AN EXPLORATION OF MOLECULAR MARKERS IN PROGNOSIS OF CERVICAL INTRAEPITHELIAL NEOPLASIA

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by

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Dedicated to
My parents, my husband
My daughters Ahd and Shahd
With love

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ABBREVIATIONS

bp	base pair(s)
CGH	Comparative genomic hybridization
CIN	Cervical intraepithelial neoplasia
CIN1	Cervical intraepithelial neoplasia grade one
CIN2	Cervical intraepithelial neoplasia grade two
CIN3	Cervical intraepithelial neoplasia grade three
DF	Disease free
DLEC1	deleted in lung and esophageal carcinoma 1
DNA	Deoxyribonucleic Acid
dNTP	deoxyribonucleoside 5 -triphosphate
DP	Disease persistence
EDTA	Ethylenediamine tetra-acetic acid
FHIT	fragile histidine triad gene
G6PD	glucose 6-phosphate dehydrogenase
HIV	Human immunodeficiency syndrome
HPV	Human papilloma virus
HSV 2	Herpes simplex virus type two
LLETZ	Large loop excision of the transformation zone
LOH	Loss of heterozygosity
NHSCSP	National Health Service Cervical Screening Programme
NPCR	nasopharyngeal carcinoma related protein
ORCTL	organic cationic transporter-like 4
ORFs	Open Reading Frames
Pap	Papanicolaou smear
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PG	progesterone receptor
Rb	Retinoblastoma gene
RT-PCR	reverse transcription-polymerase chain reaction
TBE	Tris-borate-EDTA buffer
TEMED	N,N,N′,N′-tetramethyl-ethylenediamine
TRIM29	tripartite motif-containing 29
TRPC6	transient receptor potential cation channel, superfamily C, member 6)
TSG	tumour suppressor gene
TZ	transformation zone
XYLB	Xylukokinase homolog

ABSTRACT

Cervical intraepithelial neoplasia (CIN) lesions of the same morphological grade show variable clinical behaviour; some progress and others regress. Currently, there are no biochemical or molecular markers which can distinguish CIN lesions with different prognosis. We have optimised several molecular methods on archival cervical smears and screened a number of molecular markers that may aid prognosis of CIN.

By systematically validating different protocols, we have established that crude DNA preparations from a small number of microdissected cells from cervical smears are adequate for various PCR-based investigations. Furthermore, the crude DNA preparations could be further purified and used for PCR-based clonality analysis of the X-linked genes.

Using PCR-based clonality analysis of the androgen receptor gene, we have shown that CIN3 and the majority of CIN2 lesions are monoclonal, whereas CIN1 lesions are polyclonal. Importantly, patients with monoclonal CIN2 show disease persistence or progression, while polyclonal CIN2 regress after treatment and remain negative during follow-up.

To further identify molecular markers that are technically easy to apply and potentially suitable for prognosis assessment of all CIN grades, we have investigated the prognostic value of gene deletions. In a pilot study, we screened 12 microsatellite markers, which showed high frequencies of loss of heterozygosity (LOH) in cervical carcinomas, and identified four, including D3S1300, D3S1260, D11S35 and D11S528, that were significantly associated with CIN persistence or progression. The 4 markers were further investigated in a larger cohort. Combined analysis of LOH at these 4 loci permitted the identification of 22-47% of CIN lesions of various histological grades, that were associated with disease persistence or progression with 100% specificity. LOH at these loci was significantly associated with HPV16 infection. Bioinformatics analysis identified several candidate genes including the fragile histidine triad and progesterone receptor gene that may be the target of deletions.

CHAPTER 1

GENERAL INTRODUCTION

Carcinoma of the uterine cervix is the second leading cause of carcinoma related death in women worldwide, with an estimated 470,000 newly diagnosed cases per year (Drain *et al*, 2002). The age of peak incidence is between 45-59 years (Stockton *et al*, 1997;Schoell *et al*, 1999). There are two main types of cervical carcinoma, squamous cell carcinoma and adenocarcinoma, with the former accounting for about 75%. Most of cervical squamous carcinomas are preceded by the pre-neoplastic stage, referred to as cervical intraepithelial neoplasia (CIN). CIN is the research subject of this thesis.

1.1 Epidemiology

1.1.1 Incidence

Approximately, 470,000 women are diagnosed with cervical carcinoma worldwide each year, accounting for 10% of all carcinomas diagnosed in women (Drain *et al*, 2002). Around, 80% of cases occur in the developing world, where the disease is usually detected at advanced stages causing 190,000 deaths each year (Pisani *et al*, 1999;Patnick, 2000;Drain *et al*, 2002).

The incidence of invasive cervical carcinoma in many Western countries has fallen due to the introduction of cervical carcinoma screening programmes. In England, since introduction of the National Health Service Cervical Screening Programme (NHSCSP) in 1988, the incidence of invasive cervical carcinoma had dropped from 16/100,000 in the mid-1980s, to 10/100,000 in 1995 with an overall decline of 35% (Quinn *et al*, 1999;Levi *et al*, 2000). The drop of the incidence occurred in every age group from 30-34 to 70-74, in particular in 50-54 age group (Quinn *et al*, 1999;Levi *et al*, 2000).

In contrast, the incidence of cervical carcinoma in developing countries remains high. For example, in India the rate was 99.1/10,000 in the period of 1982-1995, and in Cali Colombia it was 77.4/100,000 in the period of 1987-1991 (Soler *et al*, 2000; Shanta *et al*, 2000).

In contrast to the dramatic drop of the incidence of cervical carcinoma during late 1980s and 1990s in the Western countries, the incidence of CIN appears to have increased. In England, it rose from about 55 / 100,000 in 1985 to 80 / 100,000 cases in the 1995 (Quinn *et al*, 1999). This increase was particularly prominent in women aged 20-29 (Quinn *et al*, 1999), whereas women in the age 30-49 group showed no overall increase and the older women group (aged 50-84) displayed a decline (Quinn *et al*, 1999). The primary reason for the increase of CIN in women aged 20–29 might be related to the change in sexual behaviour in

women including the early onset of sexual activity and consequently, earlier age of HPV infection.

1.1.2 Mortality

The worldwide cervical carcinoma mortality rate of nearly 200,000 deaths / annum (Drain *et al*, 2002) has been declining in developed countries by up to 70% over the last few decades since introduction of the cervical carcinoma screening programmes. In the UK, before the introduction of the NHSCSP in 1988, cervical carcinoma caused 11.2/100,000 deaths/ annum (Patnick, 2000; Quinn *et al*, 1999). By 1997, the mortality rate had fallen to 3.7/100,000, accounting for 2% of carcinoma related death in women (Patnick, 2000;Quinn *et al*, 1999). The cervical carcinoma mortality rate continues to fall, by approximately 7% per annum (Patnick, 2000;Quinn *et al*, 1999).

In developing countries where cervical carcinoma is still the major health problem, the magnitude of this disease is probably much greater than recorded because there are no complete and durable tumour registries. The highest mortality rate has been reported in Africa (15.3/100,000), followed by Latin America (14.3/100,000) (Drain *et al*, 2002).

1.2 Etiology

Based on both epidemiological and laboratory studies, it is now understood that the development of cervical carcinoma is associated with several risk factors.

1.2.1 Human Papilloma Virus Infection

In the last 20 years, Human Papilloma Virus (HPV) has been at the centre of investigations in relation to the etiology of cervical carcinoma. The prevalence of HPV-DNA found in cervical carcinoma has dramatically increased with the improvement of the detection methods. In the 1980s, the use of Southern blot analysis identified HPV in 40-70% of cases (Bosch[b] *et al* , 2002). Further improvement in the probe design and testing conditions enabled the detection of HPV in nearly 100% of cases (Bosch[b] *et al*, 2002). Recent studies, using PCR on a large series of cases, have shown that HPV infection is present in 93-99% of cases (Bosch *et al*, 1995; Walboomers *et al*, 1999).

HPV represents a heterogeneous group of DNA viruses encompassing a family of more than 80 genotypes, of which about 40 are found in the genital tract. The current HPV typing is based on sequence differences, in particular in the E6/E7 and L1 regions (Southern *et al*, 1998;Soler *et al*, 2000;Sonnex, 1998;Stewart *et al*, 1996). HPV types can also be divided into cutaneous or mucosal. From a clinical perspective, mucosal HPV

types are further subdivided into low, intermediate and high-risk groups depending on the lesions they are associated with. Low-risk types such as HPV6 and 11, are associated with benign genital warts and low-grade CIN lesions (CIN1), and are rarely found in CIN2, CIN3 and invasive lesions. On the other hand, intermediate-risk types such as HPV31, 39, 52, and 58 and high-risk types, for example HPV16, 18, 33, 45 and 56, are more often seen in CIN2, CIN3 and cervical carcinoma than in CIN1 and borderline lesions (Chan *et al*, 2002;Jastreboff *et al*, 2002;Lazo, 1999).

Evidence from epidemiological studies strongly indicates that HPV infection is a critical factor in the development of cervical carcinoma (Bosch *et al*, 1995). Nearly all cervical carcinomas have detectable HPV (Walboomers *et al*, 1999). High-risk HPV types are more often seen in CIN2, CIN3 and invasive cervical carcinoma than CIN1 and borderline lesions (Chan *et al*, 2002;Jastreboff *et al*, 2002;Lazo, 1999). Longitudinal studies have shown that HPV infection precedes the development of cervical carcinoma (Dillner *et al*, 1997). The prospective case-control studies show that women with normal Pap smear test result but positive for high risk HPV types had a 16.4 times higher risk of developing cervical carcinoma than those negative for high risk HPV (Wallin *et al*, 1999). Preliminary results of a recent HPV 16 vaccine trial showed decrease in the incidence of

both HPV-16 infection and HPV related CIN lesions in women who received the vaccine but not in the control group (Koutsky *et al*, 2002).

The strong epidemiological evidence in favour of a causative role of HPV infection in development of cervical carcinoma is supported by molecular studies of the oncogenic activity of molecules associated with the virus. Two viral oncoproteins namely E6 and E7 have been shown to play an important role in the process of malignant transformation. Both proteins are consistently expressed in cervical carcinoma cell lines and can immortalize primary cervical epithelial cells and human keratinocytes in vitro (zur Hausen, 1999;Southern *et al*, 1998). Transgenic mice with E6/E7 of HPV16 show hyperplastic and dysplastic squamous lesions (Arbeit *et al*, 1994;Coussens *et al*, 1996). Molecular studies demonstrate that the HPV E6 protein binds the p53 gene product and the E7 protein forms complexes with Rb gene (Dyson *et al*, 1989;Werness *et al*, 1990), thus blocking their tumour suppresser activity (as detailed in section 1.8.1.1).

1.2.2 Sexual Behaviour And Other Infectious Agents

Epidemiological studies conducted during the past 30 years have consistently identified sexual behaviour as a major risk factor for cervical carcinoma and its association with this disease is in

many ways similar to that of other sexually transmitted diseases. Age at first intercourse, number of sexual partners, cigarette smoking, low socioeconomic status, and the acquisition of HPV are consistently linked to sexual behaviour. With the exception of HPV infection, none of the others have been shown to be significant independent risk factor (Rock *et al*, 2000;Jastreboff *et al*, 2002;Schoell *et al*, 1999;Lehtinen *et al*, 2002;Castellsague *et al*, 2002). Cervical carcinoma rarely occurs in nuns and virgins (Morin *et al*, 2000; Jussawalla *et al*, 1984). In Israel, where the lowest worldwide incidence of cervical carcinoma is registered, this is most likely related to their conservative sexual life style (Sadan *et al*, 2003).

Chlamydia trachomatis is the most common sexually transmitted bacterial infection, and has been consistently associated with cervical carcinoma and its precursors. In a case control study, Anttila et al showed that the presence of serum antibodies to Chlamydia trachomatis was associated with 6.6 fold increase in risk of cervical carcinoma development as compared to seronegative women (Anttila *et al*, 2001). However this association has commonly been attributed to be confounded by HPV infections (Wallin *et al*, 2002;Smith *et al*, 2002).

Herpes simplex virus type 2 (HSV-2) is another infectious agent associated with sexually transmitted diseases (Cherpes *et al*,

2003). The role of HSV-2 infection in development of cervical carcinoma is unclear. HSV-2 associated oncogenes are capable of transforming cells in culture (Tran-Thanh *et al*, 2003). However, the recent study with a highly sensitive and specific PCR method failed to detect HSV-2 DNA in high grade CIN and invasive cervical carcinoma (Lehtinen *et al*, 2002). The role of HSV-2 in development of cervical carcinoma, is thought to be through the induced inflammatory response (Castellsague *et al*, 2002).

