in p53 expression did not reach statistical significance ($p=0.09$). Twenty nine percent (15/52) of all cases of PDV expressed p53 compared to 13% (5/37) of all cases of PDB. Fifty nine percent (41/69) of PDV cases expressed the cyclin D1 protein compared to only 8% (3/34) of PDB, and pRb was expressed in 56% (40/72) of PDV compared to 33%(12/36) of PDB cases. There was no difference in the expression of Ki67 between PDV and PDB.

VEGF was not expressed by the Paget cells of either PDV or PDB. However, they may stimulate the surrounding epidermal and dermal cells to release VEGF. VEGF and PD-ECGF/TP were significantly higher ($p=0.001$ and $p<0.0005$ respectively) in the breast epidermal and dermal cells in PDB (61% and 95% respectively) as compared to the vulva cases (26% and 54% respectively). There appeared to be no significant differences in MVD between PDV and PDB cases

Abnormal expression levels of the cell adhesion molecules also revealed differences between PDV and PDB. Thirty six (57%) of the 63 cases of PDV expressed plakoglobin with a score of greater than >5 %; in PDB it was 26%. E cadherin was

expressed (>5%) in 98% of PDV cases and 83% of PDB, and for β catenin 69% of PDV and 52% of PDB expressed the protein.

In almost all cases the expression of the mRNA corresponded with the expression of the adhesion molecule proteins.

The results suggest that PDV and PDB demonstrate differences in the immunophenotype of various molecular markers. This could be as a result of the dysfunction of certain pathways (e.g cyclin D1 for PDV and pRb/ plakoglobin for PDB) and the angiogenic factors VEGF and PD-ECGF/TP having a more prominent role in the pathogenesis of PDB as compared to PDV.

Are there differences in the molecular pathology when PDV is associated with or without an underlying invasive adenocarcinoma?

p53 and pRb, molecular markers involved in cell cycle control were noted to have different rates of expression in PDV with invasive adenocarcinoma compared to PDV without invasive disease.

Abnormal p53 expression in PDV with invasive disease was 40% (4/10) and without invasive disease it was 26% (11/42, p= 0.62). Abnormal pRb expression was demonstrated in 70% of PDV with invasive disease compared to 40% of PDV without invasion (p= 0.16).

Similarly, E-cadherin, plakoglobin and β- catenin that are involved in cell-cell adhesiveness were found to be reduced in the intrapeidermal compartment of PDV with invasive disease as compared to those without invasive disease. E-cadherin was found to be significantly different, p =0.005. The p values for plakoglobin and for β-catenin were 0.27 and 0.21 respectively.

Disruption of cell cycle regulation at pRb checkpoints as demonstrated by abnormal

expression of the pRb protein and loss of apoptosis as seen by overexpression of the p53 protein may be mechanisms underlying disease progression in PDV.
Reduced cell-cell adhesiveness is one of the underlying mechanisms in the development of tissue invasion and metastasis. I have demonstrated reduced cell-cell adhesiveness in cases with invasive disease, which could be an underlying mechanism, involved in the progression of PDV

Are there biological molecular markers that can determine the progression of intraepidermal PDV to invasive PDV?

p53 over expression may be associated with progression of PDV towards invasive disease, since higher protein levels were found in PDV cases associated with an invasive carcinoma. Similiarly, loss of pRb expression was found in 70% of PDV cases with invasive adenocarcinoma and 40% of those without. pRb expression is present in normal tissue. Increased loss of pRb expression has been demonstrated in stage I to IV vulval cancer suggesting a possible role in tumour progression (Chan et al., 1998) The evaluation of pRb could be used as a diagnostic or prognostic marker for PDV in determining which patient will go on to develop invasive adenocarcinoma and therefore could be a helpful addition to clinicopathological investigations in the management of PDV. This could involve monitoring of patients with low pRb levels.

If there are differences in the molecular pathology between intraepidermal and invasive PDV are these reflected in different clinicopathological presentation ?

In general those patients with invasive disease have a higher risk of recurrence than those without invasive disease. However, not all cases of recurrent disease appear to be correlated with p53 expression and loss of pRb expression. One patient who had invasive disease had repeated surgery for recurrent disease but biopsies taken were

noted to be negative for p53. Loss of pRb expression was however noted from the tissue specimens. Loss of pRb expression maybe a better marker for determining which cases of Paget's disease would go on to develop recurrent disease and hence a useful prognostic marker.

FUTURE WORK

Retinoblastoma activity can be lost through mutations in the *RB1* gene or prevented by binding with the viral oncoprotein high-risk HPV E7 (Zur Hausen 1991). Further work can be carried out by assessing the presence of HPV activity in those PDV cases that have been demonstrated to have loss of pRb expression and sequencing of the RB1 gene to identify any mutations. *RB1* gene chip scanning can then be performed if there is a common mutation.

Performing mutational analysis on the PDV cases with reduced expression of Plakoglobin, E-cadherin and β catenin will allow further insight into the involvement of these molecules in the mechanism of PDV.

The continuing cooperation between those clinicians with PDV cases and the addition of these cases to the PDV database will allow for increased awareness and improved management of this rare disease, along with further detailed clinicopathological and molecular comparisons.

This has been the largest study performed on PDV and PDB. The identification and characterisation of the genes and pathways involved in the molecular mechanism of PDV and PDB will lead to a better understanding of these diseases, generating potentially new markers and allow for the development of therapeutic improvements.

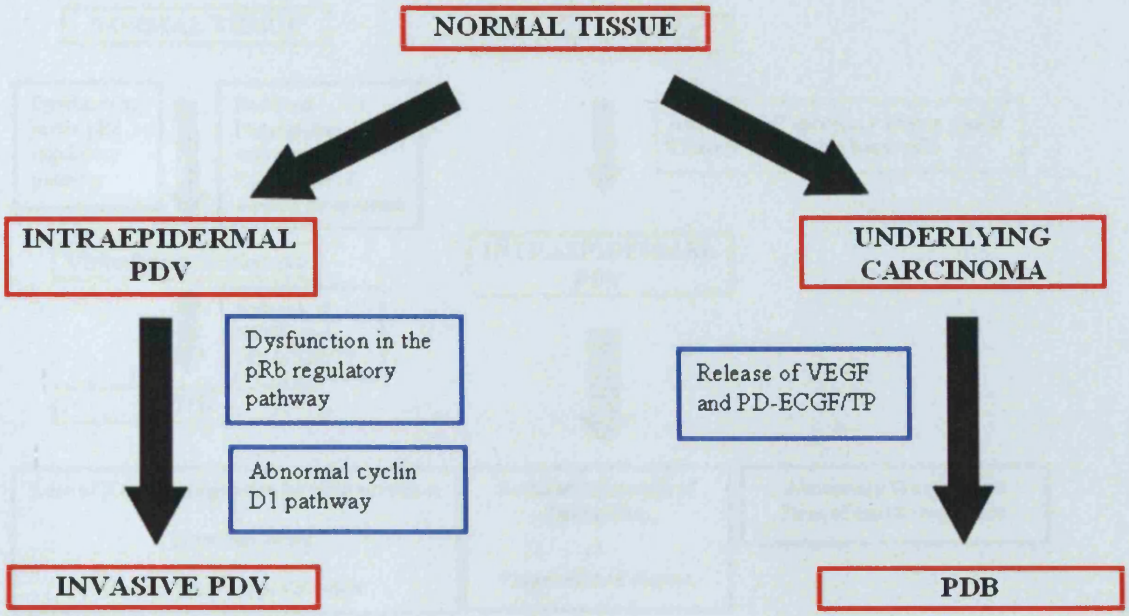

Figure 7.1 Potential pathways for the differences in the molecular pathology between PDV and PDB. The blue boxes indicate changes identified in this work.

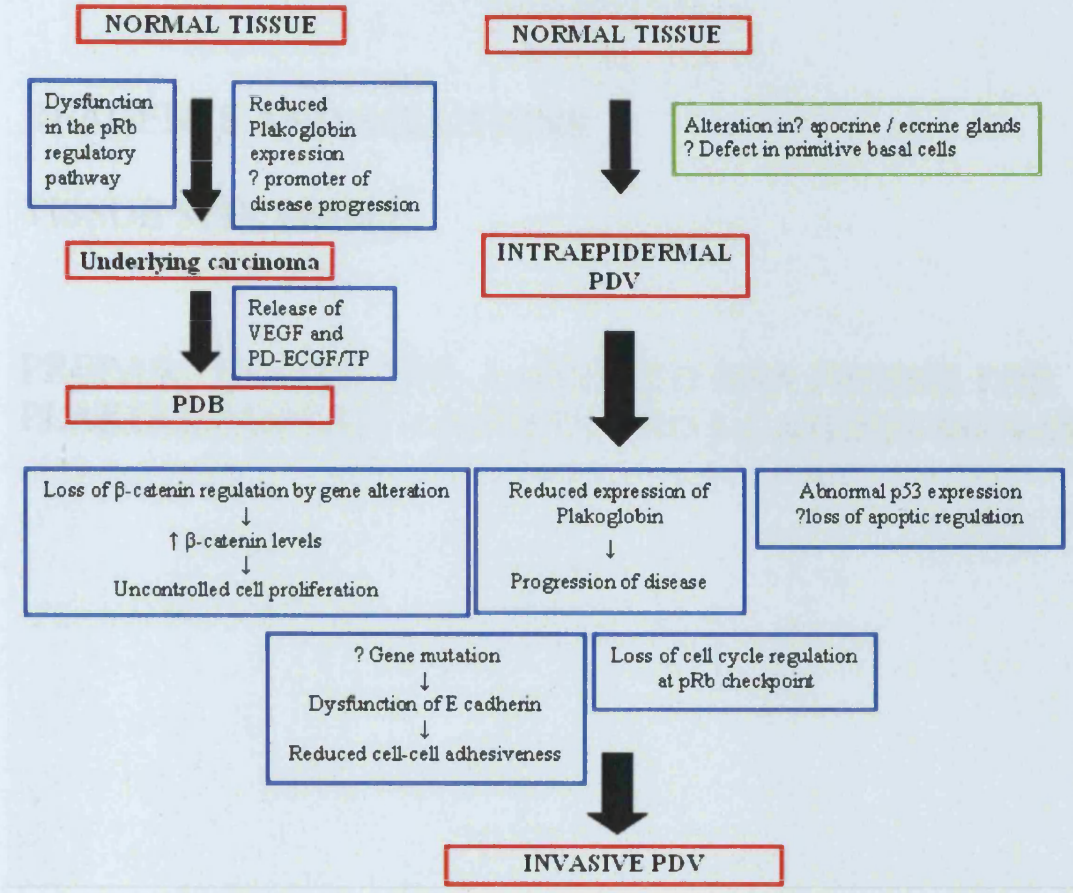

Figure 7.2 Potential mechanisms for disease progression in PDV and PDB.
The blue boxes indicate changes identified in this work. The green box indicates results from other studies.

APPENDIX I

REAGENTS AND SOLUTIONS

TISSUE SPECIMENS

PREPARATION OF DIG- LABELLED RNA PROBES FOR PLAKOGLOBIN, E-CADHERIN AND β-CATENIN MRNAS.

REAGENTS AND SOLUTIONS

3-Aminopropytriethoxysilane solution (APES)
294 of acetone with 6ml APES. Keep APES in the fridge and dilute in acetone just prior to use.

Diaminobenzidine (DAB) Solution

Add 180mg of 3,3'-diaminobenzidine tetrahydrochloride to 270ml of distilled water, 30ml TBS, 1ml imidazole solution. Add 120-µl hydrogen peroxide.

Hydrogen peroxide solution

Add 10ml 30% hydrogen peroxide to 90ml distilled water.

Phosphate Buffered Saline

Dissolve 5PBS powder satchets in 5l distilled water. pH must be 7.4

Phosphate Buffered Saline- bovine serum albumin (PBS-BSA)

100mg of bovine serum albumin is dissolved in 100mls of PBS.
0.1% BSA. Store at 4° C.

Sodium Citrate Buffer

2.1g of citric acid monohydrate is added to 950ml of distilled water and 13ml 2M NaOH. Make up to 1l. Adjust pH to 6.0. Store at 4° C. 0.01M sodium citrate. Store at 4° C.

Tris-Buffered saline (TBS)

43.83g of NaCl and 30.6g of Tris base is made up to 5l of distilled water. Add ~35ml of concentrated HCl, pH must be 7.6, store at 4° C.

10% formol saline:

40% formaldehyde (100ml)

Sodium chloride (9g)

Tap water (900ml),

10% neutral buffered formaldehyde:

40% formaldehyde (100ml)

Distilled water (900ml)

Sodium dihydrogen phosphate monohydrate (4g) and

Disodium hydrogen phosphate anhydrous (6.5g)

In aqueous solution formaldehyde exists mostly as its monohydrate, methylene glycol $[CH_2(OH)_2]$ in equilibrium with monomeric formaldehyde.

Pre-hybridisation solution (1ml)	Hybridisation solution (1ml)
200µl 20XSSC	200µl 20XSSC
100 µl 10X Denhardts	100 µl 10X Denhardts
500 µl Formamide	500 µl Formamide / 20% Dextran Sulphate
50 µl 10mg/ml t-RNA	50 µl 10mg/ml t-RNA
50 µl 10mg/ml salmon sperm DNA	50 µl 10mg/ml salmon sperm DNA
100 µl DEPC water	100 µl labelled probe

Buffer 1 (1L)

Tris-HCl pH 7.5	100ml (0.1M final concentration)
4M NaCL	37.5ml (150mM final concentration)
H_20	862.5ml

Buffer 2 (1L)

Tris-HCl pH 9.5	100ml (0.1M final concentration)
4M NaCL	25ml (0.1M final concentration)
H_20	825ml

Buffer 3(1L)

Tris-HCl pH 8.0	10ml (10mM final concentration)
0.5M EDTA	2ml (1mM final concentration)
H_20	988ml

TISSUE SPECIMENS

Tissues were processed using an enclosed automatic processing system (VIP 2000F/300E) programmed with the following processing schedule:10% neutral buffered formalin two hours at 40°C, 70% industrial methylated spirit (IMS) one hour at 40°C, 90% IMS one hour at 40°C, absolute IMS three hours at 40°C, xylene four hours at 40°C, paraffin wax for three hours at 60°C. Tissues were embedded in paraffin wax utilising the Tissue-Tex III.

Preparation of DIG-labelled cRNA probes for plakoglobin, E-cadherin and β-catenin mRNAs.

Producing the PCR product

Template cDNA was generated from a two-step reverse transcriptase-PCR using GeneAmp RNA PCR core kit (Perkin Elmer). The method was as per manufacturers instructions.

The following materials were used to produce a 50 µl PCR reaction:

10-100ng of cDNA template

5µl of 10X PCR buffer

2 µl 50Mm $MgCL_2$

0.5µl of 50mM dNTP's

1µl of forward and1µl reverse primer

Sterile water was added to make up the volume to 49µl. 1 µl of the enzyme Biotaq polymerase (Bioline)(1 unit/ µl) was then added to the solution to make a final volume of 50µl.

All PCRs were carried out using a single block PCRexpress (Hybaid).

PCR products were amplified as described with 0.8 pmoles forward primer and 1.6 pmoles reverse primer.

After the PCR reaction, 95% formamide containing 1% pararosaniline (sigma) was added in equal volume to the PCR product and DNA denatured at 95%C for 2 min.

Primer sequences and annealing temperature for PCR are described in Table 5.22.