Human Immunodeficiency Virus (HIV) positive women have consistently been shown to be at an increased risk of development of cervical carcinoma (Franceschi *et al*, 1998;Selik *et al*, 1998). Since HPV and HIV share the same route of transmission, it is difficult to determine their respective contributions to the etiology of the disease. Hence, the relationship between HIV infection and cervical carcinoma could be explained either by the frequent occurrence of HPV infection in HIV-positive women and /or by a direct suppressive effect of HIV infection on the immune system which may promote the persistence of HPV infection (Serraino *et al*, 1999).

1.2.3 Hormones And Oral Contraceptives

An increased incidence of cervical carcinoma and its precursors has been reported in women using oral contraceptive (Moreno *et al*, 2002). A case control study carried out by International

Agency For Research on Carcinoma (IARC) (cases and controls were positive for HPV DNA), showed that the use of oral contraceptives for five or more years is a cofactor that increases the risk of cervical carcinoma up to fourfold (Bosch[a] *et al*, 2002;Moreno *et al*, 2002). Mittal et al showed that HPV expression in human ectocervical cells lines was enhanced when progesterone or glucocorticoids were added to the culture (Mittal *et al*, 1993). It has been shown by both in vitro and in vivo studies that steroid hormones enhance the transcription of the HPV E6/E7genes (Moodley *et al*, 2003).

1.2.4 High Parity

High parity has consistently been found to be associated with cervical carcinoma in most case- control studies. In the IARC multicenter study, HPV positive women who reported seven or more full term pregnancies had a fourfold increased risk of cervical carcinoma compared with similar HPV positive women who were nulliparous (Munoz *et al*, 2002;Hildesheim *et al*, 2001;Schiffman *et al*, 1993).

1.2.5 Smoking

Epidemiological studies have implicated cigarette smoking as a possible contributing factor in the development of cervical carcinoma (Southern *et al*, 1998;La Vecchia *et al*, 1986). It has been shown that smokers have a 4-fold increased risk of

developing cervical carcinoma compared to non-smokers (Winkelstein, 1990). The actual mechanism by which smoking leads to an increased risk of cervical carcinoma is not fully understood. The constituents of smoke and their derivatives can enhance the proliferation of HPV transformed cervical cells (Morelli *et al*, 1993;Waggoner *et al*, 1994). Nicotine and cotinine are found at high levels in cervical mucus of smokers, and are believed to have local immunosuppressive and mutagenic effects (Castellsague *et al*, 2002). Reduction in number and function of Langerhans cells and helper/inducer T lymphocytes has been observed in the squamous epithelium of the cervical transformation zone in female smokers (Morelli *et al*, 1993;Waggoner *et al*, 1994).

1.3 Histology

1.3.1 The Normal Cervix

The uterus consists of the corpus and the cervix. The cervix is covered on its outer surface (ectocervix) by stratified squamous epithelium (Figure 1.1), and on its inner surface (endocervical canal) by the mucus secreting columnar epithelium (Figure 1.2) and associated crypts.

A B

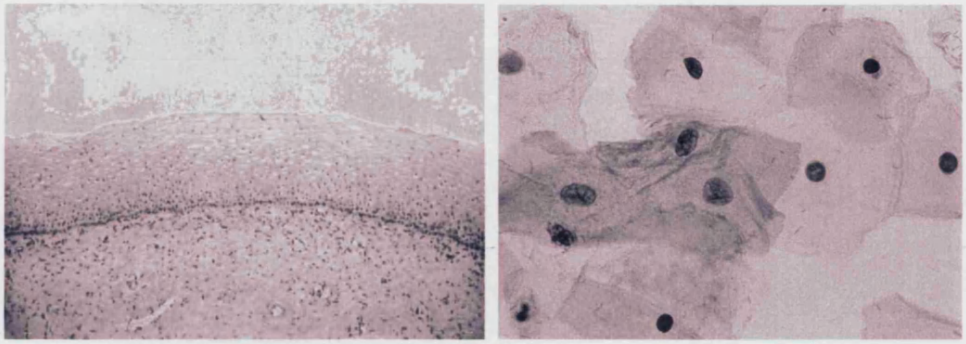

Figure 1.1: Normal ectocervical squamous epithelium, A: Histological section, shows stratified squamous epithelium. B: Pap smear shows superficial squamous epithelial cell and intermediate cells.

A B

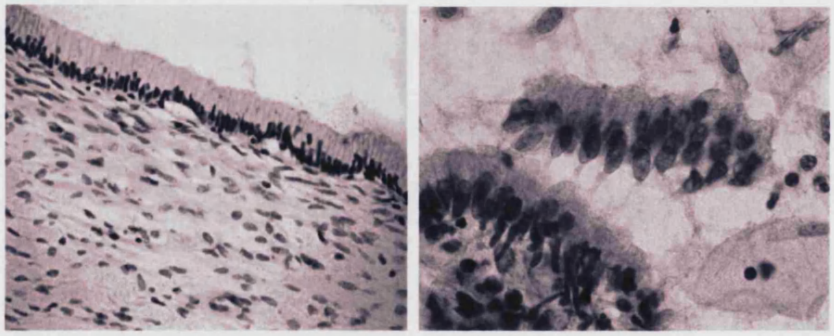

Figure 1.2: Normal endocervical columnar epithelium. A: Histological section shows normal endocervical columnar epithelium, B: Pap smear shows endocervical columnar epithelium.

The junction between the two different epithelia is known as the squamocolumnar junction (Figure 1.3).

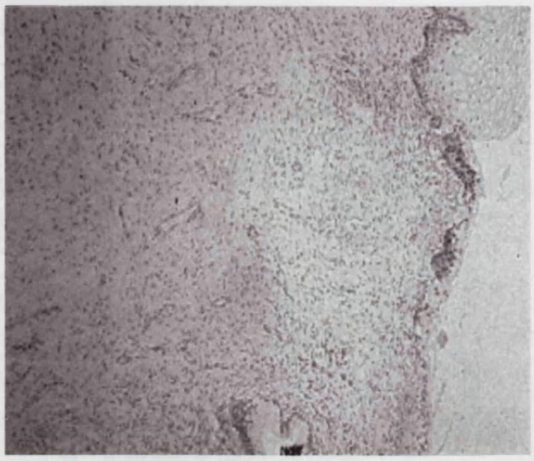

Figure 1.3: Histology section showing squamocolumnar junction, with stratified squamous epithelium and endocervical glands.

The location of the squamocolumnar junction varies throughout life. Before puberty, it is usually located within the endocervical canal. With the onset of the menstruation or at the time of the first pregnancy, increase in volume of the cervix leads to eversion of the columnar epithelium (ectopy), so that the squamocolumnar junction is located distal to the external os. In the second and third decade of life, the squamocolumnar junction is located near the external os, because the ectopic columnar epithelium undergoes metaplastic change to a non-keratinizing stratified squamous epithelium. The process of metaplastic change is patchy, occurring at several sites in the ectopic columnar epithelium at different times. As the menopause approaches, the cervix atrophies and the location of the squamocolumnar junction recedes into the endocervical canal.

The columnar epithelium of the external os is susceptible to metaplastic changes. This metaplastic change is a normal

physiological protective mechanism, where the fragile monolayer of the columnar epithelium is replaced by a protective multilayered squamous epithelium, upon exposure to the acidic pH of the vagina or chronic infections. During this metaplastic process, the epithelium seems to be particularly vulnerable to malignant transformation and the region showing metaplastic changes is commonly refereed to as the transformation zone (TZ).

The ectocervix (Figure 1.1) is covered by stratified squamous epithelium, which is composed of four distinct layers. Basal layer consists of a single row of cells and rests on a thin basement membrane. It is responsible for the continuous epithelial regeneration through active mitosis. Midzone is the dominant portion of the epithelium, the lower one third of the midzone known as parabasal layer, also contributes to the epithelial growth. Cells that are involved in ascending maturation occupy the upper portion of the midzone. These cells appear in the Pap smear as large cells and known as intermediate cells. Superficial zone contains mature cells, which appear in the Pap smear as large cells with large amount of cytoplasm and small, pyknotic nuclei, they are known as superficial squamous epithelial cells.

1.3.2 Pre-Neoplastic Lesions

Most cervical carcinomas are believed to derive from pre-neoplastic epithelial lesions known as CIN, in which the abnormal cells are confined to the epithelial layer. The intraepithelial neoplasm may be of squamous or columnar cell type. Early studies on mapping of CIN and the surrounding epithelium in cone biopsies showed that only 3.1% of CIN were found in the ectocervix, 9.7% were surrounded by endocervical epithelium and the remaining 87.2% occurred within the transformation zone (Abdul-Karim *et al*, 1982).

In Europe, CIN is classified using a three-tier (CIN1-3) (Arends *et al*, 1998) system classification. The division of CIN into different grades is purely on morphological grounds. The grading depends on the proportion of epithelial thickness occupied by dyskaryotic cells and the level of their pleomorphism and mitoses. The histological criteria used for diagnosis of different grades of CIN lesions are summarized below:

CIN1 (mild dysplasia): CIN is a full thickness change but proliferating cells and mitoses are confined to the lower one third of the epithelium (Figure 1.4).

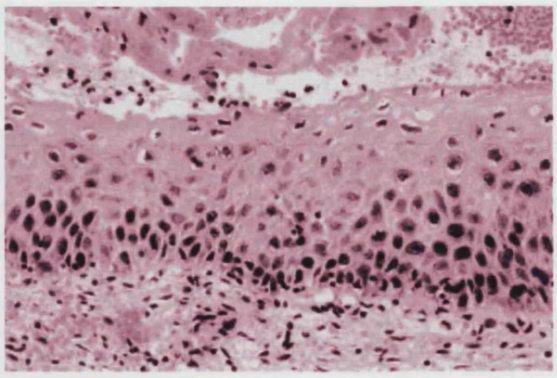

Figure 1.4: Histology of CIN1: Abnormal cells are restricted to the lower one third of the epithelium.

CIN2 (moderate dysplasia): CIN is a full thickness change but proliferating cells and mitoses are confined to the lower two-thirds and may be abnormal (Figure 1.5).

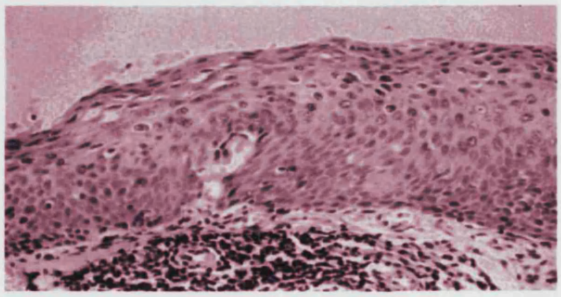

Figure 1.5: Histology of CIN2: Abnormal cells are restricted to the basal two thirds of the epithelium.

CIN3 (severe dysplasia and carcinoma in situ): Atypical cells transect the entire epithelium and proliferating cells are found in all three layers but do not invade the basement membrane (Figure 1.6). Nuclear crowding and pleomorphism are usually more

marked than those of CIN1 and CIN2. Mitoses are frequent, present in the upper one third of the epithelium and are often abnormal.

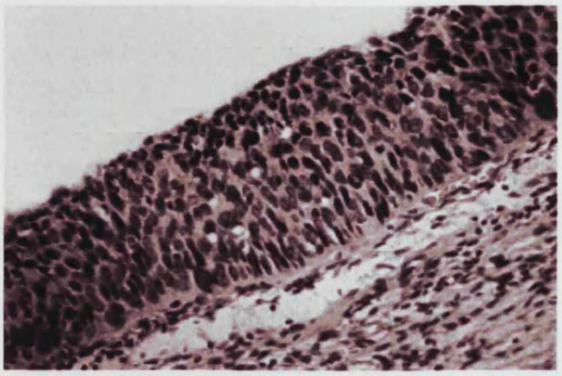

Figure 1.6: Histology of CIN3: Abnormal cells transect the entire epithelium.

Cytological grading of CIN lesions relies mainly on the cell morphology, assuming that the sample collected from the surface reflects the changes that occur throughout the epithelium. These abnormal cytological changes are termed " dyskaryosis". Dyskaryotic cells are classified into subgroups to enable cytopathologists to predict the underlying histological changes of the cervix. Three grades of dyskaryosis (mild, moderate and severe) are recognized according to the nucleo-cytoplasmic ratio. Mild dyskaryosis correlates to CIN1. Dyskaryotic cells show enlarged nuclei, which occupy less than half of the cell diameter, with irregular nuclear outline, abnormal chromatin pattern and hyperchromasia (Figure 1.7).

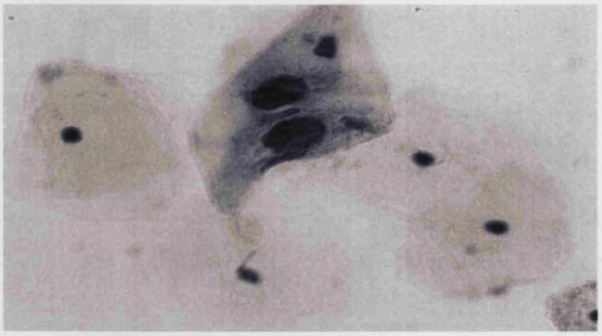

Figure 1.7: Cytology of mild dyskaryosis: Dyskaryotic cells show enlarged hyperchromatic nuclei, which occupy less than half of the cell diameter.

Moderate dyskaryosis correlates to CIN2. The nucleus of dyskaryotic cells occupies half to two-thirds of the cell diameter (Figure 1.8).

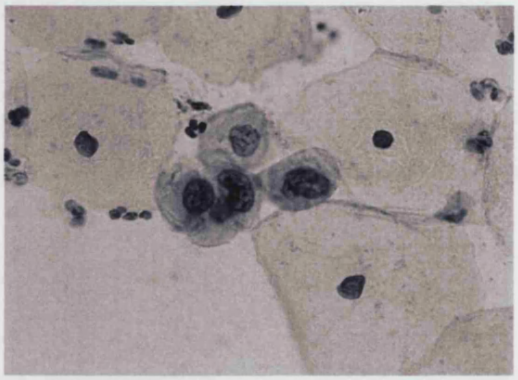

Figure 1.8: Cytology of moderate dyskaryosis: Dyskaryotic cells show enlarged hyperchromatic nuclei, which occupy half to two-thirds of the cell diameter.

Severe dyskaryosis correlates to CIN3. The nuclei of dyskaryotic cells occupy more than two-thirds of the cell diameter with only

a thin rim of cytoplasm remaining (high nucleo- cytoplasmic ratio) (Figure 1.9).

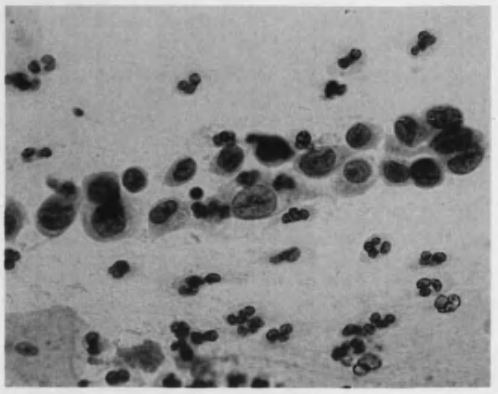

Figure 1.9: Cytology of severe dyskaryosis: Dyskaryotic cells show enlarged hyperchromatic nuclei, which occupy more than two-thirds of the cell diameter with only a thin rim of cytoplasm remaining.

In addition, a separate category describing changes lesser than CIN1 is called borderline changes. The term borderline is used when there is uncertainty if the nuclear changes are reactive or dysplastic, for example distinction between borderline nuclear changes and mild dyskaryosis when HPV infection is present.

1.3.3 Invasive Cervical Carcinoma

Most invasive carcinomas of the cervix develop when the dyskaryotic cells from an intraepithelial lesion invade the basement membrane as a consequence of uncontrolled cell division. Histologically, invasive cervical carcinomas are classified as squamous cell carcinoma, adenocarcinoma, adenosquamous carcinoma and undifferentiated carcinoma.

Among these different types, squamous cell carcinoma is the most common, accounting for 75% of all cases. As this thesis focuses on the squamous lesions, only the histology of squamous carcinoma is described below.

1.3.3.1 Histology Of Squamous Carcinoma

WHO classified cervical squamous cell carcinoma into two categories, keratinizing and non-keratinizing.

Keratinizing carcinomas: The tumours are composed of characteristic epidermoid cells, the most striking features of which are the circular whorls of cells with central nests of keratin (epithelial pearls). The nuclei are large and hyperchromatic with coarse chromatin. Mitotic figures are not frequent.

Non-keratinizing carcinomas: The tumour cells are generally recognizable as squamous by their polygonal shape and evidence of keratin formation and individual cell keratinization, but keratin pearls are not seen. Mitotic figures are usually numerous.

1.3.3.2 Histological Features That Suggest Invasive Carcinoma

Invasion of the basement membrane and beyond by neoplastic cells.

Pleomorphism: The morphology of carcinoma cells is usually different from that of CIN3 cells. They usually vary in size and shape and show giant cells with one or more nuclei. The nucleus

of a carcinoma cell is often larger and hyperchromatic, and usually contains one or more prominent nucleoli. Some malignant cells may contain large cytoplasmic vacuoles, which produce a "signet ring" appearance.

High mitotic activity: Invasive carcinoma cells usually have high mitotic activity and often show abnormal mitotic figures such as tripolar mitosis.

1.3.3.3 Staging Of Cervical Carcinoma

Cervical carcinoma is commonly staged according to the international Federation of Gynecology and Obstetrics (FIGO) system (Nguyen *et al*, 1999):

Stage I: Tumour is strictly confined to the cervix, and is further subdivided into:

Stage Ia: Preclinical carcinoma of the cervix, diagnosed only by histological examination of biopsies.

Stage Ia1: Lesions with minimal microscopic evidence of stromal invasion.

Stage Ia2: Microscopic lesions with the depth of invasion less than 5mm and the horizontal spread less than 7mm.

Stage Ib: Lesions greater than stage Ia2, whether seen clinically or not.

Stage II: Tumour extends beyond the cervix, but not yet involves the pelvic wall. The carcinoma may involve the vagina, but not the lower third.

Stage IIa: No obvious parametrial involvement.

Stage IIb: Obvious parametrial involvement.

Stage III: Tumour extends to the pelvic sidewall or the lower third of the vagina and all cases including those with hydronephrosis or nonfunctioning kidney.

Stage IIIa: No involvement of the pelvic wall.

Stage IIIb: Involvement of the pelvic wall, hydronephrosis or nonfunctioning kidney.

Stage IV: Tumour extends beyond the pelvis or clinically involves the mucosa of the bladder or rectum.

Stage IVa: Spread to adjacent organs.

Stage IVb: Spread to distant organs.

1.4 Detection Of CIN By Cytology

In 1928 George Papanicolaou, reported that tumour cells from women with cervical carcinoma could be detected in vaginal smears. The idea was not readily received until 1941 when Papanicolaou and Trout published their monograph, in which he and his colleague described the range of cells seen in vaginal smears. The potential of this technique as a screening method for cervical carcinoma and detecting it at its earliest and most treatable stage was realized and the cervical carcinoma screening programme was introduced in Europe and North America in 1960.

1.4.1 Specimen Collection And Preparation Of Cervical Smears

The most common tool for sampling the cervix is the cervical scrape, which is done by a specially designed wooden or plastic spatula.

The cervix is clearly visualized and the end of the spatula is inserted into the cervical os and rotated for 360°. The cervical mucus and cells adhering to the spatula are spread evenly across a glass slide. The slide is fixed immediately either by immersion in 95% ethanol for at least 30 min or by spray fixative (polyethylene coating fixative). The slides are then stained with Papanicolaou stain (Pap).

1.4.2 The NHS Cervical Screening Programme (NHSCSP)

The application of Papanicolaou smear test as a screening method for cervical carcinoma in the past decades has led to a dramatic decrease in both its incidence and mortality. The steady decline is largely attributed to early diagnosis and successful treatment of CIN lesions thus preventing them to develop into invasive carcinomas. Papanicolaou smear screening is capable of detecting precarcinomaous lesions as well as asymptomatic invasive squamous carcinomas, both of which can be treated effectively. The NHSCSP was introduced in 1988 in the UK (Cuzick *et al*, 1999 Herbert, 1997).

1.4.2.1 The Targeted Population

In England and Wales, women aged between 20 and 64 years are invited for cervical screening every 3-5 years. In Scotland the targeted age group is between 20 and 60 years, at 3 yearly intervals (Cuzick *et al*, 1999;Patnick, 2000). There are approximately 15 million women within these age groups in Britain (Patnick, 2000).

1.4.2.2 The Screening Interval

The effectiveness of the screening programme can be judged by coverage. This is the percentage of women in the target age group (25-64) who have been screened in a given five-year period. The coverage has improved from about 50% in the mid-1980s to 85% in 1998 and has stabilized at this level (Cuzick *et al*, 1999;Patnick, 2000).

1.4.2.3 Evidence That Cervical Screening Reduces Cervical Carcinoma

Both the incidence and mortality rate of cervical carcinoma in the UK have fallen since the introduction of the NHSCSP. The incidence fell from 16/100,000 in the mid-1980s to 10/100,000 in 1995, whilst the mortality rate dropped from 11.2/100,000 in the mid-1980s to 3.7/100,000 in 1997 (Patnick, 2000;Quinn *et al*, 1999).

1.4.2.4 Problems With The Cervical Smear Test

Although it has proved to be successful, cervical cytology screening is not without its problems. It is highly labour intensive and requires highly skilled personal (Baldwin *et al*, 2003). Some practical difficulties associated with conventional cytological screening are summarized below.

1) Sampling errors: A limited number of cells are collected which may not be representative of the lesion (lesions high up in the endocervical canal should be sampled by endocervical brush).

2) Errors related to smear preparation: Uneven spreading of the material on the slide, poor cellular preservation due to delay in fixation may lead to air-drying artifact or poor quality staining.

3) Errors related to interpretation: False negative and false positive results are the main concern. Excessive presence of inflammatory cells can obscure the abnormal cells. High workload may affect the performance of individual cytologists. The reported sensitivity of a single cervical smear test (the proportion of disease positives that are test positive) is low and shows a wide variation (30-87%), and the specificity of a single Pap test (the proportion of disease negatives that are test negative) might be as low as 86% (Baldwin *et al*, 2003). Sensitivity and specificity could be improved by reducing false positive and false negative results through improving the sampling quality, reducing the laboratory errors and elimination of interobserver variations.

1.4.2.5 Improvement Of Cervical Smear Test

To reduce the false positive and false negative results of conventional smears screening, several new technologies have been developed and some of them are already incorporated into clinical practice.

1.4.2.5.1 Monolayer (Liquid-Based) Cytology Preparations

The cells collected from the cervix are first put into a liquid transport medium and later plated out as a thin-layer of cells on glass slides in laboratories. There are a number of advantages for this approach.

1) A uniform thin-layer of approximately 80000 cells provides better morphology;

2) Smaller slide area to be screened: 17×20mm2 versus 24×50mm2 for conventional slides;

3) Suitable for automated screening: It is hoped that a high-speed camera can be used to scan about 200 conventional smears daily. The advantage of this method is that it is cost effective.

4) Excess blood and inflammatory cells are lysed;

5) Several slides can be generated from one sample, thus allowing for additional tests, such as immunocytochemistry and molecular investigation (Lin[a] *et al*, 2000).

1.4.2.5.2 HPV Typing

HPV typing is now increasingly used to assist the interpretation of a smear test. HPV infection is strongly associated with development of CIN. The HPV is positive in 80% CIN lesions but only in 11-20% of normal smears (Kjaer *et al*, 2002;Stanley, 2001). Moreover high risk HPV is associated significantly with high-grade CIN lesions and cervical carcinoma (Chan *et al*, 2002;Jastreboff *et al*, 2002;Lazo, 1999). Although HPV infection may be transient, persistent infection of high risk HPV has been shown to be closely associated with persistence and progression of CIN lesions (Nobbenhuis *et al*, 1999).

Conventional smear test together with HPV typing, detects 90-100% of high-grade CIN lesions (Rock *et al*, 2000). When liquid-based cytology is combined with HPV typing, 100% of high-grade CIN lesions are hoped to be detected (Rock *et al*, 2000).

1.4.2.5.3 Immunocytochemistry Of Cell Proliferation Markers

1) Proliferating cell nuclear antigen (PCNA) expression; PCNA functions as an auxiliary factor for DNA polymerases, and is involved in DNA repair and replication. However, PCNA expression in normal cervix is restricted to the basal layer. In CIN, PCNA is expressed at various levels of the epithelium (Demeter *et al*, 1994;Baldwin *et al*, 2003). PCNA expression generally correlates with the grade of CIN, mitosis and the copy number of HPV infection as well as the E6/E7 transcript level

(Dollard *et al*, 1992;Shurbaji *et al*, 1993;Baldwin *et al*, 2003;Demeter *et al*, 1994). Staining for PCNA can be affected by a variety of factors, including fixation time (Rowlands *et al*, 1991). The sensitivity and specificity of PCNA staining for detection of dyskaryotic cells have been shown to be low when applied to cervical smears (Williams *et al*, 1998).