Gene	Forward primer	Reverse primer	Anneal Temp/°C
Plakoglobin	GCCTGCCTTCTTCTTGTGTC	CTGAAGCTTTAGTGGCCAGG	60
β-catenin	GGAAGGTCTCCTTGGGACTC	ACTAGTCGTGGAATGGCACC	60
E-cadherin	TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGAC	60

Table 5.22 Primer sequences and annealing temperature for PCR.

Analysis of the PCR product using agarose gel electrophoresis

3 µl of PCR product was analysed by alongside 3µl of DNA marker using agarose gel electrophoresis. Agarose gels were made by dissolving 1% agarose (sigma) in 1xTBE (45mM Tris-borate, 2mM EDTA) and then heating the solution in the microwave. After cooling to below 65°C, Et Br (final concentration 20 µg /ml) was added and the gel poured into a casting tray. DNA products were loaded onto the gel using 6x loading dye and run in a submarine tank (BioRad) containing 1xTBE. For size comparison the 3 µl of known DNA was used as a marker ladder and loaded onto each gel. Products were visualised using a UV transilluminator (Alpha Innotech corporation).

Purification of the DNA from the agarose gel was performed using a QIAgen Gel Extraction kit and carried out as per manufacturer's instruction.

Linearisation of the plasmid and ligation of the PCR product

Approximately 1 µg of DNA was subjected to restriction endonuclease treatment for 1-2hrs (as specified by the manufacturer (NEB) using 1-5 units of enzyme in a total reaction volume of 20 µl containing 1x buffer (also specified by manufacturer). The product was subsequently phenol-chloroform extracted and ethanol precipitated before resuspension in distilled water.

Ligation of the vector and purified insert DNAs were carried out using T4 DNA ligase (NEB {2 units per reaction}) in supplied reaction buffer in total volume of 15µl. Ligations were carried out overnight at 16°C and 3 µl of the reaction used to transform chemically competent bacteria using the TOPO Cloning Reaction (Invitrogen).

Growing the bacterial colonies with the DNA sequence required using the TOPO Cloning Reaction

This reaction provides the direct insertion of Taq polymerase-amplified PCR product in a plasmid vector. The plasmid vector (pCR II-TOPO; (Invitrogen) is linked to Topoiosmerase I, which catalyses the insertion of the PCR fragment utilising the A overhangs generated by Taq polymerase during PCR amplification.

The following materials and reagents were used to perform the TOPO cloning reaction as per manufacturer's instruction:

3 µl fresh PCR product

1 µl of sterile water

1 µl of TOPO vector (pCR II-TOPO)

1 µl of salt solution (1.2 M NaCl; 0.06M $MgCl_2$) which was added last

The 6 µl mixture was then gently mixed and incubated at room temperature for 5 min. The reaction was then left on ice and the one shot chemical transformation was performed.

One-shot chemical transformation

2 µl of the TOPO cloning reaction from above was mixed in a vial with chemically competent E. coli . The mixture was incubated on ice for 5-30 min and then heat shocked for 30s at 42 °C . This was followed by immediately transferring the tubes to

ice. 450 µl of Luria-Bertani (LB) broth was added to the mixture and it was then incubated at 37 °C for 1 ½h

3-5 µl of the mixture (100 µl from each transformation) was spread onto a prewarmed selective plate (agar gel, 50 µg /ml ampicillin and LB) with a glass rod and incubated overnight at 37 °C.

A colony was picked and cultured overnight in LB medium containing 50ng/ml ampicillin broth.

Isolating the DNA plasmid using miniprep protocol (Qiagen)

This system is based on the alkaline lysis of bacteria and differential precipitation of small plasmid DNA. The method is described as per the manufacturer's (Qiagen) instructions.

The plasmid DNA was isolated by spinning the solution of LB ampicillin and culture down until pellet of bacteria is seen.

The solution of LB ampicillin and culture was placed in to a microcentrifuge tube and centrifuged at a speed of 8,000rpm for 1 minute and excess fluid was removed. The pelleted bacterial cells were then resuspended in 250 µl of buffer P1 (resuspension buffer) and transferred into a microcentrifuge tube. This was followed by the addition of 250 µl of buffer P2 (lysis buffer) and mixed and then 350 µl of buffer N3 (neutralisation buffer). The mixture was then centrifuged at a maximum speed (13,000 rpm) for 10 min. The supernant was removed and placed into centrifuge columns and spun for 1 min. The supernant fluid was again removed and mixed with 750 µl of the buffer PE (wash buffer) and spun for 1 min. The supernant removed was respun for a further 1 min to remove residual wash buffer. To elute DNA 50 µl

of water was added and allowed to stand for 1 min and then centrifuged for a further 1 min.

Linearisation of the template DNA using the restriction enzymes EcoRI and Bam H1

As previously described, the DNA inserts were cloned into the pCRII-TOPO vector which have promoters, T7 and SP6 either side of the cloning site. This enables both antisense and sense probes to be synthesised using one transcription vector but two different RNA polymerases: T7 RNA polymerase for antisense RNA (cRNA) and SP6 RNA polymerase sense RNA (mRNA).

In order to synthesise antisense RNA probes, the DNA was linearised with the restriction endonuclease EcoR1 to initiate transcription from the T7 promoter. For the sense probe, the DNA was linearised with the restriction endonuclease Bam H1 to initiate transcription from the SP6 promoter.

The following materials were used to for a 50 µl reaction mixture

5 µl of restriction buffer (10x)

10 µl DNA template

34 µl sterile water

1 µl of restriction enzyme (Eco R1 or Bam H1)

The mixture was incubated at 37 °C for 2 h and then the DNA was purified using 200 µl of phenol:chloroform extraction mix , 80 µl of water and 20 µl of NaOH. This was followed by centrifuging for 7-10 min at 13,000rpm.

180 µl of the top phase (aqueous) of the mix was removed and placed into a DEPC-treated tube with 500 µl of 100% cold ethanol. This was incubated over night at room temperature. The mixture was centrifuged for 10 mins at 13,000rpm and then the

ethanol was removed. 170 µl of 70% ethanol was added and the mixture was centrifuged for 3 min at room temperature. The 70% ethanol was removed and the remaining solution was dried at room temperature. This was followed by resuspending the mix in 100 µl DEPC water.

Transcription of the RNA probes

This was performed using the DIG RNA labelling kit (Boehringer Mannheim). This uses DIG to label RNA transcribed by either T7 or SP6 polymerase from the plasmid template. Plasmid templates with specific inserts to hybridise to plakoglobin, E-cadherin and β-catenin mRNAs were constructed.

The following reagents were added to a sterile RNAse- free microfuge tube (on ice):

4 µl of purified template

2 µl of NTP labelling mixture

2 µl transcription buffer

1 µl of RNAse inhibitor (placental)(20units/ µl)

2µl RNA polymerase (20units/ µl) (SP6 and T7)

The mixture was vortexed and centrifuged briefly for a few seconds and then incubated for 2 hr at 37°C. Following this 2µl of 0.2M EDTA solution was used to stop the reaction. The mixture was centrifuged for 15 min at 13000 rpm. The remaining pellet was washed with 50µl 70% cold ethanol and centrifuge for 2-3 min at 13000rpm. Excess alcohol was pipetted off and the remaining pellet was dried briefly under vacuum and dissolved in 30µl RNAase-free water.

As the amount of DIG-labelled RNA transcript greatly exceeds the amount of DNA template, removal of the DNA template (by DNAase I) was unnecessary in this case.

Quantification of the probe by a dot blot test

To ensure an excess of probe and also that an approximately equal amount of label was added to both sense and antisense reactions, the labelling of RNA was quantified.

Using DIG labelled RNA control (100μg/ μl), serial dilution of the sense and antisense probes and the control RNA were performed. 1 μl of control RNA was placed into a RNase free tube along with 9 μl of DEPC water. From this tube 1 μl of the solution was placed into another tube with DEPC water (1/100 dilution). This is repeated until a final dilution of 1/10000 is obtained. A spot of probe or control RNA was placed onto Hydrobond XL nucleic acid membrane and cross- linked using UV radiation (120mJ) for 5 min.This binds the RNA onto the membrane. The membrane was placed in 5% milk diluted in Tris-buffered saline-Tween mild detergent (TBS-T) and left for 1h on the rotator at room temperature. The milk protein prevents non-specific binding. The excess milk was removed and the anti- DIG antibody (1:500 dilution) was added. This was placed into a sealed plastic bag and placed on the rotator for 30 min. Colour development was produced by adding 2ml of NBT/BCIP into the sealed plastic bag containing the membrane. This was placed on the rotator for approximately 30 min until colour developed and washed with TBS-T and allowed to dry. Newly-labelled RNA was compared to pre-quantified control labelled RNA to estimate the concentration of labelled probe RNA.

APPENDIX II

MOLECULAR DETAILS FOR EACH PATIENT

PDV	p53	Ki67	Cyclin D1	pRb	E- cadherin	B-catenin	Plakoglobin		
CASE 001		10%	0	0	m<100% s	n <5% 0	m <100% s	Nested	
CASE 002	0	0	0	0	m<75%s	m<25% mi	m<25% s	Nested	
CASE 003	0	0	50%	75%		mc<100% s	nu<100% m<25*	Single cell	
CASE 004	0	0	0	0	m <25% s	0 <5% 0	m <75% s	nested lichenoid	
CASE 005	0	10%	20%	0	m <75% s	0 <5% 0	m <75% s	nested lichenoid	
CASE 006	0	0	0	<10%					
CASE007inv	0	0	0	50%	m<100%s	n<5% 0	m<25%s	nested	
CASE 008	0	40%	40-50%	>75%	m <5% 0 mi	0 <5% 0	m <5% mi	single cell	
CASE 009	0	10%	<10%	0					
CASE010inv	0	50%	10%	0	m<25%mi	m<75%s	n<5% 0	single cell,	lichenoid
CASE 011	10%	10%	0						
CASE 012	10%	5%	75%	25%					
CASE 013	0	10%	20%	0	m<100% s	n <5% 0	m <75% mi	nested	
CASE014inv	0	0	<10	10%					
CASE 015	0	5%	75%	<10%	m <75% s	m <25% s*	n <5% 0	single cell	
CASE 016	0	50%	0		m <100% s	c <5% mi	nu <50% mi	nested lichen	
CASE 017	>75%	5%	<10%	>75%	m <75% s	m <50% mi	c <5% mi	nested	
CASE 018	<10%	5	50%	0	m<50 mi	c <75mi	n<5%0	nested	
CASE 019	75%	75%	50-75%	>75%					
CASE 020	0	0	0	0					
CASE 021	0	0	50%	<10%	m<100%s	n<5% 0	m<75%s	single cell	
CASE022inv	25-40%	>75%	<10%	>50%	0 <5 0	0 <5 0	0 <5 0	licheniod	
CASE 023	75%	20%	75 %	0	m<100%s	m<100%s	m<5%mi	single cell	
CASE024inv	25-40%	5	10%	0	m<50% s	c<25% mi	c<25% mi	infiltrative	
CASE 025	0	0	40%	50	m<100%s	c <5% mi	0 <5% 0	single cell	
CASE 026	0	0	15%	>75%					
CASE 027	<10%	0	75%						
CASE028inv	30-40%	0	50%	0	m<25% mi	N<5% 0	n<5% 0	nested	
CASE 029	<10%	50%	50%	50-75%	m<75%s	M<50%s	c<5% mi	single cell	
CASE030inv	75%	0	0	0					
CASE031	0	0			m<50% s	M<5%mi	n<5% 0	single cell	
CASE 032	10%	5%	<10%	>1%	m<75%s	M<50%s	m<5%mi	nested	
CASE 033	50%	50%	75%	>75%	m<100%s	M<75%s	m<25%mi	nested	lichenoid
CASE 034	25-40%	50%	10%						
CASE 035	<10%	20%	20-40%	0	m<75%s	M<25%mi	n<5% 0	single cell	lichenoid
CASE 036	10%	0	10%	20-40%					
CASE 037	75%	40%	75%	0					
CASE 038	<10%	0			m<75%s	M<50%s	m<25%mi	single cell	lichenoid
CASE 039	50-75%	0	75%	50%	m<75%mi	c<25%mi	m<50%s	nested lichen	lichenoid
CASE 040	0	0	0%	>75%	m<100%s	M<50%s	m<75%s	single cell	lichenoid
CASE 041	10%	25%	<10%						
CASE 042	75%	75%	0	>75%	m<100%s	M<25%mi	m>50%s	single cell	lichenoid tissue
CASE 043	75%	50%	75%		m<50%s	M<25%s	m<100%s	single cell	
CASE 044	0	50%	<10%		m<75%s	M<25%s	m<50%s	nested	single cell
CASE045inv	0	5%	0	0	m100% s	0<5% 0	m<100% s	nested	

PDV	p53	Ki67	Cyclin D1	pRb	E- cadherin	B-catenin	Plakoglobin		
CASE 046	0	0							
CASE 047		5%	50%	>75%	m<75%s	M<25%s	m<25%s	single Foll+licen	lichenoid
CASE048inv	0	0	10%	0					
CASE049inv	0	50%	20-40%	0					
CASE 050	>75%	0		20-40%					
CASE 051	0	75%	0	0	m<50%s	M<5%mi	m<25%mi	single cell	
CASE 052			50%	75%	m<100%s	c <5% mi	m<75% s	single cell	
CASE 053			0	20%	m<100%s	c<25% mi	n<5% 0	single cell	lichenoid
CASE 055	20-40%	0	50-75%	0					
CASE056	16/3.0		50%	20-40%					
CASE 057	0	10-20%	75 %	50-75%	m<100%s	M<50%s	m<25%mi	nested pat. Li	lichneoid
CASE 058				<10%	m<25%mi	P<25%s	m<25%s	single cell lichenoid	
CASE 059	0	0	>75%	>75%					
CASE 060			50%	50%	m<100%s	M<50smi	m<25%mi	single cell	
CASE 061				0	m<75% s	c<50% mi	c<25% mi	single cell	
CASE 062	0			>75%	m<100%s	m<25% mi	c <25% mi	lichenoid	
CASE 063	<10%	50%	10-25%	0	m<50% mi	M<25% mi	c<75% s	single cell	lichenoid
CASE 064	20-40%	20-40%	0	>75%	m<25%mi	M<50%s	m<50%s	single cell	
CASE 065	<25%	0		<10%	m<75%s	M<25%s	m<75%s	single cell	
CASE 066	0	0	0	0	m<75%s	M<25%mi	c<5%mi	single cell	
CASE 067	10-20%	0		<10%	m<100%s	N<5%0	m<75%s	single cell	
CASE 068	<10%	<10%	50%	50%					
CASE 069	0	0			m<75%s	N<5%0	m<50 mi	single cell	
CASE 070	5.3/2.3		<10%	0	m<50%s	P<25%s	m<5%mi	single cell	
CASE 071	<10%	<10%		0	m<75%s	P<5%s	c<5%0mi	singlecell	
CASE 072	0	0	<10%	0	m<75%s	M<50%m	n<5% 0	nested follicular+eccrine duct ext	
CASE 073			<10%	>75%	m<75%s	M<5%s	n<5% 0	singlecell	lichenoid
CASE 074	0	0	10%	0	no tissue	N<5%0	m<5%mi	single cell	
CASE 075	>75%	0	>50%	20-40%	m<75%s	M<5%mi	m<50% s	single cell	Lichenoid
CASE 076				0	m<100%s	M<25%s	m<75%s	single cell	
CASE 077	75%	50%	75%	75%	m<100%s	M<25%s	m<5%mi	single cell	
CASE 078				0	m<75%s	M<25%mi	n<5% 0	single cell	Lichenoid
CASE 079	0	0		20-40%	m<100%s	M<25%mi	n<5% 0	single cell	
CASE 080	0	0	50 %	<10%	m<50%s	P<25%s	m<5%mi	nested follicular+eccrine duct ext	
CASE 081	0	0	0	0	m<75%s	M<25%s	c<25%mi	single cell	Lichenoid
CASE 082	0	0			m<50%s	M<25%s	n<5%0	single cell	
CASE 083	0	75%	0						
CASE 084	50%	0%							