2) Ki-67 expression; Ki-67 is a nuclear antigen, which is used as cell proliferation marker. Similar to PCNA, Ki-67 is expressed only in basal cells of normal cervix, but shows increased frequency and persistence of expression in CIN and squamous cell carcinoma (Konishi *et al*, 1991). Although some studies showed that Ki-67 expression in superficial epithelial layers positively correlates with the grade of CIN and presence of high risk HPV (Kruse[a] *et al*, 2001;Kruse[b] *et al*, 2001;Baisch, 1987;Verheijen *et al*, 1989), the sensitivity and specificity of Ki-67 staining for detection of dyskaryotic cells have been shown to be low, when applied to cervical smears (Williams *et al*, 1998).

3) Minichromosome maintenance proteins (Mcm) and Cdc6; The Mcm and Cdc6 proteins are members of the pre-replication complex that is essential for licensing DNA replication (Lei *et al*, 2001). Staining for Mcm and Cdc6 proteins have shown high sensitivity and specificity for detection of dyskaryotic cells in both cervical smears and biopsies (Freeman *et al*, 1999;Williams *et al*, 1998). In addition, the antibodies for Mcm and Cdc6 work

on both fresh and fixed specimens thus can be applied to both cytological and histological specimens.

Antibodies against Mcm and Cdc6 showed high percentage of immunostained cells in high grade CIN (78-100%) and in low grade CIN (8-70%). Whereas, antibodies against PCNA and Ki-67 showed low percentage (5-20%), (3-10%) in high and low grade CIN respectively (Williams *et al*, 1998).

1.5 Natural History Of Cervical Intraepithelial Neoplasia

Despite the apparent morphological homogeneity within each sub-group, CIN lesions show variable clinical behaviour. In reviewing the literature over the past 40 years, Ostor, Duggan et al, found that in absence of any treatment, 60% of CIN1 regressed, 30% persisted, whereas 10% progressed to a higher-grade lesion (Duggan *et al*, 1998;Ostor, 1993). Around 40% of CIN2 regressed, whereas 40% and 20% showed persistence or progression to a higher-grade lesion respectively (Duggan *et al*, 1998;Ostor, 1993). Similarly, 32% of CIN3 regressed, while 56% and 12% showed persistence or progression to carcinoma in situ or invasive carcinoma respectively (Duggan *et al*, 1998 ;Ostor, 1993).

The risk of progression from low to high grade CIN has been shown to be greater in patients with persistent high-risk HPV

infection. Follow up studies of women with and without cervical abnormalities have indicated that the persistent presence of high-risk HPV infection is necessary for the development, maintenance, and progression of CIN lesions (Koutsky *et al*, 1992;Nobbenhuis *et al*, 1999). Moreover women with a low grade lesion, who are negative for high-risk HPV DNA, are unlikely to develop CIN2/3 and their cytological finding is likely to return to normal (Nobbenhuis *et al*, 2001;Zielinski *et al*, 2001).

The malignant potential of high grade CIN is strongly supported by the study of McIndoe WA, et al from the National Women's Hospital in New Zealand (McIndoe *et al*, 1984). They followed up untreated women with persistent abnormal cytology following diagnosis of carcinoma in situ in 1970s, and showed that 22% of them developed invasive cervical carcinoma.

The epidemiological studies show that the mean age of patients with carcinoma in situ is 15.6 years younger than that of patients with invasive carcinoma (Cramer *et al*, 1974). The data suggest that on average, it probably takes more than 10 years for a CIN lesion to develop into an invasive carcinoma. Richart and Barron reported that 58 months were needed for a CIN1 lesion to develop into a carcinoma in situ, 38 months for CIN2, and 12 months for CIN3 (Barron *et al* 1970). They predicted that 66% of all dysplasia would progress to CIS within 10 years in the absence of treatment.

1.6 Management Of CIN Lesions

1.6.1 Management Of CIN1

There is no absolute international agreement on management of women with CIN1. In general, each case needs to be decided on an individual basis. Due to the high spontaneous regression rate and a long incubation period for CIN1 lesions to progress into a high-grade lesion (Duggan *et al*, 1998;Ostor, 1993), a repeat cervical smear in 6 months is usually recommended in the first instance. Colposcopy is recommended if the repeat smear continues showing abnormal cells (NHS ABC decument, 2000). If 2-3 consecutive smears show negative results within 12-18 months of the follow up, the women may be returned to a routine cervical screening. High risk HPV typing has been included in the management of CIN1. If a high-risk HPV infection persists, patients who are under 30 years of age should be followed by colposcopy, whereas those who are over 35 years should be treated. Patient may return to a routine screening if the follow-up smear and high-risk HPV are negative.

1.6.2 Management Of CIN2 And CIN3

There is no international agreement on treatment of CIN2 and CIN3 lesions, but the following guidelines are recommended. Patients with CIN2 or CIN3 lesions are recommended for colposcopic examination within 4 weeks of diagnosis and are

usually treated during colposcopic examination by "select and treat" or "see and treat" strategy. In the strategy of select and treat, biopsy will be taken for histology and high-risk HPV typing. If the biopsy shows high-grade lesion, lesion should be removed using large loop excision of the transformation zone (LLETZ). On the other hand if the biopsy shows a low-grade lesion, patient is recommended for colposcopic examination in 6 months. If high-risk HPV infection persists, the lesion should be treated by LLETZ. In see and treat strategy, the lesion is removed by LLETZ and sent for histological examination as well as for high-risk HPV typing. The patient should be followed up by colposcopy in 6/12 months.

1.7 Treatment

1.7.1 Treatment Of CIN

Several modalities of treatment are available for patients with CINs. The goal of these treatment modalities is local control and prevention of invasive lesions.

1.7.1.1 Large Loop Excision Of The Transformation Zone (LLETZ): It is a widely used procedure for diagnosis and treatment of CIN.

Procedure: During colposcopy, a wire loop electrode is placed into the cervix to a depth of 5 to 6mm under local anesthesia. From one lateral margin of the transformation zone, the loop is pulled across and out at the opposite side so that the entire transformation zone can be removed (Cirisano, 1999).

Advantage: It is a potentially curative procedure, which can be performed in an outpatient clinic. It also provides specimen for histological diagnosis (Chan *et al*, 1997).

Disadvantage: Bleeding during or post-operation has been reported in a range of 1% to 8%. Cervical stenosis occurs in approximately 1% of patients treated by LLETZ (Bigrigg *et al*, 1990). LLETZ has a 97% curative rate and is the most common method used for treatment of CIN (Dey *et al*, 2002).

1.7.1.2 Excisional Cone Biopsy: Several types of cone biopsies including broad deep cone, cylindrical cone and mini cone are used for treatment of CIN. Conization can be performed either with a surgical scalpel or with a laser. Laser cone biopsy has fewer complications than surgical (scalpel) cone biopsy, is far more precise and causes less distortion to the cervix.

Procedure: An incision is made into the mucous membrane of the ectocervix, which includes all the abnormal area.

Advantage: The method can be used as both diagnostic and treatment procedure, with approximately 96% success rate.

Disadvantage: This treatment method is associated with a high rate of complications including haemorrhage, infection, and

subsequent deformity or incompetence of the cervix, which has been reported in up to 12% of the patients (Luesley *et al*, 1985).

1.7.1.3 Electrocoagulation Diathermy

Procedure: Under general anesthesia, the cervix is dilated to expose the distal canal; an electrode is applied to the abnormal tissue, allowing thermal destruction.

Advantage: The method has 90% success rate in treatment of CIN lesions (Chanen, 1983;Benedet *et al*, 1987;Creasman *et al*, 1984).

Disadvantage: General anesthesia and its complications. Vaginal discharge or frank bleeding may happen 3-4 weeks following the procedure. Minor burn to the vulvar skin may occur. No histology is available.

1.7.1.4 Cryosurgery:

This technique can be performed as an outpatient procedure.

Procedure: A liquid refrigerant such as nitrous oxide is applied to the CIN lesion through cryoprobe. An ice ball will develop after a freeze period of several minutes. This can be repeated until the abnormal tissue is destroyed completely.

Advantage: The method is simple and efficient, with a 94% of success rate (Benedet *et al*, 1987;Creasman *et al*, 1984).

Disadvantage: Patients may feel faintness, mild abdominal cramps during the procedure. Watery vaginal discharge may occur 2 to 3 weeks following the operation (Paraskevaidis *et al*, 2001). No histology is available.

1.7.1.5 Carbon Dioxide Laser Ablation:

Procedure: The carbon dioxide laser beam is invisible and is usually guided by a second laser that emits visible light. The energy of the laser is absorbed by water, and the tissue is destroyed by vaporization. The laser is mounted to and directed by a colposcope.

Advantage: The method can be used for large lesions (Gunasekera *et al*, 1990). Success rates are ranged from 83% to 97% for CIN1-2, and 77% to 96% for CIN3.

Disadvantage: It is a painful procedure, and bleeding can be a problem as the depth of tissue destruction increases.

1.7.1.6 Hysterectomy:

Total hysterectomy may be performed for pre-invasive lesions of the cervix, if clinical circumstances are so negative.

Procedure: The surgical approach is based on patient selection and may be either total vaginal hysterectomy or abdominal hysterectomy. If ovarian removal is indicated, patients may be considered for laparoscopic assisted vaginal hysterectomy.

Advantage: This treatment procedure has the lowest rate of CIN recurrence.

Disadvantage: It is too radical. Fertility is lost and the patients still need to be followed up by cervical smears because recurrence does occur, although the rate is low.

Because of the concern over the possibility of missing invasive cervical carcinomas, excisional methods for CIN treatment allowing examination of tissue are now being widely used and replaced the ablative techniques. LLETZ is the most common method used, with its high success rate. (Bigrigg *et al*, 1994; Luesley *et al*, 1990).

Although CIN lesions can be effectively treated by the mentioned methods, 10-15% of cases show disease recurrence (Bistoletti *et al*, 1988;Elfgren *et al*, 1996). One of the major difficulties in the clinical management of patients with CIN lesions is to distinguish between those that will progress or persist from those that will be cured.

1.7.2 Treatment Of Invasive Cervical Carcinoma:

Invasive cervical carcinomas are treated by surgery, radio-or chemotherapy alone or by combination. The choice of these different treatment methods is influenced by many factors including women's age, health and extent of the disease. Treatment is governed by the staging of the disease. Patients with stage Ib disease are commonly treated with wertheim's

hysterectomy or radiotherapy with a 5-year survival in 70-90%. Radiotherapy gives similar results to surgery but the radiation side effect makes surgery the preferred option in the majority of cases (Donato, 1999). Stages IIa and IIb diseases are treated with radiotherapy with a 5-year survival rate in 70% of cases. Patients with stages IIIa and IIIb disease are treated with radiotherapy with a 5-year survival rate of 40%. Stages IVa and IVb disease are treated with radiotherapy with 5-year survival rate only 15%. Radiotherapy is given after surgery when there is lymph node spread or incompletely excision of the primary tumour. Chemotherapy prior to surgical treatment is used to cause tumour shrinkage, thus facilitating the surgical excision of the tumour. Chemo-radiotherapy (cisplatin-based) has been used in treatment of large cervical lesions where the dose of radiation needed to achieve high rates of tumour control exceeds the dose tolerated by normal tissues in the pelvis (Green *et al*, 2001;Rose, 2002).

1.8 Molecular Genetics

Despite the important role of HPV associated oncoprotein in the genesis of cervical carcinoma, there is strong evidence that HPV infection alone is insufficient to cause malignant transformation (Southern *et al*, 1998). Evidence is now emerging that genetic changes play a critical role in the transformation of HPV infected cells and may account for the irreversible clinical behaviour of

CIN lesions. These genetic changes include activation of cellular oncogenes and loss of tumour suppressor genes.

1.8.1 Oncogenes

1.8.1.1 HPV Associated Oncogenes

All HPV types studied so far encode 8 Open Reading Frames (ORFs), which are sequences coding for proteins synthesized "early" (E) or "late" (L) in viral replication (early and late refer to the time of viral protein expression after infection). The ORFs can be grouped according to their function.