Inv= invasive PDV, m= membraneous, c= cytoplasm, nu= nuclear, n=nil, p= paranulcear, mi= mild, s= strong

PDB	p53	Ki67	Cyclin D1	pRb	C-erBb-2	E- cadherin	β-catenin	Plakoglobin
CASE 087	0	0	0	0	>75%	m<75% s	c<25% mi	n<5% 0
CASE 088 DCIS			0	0		n <5% 0	n <5% 0	n <5% 0
CASE 089 CA			0	0	>75%	n<5% 0	no staining	n<5% 0
CASE 090		0	0	0	0			
CASE 091DCIS+CA				20-40%	0	m<100% s	c <5% mi	m <5% s
CASE 092 CA	>50%	0	0	0				
CASE 093	0		25-40%	0	>75%	m<100%s	c<5% mi	c<5% mi
CASE 094 DCIS	>20%	50%	0	0		m<75% s	n <5% 0	c<25% mi
CASE 095 DCIS	0	0	0	0	>75%	m<25% mi	m<50% mi	c<5% mi
CASE 096 DCIS+CA	>10%	10%	>20 %	10%	0	m<100%s	m<50% s	m <75% s
CASE 097 CA			0	0	>75%			
CASE 098			0	0	>75%			
CASE 099 DCIS	0	0	0	0	0	m<100% s	c<5% mi	m<75% mi
CASE 100 CA			0	0	0	m <75% s	m <75% s	n <5% 0
CASE 101				0	0	m <100s	0	c <100% s
CASE 102 DCIS			0	0	>75%			
CASE 103 CA			0	0	0	m<100% s	m<5% mi	n <5%0
CASE 104 DCIS			0	0				
CASE 105			0	0	50-75%			
CASE 106	<10%	0	0	0	0			
CASE 107	0	0	0	10%	>75%			
CASE 108 DCIS		0	0	0	>50%	m<100%s	m<25% mi	n<5% 0
CASE 109 DCIS	0	0	0	0	75%			
CASE 110		<10%		0				
CASE 111	0		0	0	<10%			
CASE 112 DCIS			0			m<25% mi	m<50% mi	c<5% mi
CASE 113 DCIS+CA	0			0	0	M<100%s	0	C<100%s
CASE 114 CA	0	<10%	<10%	0	25-40%			
CASE 115	0		0	40 %	75%			
CASE 116 DCIS+CA	0	0	0	>10 %	0	c<75% s	c<75% s	c<5% mi
CASE 117		<10%	0	0	>75%	m<75% s	c<25% mi	n<5% 0
CASE 118	0	0	0	0	>75%			
CASE 119								
CASE 120			0	>10%	>75%			
CASE 121								
CASE 122 DCIS			0	0	>75%	n <5% 0	n <5% 0	N <5%0
CASE 123 DCIS+CA	0		0	50-75%	0	m <100% s	m<75% s	M <75% s
CASE 124 DCIS	<10%		0	10				
CASE 125DCIS			0	0	>75%	m<100% s	c<25% mi	c <5% mi
CASE 126 DCIS+CA	\z>10%			>10%	>10%	m<75% s	m<75% s	M <5% s
CASE 127				<10%		m <100% s	m<75% s	M <75% s
CASE 128 CA	<10%	<10%	75%	<10%	>75%	m<25% mi	c<5% mi	mi<5%mi
CASE 129 DCIS	0	0	0	10%	>50%	m<50% mi	m<75% s	C< 25% mi
CASE 130	>10%			0	>75%			
CASE 13 1DCIS	0	<10%	0	0	0			
CASE 132 DCIS	>50%	>75%		15%	0	m<75% mi	m <75%s	c <5% mi
CASE 133	>50%		0		>75%	m<100%s	c<5% mi	c<5% mi
CASE 134	0	0	0		0			
CASE 135								
CASE 136					>75%	n <5% 0	m <5% mi	n <5% 0
CASE 137					>75%			

PDB	p53	Ki67	cyclin D1	pRb	C-erBb-2	E- cadherin	β-catenin	Plakoglobin
CASE 138								
CASE 139		0		>75%		m<100%s	c<5% mi	nu<25% mi
CASE 140						m<100%s	c<5% mi	nu<25% mi
CASE 141				10%				
CASE 142				0				
CASE 143				0		m<75%s	M<75% s	n<5% 0

PDV	HVDx200	AVDx200	HVDx400	AVD x400	VEGF	PD-ECGF	(intensity of staining)
CASE 001	17	13.3	5	3.7	0	>75%	2(e), 1(d)
CASE 002					7(d) 3(b)	,10%	1(e),1(d)
CASE 003	46	30.3	5	3	0	50%	1(e),3(d)
CASE 004	42	32	8	5.7	5(d)	10%	2(e), 2(d)
CASE 005	21	14.3	12	7.3	0	0	3(e), 1(d)
CASE 006	8	6.7	8	5.7	0	0	
CASE007inv	3	1.3	17	14	0	50%	2(e), 2(d)
CASE 008	26	20.3	14	9.6	0	<5%	0(e), 2(d)
CASE 009	7	4	9	7.3	2(e)	75%	3(e), 2(d)
CASE010inv					1(e),2(d)	75%	3(e), 3(d)
CASE 011					3(e)	75%	3(e), 2(d)
CASE 012	32	26.7	8	6.3	1(d)	<10%	
CASE 013	22	15.3	9	5.3	0	>75%	2(e), 2(d)
CASE014inv	22	14.7	11	8.7	1e)	<10%	
CASE 015	27	18.3	6	5.3	1e)	<10%	
CASE 016	25	16	16	11.7	2(d)	50%	1(e), 2(d)
CASE 017	26	15.3	6	4.6	1(e),2(d)	<10%	
CASE 018	100	98.3	12	6.3	3(e)	50%	
CASE 019	50	34.7	13	9.3	0		
CASE 020					1(e)		
CASE 021	14	4.7	7	2.3	1(d)		
CASE022inv							
CASE023	3	2	2	1.3	0	0	
CASE024inv	20	19.7	6	5.7	4(d)	10-25%	
CASE 025	16	14.6	13	12.3	2(d)	50-75%	
CASE 026	17	13.7	8	6		50-75%	3(e), 2(d)
CASE 027	30	22.3	22	11	5(d)	0	
CASE028inv	60	38.6	33	19	7(e+d)	50%	
CASE 029	30	24.7	13	6.7	0	0	3(e)
CASE030inv	18	10	11	7	1(d)	10%	
CASE 031	24	13	6	4.6	1(d) 3(b)	50%	
CASE 032	22	13	5	3.7		40%	
CASE 033	31	23	12	10.3	1(d)		
CASE 034	30	23.3	13	7.7	2(e)		
CASE 035	15	10.7	5	3	0		
CASE 036	22	13.7	23	9			
CASE 037	17	15	8	6.3			
CASE 038	26	13	23	12.3	0	0	
CASE 039	32	26.3	20	11	0	0	
CASE 040	18	12.7	8	7		0	
CASE 041	28	22	15	12.3			
CASE 042	3	2	5	3.3			
CASE 043						10-25%	
CASE 044							
CASE045inv	3	2.3	4	2.3	0	<10%	1(e), 2(d)
CASE 046					0		
CASE 047	68	49.7	15	12.7	0	<10%	2(e), 2(e)
CASE048inv	3	1.3	9	7.7	0		
CASE049inv	34	25	6	5	0		
CASE 050	47	27.3	20	11.3		0	

PDV	HVDx200	AVDx200	HVDx400	AVD x 400	VEGF	PD-ECGF	(intensity of staining)
CASE 051	34	22.7	18	13.3		0	1(e)
CASE 052					2(e), 0(d)	<10%	
CASE 053	60	39.3	8	6		<10%	
CASE 054	25	22	9	7.3		75%	
CASE 055	30	20	20	6.6		75%	
CASE 056	16	5.3	3	1	0	75%	
CASE 057	57	38.3	10	5		20-40%	
CASE 058	22	15	5	4.3	4(d)	>75%	
CASE 059	29	12	5	3.3		>75%	
CASE 060					0		
CASE 061						>75%	
CASE 062	38	12.7	20	13.7		25-40%	
CASE 063	38	26	10	5.3		0	3(e)
CASE 064	22	9.6	26	18.6		10%	3(e)
CASE 065					0	>75%	3(e), 2(d)
CASE 066	20	17.7	4	3.3		<10%	
CASE 067	17/	13.3	5	3.7			
CASE 068					3(d)	>5%	2(e),1(d)
CASE 069					0		
CASE 070	9	5.3	3	2.3	0		
CASE 071	11	7	11	8.6	0	>50%	2(e), 3(d)
CASE 072	20	13.7	7	7	0		
CASE 073	12	9.7	7	6.3		20-40%	
CASE 074	54	43	14	12.7	3(e) 2(d)	20-40%	
CASE 075	16	12	5	3	2(e) 5(d)		
CASE 076	35	19.6	2	1.3	1(e), 3(d)	<10%	1(e), 1(d)
CASE 077	30	24.7	10	4.6		0	2(e)
CASE 078							
CASE 079	38	28.3	12	8.3		>75%	
CASE 080	32	22.6	2	1.6		0	2(e)
CASE 081	36	24.6	17	12	2(e),0(d)	>75%	3(e), (2)d
CASE 082	50	28	4	3		>75%	1(e)
CASE 083					1(d)		
CASE 084							
CASE 085	24	17	7	5.3			
CASE 086							
CASE 087	6	3.3	2	1.3	0	75%	1(e),2(d)
CASE 088	24	16.7	10	6.7	3(d)		
CASE 089					2(d)	<5%	

Inv = invasive PDV, e=epidermis, d= dermis

PDB	HVDx(200)	AVD(200)	HVDX(200)	AVDX(400)	VEGF	PD-ECGF/TP
DCIS						
CASE112					1(e)	50% 1(e)2(d)
CASE132	40	24.7			0	50%3(e)2(d)
CASE129	12	4.3			0	75%2(e)3(d)
CASE124	24	16.7	10	6.7	1(e)1(d)	0
CASE131					1(d)	40% 1(e)2(d)
CASE 094	30	11.7	10	6.7	2(e)	75% 2(e)3(d)
CASE 095	30	19.3	7	6	3(e)	01(e)1(d)
CASE102	51	46	15	13.6	3(e)	01(e)1(d)
CASE104	14	11.7	13	10.6	1(d)	0
CASE108	14	9.3	14	9.7	4(d)	2(e)3(d)
CASE109	34	26.7	14	11	4(d)	01(e) 1(d)
CASE122	20	11.6	7	3.6	2(d)	0
CASE125	9	5.6	14	10	2(d)	0
DCIS +CA						
CASE116					0	01(e) 2(d)
CASE126	24	18	5	4.3		0
CASE123	8	6			1(e)1(d)	20%2(e)2(d)
CASE113	26	23.3	8	6.3	0	01(e)1(d)
CASE099	3	1.3			1(e)	50%2(e)1(d)
CASE096	12	10	14	11.7	0	50%3(e)3(d)
CA						
CASE 097					2(d)	0
CASE 092	21	15.3	40	33.7	1(d)	75%2(e)1(e)
CASE100	20	14	8	5.6	2(d)	0
CASE103	22	16.7			4(d)	75%1(e)1(d)
CASE128	30	22.3	15	11.3		75%2(e)3(d)
CASE114	22	16	15	14		50%3(e)3(d)

DCIS = ductal carcinoma in situ , CA= carcinoma

REFERENCES

Aberle H., Bierkamp C., Torchard D., Serova O., Wagner T., Natt E., Wirsching J., Heidkamper C., Montagna M., Lynch H. (1995) The human plakoglobin gene localizes on chromosome 17q21 and is subjected to loss of heterozygosity in breast and ovarian cancers. Proc Nat. Acad Sci **92**: 6384-6388.

Aberle H., Bauer A., Stappert J., Kispert A., Kemler R.(1997) Beta- catenin is a target for the ubiqutin-proteasome pathway. EMBO J. **16**: 3797-3804.

Agerbaek M., Alsner J., Marcussen N., Lundbeck F., von der Masse H. (2003). Retinoblastoma protein expression is an independent predictor of both radiation response and survival in muscle invasive bladder cancer. Br J Cancer **89**: 298-304.

Adcock L.L , Julian T. M., Okagaki T., Jones T.K. Prem. K.A., Twiggs L.B., Potish R.A., Phillips G.L. (1982). Carcinoma of the uterine cervix FIGO stage IB gynecol Oncol. **14**: 199-208.

Ai J., Tan Y., Ying W., Hong Y., Liu S., Wu M., Qian X., Wang H., (2005). Proteome analysis of hepatocelluar carcinoma by Laser capture microdissection. Proteomics **9**

Allred DC, Clark GM, Elledge R, Fuqua SW, Brown RW, Chamness GC, Osborne CK, McGuire WL. (1993). Association of p53 protein expression with tumour cell proliferation rate and clinical outcome in node negative breast cancer. J Natl Cancer Inst **85**:201-206.

Andersen S.N, Rognum T.O, Bakka A, Clausen O.P.F (1998). Ki67: a useful marker for the evaluation of dysplasia in ulcerative colitis. J Clin Pathol. **51**:327-332

Anderson WR.,(1979) Bilateral Paget's disease of the nipple: case report.Am J Obstet Gynecol **134**: 877-878.

Anelli A, Anelli T.F.M, McCmormick B, et al. (1995). Conservative management of Paget's disease of the nipple. Proc. Am Soc Clin Oncol. **14**:100.

Archibald RG.,(1922) A case of Paget's disease associated with carcinomatous infiltration of the breast of a male native of Sudan Am J Trop Med **2**:133-137.

Arima J, Imazono Y, Takebayashi Y, Nishiyama K, Shirahama T, Akiba S, Furukawa T, Akiyama S, Ohi (2000). Expression of thymidine phosphorylase as an indicator of poor prognosis for patients with Transitional cell Carcinoma of the bladder. Cancer **88**:1131-1138.

Ascensao A.C, Marques M.S.J, Capitao- Mor M. (1985). Paget's disease of the nipple. Clinical and Pathological review of 109 female patients. Dermatologica **170**:170-179.

Ashikari R, Park K, Huvos A.G, Urban J.A. (1970). Paget's disease of the breast. Cancer **26**: 680-685.

Bali A, O'Brien P., Edwards L., Sutherland R., Hacker N., Henshall S.(1996). Cyclin D1, p53 and p21Waf1/Cip1 expression is predictive of poor clinical outcome in serous epithelial ovarian cancer. Clin. Cancer Res. **10**: 5168-5177.

Bancher-Todesca D, Obermair A, Bilgi S, kohlberger P, Kainz C, Breitenecker G, Leodlter S, Gitsch G. 1997. Angiogenesis in vulvar intraepithelial neoplasia. Gynecol Oncol **64**:496-500.

Bankfalvi A., Navabi H., Bier B., Bocker W., Jasani B., Schmid K.W. (1994) Wet autoclave pre-treatment for antigen retrieval in diagnostic immunohistochemistry. J. Pathol. **174**: 223-228.

Banerjee S.N, Estabrook A, Schnabel F.R. (1997) Surgical treatment of Paget's disease. Inc: Silverstein MJ (ed). Ductal Carcinoma in situ of the breast. Baltimore.pp.551-554.