E1 gene encodes a phosphoprotein that is the major viral DNA replication factor and is necessary for the initiation of viral DNA synthesis. E1 has intrinsic helicase, ATPase activities, and specific DNA binding ability (Lusky *et al*, 1991). The E1 protein, in association with E2, is essential for viral replication and the E1-E2 complex binds through E2 to specific sites in the upstream regulatory region implicated in viral replication and gene expression. E2 has the ability of transcription repression of E6/E7, and its expression may result in cell apoptosis (Desaintes *et al*, 1997). Integration of the HPV genome into the host chromosome in the carcinomas often results in the disruption of the viral E1 or E2 genes (Southern *et al*, 1998). The E2 gene is commonly disrupted as a result of viral integration, leading to loss of its function (Choo *et al*, 1987; Corden *et al*, 1999). Several

studies observed that the loss of E2 expression in cervical carcinoma cells is important in HPV-associated cervical carcinogenesis. A decrease in HPV-18 E6/E7 transcripts has been detected in Hela cells after the introduction of E2 into the cells (Dowhanick *et al*, 1995;Goodwin *et al*, 1998;Hwang *et al*, 1993). The introduction of E2 results in the growth arrest of the HPV-positive carcinoma cell lines but not the HPV-negative carcinoma cell lines (Dowhanick *et al*, 1995;Goodwin *et al*, 1998;Hwang *et al*, 1993).

E3 and E4 encode proteins, which can disrupt the host cytoplasmic keratin network, producing the classical halo effect in infected keratinocytes. The function of E3 and E4 is not yet fully understood (Dowhanick *et al*, 1995;Goodwin *et al*, 1998;Hwang *et al*, 1993).

E5: It stimulates cell growth by interaction with the epidermal growth factor receptor (Hwang *et al*, 1995;Zhang,B *et al*, 2002). E5 has been shown to have a transforming function in HPV types 1, 6 and 16 (Chen, *et al* 1990;Crusius *et al*, 1997). Moreover E5 has been shown to prevent apoptosis following DNA damage (Zhang,B *et al*, 2002). However, as HPV-infected lesions progress to cervical carcinoma, the episomal viral DNA frequently becomes integrated into host cell DNA, and a substantial part of genome, commonly including the E5 coding sequence is deleted (Schwarz *et al*, 1985).

E6 and E7 are the most important HPV associated oncogenes. E6 and E7 can immortalize human cells, for example transfection of human foreskin keratinocytes with high-risk HPV DNA, but not with low-risk HPV DNA (Barbosa *et al*, 1991), have been demonstrated to immortalize these cells (Hawley-Nelson *et al*, 1989;Woodworth *et al*, 1989). Persistent expression of E6 and E7 appears necessary to maintain the transformed phenotype. The application of synthetic anti E6 and E7 oligonucleotides to cervical carcinoma cell lines containing HPV-18 significantly inhibits cell growth (Steele *et al*, 1993). The E6 oncoprotein binds to the p53 tumour suppressor protein and mediates its degredation (Werness *et al*, 1990). The interaction of high risk HPV/E6 with p53 is not direct but is mediated by a cellular protein called the E6-associated protein (E6AP), which is an ubiquitin protein ligase (Hubbert *et al*, 1992;Scheffner *et al*, 1993;Talis *et al*, 1998). In addition, E6 induces telomerase activity via transcriptional activation of telomerase reverse transcriptase (hTERT) expression (Klingelhutz *et al*, 1996).

E7 binds the retinoblastoma gene product (Rb) and causes its inactivation which leads to cell proliferation through the activation of genes under the control of the E2F transcription factor (Stanley, 2001). When E2F protein complexes with Rb, Rb acts as a transcriptional repressor and suppresses E2F transcription activity (Stanley, 2001). E7 binds to Rb, thus

freeing E2F and permitting its transcriptional activity. In the normal life cycle of HPV, the binding of E7 to Rb is apparently essential for the activation of the cell cycle DNA replication machinery in differentiated keratinocytes that had otherwise exited the cell cycle. HPV depends on the host cell DNA polymerases and replication machinery for the replication of the viral genome. Because this machinery is only expressed in the S phase of the cell cycle, these viruses must stimulate cellular proliferation and drive the cell into S phase through binding of E7 to Rb to free the E2F family of transcription factors.

The integration of viral DNA into the host genome does not occur at specific chromosomal sites. However, it has been found that HPV DNA may be integrated in the vicinity of known oncogene or tumour suppresser gene (Sonnex, 1998). For example, in the HeLa cell line, integration of the HPV-18 genome is within approximately 50 kilobases of the c-myc oncogene on human chromosome 8 (Durst *et al*, 1987), which may lead to increased c-myc expression seen in a proportion of cervical carcinoma. HPV16 integrations in cervical carcinomas preferentially target common fragile sites including FRA3B (3p14.2) where the fragile histidine triad (FHIT) gene, a putative tumour suppresser gene, is located, and are accompanied by deletion of cellular genes (Wilke *et al*, 1996;Thorland *et al*, 2003). Such an integration event may lead to oncogene activation or tumour suppresser gene

inactivation, thus contributing to development of cervical carcinoma.

1.8.1.2 Ras, c-myc

The Ras family is a class of molecules (G proteins), which are involved in transmission of growth stimulatory signals from the cell surface membrane to the nucleus. The gene contains four exons, which code for a 21-Kd protein that localizes to the inner cell membrane (McGrath *et al*, 1984). Ras gene activation is often caused by gene mutation or gene amplification. Several studies have investigated H-ras and K-ras gene mutation in cervical carcinoma since they are frequently mutated in other carcinomas. Collectively, these studies show that 14-35% of cervical carcinomas harbour mutation in one of the H-ras hot-spots (codons 12,13 and 61) with the majority occurring at codon 12 (Lee *et al*, 1996;Wong *et al*, 1995). Mutations in these hot-spots are known to constitutively activate ras gene activity (Barbacid, 1987). Alonio et al reported Ha-ras mutation in 20% of CIN3 lesions and 41% invasive carcinomas and showed that the mutation was associated with disease progression in 61% of cases (Alonio *et al*, 2003). In line with this finding, cervical carcinoma which over-express ras p21 protein has been shown to be associated with a poor prognosis (Sagae *et al*, 1990). Whether ras gene mutation is an early event during cervical carcinoma development remains unclear as conflicting data on ras gene

mutation in CINs have been reported (Grendys *et al*, 1997;Lee *et al*, 1996). Several studies found no ras mutations or amplifications in CIN and invasive carcinoma (Iwasaka *et al*, 1992;Van Le *et al*, 1993;Willis *et al*, 1993).

The c-myc gene encodes a protein product predominantly located in the cell nucleus and its constitutive expression generally enforces unscheduled proliferation, prevents differentiation or sensitizes cells to apoptosis (Donzelli *et al*, 1999;Facchini *et al*, 1998;Galaktionov *et al*, 1996;Koskinen *et al*, 1993). Several studies have shown that c-myc gene over-expression is common and may correlate with the clinical behaviour of cervical carcinoma. Iwasaka et al reported an over-expression of c-myc gene as shown by immunohistochemistry in 44% of invasive cervical carcinomas and an association of c-myc over-expression with poor prognosis (Iwasaka *et al*, 1992). Riou et al reported that patients whose tumour showed c-myc over-expression had an eight-fold greater incidence of early relapse than those without c-myc over expression (Riou *et al*, 1987). C-myc gene amplification has been found in 42% of high-grade CIN lesions (Golijow *et al*, 2001). In some studies, the gene amplification correlated with poor prognosis in early stage cases (Bourhis *et al*, 1990). As discussed previously, integration of the HPV genome frequently occurs near the c-myc gene locus. This may deregulate c-myc transcriptional regulation and account for the cervical

carcinomas that show c-myc protein over-expression but lack evidence of the gene amplification (Couturier *et al*, 1991).

1.8.2 Tumour Suppressor Genes

1.8.2.1 The Retinoblastoma Gene

The retinoblastoma gene is located on chromosome 13q14 and encodes a nuclear phosphoprotein (Rb). Rb is one of the major components that regulate the G1/S transition and its checkpoints. It functions by inhibiting the transcriptional activity of E2F, which controls the expression of genes necessary for progression into the S phase of the cell cycle. The activity of the Rb is mediated by its phosphorylation state. Rb is only able to complex with E2F and blocks its transcriptional activity when it is in a hypophosphorylated state. When cells are stimulated to proliferate, Rb becomes phosphorylated by the cyclin D1/CDK4 (cyclin dependent kinase) complex, which controls the transition from G1 to S phase. This is normally inhibited by cyclin-dependent kinase inhibitors (CDKI) such as p15 (WAF1), p16 (INK4A), p21 and p27 (KIP1) proteins (Arends *et al*, 1998;zur Hausen, 2002;Baldwin *et al*, 2003). Hyperphosphorylated Rb dissociates from E2F, permitting its transcriptional activity.

Released E2F transactivates a set of genes necessary for progression into the S phase of the cell cycle. These genes include cyclin E, cyclin A, Cdk2, and E2F-1 (one of the E2F

family) (Arends *et al*, 1998;zur Hausen, 2002;Baldwin *et al*, 2003). Interestingly, E2F-1 also induces the expression of p19ARF, which results in the stabilization of p53 (Hickman *et al*, 2002). The transcriptional activation of E2F-1, as well as cyclin E, constitutes a feed-forward mechanism: the additional cyclinE/Cdk2 activity ensures the full phosphorylation of Rb so that more E2F molecules are in the free active form, which in turn results in an enhanced E2F1 and cyclin E transcription (Sano *et al*, 2002;zur Hausen, 2002; Murphy *et al*, 2003).

1.8.2.2 p53 Gene

The p53 gene, located on chromosome 17p13.1, encodes for a transcriptional factor which plays a critical role in maintaining the integrity of genome, guarding against genome instability and oncogene expression, by inducing both cell cycle arrest and apoptosis (Hickman *et al*, 2002). Activation of p53 can be triggered by both DNA damage agents such as ultraviolet or γ radiation, carcinogens, chemotherapeutic drugs and non-DNA damaging conditions, including hypoxia and disruption of cell adhesion (Hickman *et al*, 2002). p53 activation can also be triggered by deregulated expression of certain oncogenes such as p14/19ARF (Hickman *et al*, 2002). These various signals activate p53 through several pathways. Once activated, p53 transactivates a set of genes to induce cell cycle arrest or apoptosis (Hickman *et al*, 2002). The gene involved in cell cycle arrest is mainly

p21WAF1, which binds and inhibits CDK particularly CDK2 activity (Harper *et al*, 1993). The genes involved in p53 mediated apoptosis include BAX, NOXA, p53AIP, APF1, Puma and Fas (Miyashita *et al*, 1994;Owen-Schaub *et al*, 1995;Miyashita *et al*, 1995;Moroni *et al*, 2001;Oda[a] *et al*, 2000;Oda[b] *et al*, 2000;Nakano *et al*, 2001).

In normal cells, the level of p53 expression is relatively low and is regulated by its regulator, MDM2. MDM2 binds to p53 and acts as an ubiquitin ligase, thus leads to p53 degradation (Skomedal *et al*, 1999). Dissociation of p53 from MDM2 can be induced by phosphorylation of p53 and/or MDM2 on several key residues by stress or DNA damage activated kinases (Prives, 1998). In addition, MDM2 can be sequestrated by p14ARF (Michael *et al*, 2002).

Inactivation of p53 function could result in inappropriate replication of damaged DNA, inappropriate cell survival after cellular stress or abnormal segregation of chromosomes during mitosis, thus contributing to malignant transformation. Loss of p53 function is the most common genetic defect found in human carcinoma, and is commonly caused by mutation in one allele and loss of the other, or increased degradation by both cellular and viral products. However, P53 mutations are very rare in cervical carcinoma (Scheffner *et al*, 1993;Talis *et al*, 1998).

In cervical carcinoma, the E6 oncoprotein, as described in section 1.8.1.1, binds to the p53 tumour suppressor protein and mediates its degeneration (Scheffner *et al*, 1993;Talis *et al*, 1998).

1.8.3 Chromosomal Loss In CIN And Cervical Carcinoma

There is no doubt that HPV infection plays a critical role in the genesis of cervical carcinoma. However, HPV infection alone is insufficient to cause malignant transformation. The evidence in support of this notion includes: only a small percentage of HPV positive women develop cervical carcinoma (Rome *et al*, 1987;Syrjanen, 1987); and a long latency (up to 17 years) between HPV infection and emergence of CIN (Meijer *et al*, 2000).

Growing evidence indicates that genetic changes play a critical role in the transformation of HPV infected cells. Larson et al have shown a strong association between the number of chromosomal regions showing loss of heterozygosity (LOH) and the grade of CIN lesions, which supports the concept of progressive accumulation of abnormalities affecting various tumour suppressor genes (TSG) during development of cervical carcinoma (Larson[a] *et al*, 1997). A number of chromosomal regions have shown LOH in cervical carcinoma. They include 3p14.1-p22, 4p16, 4q21-35, 5p13-15, 6p21-22, 6q21-25, 11p15, 11q23, 13q12.3-q13, 17p13.3 and 18q12.2-22 (Mitra *et al*,

1994;Mullokandov *et al*, 1996). Of these regions, 3p14.2, 3p22-21, 6p21 and 11q23 have shown frequent LOH in cervical carcinoma by a number of independent studies, suggesting that these regions may contain TSGs (Kersemaekers *et al*, 1999; Chung *et al*, 2000;Guo[a] *et al* , 2000Chatterjee *et al*, 2001;;Harima *et al*, 2001; *et al*; Acevedo *et al*, 2002). Among them, a number of chromosomal regions at 3p (3p14.1, 3p21-22) are the most frequently deleted in CIN and cervical carcinoma although there are discrepancies in the frequency and minimum region of deletion observed (Guo[a] *et al*, 2000). The frequency of LOH at 3p14.2 has been variously reported in different grades of CIN lesions (30%-58%) and in cervical carcinoma (37-73%) (Kohno *et al*, 1993; Rader *et al*, 1998;Yoshino *et al*, 1998;Butler *et al*, 2000;Lin[b] *et al*, 2000;Butler *et al*, 2002). LOH at 3p14.2 occurs more frequently in invasive and high-grade CIN lesions than CIN1 (Butler *et al*, 2002). Guo et al pointed out that chromosome 3p deletions were observed more frequently in CIN with coexisting carcinoma than in CIN without coexisting carcinoma (Guo[a] *et al*, 1998). A close association between HPV infection and LOH on chromosome 3p has been reported (Muller *et al*, 1998;Yoshino *et al*, 1998; Butler *et al*, 2000). In vivo study shows that HPV can insert its genes into chromosome 3 fragile site FRA3B located at 3p14.2, deleting a piece of DNA including the Fragile Histidine Triad gene (FHIT) (Thorland *et al*, 2003;Wilke *et al*, 1996).

FHIT gene is a candidate TSG (Ohta *et al*, 1996). Immunohistochemistry studies have shown that FHIT protein expression is reduced or absent in 50% of CIN3, and 78% of cervical carcinomas (Butler *et al*, 2002). RT-PCR studies have shown absent FHIT transcripts in cervical carcinoma cell lines (Greenspan *et al*, 1997). Moreover, LOH correlated with reduced FHIT expression and both LOH and reduced FHIT expression positively correlated with the histological grade of CIN (Birrer *et al*, 1999;Connolly *et al*, 2000;Butler *et al*, 2002). FHIT LOH has also been reported to be significantly higher in recurrent CIN lesions (Wilke *et al*, 1996; Muller *et al*, 1998; Yoshino *et al*, 1998; Lin[b] *et al*, 2000;Butler *et al*, 2000;Thorland *et al*, 2003).

Frequency of LOH at 3p21-22 has been reported to be between 56%-75% in cervical carcinoma (Karlsen *et al*, 1994;Huettner *et al*, 1998). LOH at 3p21 positively correlates with mitotic activity of tumour cells (Kersemaekers[a] *et al*, 1998). Putative TSG, β catenin is mapped to 3p21 and The Transforming Growth Factor-β Receptor (TβR) type II is mapped to 3p22. Both have been investigated and so far there is no convincing data to show that they are the deletion targets in these regions (Lazo, 1999). However, increasing evidence suggests that gene deletion in these regions is important in the genesis and progression of cervical

carcinoma (Wistuba *et al*, 1997;Guo[a] *et al*, 2000;Chatterjee *et al*, 2001).

A role of chromosome 4 in the development of cervical carcinoma has been suggested through the ability of the whole chromosome to revert immortal HeLa cells to a senescent phenotype (Ning *et al*, 1991). Comparative genomic hybridization (CGH) and LOH studies have revealed deletions at specific regions on chromosome 4. Four minimal regions of deletions have been identified and they include 4q34-q35 (LOH frequencies of 76%), 4q25-q26 (48%), 4p15.1-p15.3 (36%) and 4p16 (26%) (Sherwood *et al*, 2000). Different patterns of LOH have been shown in different histological variants. LOH at 4q34-q35 predominantly occurred in squamous cell carcinoma (83%), while LOH at 4p15.3 frequently occurred in adenocarcinoma (50%) of the cervix (Sherwood *et al*, 2000).

High frequencies of LOH have also been observed in the short arm of chromosome 5. Mitra et al showed frequent LOH at 5p15.1-15.3 in both CIN (60%) and cervical carcinoma (50%) (Mitra *et al*, 1995;Mitra, 1999). Deletion at these regions appeared to be independent of high risk HPV infection (Mitra, 1999). Candidate TSG programmed cell death gene (PDCD6), and thyroid receptor interacting protein 13 (TRIP13) have been identified at 5p15.3. However, studies of their expression did not

provide evidence of down-regulation in cervical carcinoma cell lines (Arias-Pulido *et al*, 2002).

Several regions of chromosome 6 have been shown LOH (Chuaqui *et al*, 2001). 6q21-q25.1 have been shown LOH in 30% of CIN1, 37% of CIN2, 40% of CIN3, and 55% of cervical carcinoma (Chuaqui *et al*, 2001).

On the short arm of chromosome 6, 6p23-25 and 6p21.3 have been shown high frequencies of LOH in cervical carcinoma and CIN lesions.

6p25 and 6p21.3 was found in 74% and 87% of cervical carcinomas respectively, 91.7% of CIN2 and CIN3, and 50% of CIN1 lesions (Chatterjee *et al*, 2001). Deletion at 6p21 was significantly associated with poor overall and disease-free survival in patients with cervical carcinoma (Harima *et al*, 2001), and also associated with disease recurrence after radiotherapy (Harima *et al*, 2000).

The minimum region deleted at 6p25 contains several putative TSGs including TUBB, P16, ELANH2, FKHL6, and FKHL7 (Chatterjee *et al*, 2001). 6p21.3 is also highly gene rich and harbors at least 64 genes, including a cluster of 12 histone family genes, 5 butyrophilin family genes, and HLA class I genes (HLA-A, HLA-C, HLA-E). Mutation analysis of some of these genes (IER3) did not reveal any pathogenic alterations (Chatterjee *et al*,

2001). Although the role of these genes in cervical carcinogenesis remains unknown, the HLA class I genes have been proposed as targets of 6p21.3 deletions in cervical carcinoma. Loss of HLA class I antigen expression is a common phenomenon in most cervical carcinomas (Keating *et al*, 1995), and the loss of HLA-A2 and B7 expression is associated with poor prognosis of cervical carcinoma (van Driel *et al*, 1996).

On chromosome 9, LOH has been found at 9pter-p22 in 14% of cervical carcinomas (Huettner *et al*, 1998), and at 9pter-p13 in 67.5% of cervical carcinomas (Manolaraki *et al*, 2002). Dellas et al showed that LOH at these regions was significantly more frequent in carcinomas with lymph node metastases than in node-negative tumours (Dellas *et al*, 1999).

Functional studies have shown that chromosome 11 carries a gene or genes that can suppress the tumourigenic properties of human cervical carcinoma cell lines (Srivatsan *et al*, 2002;Saxon *et al*, 1986). LOH has been found at several regions of chromosome 11 including 11p12-13, 11p15, 11q13, and 11q22-23. The observed frequencies of LOH at these regions varied between studies, ranging from 14% to 33% at 11p15 and from 27% to 60% at 11q23.1-q23.2 in cervical carcinoma (Pulido *et al*, 2000;Steenbergen *et al*, 1996).

A candidate TSG (PPP2R1B), which maps to 11q22-23, has been found to be mutated in 15% of lung and colon carcinoma. However, mutational analysis of the PPP2R1B gene in cervical carcinoma did not reveal any evidence of somatic mutation (Steenbergen *et al*, 1996; Pulido *et al*, 2000). Huettner et al have shown a correlation between LOH at 11q23.3 and the extent of lymphovascular space invasion in radical hysterectomy specimens (Huettner *et al*, 1998). A significant association between tumours with LOH at 11q21.2, and poor survival has also been reported (Kersemaekers[a] *et al*, 1998;Harima *et al*, 2001).

Although a number of studies have investigated LOH in cervical carcinoma and cervical intraepithelial neoplasia, there are relatively few data relating LOH to clinical behaviour or prognosis (Table1.1), particularly of the CIN lesions.

Table 1.1: Common LOH in CIN and cervical carcinoma.

Chromosomal Location	LOH Frequency in CIN	LOH Frequency in cervical carcinoma	Correlation with prognosis	Reference
3p13-14.3	-	*75%*	-	*Jones&Nakamura, 1992*
3p13-21.3	-	*45%*	-	*Kohno et al, 1993*
3p14.1-12	*30%*	*37%*	-	*Rader et al, 1998*
3p14.2	-	*56%*	-	*Wistuba et al, 1997*
3p14.2	-	*59%*	-	*Yoshino et al, 1998*
3p14.2	*52%*	*73%*	-	*Butler et al, 2002*
3p14.2	-	*44%*	-	*Yoshino et al, 2000*
3p14.2	*58%*	-	*High rate in recurrent CIN*	*Lin, M.W, et al, 2000*
3p21	-	*57%*	-	*Wistuba et al, 1997*
3p24.2-22	*36%*	*80%*	-	*Guo et al, 2000*
5p15.1-15.2	*60%*	*48%*	*Associated with progression*	*Mitra,A.B.etal, 1995*
6p21.2	-	*46.80%*	*Associated with recurrence after radiotherapy*	*Harima.A,etal, 2001*
6p23	*21%*	*33%*	-	*Rader et al, 1998*

1.8.4 Chromosomal gain in CIN and cervical carcinoma

The common chromosomal gains in CIN and cervical carcinoma are summarized in Table 1.2. CGH studies have shown that the most frequent and consistent finding in cervical carcinoma and CIN is 3q amplification (Heselmeyer *et al*, 1996). It is believed that 3q amplification plays an important role in the transition from severe dysplasia (CIN3) to invasive carcinoma (Heselmeyer *et al*, 1996). Amplification at 3q has also been observed frequently in other solid tumours including carcinoma of the ovary, lung, head and neck, suggesting the presence of an oncogene important for multiple carcinoma types (Speicher *et al*, 1995;Arnold *et al*, 1996;Brass *et al*, 1996; Kirchhoff *et al*, 1999). Using Southern blot and fluorescence in situ hybridization (FISH), the region targeted for amplification has been mapped within band 3q26.3 (Sugita *et al*, 2000). P13 kinase/AKT (PIK3CA) and hTR located in the centre of the amplification domain at 3q26.3 (Soder *et al*, 1997).

PIK3CA encodes the p110α catalytic subunit of phosphatidylinositol 3-kinase, which plays an important role in regulating cell growth and apoptosis. The gene is frequently amplified in cervical carcinoma and this correlates positively with 3q26.3 amplification (Shayesteh *et al*, 1999; Ma *et al*, 2000). In cervical carcinoma cell lines, PIK3CA amplification is associated with increased protein expression and P13-kinase activity (Ma *et*

al, 2000). Furthermore, treatment with a P13-kinase inhibitor significantly suppresses cell proliferation and promotes apoptosis (Ma *et al*, 2000). These observations suggest that PIK3CA amplification may be one of the genes targeted by 3q26.3 amplification.

Chromosomal gain has also been detected on 1q as 22% and 42% in CIN3 and cervical carcinoma respectively, where is on 1p as 22% and 33% (Umayahara *et al*, 2002).

Table 1.2: Common chromosomal gains in CIN and cervical carcinoma.

Chromosomal Location	Gain Frequency in CIN	Gain Frequency in cervical carcinoma	Correlation with prognosis	Reference
3q	*61%*	67%	-	*Umayahara, 2002*
3q	*35%*	*72%*	-	*Kirchhoff, 1999*
3q	-	*77%*	*Carcinoma progression*	*Heselmeyer, 1996*
1q	-	*47%*	*Carcinoma progression*	*Heselmeyer, 1996)*
1q	-	*45%*	-	*Kirchhoff, 1999*
1q	*22%*	*42%*	-	*Umayahara, 2002*
1P	*22%*	33%	-	*Umayahara, 2002*

1.8.5 Aneuploidy

Aneuploidy is commonly associated with human malignancies and certain premalignant conditions. It occurs in most if not all invasive cervical carcinomas. Aneuploidy also occurs in CIN lesions and its frequency correlates with the CIN grade, being found in 14% of CIN1 but 55% and 94% of CIN2 and CIN3 respectively (Monsonego *et al*, 1997). Furthermore, aneuploidy appears to be associated with progression and poor prognosis of CIN lesions (Fu *et al*, 1981;Dudzinski *et al*, 1987). However, studies by Hanselaar et al and Atkin et al showed that diploidy in CIN lesion did not necessarily indicate good prognosis (Hanselaar *et al*, 1988;Atkin *et al*, 2004). Thus, it remains to be investigated whether DNA ploidy status can be used for CIN prognosis.