Bargmann C.I,Hung M.C, Weinberg R.A (1986) The neu oncogene encodes an epidermal growth factor receptor-related protein. Nature **319**:226-230.
Becker K.F, Atkinson M.J, Reich I., Nekarda H., Siewert J.R, Hofter H.(1994). E-cadherin gene mutations provide clues to diffuse type gastric carcinomas. Cancer Res **54**: 3845-3852.

Baker S.J, Fearon E.R., Nigro J., Hamilton S., Preisinger A.C, Jessup J.M., van Tuinen P., Ledbetter D.H., Barker D.F., Nakamura Y., Whyte R., Volgelstein B.,(1989). Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science **244**: 217-221.

Baker S.J, Markowitz S, Fearon ER, Willson J.K.V, Vogelstein B (1990): Suppression of Human colorectal cell growth by wild type p53. Science **249**: 912-915.

Barker N., Morin P.J., Clevers H.,(2000) The Yin- Yang of TCF/beta-catenin signalling. Adv. Cancer Res **77**: 1-24.

Barth AL., Nathke IS., Nelson WJ. (1997) Cadherins, catenins and APC protein: interplay between cytoskeletal complexes and signalling pathways. Curr Opin Cell Biol. **9**: 683-690.

Bartkova J., Lukas J., Muller H., Lutzhoft D., Strauss M., Bartek J.(1994) Cyclin D1 protein expression and function in human cancers. Int J Cancer **57(3)** :353-361.

Bates S., Parry D., Bonetta **et al.**,(1994). Absence of cyclin D cdk complexes in cells lacking functional retinoblastoma protein. Oncogene **9**:1633-1640.

Beckstead J.H. (1994) Improved antigen retrieval in formalin-fixed paraffin embedded tissues. Appl. Immunohistochem **2**: 274-281.

Behrens J., Mareel M.M.,Van Roy F.M., Birchmeier W. (1989) Dissecting tumour cell invasion : epithelial cells acquire invasion properties after the loss of uvomorulin-mediated cell-cell adhesion. J. Cell Biol **108**: 2435-2447.

Ben-Ze'ev A. (1997). Cytoskeletal and adhesion proteins as tumour suppressors. Curr Opin. Cell Biol **9** :99-108.

Ben-Ze'ev A and Geiger B. (1998). Differential molecular interactions of β- catenin and plakoglobin in adhesion, signalling and cancer. Curr Opin. Cell Biol **10**: 629-639.

Berenson JR, Koga H., Yang J, Pearl J., Holmes EC, Figlin R, (1990). Frequent amplification of the bcl-1 locus in poorly differentiated squamous cell carcinoma of the lung. The lung cancer study group. Oncogene **5**:1343-1348.

Bergen S, DiSia P.J, Liao S.Y., Berman M. (1989).Conservative Management of Extramammary Paget's disease of the Vulva. Gynecol Oncol.**33**: 155-156.

Berns EM., Van Staveren IL., Look MP., Smid M., Kiljn SG., Foekens JA. (1998). Mutation residues of TP53 that directly contact DNA predict poor outcome in human primary breast cancer. Br J Cancer **77**:1130-1136.

Bershadsky A.D, Yehuda-Levenberg S, Geiger B. (1995). Molecular interactions in the submembrane plaque of cell-cell and cell-matrix adhesions. Acta Anat. **154**: 46-62.

Berx G., Cleton-Jansen A.M., Nollet F., De Leeuw W.J, Van de Vijver M., Cornelisse C., Van Roy F: E-cadherin is a tumour /invasion suppressor gene mutated in human lobular breast cancers EMBO J. **14**: 6107-6115.

Bieche I, Lidereau (2000). Loss of heterozygosity at 13q14 correlates with RB1 gene underexpression in human breast cancer. Mol. Carcinogen. **29**: 151-158.

Birchmeier W,and Behrens J.(1994). Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. Biochim Biophys Acta **1198**:11-26.

Bodner S.M,Minna J.D, Jensen S.M.,et al. (1992). Expression of mutant p53 protein in lung cancer correlates with the class of p53 gene mutation. Oncogene **7**:743-749.

Boehm F, Morris JMCL. (1971). Paget's disease and apocrine gland carcinoma. Obstet. Gynaecol **38**: 185-192.

Boller K.,Vestweber D., Kemler R.(1985) Cell adhesion molecule uvomorulin is localised in the intermediate junctions of the adult intestinal epithelial cells. J Cell Biol **100**:327-332.

Bosari S., Lee., AKC. Delellis RA., Wiley BD. Heatley GJ . Silverman ML(1992) Microvessel quantification and prognosis in invasive breast carcinoma. Hum Pathol **23** : 755-761.

Bosari S., Arthur L., Del-Ellis R., Wiley B., Heatley G., Silverman M., (1992) Microvessel Quantification and prognosis in invasive breast carcinoma. H. Pathol. **23**: 755-761.

Bouck N.P. (1990) Tumour angiogenesis: the role of oncogenes and tumour suppressor gene. Cancer cells **10**:179-185.

Bouzubar N, Walker KJ, Griffiths K, Ellis, IO, Elston CW, Robertson JFR, Blamey RW, Nicholson RI (1989). Ki67 immunostaining in primary breast cancer: pathological and clinical associations. Br J Cancer **59**: 943-947.

Bradley RS.,Cowin P., Brown AM. (1993) Expression of Wnt-1 in PC12 cells results in modulation of plakoglobin and E-cadherin and increased cellular adhesion. J.Cell Biol **123**: 1857-1865.

Brem S.S. and Medina D., (1977) Angiogenesis :A marker for neoplastic transformation of mammary papillary hyperplasia . Science **19**: 880-882.

Bringuier P.P., Umbas R., Schaafsma E.,(1993) : Decreased E-Cadherin immunoreactivity correlates with poor survival in patients with bladder tumours Cancer Res. **52**: 5104-5109.

Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Dvorak HF, Senger DR. Increase expression of vascular permeability (vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas. Am J Pathol **143**:1255-1262 (1993).

Brown N.S, Bicknell R., (1998) Thymidine phosphorylase, 2-deoxy-D-Ribose and angiogenesis. Biochem J. **334**: (Pt 1):1-8.

Bukholm IK, Nesland JM, Karesen R, et al (1995). E-cadherin and α–,β- and γ-catenin protein expression in relation to metastasis in human breast carcinoma J Pathol **185**:262-266.

Bulens P. Vanuystel L, Rijnders A, et al.(1990). Breast conserving treatment of Paget's disease. Radiother Oncol **17**: 305-309.

Bussolati G and Pich A. (1975). Mammary and extramammary Paget's disease.. An immunohistochemical study. Am J Pathol. **80**: 1013-1023.

Burke T. W., Hoskins W.J., Heller P.B, Bibro M.C., Weiser E.B., Park R.C. (1987). Prognostic factors associated with radical hysterectomy failure. Gynecol **26**: 153-159.

Burrows N.P, Jones D.H. Hudson P.M, Pyre R.J. (1995) Treatment of extramammary Paget's disease by radiotherapy **132**: 970-972.

Caca K., Kolligs F., Ji X., Hayes M., Qian J., Yahanda A., Rimm D., Costa J., Fearon E., (1999) β- and γ catenin mutations , but not E-cadherin inactivation , underlie T-cell factor/lymphoid enhancer factor transcriptional deregulation in gastric and pancreatic cancer. Cell Growth Differ **10**: 369-376.

Carnochan P., Briggs JC., Westbury G., Davies AJ (1991). The vascularity of cutaneous melanoma a quantitive histologic study of lesions 0.85-1.25mm in thickness. B.J Cancer **64**:102-107.

Cattoretti G., Pileri S., Parravicini C., Becker M.H.G., Poggi S., Bifulco C., Key, G. et al. (1993) Antigen retrieval unmasking on formalin-fixed, paraffin-embedded sections. J. Pathol. **171**: 83-98.

Chan MK., Cheung TH., Chung TKH., Bao SY., Zhao CL., Nobori T. (1998) Expression of p16 INK4 and retinoblastoma protein in vulval lesions of Chinese women. Gynecol Oncol **68**: 156-61.

Chanda J.J. (1985) Extramammary Paget's disease: Prognosis and relationship to internal malignancy. J. Am Acad Dermatol **113**:1009-10013.

Charpin C., Garcia S., Bonnier P., et al.(1997) Reduced E-cadherin immunohistochemical expression in node-negative breast carcinomas. Am J Clin Pathol **109**:431-438.

Chaudary M.A, Mills R.R, Lane B, Miller N.A. (1986) Paget's disease of the nipple :a ten year review including clinical, pathological, and immunohistochemical study. Am J Pathol **80**: 1013-1023.

Choi WW, Lewis M, Lawaon D, Yin-Goeon Q, Birdsono GG, Cotsonis GA, Cohen C, Young AN. (2005). Angiogenic and lymphangiogenic microvessel density in breast carcinoma: correlation with clinicopathologic parameters and VEGF-family gene expression. Mod Pathol. **18(1)**:143-152.

Chulvis do Val K., Almeide Filho GL., Valiante PM., Gondim C., Takiya LM., Cervalho Mada G., (2004). VIN p53 expession /P53 gene mutation and HPV in recurrent /progressive cases. J Repro. Med **49**:868-874.

Cohen C., Guarner J., Derose P.B. (1993) Mammary Paget,s disease and associated carcinoma. An immunohistochemical study. Arch. Pathol. Lab Med **117**: 291-294.

Colcock B.P., Sommers S.C,(1954). Prognosis in Paget's disease of the breast. Surg Clin N Am **35**:733-783.

Conman C.R (1961). Adhesiveness and stickiness: two independent properties of the cell surface.Cancer Res **21**:1436-1438.

Coussens L.,Yang-Feng T.L.,Liao Y-C.,Chen E., Gray A., McGrath J., Seeberg P.H., Libermann T.A., Schlessinger J.,Francke U., Levinson A., Ullrich A.(1985) Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science **230**:1132-1139.

Cowie A.T. (1974). Overview of the mammary gland. J Invest Dermatol **63**: 2-9.

Cowin P., Kapprell H., Franke, Tamkun J.,Hynes O. (1986). Plakoglobin : a protein common to different kinds of intercellular adhering junctions. Cell **46**: 1063-1073.

Cowin P., and Burke B. (1996) Cytoskeleton-membrane interactions. Curr. Opin. Cell Biol, **8**: 56-65.

Conn G., Soderman D., Schaffer M-T., Wile M., Hatcher V., Thomas K. (1990). Purification of a glycoprotein vsascular endothelial cell mitogen from a rat glioma cell line. Proc Natl Acad Sci USA **87**: 1323.

Conn G., Bayne M., Soderman L., Kwok PW., Sullivan KA., Palisi TM, Hope DA., Thomas KA. (1990) Amino acid and cDNA sequence of a vascular endothelial cell mitogen homologous to platelet-derived growth factor. Proc Natl Acad Sci USA **87**: 2628.

Crawford D , Nimmo M., Clement P. Thomson T., Benedet J L. Miller D., Blake Gilks C (1999). Prognostic factors in Paget's disease of the vulva: A study of 21 cases. Int. J Gynecol Pathol **18**:351-359.

Crawford H., Fingleton B., Rudolph –Owen L., Goss K., Rubineld B., Polakis P., Matriian L. (1999) The metalloproteinase matrilysin is a target of β- catenin transactivation in intestinal tumours. Oncogene **18**: 2883-2891.

Crocker H.R. (1889) Paget's disease affecting the scrotum and penis. Transactions of the Pathological Society of London 1888-1889 **40**: 187-191.

Culberson J.D and Horn R.C.(1956). Paget's disease of the nipple: Review of 25 cases with special reference to melanin to pigmentation of 'Paget's' cells. Arch Surg. **72**:224-231.

Curtin J.P, Rubin S.C Jones, W.B Hoskins W.J and Lewis J.L (1990). Paget's disease of the vulva, Gynecol. Oncol. **39**:374-377.

Dameron K.M., Volpert O.V, Tainsky M.A., Bouck N.P., (1995) Control of angiogenesis in fibroblasts by p53 regulation of Thrombospondin-1. Science **265**: 1582-1584.

Daiz de Leon E., Carcangiu M.L., Prieto V., McCue P., Burchette J.L. To G., Norris B., Kovatich A.J., Sanchez R.L., Krigman H., Gatalica Z. (2000). Extramammary Paget disease is characterised by the consistent lack of oestrogen and progesterone receptors but frequently expresses androgen receptor.Am J.Clin Pathol **113**:572-575.

Defegu S., O'Quinn. A.G., and Dhurandhar, H.N. (1986). Paget's disease of the vulva and urogenital malignancies: A case report and review of the literature, Gynecol. Oncol. **25**:347.

Demopoulos R. (1971). Fine structure of the extramammary Paget's cells. Cancer **27**: 1202-1209.

De Potter CR. Eeckhout I, Schelfhout AM.: Keratinocyte induced chemotaxis in the paththogenesis of Paget's disease of the breast.(1990). Histopathology **17**:243.

Di Fiore P.P.,Pierce J.H.,Kraus M.H., Segatto O., King C.R., Aaronson S.A.,(1987) erbB-2 is a potent oncogene when over-expressed in NIH/3T3 Cells Science **237**:178-182.

Dobbs SP. Hewett PW, Johnson PW, Johnson IR, Carmaichael J. Murray JC. (1997) Angiogenesis is associated with vascular endothelial growth factor expression in CIN. Br J Cancer 76:1410-1415.

Doldi N, Origoni M, Bassan M, Ferrari D, Rossi M, Ferrari A. (1996) Vascular endothelial growth factor expression in human vulvar neoplastic and non neoplastic tissues. J Reprod Med. **41**:844-848.

Dong M., Nio Y., Yamasawa K., Toga T., Yue L., Harada T., (2003) Journal of Surgical Oncology **82**: 111-120.

Dowell S.P., Wilson P.O.G., Derias N.W., Lane D.P., Hall P.A., (1994). Clinical utility of the immunocytochemical detection of p53 protein in cytological specimens. Cancer Res. **54**:2914-2918.

Drach J, Gattringer C, Glassl H, Drach D, Huber H., (1992) The biological and clinical significance of the Ki67 growth fraction in myeloma . Hematol Oncol **10**:125-134.

Dubreuilh W. (1901) Paget's disease of the vulva. Br J. Dermatol.**13**: 407-413.

Duffy MJ. (1993) Cellular oncogenes and suppressor genes as prognostic markers in cancer. Clin Biochem **26**: 439-437.

El-Sharkawi A and Waters J.S. (1992) The place of conservative management of Paget's disease of the nipple. Eur J Surg Oncol. **18**: 301-303.

Eusebio R.B, and Deckers P.J. (1992) Paget,s disease of the nipple-areola complex: A plea for conservatism. Contemp Surg **40**: 13-15.

Evans, T. Rosenthal E.T, Youngbloom J., Distel D., Hunt., T (1983) Cyclin:a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage divison.Cell **33**: 389-396.

Falette N., Paperin MP., Treilleux I., Gratador AC., peloux N., Mignotte H., Tooke N., Lofman E., Inganes M., (1998) Prognsotic value of p53 gene mutations in a large series of node-negative breast cancer patients Cancer Res.**58**:1450-1455.

Fanning J., Lambert H.C.L, Hale T.M, Morris P.C, Schuerch C, (1998) Paget's disease of the vulva: Prevalence associated vulvar adenocarcinoma, invasive Paget's disease, and recurrence after surgical excision. Am J Obstet Gynecol **180**: 24-28.

Farquhar M., Palade G. (1963) .Junctional complexes in various epithelia. J Cell biol **7**: 375-412.

Fett J.W. et al., (1985). Isolation and charactrisation of angiogenin , an angiogenic protein from human carcinoma cells. Biochem. **24**: 5480-5486.

Ferrara N. Henzel W.J.(1989). Pituitary follicular cells secrete a novel heparin – binding growth factor specific for vascular endothelial cells. Biochem Biophys. Res. Commun **161**: 851-855.

Feuer G.A, Shevchuk M, Calanog A (1990) Vulvar Paget's disease: The need to exclude an invasive disease. Gynecol Oncol.**38**: 81-89.

Fina L., Molgaard HV., Robertson D., Bradley NJ., Monaghan P., Delia D., (1990). Expression of the CD34 gene in vascular endothelial cells. Blood **75**: 2417-2426.

Fisher D.E, (1994) Apoptosis in cancer therapy: crossing the threshold. Cell **78**: 539-542.

Fligiel Z., Kaneko M., (1975) Extramammary Paget's disease of the external ear canalin association with cerumninous gland carcinoma. Cancer **36**:1072-1076

Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease.(1995) Nat Med **1**: 27-31.

Folkman J. What is the evidence that tumours are angiogenesis dependent ? (1990) J Natl Cancer Inst. **82**:4-6.

Folkman J., Watson K., Ingber D., Hanahan D., (1989) Induction of angiogenesis during the transistion from hyperplasia to neoplasia. Nature **339**: 58-61.

Folkman J. Tumour angiogenesis. (1994) In : Mendelsohn J., Howley P., Liotta L., Israel, M., eds. The molecular basis of cancer, W.B Saunders Company, Philadelpia, in press.

Folkman J. Angiogenesis and breast cancer. (1994) J.Clin. Oncol.**12**: 441-443.

Folkman J. ,Shing Y.Angiogenesis (1992) J. Biol. Chem. **267**: 10931-10934.

Folkman J and Klagsbrun M., (1987). Angiogenic factors Science **235**: 444-447.

Fox S.B., Moghaddam A., Westwood M., Turley H., Bicknell R., Gatter K., Harris A.(1995) . A platelet –derived endothelial cell growth factor /thymidine phosphorylase expression in normal tissues: an immunohistochemical study. J.Pathol. **176**:183-190.

Friedrich EG. Wilkinson FEJ. Steingraeber PH., Lewis JP. (1975): Paget's disease of the vulva: a clinicopathologic study of eight cases. Obstet Gynecol **45**: 73-78.

Frixen U.H, Behrens J., Sachs M., Eberle G., Voss B., Warda A., Lochner D., Birchmeir W. (1991). E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J. Cell. Biol. **113**:173-185.

Fu W., Lobocki CA., Silberberg BK., Chelladurai M., Young SC. (2001). Molecular markers in Paget's disease of the breast. J Surg Oncol. **77(3)**: 171-178.

Fukushige S-I., Matsubara K-I., Yoshida M., Sasaki M.,Suzuki T., Semba K, Toyoshima K., Yamamoto T., (1986) Localisation of a novel v-erbB- related gene, c-erbB-2, on human chromosome 17 and its amplification in a gastric cancer cell line. Mol Cell Biol **6**: 955-958.

Fujimoto J, Sakaguchi H, Aoki I, Tamaya T. (2000) The value of Platelet-derived Endothelial Cell Growth Factor as a novel predictor of advancement of uterine cervical cancers. Cancer Res **60**:3662-3665.

Fuller L.C, Allen M.H, Montesu M., Barker J.N., Macdonald D.M., (1996) Expression of E-cadherin in human epidermal non-melanoma cutaneous tumours. Br. J. Dermatol. **134**: 28-32.

Funayama N., Fagotto F., McCrea P., Gumbiner BM. (1995). Embryonic axis induction byt eh armadillo repeat domain of beta-catenin: evidence ofr intracellular signalling. J.Cell.Biol. **128**:959-968.

Furukawa T, Yoshimura A, Sumizawa T, Haraaguchi M, Akiyama SI. Angiogenic Factor. Nature **356**:668,1992.

Gasparini G, Toi M, Gion M. (1997) Prognostic significance of vascular endothelial growth protein in node-negative breast carcinoma. J Natl Cancer Inst **89**: 139-47.

Gasparini G, Toi M, Miceli R. (1999). Clinical relevance of vascular endothelial growth factor and thymidine phosphorylase in patients with node-positive breast cancer treated with either adjuvant chemotherapy or hormone therapy. Cancer J Sci Am **5**: 101-111.

Geiger B., (1989) Cytoskeleton- associated cell contacts. Curr.Opin. cell Biol. **1**:103-109.

Gerdes J: Ki67 and other proliferating markers useful for immunohistological diagnostic and prognostic evaluations in human malignancies (1990): Semin.Cancer Biol **1**:99-206.

Gasparini G., Weidner N., Maluta S., Pozza F., Boracchi P., Mezzetti M., Testolin A., Bevilacqua (1993). Intramural Microvessel Density and p53 protein: correlation with metastasis in head-and-neck squamous-cell carcinoma. Int J Cancer **55**:739-744

Going J., Lamb R (1996). Practical Histological Microdissection for PCR analysis J.Pathol. **179**:121-124.

Goldblum and Hart (1997) Vulvar Paget's Disease: A clinicopathologic and Immunohistochemical Study of 19 cases. Am J.Surg. Pathol. **21**:1178-1187.

Gnarra J. R.et al. (1996) Post- transcriptional regulation of vascular endothelial growth factor mRNA by the product of the VHL tumour suppressor gene. Proc . Natl. Acad. Sci. USA **93**: 10595- 100599.

Graeber T.G et al. (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature **379**: 88-91.

Graham H. (1939) The story of Surgery. New York, Doubleday, Doran & Co, Inc. Hughes E.S.R. (1950) The development of the mammary gland . Ann R Col Surg Engl **6**: 99-119.

Gras E., Pons C., Machin P., Matias-Guiu X., Prat J. (2001) Loss of heterozygosity at the RB-1 locus and pRb immunostaining in epithelial ovarian tumours: a molecular, immmunohistochemical, and clincopathological study. Int J Gynecol Pathol 2001; **20** : 335-340.

Gredts J., Ingram CD (2000). Abnormal expression of cell cycle regulatory proteins in ductal and lobular carcinomas of the breast. Mod Pathol **13**: 945-953.

Guidi AJ, Abu-Jawdeh G, Berse B, et al. (1995) Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in cervical neoplasia. J Natl Cancer Inst.**87**:1237-1245.

Gumbiner B. (1987). Structure , biochemistry and assembly of epithelial tight junctions. Am. J. Physiol (Cell Physiol.) **253**: C749-C758.

Gumbiner BM, (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell **84**:345-347.

Gumbiner B., (1997) Carcinogenesis: a balance between β- catenin and APC. Curr Biol. **7**: R443-446.

Gunn, G. A., and Gallager, S. H. (1980). Vulvar Paget's disease: A topographic study, Cancer **46**:590-594.

Haagensen C.D. (1971) Diseases of the Breast, 3rd ed. Philadelphia, W.B saunders, **22**: 979-982.

Habour JW, Lai SL,Whang PJ et al (1988) Abnormalities in structure and expression of the human retinoblastoma gene in small cell lung cancer.Science **241**:353-357.

Hall NR., Fish DE., Hunt N Golding RD., Guilou PJ., Monson JRT (1992). Is the relationship between angiogenesis and metastasis in breast cancer real ? Surg Oncol. **1**:223-229.

Hamada J., Cavanaugh P.G, Lotan O Nicholson G. (1992) Separable growth and migrator factors for large- cell lymphoma cells secreted by microvascular endothelial cells derived from target organs for metastasis. Br J Cancer **66**:349-354.

Hanahan D., and Folkman J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumourigenesis. Cell **86**: 353-364.

Hanahan D. and Weinberg RA (2000). The Hallmarks of cancer. Cell **100**: 57-70

Hantschmann P., Sterzer S., Jeschke U., Friese K., (2005) P53 expression in vulvar carcinoma, VIN, squamous cell hyperplasia and Lichen sclerosis. Anti Cancer Res **25** (3A): 1739-1745

Hansen S., Sorensen, Vach W., Grabau D., Bak M., Rose C., (2004). Microvessel density compared with the Chalkley count in a prognostic study of angiogenesis in breast cancer patients. Histopathology **44**: 428-436

Haraguchi M., Miyadera K., Uemura K., Sumizawa T., Furukawa T., Yamada K. et al. (1994) Angiogenic activity of enzymes. Nature **368**:198.

Hashimoto T., Tokuchi Y., Hayashi M., Kobayashi Y., Nishida K., Hayashi S., Ishikawa Y., Tsuchiya S., Nakagawa K., Hayashi J., Tsuchiya E., (1999) p53 null mutations undetected by immunohistochemical staining predict a poor outcome with early stage non-small cell lung carcinomas. Cancer Res. **59**: 5572-5577

He T., Sparks A., Rago C., Hermeking H., Zawel L., da Costa L., Morin P., Volgestin B., Kinzler K. (1998). Identification of c-MYC as a target of the APC pathway. Science **281**: 1509-1512.

He T., Chan T., Volgelstein B., Kinzler K., (1999) PPARδ is an APC- regulated target of nonsteriodal anti-inflammatory drugs Cell **99**: 335-345.

Healey E, Angus B, Lawrence CM, Rees JL. (1995) Prognostic value of Ki67 antigen expression in basal cell carcinomas. Br J Dermatol **133**: 737-741.

Heffelfinger SC, Miller MA, Yassin R, Gear R., (1999) Angiogenic growth factors in preinvasive breast disease. Clin Cancer Res **5(10)**: 2867-2876.

Heichman K.A and Roberts J.M (1994). Cell **79**:557.

Helwig EB, Graham JH (1963). Anogenital(extramammary) Paget's disease: a clinicopathological study. Cancer **16**:387-403.

Hendricks JB, Wilkinson EJ, Kubilis P, Drew P, Blagdes SM, Munakata S.(1994) Ki67 expression in vulvar carcinoma. Int J Gynecol Pathol **13**: 205-210.

Hietanen SH, Kurvinen K, Syrjanen K, Grenman S, Carey T, McClatchey K, Syrjanens (1995). Mutation of tumour suppressor gene p53 is frequently found in vulvar carcinoma cells. Am J Obstet Gynaecol **173**: 1477-1482.

Hollstein M, Sidransky D, Vogelstein B, Harris C (1991). p53 mutations in human cancers. Science **253**: 49-53.

Hollstein M., Rice K., Greenblatt, MS, Soussi T., Fuchs R., Sorlie, T. et al. (1994). Database of p53 gene somatic mutations in human tumours and cell lines. Nucleic Acids Res, **22**: 3551

Hollingsworth HC, Kohn EC, Steinberg SM, Rothenberg ML, Menoir MJ. (1995). Tumour angiogenesis in advanced stage ovarian carcinoma. Am J Pathol. **147**:33-41

Holmgren L., O'Reilly, M., Folkman J., (1994) Cell **79(2)**: 315-328

Hong MK, Laskin WB, Herman BE, Johnson MH, Vargo JJ, Steinberg SM, (1995). Allegra CJ, Johnson PG. Expansion of the Ki67 proliferative compartment correlates with degree of dysplasia in Barrett's Esophagus. Cancer **75**: 423-429.

Horak E., Leek R., Klenk N., Lejeune S., Smith K., Stuart N., Greenall M., Stepniewska K., Harris A. (1992) Angiogenesis,` assessed by platelet/ endothelial cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer. Lancet **340**:1120-1123.

Howley PM, Munger K, Romanczuk H, Scheffner M, Huibregtse JM.(1991) Cellular targets of the oncoproteins encoded by the cancer associated human papillomaviruses. Princess Takamatsu Symp **22**:239-248.

Hu CK., McCall S., Madden J., Huang H., Clough R., Jirtle RL., Ansher MS.,(2005). Loss of heterozygosity of M6P/IGF2R gene is an early event in the development of prostate cancer. Prostate Cancer Prostatic Dis. **22**: 62-70.

Hultborn KA., Larsson LG., Ragnhult I. (1955). The lymph drainage from the breast to the axillary and parasternal lymph nodes: Studied with the aid of colloid Au-198. Act Radiol **43**: 52-64.

Hultborn AL., Hulten B., Roos M. (1974). Effectiveness of axillary lymph node dissection in modified radical mastectomy with preservation of pectoral muscles. Ann Surg **179**: 269-272.

Huber O., Bierkamp C., Kemier R., (1996). Cadherins and catenins and APC protein: interplay between cytoskeletal complexes and signalling pathways. Curr Opin Cell Biol 1996 **8**: 685-691.

Hui R., Cornish AL. McClelland RA . (1996). Cyclin D1 and oestrogen receptor messenger RNA levels are positively correlated in primary breast cancer. Clin Cancer Res **2**:923-928.

Iliopoulos O., Levy A.P., Jiang C., Kaelin W.G., Goldberg M.A (1996).Negative regulation of hypoxia-inducible genes by the Von Hippel-Lindau disease-associated and sporadic hemangioblastomas. Cancer Res. **55**: 1358-1364.

Ingis K, (1946) Paget's disease of the nipple:with special reference to the changes in the ducts. Am J Pathol **22**:1-33.

Ingis K, (1952) The essential difference between the epidermal changes in Paget's disease of the nipple and those in "Bowen's precancerous dermatosis" J Pathol Bacteriol **64**: 637-643.

Innis M.A, Myambo K.B, Gelfand D.H, et al., (1988) DNA sequencing with Thermus –aquaticus DNA polymerase and direct sequencing of polymerase chain reaction amplified DNA. Proc Natl Acad Sci USA **85**: 943-960.

Innis M.A, Gelfand D.H. (1990) Optimisation of PCRs. In: Innis M.A, Gelfand D.H Sninsky J.J, et al., eds. PCR protocols a guide to methods and applications. New York, Academic Press, :185-207.

Ioachim E, Kamina S, Athanassiadou S, Agnantis NJ.(1996) The prognostic significance of epidermal growth factor receptor (EGFR), C-erbB-2, Ki67 and PCNA expression in breast cancer. Anticancer Res **16**:3141-3148.

Ishikawa F., Miyazono K., Hellman U., Drexler H., Wernstedt C., Hagiwara K., Usuki K., Takaku F., Risau W., Heldin C., (1989). Identification of angiogenic activity and the cloning and expression of Platelet –derived endothelial cell growth factor. Nature **338**: 557-562.

Isola JJ, Helin HJ, Helle MJ, Kallioniemi O. (1990) Evaluation of cell proliferation in breast carcinoma. Comparison of Ki67 immunohistochemical study, DNA Flow Cytometric analysis, and mitotic count. Cancer **65** :1180- 1184.

Ito S., Ohgo T., Saeki H., NakamuraT., Watanabe M., Tanaka S., Kakeji Y., Maehara Y.(2005). P53 mutations profiling of multiple oesophageal carcinoma using laser capture microdisseection to demonstrate field carcinogenesis. Int J Cancer **113**: 22-28

Jackman M.R, Pines J.N. (1997) Cyclins and the G2 M transition. Cancer Surv **29**:47-73.

Jackson D.P., Hayden J.D., Quirke P., (1992) Extraction of nucleic acids from fresh and archival material. In: Mcpherson M.J Quirke P., Taylor G.R eds. PCR a practical approach. Oxford IRL Press : 29-50.

Jacobaeus HC. (1904) Paget's disease und sein verhaltniss zum milchdrusenkarzinom. Virchow's Arch [A] **178**: 124-142.