1.8.6 Telomerase

Telomere is a special DNA sequence element at both ends of a linear chromosome, which is composed of several hundred repeats of a simple hexanucleotide sequence (TTAGGG) extending up to 30 kb. It plays an important role in the structural integrity and function of chromosomes by protecting them from end-to-end fusions, rearrangements, degradation, and chromosomal loss (Rhyu, 1995;Kyo *et al*, 1997; Wisman *et al*, 2001;Reesink-Peters *et al*, 2003). Telomere also acts as a buffer zone, which allows loss of terminal DNA sequence but not disruption of vital protein coding sequence during chromosome replication. Replication

related shortening of telomeric DNA to a critical length signals cells to stop division and to enter a senescent state (Counter *et al*, 1992;Gorham *et al*, 1997). As a result of this "end-replication" problem, somatic cells have a limited proliferative capacity. Maintenance of telomere length is therefore considered to be necessary for continued cell division (Counter *et al*, 1992;Counter *et al*, 1994).

Telomere length is maintained by telomerase, which is a specialized RNA-protein complex containing RNA template (hTR) and protein components, the telomerase associated protein (TP1) and the catalytic subunit (hTERT) (Meyerson *et al*, 1997;Nakamura *et al*, 1997). Telomerase directs the synthesis of telomeric repeats at chromosomal ends, so its activation is essential for halting telomere shortening and attaining cellular immortality (Counter *et al*, 1992;Counter *et al*, 1994). Telomerase expression is low or absent in most human somatic tissues, with its expression principally restricted in the adult to activated lymphocytes, germ cells, and tissue stem cells (Broccoli *et al*, 1995;Hiyama *et al*, 1995).

Similar to other somatic tissues, normal cervix is negative for telomerase activity as shown by PCR-based telomerase repeat amplification assay (TRAP) (Kawai *et al*, 1998). However, most cervical carcinomas are positive for telomerase activity (Anderson, 1997;Pao *et al*, 1997;Wisman *et al*, 1998). In line

with this, amplification or increased copy number of the hTR and hTERT gene in a subpopulation of tumour cells has been detected in all cervical cancers (Soder *et al*, 1997). Moreover, hTERT gene amplification is associated with enhanced hTERT protein expression (Zhang,A *et al*, 2002). Although the hTR and TPI (telomerase associated protein) are commonly expressed in the normal cervix (71% to 100%) as well as in cervical carcinomas (43% to 100%) (Nakano *et al*, 1998;Snijders *et al*, 1998;Takakura *et al*, 1998;Yashima *et al*, 1998), hTERT mRNA is expressed in 33% of normal cervices but in 80-100% cervical carcinomas (Nakano *et al*, 1998;Snijders *et al*, 1998;Takakura *et al*, 1998). Telomerase activity is detected in 6% of normal cases, 31% of CIN1, 80% of CIN2, 71% of CIN3, and 88% of cervical carcinoma (Kawai *et al*, 1998). Taken together, telomerase activation may play an important role in the development of cervical carcinoma.

Although some studies suggested that telomerase assay or detection of hTERT mRNA might be a useful screening method for detection of neoplastic cells in cervical lesions (Kyo *et al*, 1997;Takakura *et al*, 1998), others showed that detection of telomerase or its components in CIN lesions is neither of diagnostic nor prognostic value (Rhyu, 1995;Kyo *et al*, 1997; Wisman *et al*, 2001;Reesink-Peters *et al*, 2003).

The aim of this thesis is to isolate and characterize prognostic markers that distinguish between CIN lesions that will progress to higher grade and those that are eradicated after treatment. Additionally, the prognostic markers would then be clinically evaluated by correlating them to various clinical parameters such as follow up time, HPV status and treatment.

Chapter 2

Materials And General Methods

2.1 Materials

2.1.1 Reagents

2.1.1.1 Chemicals

Sources of all reagents and suppliers used in this thesis are listed below in Table 2.1.

Table 2.1: Sources of the chemical reagents used in this thesis.

Chemicals	Source
Agarose	Sigma
Ammonium persulphate	Sigma
Boric acid	Sigma
Bromophenol blue	Sigma
Chloroform	Merck
deoxyribonucleoside 5 -triphosphate (dNTP)	Promega
Ethylenediamine tetra-acetic acid (EDTA)	Sigma
Ethanol	AnalaR
Ethidium bromide	Sigma

Chemicals	Source
PCR primers	Genosys or Thermo Hybaid
Isopropanol	Merck
Fluorescently labeled PCR primers	Applied Biosystems
Magnesium chloride	Life Technologies
Mineral oil	Sigma
PCR buffer	Life Technologies
Phenol	Sigma
PhiX174 DNA/Hinf I markers	Promega
Polyacrylamide	Promega
Proteinase k	Promega
Rox size standard	Applied Biosystems Inc.
N,N,N´,N´-tetramethyl-ethylenediamine (TEMED)	Sigma
Tris (Trizma base)	Sigma
Water (HPLC pure)	Sigma
Xylene	Merck

2.1.1.2 Enzymes And Assay Kits

Table 2.2: Sources of biological reagents and assay kits.

Chemicals	Source
Ambion Kit	AMS Biotechnology
HpaII Restriction endonuclease	Promega
Protein Precipitation Solution	Promega
RNA Storage Solution	Ambion Ltd
RT-PCR Kit	QIAGEN
Silver staining kit	Pharmacia-Amersham
Taq polymerase	Life Technologies

2.1.2 Buffers And Solutions

Proteinase K digestion buffer: 10 mM Tris (pH 9), 50 mM KCl, 0.1% Triton X, 100μg/mL proteinase K.

Electrophoresis loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 6×TBE, 40% sucrose.

10× TBE buffer (pH 8.*0*): 89mM Tris base, 89mM boric acid, 2mM EDTA, dissolved in 1 litre of distilled water.

10× PCR buffer: 100 mM Tris HCl (pH 9), 500 mM KCI, Triton-X 100.

10% Ammonium Persulphate (APS): 0.1g of APS powder dissolved in 1ml distilled water.

Blue Dye mix: 1 part of (100mg/ml blue dextran, 25mM EDTA) dissolved in 12 parts of deionized formamide.

2.1.3 Patient Materials

130 archival cervical Pap smears (18 CIN1, 56 CIN2, 56 CIN3), 137 archival formalin-fixed, paraffin-embedded cervical biopsies (56 CIN1, 43 CIN2, 23 CIN3 and 15 cervical squamous cell carcinoma) and 2 male tonsil biopsies were retrieved from the surgical files of the Department of Histopathology, University College London Hospital NHS Trust. The morphology of the cervical smears was reviewed by Dr Gabrijela Kocjan, Department of Histopathology, University College London. The use of these archival specimens for research was approved by the joint UCL/UCLH committees on the ethics of the human research.

2.2 Methods

The methods that were commonly used for investigations in different result chapters were described below, while those only used for a specific result chapter were described in appropriate chapters respectively.

2.2.1 Preparation Of Archival Specimens For Microdissection

For Pap smears, the dyskaryotic cells were identified and marked with a diamond pen on the reverse side of the slides. The slides were then incubated in xylene for 2-5 days to remove the coverslips and then washed in 100% and 70% ethanol.

For formalin fixed and paraffin embedded tissue specimens, 3-5μm sections were cut using a microtome and dried in an oven over-night. Sections were deparaffinized in xylene for 5 minutes twice and processed through 100%, 90% and 70% ethanol solution. The sections were then rinsed in running water for 1 min, and briefly stained with haematoxylin, washed with running water for 2-3 min and processed in 70%, 90% and 100% ethanol solution.

2.2.2 Microdissection

Normal and dyskaryotic cells were microdissected separately using a glass transfer pipette as described previously by Pan et al (Pan *et al*, 1994). Briefly, the respective cells were identified under a microscope and a drop of 50% ethanol was laid on top of them (Figure 2.1). The cells were then scraped off the slide, aspirated into a glass transfer pipette, and then transferred into a clean microtube. In some cases, where the neoplastic cells were surrounded by normal cells, the surrounding normal cells were scraped off the slides first.

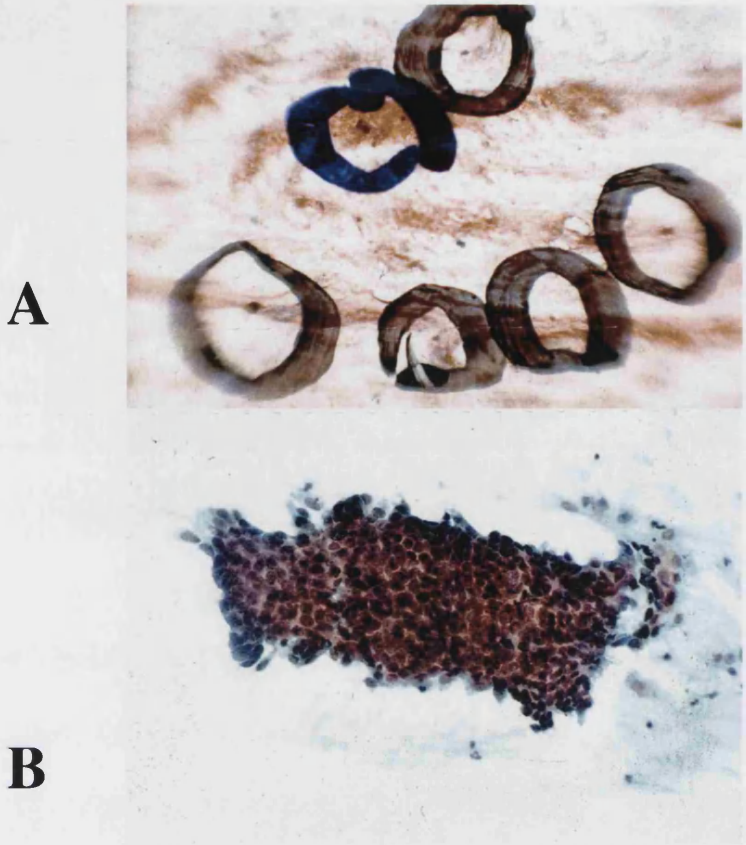

Figure 2.1: Microdissection of dyskaryotic cells from archival smears. A) Routinely stained and prepared cervical smear glass slide with manually drawn circles surrounding the areas containing dyskaryotic cells. B) Within the circle, a cluster of severely dyskaryotic cells indicating a CIN 3 lesion, which was selectively removed from the glass.

By such approach, highly enriched tumour cells population and normal cells were obtained in each case. The microdissected cells were pelleted down by centrifugation, the tube was left open at room temperature to allow the ethanol to dry, before proceeding to DNA extraction.

A number of precautions were taken during microdissection in order to avoid potential contamination:

1- Section cutting and microdissection were always carried out in a laboratory free of PCR and cloning products;

2- New pipettes were used for microdissecting different cell populations in each case.

3- Fresh 50% of ethanol was prepared and aliquoted into clean microtubes prior to microdissection. An aliquote was used for microdissection of each sample.

4- Normal and tumour cells were always microdissected separately and transferred to separate clean microtubes.

2.2.3 DNA Preparations

2.2.3.1 Crude DNA Preparations

Cells microdissected from Pap smears were washed in 1ml of 10mM Tris (pH 8.0) and 1 mM of EDTA at 4°C for 1 hour to remove the stain and then centrifuged at 13,000g for 5 minutes. The cell pellets were then digested with proteinase K (100μg/mL) in 50-150μL PCR buffer containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl and 1.0% Triton X-100). The volume of PCR buffer used for digestion depended on the total number of the microdissected cells. The digestion was carried out for 16-20 hours at 56°C. The digests were then heated at 95°C for 10min to inactivate proteinase K and centrifuged to remove cell debris. The resulting

supernatants were stored at -20°C and used for PCR. Similar crude DNA preparations were carried out on cells microdissected from archival cervical tissue biopsies.

2.2.3.2 DNA Purification

To purify the crude DNA preparation, protein was precipitated and removed. Different protein precipitation methods including precipitation with saturated NaCl and Protein Precipitation Solution (Promega, Southampton, U.K.) were tested. For DNA purification with saturated NaCl, 50μL NaCl (6M) was added to the DNA pallet followed by centrifugation at 2,000g for 10 minutes to remove the pelleted protein. The DNA samples purified failed to show complete digestion with restriction endonuclease, thus cannot be used for endonuclease digestion based study.

For DNA purification with Protein Precipitation Solution (Promega, Southampton, U.K.), 70μL Protein Precipitation Solution was added to each 200μL DNA crude preparation. Precipitated protein was then removed by centrifugation at 25,000g for 20 minutes at 4°C. DNA in the supernatant was then precipitated with isopropanol, washed in 70% ethanol, air dried and finally dissolved in 20μL distilled water. DNA samples were similarly purified from formalin-fixed and paraffin-embedded tissues.