Jacquemier J, Moles J.P, Penault- Llorca F, Adelaide J, Torrente M, Viens P, Birnbaum D, Theillet C. (1994). p53 immunohistochemical analysis in breast cancer with four monoclonal antibodies: comparison of staining and PCR-SSCP results. Br. J Cancer **69**:846-852.

Jacquemier J., Penault- Llorca F., Bertucci F., Sun Z. et al. (1998). Angiogenesis as a prognostic marker in breast carcinoma with conventional adjuvant chemotherapy: A multiparametric and immunohistochemical analysis. J Pathol. **184**:130-135.

Jahn H, Osther P.J, Nielsen E.H et al. (1995). An electron microscopic study of clinical Paget's disease of the nipple. APMIS **103**: 628-634.

Jiang W., Zhang Y-J., Khan SM., Hollstein, M.C., Santella., R.M Lu, S-H., Harris cc., Montesano R., Weinstein I.B.(1993). Altered expression of the cyclin D1 and retinoblastoma genes in human oesophageal cancer. Proc. nat. Acad. Sci (Wash) **90**: 9026-9030.

Johnson BA, Haines GK, Harlow LA., Koch AE. (1993) Adhesion molecule expression in human synovial tissue. Arthritis Rheum **36**: 137-146.

Johnson T.J, Tomek C.S, Patterson K.B, Buckley J.F (1987) Paget's disease of the male breast. Nebr Med J **12**: 10-13.

Kandel J. et al. (1991) Neovascularisation is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. Cell **66**: 1095-1104.

Kanitakis J, Thivolet, Claudy A (1993): p53 protein expression in Mammary and Extramammary Paget's disease. Anticancer Res **13**: 2429-2434.

Kariniemi A.L., Forsman L. Wahlstrom T.,Vesterinen E., Andersson L.(1984) Expression of differentiation antigens in mammary and extramammary Paget's disease. Br J Dermatology **110**: 203-210.

Kariniemi A.L, Ramaekers F, Lehto V-P., Virtanen I. (1985) Paget cells express cytokeratin typical of glandular epithelia. Br J Dermatology **112**: 179-183.

Karnovsky A., Klymkowsky M.W (1995). Anterior axis duplication in xenopus induced by the over-expression of the cadherin-binding protein plakoglobin. Proc Natl Acad Sci USA **92**: 4522-4526.

Khaled H., Bahnassi A., Zekri A., Kassem H., Mokhtar N. (2003). Correlation between p53 mutations and HPV in bilharzial bladder cancer. **155**:334-341.

Kirkham N, Berry N, Jones D.B, Taylor-Papadimitriou (1985) Paget's disease of the nipple. Immunohistochemical localisation of milk fat globule membrane antigens. Cancer **55**: 1510-1512.

Kister S.J and Haagensen C.D, (1970) Paget,s disease of the breast. Am J Surg **119**: 606-609.

Kitadai Y, Onogawa S, Kuwai T, Matsumura S, Hamada H, Ito M, Tanaka S, Yoshihara M., Chayama K (2004). Angiogenic switch occurs during the precancerous stage of human oesophageal squamous cell carcinoma Oncol Rep **11 (2)**:315-319.

Kocher T.D, Wilson A.C, (1991) DNA amplification by the polymerase chain reaction. In: Brown T.A, ed. Essential molecular biology a practical approach. Vol II Oxford :IRL Press: 1-14.

Kodoma S., Kaneko T., Saito M., Yoshiya N., Honma S., Tanaka K.(1995) A clinicopathologic Study of 30 patients with Paget's Disease of the vulva. Gynecol Oncol. **56**:63-70.

Kollermann J, Helpap B(2001) Expression of Vascular Endothelial Growth Factor (VEGF) and VEGF Receptor Flk-1 in Benign, Premalignant and Malignanat Prostate TissueAm J Clin Pathol **116**:115-121.

Kolligs F., Kooigs B., Hajra K., Hu G., Tani M., Cho K., Fearon E. (2000) γ catenin is regulated by the APC tumour suppressor and its oncogenic activity is distinct from that of β- catenin. Genes Dev **14**:1319-1331.

Korinek V., Barker N., Morin. P., van Wichen D, de Weger R., Kinzzler K., Vogelstein B., Clevers H., (1997) Constitutive transcriptional activation by a β-catenin-Tcf complex in APC-/-colon carcinoma. Science **275** 1784-1787.

Kouraklis G., Theocharis S., Vamvakas P., Vagianos C., Glinavou A., Sioka C.(2006). Cyclin D1 and Rb protein expression and their correlation with prognosis in patients with colon cancer. World J Surg Oncol. **20**: (4) 5.

Kraus M.H.,Issing W., Miki T., Popescu N.C, Aaronson S.A (1989) Isolation and characterisation of *ERB*B3, a third member of the *ERB*B/epidermal growth factor receptor family:evidence for overexpression in a subset of human mammary tumours. Proc Natl Acad Sci USA **86**:9193-9197.

Krenacs T., Krenacs L., Bagdi E. (1996) Diagnostic immunohistochemistry in Araldite-embedded bone marrow biopsies. J. Cell. Pathol **1**: 83-88.

Krenacs L., Harris C.A., Raffeld M., Jaffe E.S. (1996) Immunohistochemical diagnosis of T- cell lymphomas in paraffin sections. J. Pathol. **1**:125-130.

Kurzrock R, Ku S, Talpaz. (1995) Abnormalities in the PRAD1 (CYCLIN D1/BCL-1) Onogene are frequent in Cervical and Vulvar Squamous Cell carcinoma Cewll Lines. Cancer **75(2)**: 584-590.

Lagios M.D, Westdahl P.R, Rose M.R. Concannon S (1984). Paget's disease of the nipple. Alternative management in cases without or with minimal extent of underlying breast carcinoma. Cancer **50**:1304-1309.

Lancer H.A and Moschella S.L (1982) Paget's disease of the male breast. J. Am Acad Dermatol **7**: 393-396.

Lane DP, Crawford LV. (1979) T antigen is bound to a host protein in SV40-transformed cells. Nature **278**:261-263.

Lammie GA, Barnes DM., Millis RR., Gullick WJ (1989). An immunohistochemical study of the presence of c-erbB2 protein in Paget's disease of the nipple. Histopathology **15**: 505-504.

Lee SC, Roth LM, Ehrlich C, Hall JA (1977). Extramammary Paget's disease of the vulva. A clinicopathologic study of 13 cases. Cancer **39**:2540-2549.

Lee W.H, Bookstain R, Hong F, Young L.J, Shew J.Y, Lee EYHP (1987). Human retinoblastoma susceptibility gene: cloning, identification and sequence. Science **235**:1394-1399.

Lee EY, To H, Shew et al (1988) Inactivation of the retinoblastoma susceptibility gene in human breast cancers. Science **241**: 218-221.

Lee W.H, Shew J.Y, Hong F.D et al. The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity (1987). Nature **329**: 642-645.

Leedy DA., Trune DR., Kronz JD., Weidner N., Cohen JL (1994). Tumour angiogenesis, the p53 antigen and cervical metastasis in squamous carcinoma. Otolaryngol. Head Neck Surg. **111**: 417-422.

Levy A., Tamargo R., Brem H., Nathans D. (1989) An endothelial cell growth factor from the mouse neuroblastoma cell line NB41. Growth Factors **2**:9
Lloyd J and Flanagan A.M (2000). Mammary and Extramammary Paget's disease. J Clin Pathol **53**: 742-749.

Lien HC., Lin CW., Mao TL., Kuo SH., Hsiao CH., Huang CS., (2004) p53 overexpression and mutation in metaplastic carcinoma of the breast: gentic evidence for monoclonal origin of both the carcinomatous and the heterogenous sarcomatous components. J. Pathol. **204**: 131-139.

Lichter A.S, and Lippman M.E. (1988). Special situations in the treatment of breast cancer.In : Lippman M.E, Lichter A.S, Danforth D.N (eds). Diagnosis and management of breast cancer. Philadelpia, WB Saunders pp.422-423.

Lipponen PK.and Lukkonen TJ (1995). Reduced expression of retinoblastoma gene is related to cell proliferation and prognosis in transisitonal-cell bladder cancer. J Cancer Res Cli Oncol **121**:44-50.

Lipponen P., Saarelainen E., Ji H., et al. Expression of E-cadherin (E-CD) as related to other prognostic factors and survival in breast cancer. J.pathol **174**:101-109.

Locopo N, Fanelli M, Gasparini G. (1998) Clinical significance of angiogenic factors in breast cancer. Breast Cancer Res Treat **52(1-3)**:159-73.

Loden M., Perris F., Nielson NH., Emdin SO., Landberg G., (2003). C-erbB-2, p27 and G1/S aberrations in human primary breast cancer. Anticancer Res. **23**(3A): 2053-2061.

Lowe S.W., Schmitt E.M, Smith S.W, et al. (1993) p53 is required for radiation - induced apoptosis in mouse thymocytes. Nature **362**: 847-849.

Lowe S.W., Ruley H.W, Jacks T.,et al.(1993) p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell **74**: 957-967.

Lowe S.W., Bodis S., McCarthy A., et al. (1994) p53 status and the efficacy of cancer therapy in vivo Science **266**: 807-810.

Macchiarini P., Fontanni G., Hardin MJ.,Squartin F., Angletti CA. (1992). Relation of neovasculature to metastasis of non small cell lung cancer. Lancet **340**:145-146.

Maciag T., Mehlman T., Friesel R., Schreiber A., (1984). A Heparin binds endothelial cell growth factor, the principle endothelial cell mitogen in bovine brain. Science **225**: 932-935.

MacLean A.B. (2000). Paget's disease of the vulva. J. Obstet. Gynecol. **1**: 7-9

MacLean AB, Reid WMN, Rolfe KJ, Gammell SJ, Pugh HEJ, Gatter KC, Wong Te Fong AC, Crow JC, Perrett CW. (2000). The role of angiogenesis benign, premalignant and malignant vulvar lesions. J Reprod Med **45**:609-612.

Maestro R., Dolcetti R., Gastparotto D. et al. (1992) High frequency of p53 gene alterations associated with protein overexpression in human squamous carcinoma of the larynx.Oncogene **7**: 1159-1166.

Maguire WL.(1991). Breast cancer prognostic factors: evaluation guidelines. J Natl Cancer inst **83**: 154-155.

Maier W.P.,Rosemont G.P, Harasym E.L, Al- Saalem P, Tassoni E.M, Schor S. (1969).Paget's disease of the female breast. Surg Gynecol Obstet **128**: 1253-1263.

Malkin D., Li FP Strong LC., et al. (1990). Germ line mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science **250**:1233-1238.

Malkin D., (2001) The role of p53 in human cancer. J Neurooncol. **51**: 231-243.

Marchetti P., Cannita K., Ricevuto E., De Galitiiis F., Di Rocco ZC, Tessitore A.(2003). Prognostic value of p53 molecular status in high-risk primary breast cancer. Ann Oncol **14**: 704-708.

Mariani- Constantini R, Andreola S, Rilke F (1985). Tumour- associated antigens in mammary and extramammary Paget's disease. Virchows Arch **405**: 33-40.

Martin VG, Pellettiere EV, Miller AW(1994) : Paget's disease in an adolescent arising in a supernumerary nipple. J Cutan Pathol **21**:283.

McManus M.J and Welsch C.W.(1984) The effect of oestrogen , progesterone thyroxine and human placental lactogen on DNA synthesis of human breast ductal epithelium maintained in athymic nude mice. Cancer **54**: 1920-1927.

Mendez-Fernandez M.A, Henly W.S, Geis R.C, Schoen F.J, Hausner R.J (1980) Paget's disease of the breast after subcutaneous mastectomy and reconstruction with a silicone prosthesis.Plast Reconstr Surg **65**: 683-685.

Menzies D, Barr L, Ellis H.(1989) Paget's disease of the nipple occurring after wide local excision and radiotherapy for carcinoma of the breast. Eur J Surg Oncol.**15**: 271-273.

Merriam JM., Rubenstein AB., Klymkowsky MW.(1997) Cytoplasmically anchored plakoglobin induces a WNT-like phenotype in xenopus. Dev Biol **185**: 67-81.

Miettinen M, Lindenmayer AE, Chaubal A. (1994) Endothelial cell marker CD31, CD34 and BNH antibody to H- and –Y antigens: evaluation of their sensitivity and specificity in the diagnosis of vascular tumours and comparison with von Willebrand factor. Mod Pathol **7**: 82-90.

Miguel-Garcia A, Matutes E., Tarin F, Garcia- Talavera J, Miguel-Sosa , Carbonell F., Catovsky D. (1995) Circulating Ki67 positive lytmphocytes in multiple myeloma and benign Monoclonal gammopathy. J Clin Pathol 48:835-839.

Miller K., Auld J., Jessup E., Rhodes A., Ashton-Key M., (1995) Antigen unmasking in formalin-fixed routinely processed paraffin wax-embedded sections by pressure cooking: a comparison with microwave oven heating and traditional methods. Adv. Anat. Pathol. **2**: 60-64.

Miyake M., Tanaka K., Kikuchi- Yanoshita R., Muraoka M., Konishi M., Takeichi M (1995) Increased cell-substratum adhesion, and decreased gelatinase secretion and cell growth, induced by E-cadherin transfection of human colon carcinoma. Oncogene **11**: 2547-2552.

Miyata M., Shiozaki H., Iihara K., Shimaya K., Oka H., Kadowaki T., (1994) Relationship between E- cadherin expression and lymph node metastasis in human oesohageal cancer Int. J. Oncol **4**: 61-65.

Moghaddam A., Zhang ,H-T., Fan T-P., Hu D-E., Lees V.C., Turley H., Fox, S.B, Gatter ,K.C., Harris A.L., Bicknell R. (1995) Thymidine phosphorylase is angiogenic and promotes tumour growth. Proc Natl. Acad. Sci. 92: **998**-1002.

McColl B ., Stacker S., Achen M., (2004). Molecular regulation of the VEGF family –inducers of angiogenesis and lymphangiogenesis. APMIS **112**: 463-480.

Monaghan P., Perusinghe N.P., Cowen P., et al.(1990) Peripubertal human breast development. Anat Rec. **226**: 501-508.

Morgan D.O. (1997). Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol **13**:261-291.

Morgan D.O (1995). Principles of CDK regulation. Nature **374**, 131-134.

Morgan J.M., Navabi, H., Schmid K.W., and Jasani, B. (1994). Possible role of tissue-bound calcium ions in citrate- mediated high temperature antigen retrieval J. Cell. Pathol. **174**:301-307.

Morgan J.M. Jasani B., and Navabi H. (1997) A mechanism for high temperature antigen retrieval involving calcium complexes produced by formalin fixation. J. Cell. Pathol **2**: 89-92.

Morin P., Sparks A.,Korinek V., Barker N., CleversbH., Vogelstein B., Kinzler K., (1997). Activation of β- catenin-Tcf signalling in colon cancer by mutations in β-catenin or APC. Science **275**:1787-1790.

Motokura T.,Bloom T., Kim H.G., Juppner H., Rudderman J.V., Kronenberg H.M., Arnold A., (1991) A novel cyclin encoded by a bcl1-linked candidate oncogene Nature **350**: 512-515.

Mullis K., Faloona F., Scharf S. et al.(1986) Specific enzymatic amplification of DNA in vitro. The polymerase chain reaction. Cold Spring Harb Symp Quant Biol **51** :263-273.

Muir R.(1939). Further observations on Paget's disease of the nipple. J Pathol. **49**: 299-312.

Nakgaoka H, Lino Y, Takei H, Movishita Y (1998). Platelet-derived endothelial cell growth factor/thymidine phosphorylase expression in macrophages correlates with tumour angiogenesis and prognosis in invasive breast cancer. Int J Oncol. **13(3)**: 449-454.

Nakane P.and Pierce G. (1966) Enyzme-labeled antibodies: preparation and application for the localisation of antigens. J. Histochem. Cytochem. **14**: 929-931.

Nakamura G, Shikata N, Shoji T, Hatano T, Hioko K, Tsubura (1995). A: Immunohistochemical study of mammary and Extramammary Paget's disease. Anticancer Res **15**: 467-470.

Nenutil R., Smardovav J., Pavlova S., Hanzelkova Z., Muller P., Fabian P.(2005). Discriminating functional and non-functional p53 in human tumours by p53 and MDM2 immunohistochemistry. J Pathol. **207(3)** : 251-259.

Neufeld G., Cohen T., Gengrinovitch S., Poltorak Z., (1999) Vascular endothelial growth factor (VEGF) and its receptor. FASEB J. **13**: 19-22.

Nicosia R.F., Tchao R., Leighton J., (1986) Interactions between newly formed endothelial channels and carcinoma cells in plasma clot culture. Clin exp. Metastasis **4**: 91-104.

Nielsen NH., Emdin SO., Cajander J., Landberg G.(1997). Deregulation of cyclin E and D1 in breast cancer is associated with inactivation of the retinoblastoma protein. Oncogene **23**: 295-304.

Norton A.J, Jordon S., Yeomans P. (1994) Brief, high-temperature heat denaturation (pressure cooking): a simple and effective method of antigen retrieval J. Pathol. **173**: 371-379.

Obermair A, Kohlberger P, Bancher-Todesca D, et al. (1996) Influence of microvessel density and vascular permeability factor/ vascular endothelial growth factor expression on prognosis in vulvar cancer. Gynecol Oncol. **63**:204-209.

Oka H., Shiozaki H., Kobayashi K., Tahara H., Tamura S., Miyata M., et al. (1992). Immunohistochemical evaluation of E-cadherin adhesion molecule expression in human gastric cancer. Virchows Arch A Pathol Anat Histopathol **421**: 149-156.

Oka H., Shiozaki H., Kobayashi K., Inoue M., Tahara H., Kobayash T. et al. (1993) Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis Cancer Res **53**: 1696-1701.

Old R.W, Primose S.B.(1994). Principles of gene manipulation. An introduction to genetic engineering, 5th ed. Oxford: Blackwell Science Ltd, :178-190.

Olofsson B, Korpelainen E, Pepper SM, Mandriota SJ, Aase K, Kumar V, Gunji Y, Jeltsch MM, Shibuya M, Alitalo K, Eriksson U (1998). Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor- 1 and regulates plasminogen activator activity in endothelial cells. Cell Biol. **95**: 11709-11714.

Olson D.J, Fujimura M., Swanson P., Okagaki T.(1991). Immunohistochemical Features of Paget's disease of the Vulva With and Without Adenocarcinoma. Int. J. Gynecol Pathol.**10**: 285-295.

Olson TA, Mohanraj D, Carson LF, Ramakrisnan S. Vascular permeability factor gene expression in normal and neoplastic human ovaries (1994). Cancer Res **54**:276-280.

Ordonez N.G (1987) Awalt H. Mackay B. Mammary and extramammary Paget's disease. An immunohistochemical and ultrastructural study. Cancer **59**: 1173-1183.

Page D.L, Dupont W.D, Rogers L.W, Landerberger M. (1982). Intraductal carcinoma of the breast: Follow up after biopsy only.Cancer **49**: 825-829.

Pallle J O., Balslev E., Blichert-Toft (1990) Paget's disease of the nipple. Acta Chir Scand **156**:343-352.

Paget J. (1874). On the disease of the mammary areola preceding cancer of the mammary gland. St. Bartholomew Hospital Reports **10**: 87-89.

Paone J.F and Barker P.R. (1982). Pathogenesis and treatment of Paget's disease of the breast. Cancer **48**: 825-829.

Parker L.P., Parker J.R., Bodurka-Bevers D., Deavers M., Bevers M.W., Shen-Gunther J.,Gershenson D.M. (2000). Paget's disease of the vulva: Pathology, pattern of involvement, and prognosis. Gynecol. Oncol. **77**: 183-189.

Parker MF, Arroyo GF, Geradts J, Sabichi AL, Park RC, Taylor RR, Birrer MJ (1997). Molecular characterization of adneocarcinoma of the cervix. Gynecol oncol. 64:242-251.

Peifer M., (1997) β- catenin as oncogene : the smoking gun. Science **275**:1752-1753.

Peison B.and Benisch B. (1985) Paget's disease of the nipple stimulating malignant melanoma in a black woman. Am J Dermatopathol **7**: 165-169.

Peterse J.L, Van Dongen J.A, Bartelink H.(1988). Recuurence of breast carcinoma after breast conserving treatment . Eur J Surg Oncol. **14**: 123-126.

Pileri S.A., Roncador G., Ceccarelli C., Piccioli M., Briskomatis, A., Sabattini J., Ascani S., Santini D., Piccaluga P.P, Leone O., Damiani S., Ercolessi C., et al. (1997) Antigen retrieval techniques immunohistochemistry: comparison of different methods. J. Pathol. **183**:116-120.

Pierce L., McMormick B.,Haffty B et al.(1996). The use of radiotherapy in the conservative management of Paget's disease. Int J Radiat Oncol Biol Phys **36** (suppl) :215.

Pierard- Franchimont C. and Pierard G.E (1984). Replication on Paget cells in mammary and extramammary Paget's disease. Br J Dermatol **111**:69-74.

Plate K.H., Breier G., Welch H.A. Risau W. (1992) Vascular endothelial growth factor is a potential tumour angiogenesis factor for in human gliomas in vivo. Nature **359**: 845-848.

De Porter and A Schelfhout (1995) The neu- protein and breast cancer. Virchows Archiv **426**: 107-115.

Polakis P. (1999) The oncogene activation of β- catenin Curr Opin Genet. Dev. **9** 15-21.

Popiolek D., Hadju S., Gal D.,(1998). Case report :Synchronous Paget,s disease of the vulva and the breast. Gynecol Oncol. **71**:137-140.

Poste G. and Filder I.J. (1980). The pathogenesis of cancer metastasis. Nature **283**: 139-146.

Plowman P.N, Gilmore OJA, Curling M, et al. (1986) Paget,s disease of the nipple occurring after conservative management of early infiltrating breast cancer. Br J Surg **73**: 45.

Plowman G.D.,Whitney G.S., Neubauer M.G., Green J.M., McDonald V.L, Todaro G.J.,Shoyab M.,(1990). Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. Proc Natl Acad Sci USA **87**:4905-4909.

Porter PL, Garcia R., Moe R., Corwin DJ., Gown AM (1991) c-erbB2 oncogene protein in in situ and invasive lobular breast neoplasia. Cancer **68**:331-334.

Proctor AJ, Coombs LM, Cairns JP, Knowles MA (1991). Amplification at chromosome 11q13 in transistional cell tumour of the bladder. Oncogene **6**:789-795.

Rak J.W., Hegmann E.J., Lu C., Kerbel R.S. (1994) Progressive loss of sensitivity to endothelium –derived growth inhibitors expressed by human melanoma cells during disease progression . J.Cell Physiol.**159**: 245-255.

Rassidakis G., Lai R., Herling M., Cromwell C., Schmitt- Graeff., Medeeiros L. (2004). Retinoblastoma protein is frequently absent or phosphorylated in anaplastic large-cell lymphoma. Am. J. Pathol.**164**:2259-2267.

Rajesh L, Joshi K, Bhalla V, Dey P, Radotra BD, Nijhawan R. (2004). Correlation between VEGF expression and angiogenesis in breast carcinoma. Anal. Quant. Cytol. Histol. **26(2)**: 105-108.

Reyniak J.V. (1979) Endocrine physiology of the breast.(1979) J Reprod Med. **22**: 303-309.

Rice A and Quinn CM (2002) Angiogenesis, thrombospondin and ductal carcinoma in situ of the breast. J Clin Pathol **55**: 569-574.

Richter King C.,Kraus M.H, Aaronson S.A.(1985). Amplification of a novel v-erbB-related gene in human mammary carcinoma. Science **229**:974-976.

Rissanen P.M and Holsti P. (1969). Paget's disease of the breast. The influence of the presence or absence of an underlying palpable tumour on the prognosis and of choice of treatment. Oncology **23**: 209-216.

Ro YS, Cooper PN, Lee DA, Quinn AG, Harrison D. (1993). p53 expression in benign and malignant skin tumours. Br J. Dermatol **128**:237-241.

Roesch A., Becker B., Meyer S., Hafner C., Wild PJ., Landthaler M., Vogt T., (2005) Mod Pathol. **18**: 565-572.

Rolfe K.J, Eva L.J, A.B MacLean, Crow J.C Perrett C.W, Reid WMN. (2001) Cell cycle proteins as molecular markers of malignant change in vulvar lichen sclerosis (2001). Int. J. Gynecol. Cancer **11**:113-117.

Rolfe K.J, Crow J.C, Benjamin E., Reid WMN, A.B MacLean, Perrett C.W,.(2001) Cyclin D1 and retinoblastoma protein in vulvar cancer and adjacent lesions(2001). Int. J. Gynecol. Cancer **11**:381-386.

Rosen PP, Lesser ML, Arroyo CD, Miliicent C, Borgen P , Norton L. (1995). p53 in node- negative breast carcinoma: An immunohistochemical study of epidemiologic risk factors, histologic features, and prognosis. J Clin Oncol **13**:821-830.

Rosenberg C.L.,Wong E., Petty E.M., Bale A.E., Tsijimoto Y., Harris N.L., Arnold A., (1991) PRAD1, a candidate BCL1 oncogene:mapping and expression in centrolytic lymphoma Proc. Nat. Acad. Sci. **88**: 9638-9642.

Rubenstein A., Merriam J., Klymkowsky MW.(1997). Localising the adhesive and signalling functions of plakoglobin Dev. Genet. **20**:91-102.

Rudolph P, Kellner U., Chassevent A., Colin F. Bonichon F., Parwaresh R., Coindre JM (1997). Prognostic relevance of a novel proliferation marker , Ki-SS, for soft tissue sarcoma: a multivariate study. Am J Pathol **150**: 1997-2007.

Russell, P (2002).Genetics. Pearson Education, Inc., San Francisco, pp 187-189

Sagebiel R.W. (1969) Ultrastructural observations on epidermal cells in Paget's disease of the breast. Am J Pathol **57**: 49-64.

Sagami S.1963) Electron microscope observations in Paget's disease. Med J Osaka Univ **14**: 173-187.

Sanger F., Nicklens S., Coulson A.R, (1977). DNA sequencing with chain terminator inhibitors. Proc.Natl. Acta. Sci. USA **74**: 5463-5467.

Sano T., Oyama T., Kashiwabara K., Fukuda T, Nakajima T,(1998). Immunohistochemical overexpresssion of p16 protein associated with intact retinoblastoma protein expression in cervical cancer and cervical intraepithelial neoplasia. Pathol. Int **48**: 580-585

Saravanamuthu J., Reid W.N., St Georg D., Crow J., Rolfe K., MacLean A., Perrett C. (2003). The role of angiogenesis in vulvar cancer, vulvar intraepithelial neoplasia and vulvar lichen sclerosus as determined by micrvessel density analysis. Gynecologic Oncology **89**: 251-258.

Satiani B, Powell R.W, Mathews W.H (1977). Paget's disease of the male breast. Arch Surg. **112**: 576-592.

Satoh S., Dagigo, Y., Furukawa., Y Kato T.,Miwa N., Nishiwaki T., Kawasoe T., Ishiguro H., Fujita M., Tokino T., (2000). AXIN 1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN 1 Nature Genet. **24** 245-250.

Schechter A.L., Stern D.F.,, Vaidyanathan L.,Decker S.J Drebin J.A., Greene M.I., Weinberg R.A., (1985) The neu oncogene: an *erb*-B-related gene encoding a 185,000 Mr tumour antigen. Nature **312**:513-516.

Schechter A.L., Hung M-C., D.F.,, Vaidyanathan L., Weinberg R.A., Yang-Feng T.A, Francke U., Ullrich A., Coussens L., (1985) The neu gene: an *erb*B-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. Science **229**:976-978.

Schwarting R. (1993). Little missed markers and Ki67. Lab Invest. **68(6)**: 597-599.

Semba K., Kamata N., Toyoshima K., Yamamoto T (1995) A v-erb related protoonvogene, c-erbB-2 is distinct from c-erbB-1/epidermal growth factor receptor gene and is amplified in a human salivary gland adenocarcinoma. Proc Natl Acad Sci USA **82**:6497-6501.

Semenza G.L.(1996) Transcriptional regulation by hypoxia –inducible factor 1-molecular mechanism of oxygen haemastasis. Trends. Cardiovasc. Med **6**:151-157.

Sheistroen M, Trope C, Kaern J, Pettersen E.O, Alfsen C.G, Nesland J.M (1997): DNA Ploidy and Expression of p53 and C-erbB-2 in Extramammary Paget's disease of the vulva.Gynecol Oncol **64**: 88-92.

Shephard V., Davidson E., Davies- Humphreys (2005) Extramammary Paget's disease. BJOG **112**: 273-279.

Sherbert GV, Patil D.(2003) Genetic abnormalities of cell proliferation, invasion and metastasis with special reference to gynaecological cancers. Anticancer Res. **23**(2B) : 1357-1371.

Sherr C.J, (1995) D-type cyclins . Trends Biol Sci **20**:187-90.

Shi S-R., Key M.E, and Kalra K.L (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissue sections: an enhancement method of immunohistchemical staining based on microwave oven heating of tissue sections. J. Histochem. Cytochem. **39**: 741-748.

Shi S-R., Imam S.A, Young L., Cote R.J., Taylor C.R., Key M.E, Kalra K.L, (1995) Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. J . Histochem. Cytochem. **43**: 193-201.

Shing Y. et al., (1984) Heparin affinity : purification of a tumour –derived capillary endothelial cell growth factor. Science **223**: 1296-1298.

Shiozaki H., Tahara H., Oka H., Miyata M., Kobayashi K., Tamura S. (1991). Expression of immuno-reactive E- cadherin adhesion molecules in human cancers Am J Pathol. **139**:17-23.

Shirahama S. ,Furukawa F., Wakita H., Takigawa M., (1996) E- and P-cadherin expression in tumour tissues and soluble E-cadherin levels in sera of patients with skin cancer. J. Dermatol. Sci. **13**: 30-36.

Shtutman M., Zhurinsky J., Simcha I., Albanese C., D'Amico M., Pestell R., Ben-Ze'ev A (1999) The cyclin D1 gene is a target of the β- catenin/ LEF-1 pathway Proc Nat. Acad. Sci. **96**: 5522-5527.

Shweiki D., Itin A., Soffer D., Keshet E. (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature **359**: 843-845.

Simpson JF., Quan DE, O Malley F' Odon-Mayon T, Clarke PE. (1997). Amplification of CCND1 and expression of its protein product cyclin D1, in ductal carcinoma in situ of the breast. Am J. Pathol. **151**: 161-168.

Sineiad F., Polverini P.J., Bouck N.P. (1989). Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. Cell **56**: 345-355.

Skaug V., Ryberg D., Kure E.H., Arab M.O, Strangeland L., Myking A.O and Haugen A. (2000). p53 mutations indefined structural and functional domains are related to poor clinical outcome in non-small cell lung cancer patients. Clin Cancer Res **6**:1032-1037.

Skomedal H., Kristensen GB., Lie AK., Holm R., (1999). Aberrant expression of the cell cycle associated proteins TP53, MDM2, p21 .p27 cdk4, cyclinD1, RB and EGFR in cervical cancers. Gynecol oncol. **73**: 223-228.

Sliutz G, Schmidtz W, Tempfer C, Speiser P, Gitsch G, Eder S, Schneeberger C, Kainz C, Zeillinger R, (1997). Detection of p53 point mutations in primary human vulvar cancer by PCR and Temperature Gradient Gel Electrophoresis. Gynecol Oncol **64**: 93-98.

Silvestrini R, Benini E, Daidone MG, Veneroni S, Boracchi P, Cappelletti V, Di Fronzo G, Veronesi U. (1993). p53 as an independent prognostic marker in lymph node-negative breast cancer patients. J Natl Cancer Inst. **85**:965-970.

Simcha I., Geiger B., Yehuda-Levenberg S., Salomon D., Ben-Ze'ev A. (1996). Suppression of tumourigenicity by plakoglobin: an augmenting effect of N- cadherin. J cell Biol. **133**: 199-209.

Smith N., Rubenstein J., Eggener S., Kozlowski M., (2003). The p53 Tumour suppressor gene and nuclear protein: Basic science review and revelance in the management of bladder cancer. J Urol. **169**: 1219-1228.

Soler A.P., Knudsen K.A Salazar H., et al.(1999) P-cadherin expression in breast carcinomas indicates poor survival. Cancer **86**:1263-1272.

Sommers CL, Gelmann EP., Kemler R., Cowin P., Byers SW., (1994) Alterations in beta-catenin phosphorylation and plakoglobin expression in human breast cancer cells. Cancer Res **54**: 3544-52.

Sparks PJ., Korinek V., Barker N., Clevers H., Volgelstein B., Kinzler KW.(1997) Activation of β- catenin –Tcf signalling in colon cancer by mutations in β- catenin or APC Science **275**:1787-1790.

Stacy, D, Burrell, M.O, and Franklin, E. W., III. (1986). Extramammary Paget's disease of the vulva and anus: Use of intraoperative frozen-section margins, Am. J Obstet. Gynecol **155**: 519-523.

Stockdale A.D.,White W.F, Brierly J.D et al. (1989) Radiotherapy for Paget's disease of the nipple: A conservative alternative. Lancet **2**:664-666.

Strahm B., Capra M.(2005). Insights into the molecular basis of cancer development. J. Curr. Paed. **15**: 333-338.

Tachibana M., Kawamata H., Fujimori T., Omotehara F., Horiuchi H., Ohkura Y., Igarashi S., Kotake K., Kubota. K. (2004). Dysfunction of p53 pathway in colorectal

cancer: analysis of p53 gene mutation and the expression of the p53- associated factors p14ARF.p33ING1, p21 WAF1 and MDM2. Int J Oncol. **25**:913-920.

Takeichi M. (1995). Morphogenetic roles of classical cadherins. Curr. Opin. Cell. Biol. **7**: 619-627.

Takeichi M.(1991) Cadherin cell adhesion receptors as a morphogenetic regulator. Science **251**: 1451-1455.

Tada H., Hatoko M., Tanaka A., Kuwahara M., Muramatsu T. (2000) Expression of desmoglein I and Plakoglobin in skin carcinomas. J.Cutan Pathol **27**: 24-29.

Tada H., Hatoko M., Muramatsu T.,Shirai T.(1996). Expression of E-cadherin in skin carcinomas. J. Dermatol. **23**:104-110.

Takahashi T., Nau M.M, Chiba I., Birrer M.J., Rosenberg R.K., Vinocour M., Levitt M., Pass H., Gazadar A.F., Minna J.D. (1989). P53 -a frequent target for genetic abnormalities in lung cancer. Science **246**: 491-494.

Takata M, Hatta N, Takehara (1997): Tumour cells of extramammary Paget's disease do not show either p53 mutation or alleic loss at several selected loci implicated in other cancers. Br J Cancer **76**: 04-908.

Takata M., Fujimoto A., Aoki H., Hatta N., Ooi A., Takehara K.(1999). erbB-2 Overexpression but No Activation of β- catenin Gene in Extramammary Paget's Disease. J. Inves Dermatol. **113**:258-262.

T'Ang A, Varley JM, Chakraborty S, Murphree AL, Fung Y-KT. (1988). Structural rearrangement of the retinoblastoma gene in human breast carcinoma. Science **242**: 263-266

Tani EM, Skoog L., (1988). Immunocytochemical detection of oestrogen receptors in mammary Paget cells. Acta Cytol. **32**: 825-828.

Taya, Y.(1997) RB kinases and RB-binding proteins: new points of view. Trends Biochem. Sci. **22**:14-17.

Taylor C.R., Shi S-R., Cote R.J. (1996) Antigen retrieval for immunohistochemistry: status and need for greater standardisation. Appl. Immunohistochem. **4**: 144-166.

Tetsu O. and McCormick F., (19990. β- catenin regulates expression cyclin D1 in colon carcinoma cells Nature **398**: 422-426.

Thor A, Moore DH, Edgarton SM, et al. (1992).Accumulation of p53 tumour suppressor protein: An independent marker of prognosis in breast cancers. J Natl Cancer Inst **84**: 845- 855.

Thomas S. and Gerdes J., (2000). The Ki67 protein. J.Cell.Pathol. **182**: 311-322.

Tidy J.A, Soutter W.P, Luesley D.M., A.B MacLean, C.H Buckley , Ridley C.M. (1996). Management of lichen sclerosis and intraepithelial neoplasia of the vulva in the UK. J Royal Soc Med 90: 699-701.

Tisher E., Gospodarowicz D., Mitchell R., Silva M., Schilling J., Lau K., Crisp T., Fiddes JC., Abraham JA., (1989). Vascular endothelial growth factor: a new member of the platelet- derived growth factor gene family. Biochem Biophys Res Commun **165**: 1198.

Toker C, (1961). Some observations of Paget's disease of the nipple. Cancer **14**: 653-672.

Toker C, (1967). Further Observations on Paget's disease of the nipple. J Natl Cancer Inst. **38**: 79-92.

Toker C, (1970). Clear cells of the nipple epidermis. Cancer **25**: 601-610.

Toi M, Hoshina S, Takayanagi T, Tominaga T. Association of vascular endothelial growth factor expression with tumour angiogenesis and with early relapse in primary breast cancer (1994). Jpn J Cancer Res **85**:1045-1049.

Toi M, Inada K, Hoshina S, Suzuki H, Kondo S, Tominaga T. (1995). Vascular endothelial growth factor are frequently coexpressed in highly vascularised human breast cancer (1995).Clin Cancer Res **1(9)**:961-964.

du Toit R.S, van Rensburg P.S, Goedhals L. (1988). Paget's disease of the breast .S Afr Med J **73**: 95-97.

Treves N. (1954) Paget's disease of the male mammary gland. A report of two cases. Cancer **7**: 325-330.

Tsutsui S., Kume TM., Eva S., (2003) Prognostic value of microvessel density in invasive ductal carcinoma of the breast. Breast **10**: 312-319.

Ueda T, Aozasa K, Tsujimoto M, Ohsaawa m, Uchida A, Aoki Y, Ono K, Matsumoto K., (1989) Prognostic significance of Ki67 reactivity in soft tissue sarcomas. Cancer Res **49**:2999-3006.

Urano Y., Oura H., Sakaki A., Nagae H., Matsumoto K., Fukuhara K., Nagae T., Arase S., Ninomiya Y., Nakanishi H. (1992) Immunohistological analysis of P53 expression in human skin tumours.

Van Diest PJ. Michalides RJ. Jannink I et al., (1997) Cyclin D1 expression in invasive breast cancer:correlation and prognostic value. Am J. Pathol **150**:705-711.

Van Mer E.G. et al. (1994) Release of an inhibitor of angiogenesis upon induction of wild type p53 expression in glioblastoma cells. Nature Genetics **8**: 171-176.

Viacara P, Nacaccardo BG, Bocci G, Fanelli G, Aretini P, Lonobile A, Evanelista G.

(2004). Angiogenesis and VEGF expression in pre-invasive lesions of the human breast. J Pathol **204(2)**:140-146.

Vallbohmer D, Peters JH, Kuramochi H, Oh D, Yang D, Shimizi D, DeMeester SR et al., (2006) Molecular determinants in targeted therapy for oesophageal adenocarcinoma. Arch Surg **141(5)**: 476-482.

Velpeau AALM. Lecons orales de clinique chirurgicale faites a l'Hopital de la Charite, recuillies et publies par Gustave Jeanselme, Vol 2. Paris. Germer- Bailliere, 1841.

Venter DJ, Bevan KL, Ludwing RL, et al (1991) Retinoblastoma gene inn deletions in human glioblastomas. Oncogene **6**:445-448.

Vleminckx K.L, Vakaet J., Mareel M., Fiers W., Van Roy F. (1991) Genetic manipulation of E-cadherin expression by epithelial tumour cells reveals an invasiveness of human carcinoma cells. J. Cell. Biol. **113**: 73-185.

Walch A., Komminoth P., Hutzler P., Aubele M., Hofler H., Werner M., (2000) Microdissection of tissue sections: Application to the molecular genetic characterisation of premalignanat lesions. Pathobiology **68**: 9-17.

Wang JM, Kumar S, Pye D, van Agthoven AJ Krupinski J, Hunter RD (1993). A monoclonal antibody detects heterogeneity in vascular endothelium of tumours and normal tissue. Int J Cancer **54**: 363-370.

Walker RW, Camplejohn RS (1988). Comparison of monoclonal antibody Ki 67 reactivity with grade and DNA flow cytometry of breast carcinomas. Br J Cancer **57**:281-283.

Warford A., Pringle J.H, Hay J. et al.(1988) Southern blot analysis of embedded tissue. J Pathol **154**: 313-320.

Watanabe S., Ohnishi T., Takashasi H., Ishibashi Y. (1993) A comparative study of cytokeratin expression in Paget cells located at various sites. Cancer **72**: 3323-3330.

Watanabe Y., Hoshiai H., Obata K., Nada K.(2002) Low dose mitomycin C, etoposide, and cisplatin. Int J Gynecol Cancer **12**:304-307.

Wedham SB., Low JA., Yang SX., Chow CK., Choyke P., Danforht D., Hewitt SM., Bernen A., Steinberg SM., (2006) Antiangiogenic and antitumour effects of bevacizumab in patients with inflammatory and locally advanced breast cancer. J Clin Oncol.**10**: 769-777.

Weidner N., Semple J., Welch W., Folkman J. (1991). Tumour angiogenesis correlates with metastsis in invasive breast carcinoma. N. Engl. J Med. **324**:1-8.

Weidner N., Folkman J., Pozza F., Bevilacqua P., Allred E. Moore D., Meli S., Gasparini G. (1992) Tumour angiogenesis. A new significant and independent prognostic factor in early stage- breast carcinoma. J. nat. Cancer inst **84**: 1875-1887.

Weidner N. Carroll P., Flax J., Blumenfeld W., Folkman J. (1993) Tumour angiogenesis correlates with metastasis in invasive prostate carcinoma. Amer J. Pathol. **143**:1-9.

Weidner N. (1995) Intratumour microvessel density as a prognostic factor in cancer. Am. J Pathol. **147**: 9-19.

Weidner N. (1998) Tumour vascularity as a prognostic factor in cancer patients: The evidence continues to grow. J Pathol **184**: 119-122.

Weinberg R.A (1995) The retinoblastoma protein and cell cycle control . Cell **81**: 323-330.

Weinstat-Saslow D Merino MJ Manrow RE, Lawrence JA, Bluth RF, Wittenbel KD., Simpson JF, Page DL, Steeg PS. (1995) Overexpression of cyclin D mRNA distinguishes invasive and in situ breast carcinomas from non-malignant lesions. Nature Med. **1**:257-260.

Werness BA., Levine AJ. Howley PM. (1990) Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science **248**: 76-79.

Whorton CM., Patterson JB. (1955) Carcinoma of Moll's glands with extramammary Paget's disease of the eye lid. **8**:1009-1015.

Wieneke R, Eckert F, Kaudewitz P, De Viragh PA, Heidl G, Volkenandt M (1994). p53 protein in benign and malignant sweat gland tumours. Am J. Dermatopathol **16**:126-129.

Wilkinson &Brown (2002) Vulvar Paget's disease of urothelial orgin: a report of 3 cases and a proposed classification of vulvar Paget's disease. Human Pathol **33**:549-554.

Willert K., Nusse R., (1998) Beta-catenin: a key mediator of Wnt signalling .Curr Opin Genet Dev **8**: 95-102.

Willis R.A (1960) Pathology of Tumours, 3rd ed. Washington, Butterworth, Inc. p247.

Withers D.A, Harvey R.C.,Faust J.B.,Melnyk O.,Carey K.,Meeker T.C(1991). Characterisation of a candidate bcl-1 gene. Mol. Cell. Biol.**11**:4846-4853.

Wizgmann-Voos S., Breier G., Risau, W., Plate K.H. (1995) Up–regulation of vascular endothelial growth factor and its receptors in Von-Hippel –Lindau disease associated and sporadic hemangioblastomas. Cancer Res **55**: 1358-1364.

Wong- Te- Fong L.F, Rolfe K.J., Crow J.C., Reid W.M.N., MacLean A.B., Perrett C.W.(2000) Vascular endothelial growth factor (VEGF) and platelet-derived endothelial cell growth /thymidine phosphorylase (PD-ECGF/TP) as potential

markers in the malignant progression of vulva intraepithelial neoplasia (VIN) and lichen sclerosus to vulval cancer. J. Obstet. Gynaecol **20**:559.

Wong- Te- Fong L.F, Rolfe K.J., Crow J.C., Reid W.M.N., MacLean A.B., Perrett C.W.(2000). Quantitative immunohistochemistry of VEGF, PD-ECGF/TP and factor VIII- related antigen in vulval carcinomas. Proc. Blair. Res. Soc. Meet.: P11

Yamasaki T., Tsuda H., Imazeki N., Matsubara O., (2005). Vascular endothelial growth factor mRNA levels quantified by reverse transcription-polymerase chain reaction in microdissected breast carcinoma tissues are correlated with histological type and grade of both invasive and intraductal components. Pathol Int., **55**: 255-263.

Yamazaki N., Yamamoto A., Wada T (1999) A case of metabolic extramammary Paget's disease that responded to combination chemotherapy. J Dermatology **26(5)**: 311-3116.

Yonenga F, Takaraki Ohi Y, Sagara Y, Akiba S, Yoshinaka H, Aikan T, Miyada K, Akiyama S, Yoshida H. (1998). The expression of thymidine phosphorylase/platelet-derived endothelila cell growth factor is correlated to angiogenesis in breast cancer. Pathol Int **11**: 850-856.

Yonish- Rouach E, Resnitsky D., Lotem J., et al. (1991) Wild type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin -6.Nature **352**: 345-347.

Zetter B. (1990). The cellular basis of site-specific tumour metastsis. N Engl. J. Med. **322**: 605-612.

Zhang C., Zhang P., Sung CJ., Lawrence WD (2003). Overexpression of p53 is correlated with stromal invasion in extramammary Paget's disease of the vulva **34**: 880-885.

Zschiesche W., Schonborn I., Behrens J., et al. (1997) Expression of E-cadherin and catenins in invasive mammary carcinomas Anticancer Res **17**:561-56.

Zur Hausen H., (1991) Viruses in Human Cancers. Science **22**: 1167-73.