2.2.4 Polymerase Chain Reaction (PCR)

2.2.4.1 Primers Design

All PCR primers (Table 2.3) used in this study except for the androgen receptor and HPV primers, were designed with the help of a computer primer design programme (GCG Wisconsin Package Primer Design).

Table 2.3: List of the primers used in this thesis.

Target	Chromosomal location	Forward Primer	Reverse Primers	Estimated size (bp)
D3S1566	3p13	5'TAATTGAGAAGTCATGCCCTG3'	5'GCTCAAAGAAATGTCCTGGA3'	170
D3S1285	3p14.1	5'CACCCTACTGTGCTATCAAACA3'	5'AGTATCCAGAGGCTGGGAAG3'	153
D3S1300	3p14.2	5'GCTCACATTCTAGTCAGCCTG3'	5'TGTCACAGAATAGTCTTTCCCA3'	165
D3S1289	3p21.1	5'GCAACTTGTAAGAGAGCATTCA3'	5'ACATTACAGAAATGTGATGCA3'	259
D3S1260	3p22.2	5'GCTACCAGGGAAGCACTGTA3'	5'GCTAAACTGAAGACCCTGCA3'	126
D3S1611	3p22.3	5'TAATCCCAGCACTTCGAGAG3'	5'AGACTACAGGCATTTGCCAC3'	117
D5S406	5p15.32	5'TGCCAATACTTCAAGAAAAACA3'	5'ACTTGGGATGCTAACTGCTG3'	188
D6S105	6p21.1	5'CCAAAGTGCTGGGATTACAG3'	5'TCAAGGAAGAGAGACCATGC3'	145
D6S265	6p21.1	5'ATCACCCCCCTCACACAC3'	5'TCTAATCGAGGTAAACAGCAGA3'	100
D6S277	6p24.3	5'AGCTGAATAACACGAGGTG3'	5'TACATTTTTGATGACAATGGAA3'	101
D11S35	11q22.1	5'GAGGAAAGTCATGAACGCAG3'	5'ATCGATTAACCAACTTCACACA3'	100
D11S528	11q23.3	5'GCCTAACTAATGGTGTCCCC3'	5'GACCCCAGTGTGAGATGAAT3'	150
HPV16	NA	5'CAGGACCCACAGGAGCGACC3'	5'CGACCGGTCCACCGACCCCT3'	398
HPV18	NA	5'CCGAGCACGACAGGAACGACT3'	5'TCGTTTTCTTCCTCTGAGTCGCTT3'	174
HPV33	NA	5'AACGCCATGAGAGGACACAAG3'	5'ACACATAAACGAACTGTGGTG3'	212
HPV45	NA	5'TTGGAATTTTGGTGTCCCTC3'	5'TCCTGCTTTTCTGGAGGTGT3'	114
HPV56	NA	5'TGATGCACGAAAAATTAATC3'	5'CGGGGATAACCCAATATTCC3'	160
P53	EXON 5	5'TTCCTCTTCCTGCAGTACTC3'	5'ACCCTGGGCAACCAGCCCTGT3'	245
	EXON 8	5'CCTATCCTGAGTAGTGGTAA3'	5'GTCCTGCTTGCTTACCTCGC3'	185

Target	Chromosomal location	Forward Primer	Reverse Primers	Estimated size (bp)
BCL 10	EXON 3	5'TTAACAAGTCACAAGATGGACAGTG3'	5'CATTAAAAATTAAAAGGCAATAAAGTG3'	460
AR	NA	5'TCCAGAATCTGTTCCAGAGCGTGC3'	5'GCTTGGGGAGAACCATCCTCAC3'	220
G6PD	NA	5'GGCAACAGATACAAGAACGTGAA3'	5'CGCAGAAGACGTCCAGGAT3'	67
			5'CCAGCTCAATCTGGTGCAG3'	151
			5'CCCTCATACTGGAAACCCACT3'	242

2.2.4.2 PCR Method

For each primer pair, PCR conditions including annealing temperature, cycling parameters and MgCl2 concentration were all systematically optimised using positive and negative controls before application to test samples as summarized in Table 2.4.

Table 2.4: PCR conditions used in this thesis

Gene targeted	PCR programme	PCR condition	MgCl2 concentration
p53 and BCL10	Hot-start touch down programme	38 cycles of denaturation at 94°C for 50 seconds, annealing temperature starting at 60°C reducing 2°C every cycle until 55°C, and extension at 72°C for 50 seconds, followed by a final extension at 72°C for 5 minutes.	1.5 mM
AR	Hot-start touch down programme	35 cycles of denaturation at 94°C for 50 seconds, annealing temperature starting at 60°C reducing 1°C every cycle until 57°C, and extension at 72°C for 50 seconds, followed by a final extension at 72°C for 5 minutes.	1.5 mM

Gene targeted	PCR programme	PCR condition	MgCl2 concentration
Microsatellite markers	Hot-start touch down programme	38 cycles of denaturation at 94°C for 50 seconds, annealing temperature starting at 60°C reducing 2°C every cycle until 55°C, and extension at 72°C for 50 seconds, followed by a final extension at 72°C for 5 minutes.	1.5 mM
HPV	Hot-start touch down Programme	35 cycles of denaturation at 94°C for 50 seconds, annealing temperature starting at 60°C reducing 1°C every cycle until 57°C, and extension at 72°C for 50 seconds, followed by a final extension at 72°C for 5 minutes.	1.5 mM

For each experiment, a master mixture including all the necessary PCR reagents except the template DNA was first prepared and aliquoted into PCR microtubes or microtiter plate wells, and DNA samples were subsequently added, followed by a drop of mineral oil. Appropriate positive and negative (without template DNA) controls were included in each experiment. A typical PCR reaction was performed in a 25 μL reaction mixture containing 2.5μL 10 × PCR buffer, 0.2μL dNTP (final concentration 200μM of each dNTP), 4pmol of each primer, 1.5 mM of $MgCl_2$, 0.38-1.0 unit Tag Polymerase, dH_2O to 22μL and 3μL DNA sample.

PCR was carried out in a thermal cycler (Hybaid,UK) using appropriate PCR programme as specified for each primer set in Table 2.4.

All PCR set-ups were performed in a room separate from product analyses and DNA extraction to avoid the risk of contamination. Other precautions taken to avoid cross contamination during PCR set up including separate storage for PCR reagents and DNA samples, use of filtered tips and use of separate pipettes for measuring PCR reagents and DNA samples.

2.2.5 Electrophoresis of PCR Products

The method used for PCR product analysis depended on the product size and the resolution required. Polyacrylamide gels were used for analysis of PCR products less than 250bp or in experiments where a high resolution separation was desired, e.g. clonality analysis.

Agarose gels were used for analysis of product over 250bp or in experiments that did not require a high resolution, e.g. to check whether PCR was successful.

2.2.5.1 Electrophoresis On Agarose Gels

1.5% agarose gels were used. 1.5 gram of agarose was dissolved in 100 mL of 1×TBE buffer by boiling in a microwave. After cooling to ~50°C, 5μL ethidium bromide (0.1μg/ml) was added to the mixture. 50 mL of Agarose were poured into an appropriate gel tray and a comb inserted. When the gel was set, the comb was carefully removed. 10μL of PCR product was mixed with 2μL of loading buffer (Bromophenol Blue and sucrose solution) before

loading into the wells. Gels were run at 80 voltages for 30 min, then removed from the apparatus and viewed under an ultraviolet light illuminator. Digital images were taken using the Kodak EDAS 290 documentation system (Anachem, Luton).

2.2.5.2 Electrophoresis On Polyacrylamide Gels

7% polyacrylamide gels were used for analysis of PCR products. To prepare the polyacrylamide gel, 1.75 mL acrylamide (40% of 29:1 acrylamide: bis-acrylamide), 2mL 5xTBE, 6.25 mL H_2O and 100µL TEMED were mixed. The gel mix was poured between the glass plates and comb inserted according to the manufacturer's instructions. The gel was left for 30 minutes to set, the wells were cleaned, 4-10 µL of PCR products were mixed with 2µL loading buffer and loaded into wells. The gels were run at 150 voltages, for 90 min. Gels were stained with ethidium bromide for 2 minutes and viewed under an ultraviolet light illuminator. For clonality analysis, gels were visulised by silver staining.

2.2.5.3 Silver Staining

7% polyacrylamide gels were stained using PlusOne Silver Staining Kit in a GeneStain Automated Gel Stainer (Pharmacia-Amersham, Little Chalfont, Buckinghamshire, UK).

Gels were fixed in a fixing solution stained in a staining solution and then developed in a solution containing 25ml Sodium carbonate (5x), 125µL Sodium thiosulphate and 125µL

Formaldehyde in 100 ml water. The gels were then soaked in stopping and preserving solution.

2.3 Analysis Of PCR Products Using An ABI 377 DNA Sequencer

Dyskaryotic/neoplastic and normal cells from each case were analyzed in parallel. The PCR products were analysed on 4.5% polyacrylamide denaturing gels (Acrylamide : N-N`-Methylene-bis-acrylamide=29:1) (National Diagnostics, Hull) in 1×TBE buffer (National Diagnostics, Hull) using an ABI 377 automated fluorescent DNA sequencer (Applied Biosystems, Foster City, California, USA), which has a four-color detection system. One µl of each PCR reaction was mixed with 1.5µL blue dye and 1µl of a ROX (for filter set D) fluorescent size marker (Applied Biosystems, Foster City, California, USA). This mix was denatured for 2 minutes at 94°C and 1µl was loaded into each well on a prewarmed gel using membrane comb method according to (Hamoudi *et al*, 2002). The gel was run for 4 hours at 150 watts power, 50 milli amps current, 1650 volts voltage, scan rate of 1200 scans per hour and 50°C temperature. Whilst the PCR products were separated during electrophoresis, the fluorescence was detected in the laser scanning region using filter set D. The data was collected and stored using the GeneScan Collection Software 2.0 (Applied Biosystems, Foster City, California, USA). The fluorescent gel data collected during the run was

automatically analysed by the GeneScan Analysis Software 3.1 (Applied Biosystems, Foster City, California, USA). Sample sheets were prepared using in house SampConvertor software. The size standard calling for each lane and the quality of data were checked automatically using in house GSQC version 1.1 software. Each fluorescent peak was quantified in terms of size (in base pairs), peak height and peak area according to (Lakhani *et al*, 1996). The results were then imported into Genotyper (version 2.1) (Applied Biosystems, Foster City, California, USA) for further analysis and printing.

CHAPTER 3

Archival Cervical Smears: A Versatile Resource For Molecular Investigations

3.1 Introduction

As detailed in the General Introduction, most cervical carcinomas are believed to derive from preneoplastic epithelial lesions known as Cervical Intraepithelial Neoplasia (CIN). Cervical smear test was introduced in 1943 and has been subsequently used as a screening method for these lesions. Nationwide screening programmes were set up in many developed countries in 1980s and have resulted in a dramatic reduction in the cervical carcinoma mortality rate (Schoell *et al*, 1999). Archival cervical smears represent one of the largest resources of pathological specimens. With the appropriate long-term clinical follow-up, they could represent the most important source for research into cervical carcinoma including molecular investigations. Despite this huge potential, only a few molecular investigations based on archival cervical smears have been reported in the literature.

Several studies have shown that archival cervical smears can be used for PCR amplification of genomic sequences (Jackson *et al*, 1990; Roda Husman *et al*, 1995;Chen *et al*, 1996; Puranen *et al*, 1996;McGoogan *et al*, 1998). These studies were based on a

relatively large number of mixed normal and dyskaryotic cells collected from cervical smears. The latter cells are of main research interest. It is not known whether PCR could be applied to a small number of dyskaryotic cells, microdissected from archival smears. In addition, it remains to be tested whether archival cervical smears are suitable for PCR-based RNA analysis. To address these issues, we prepared DNA and RNA samples from dyskaryotic cells, microdissected from archival cervical smears and systematically tested to what extent such archival material can be used for PCR-based molecular investigations.

3.2 Materials And Methods

3.2.1 Materials

Cervical smears with a storage time between 10 to 11 years from 30 patients (7 CIN1, 11 CIN2 and 12 CIN3), including 6 cases (CIN3) with matched paraffin-embedded tissues, were retrieved from the pathological files of the Department of Histopathology, University College London. Formalin-fixed, paraffin-embedded tissue from further 15 patients diagnosed as having cervical squamous cell carcinoma were also retrieved. The morphology of these cervical smears and biopsies was reviewed. Formalin-fixed

and paraffin-embedded tissues tonsils from 2 male patients were used as controls.

3.2.2 Methods

3.2.2.1 Microdissection

Dyskaryotic cells (10^5-10^7) as well as normal cells were microdissected using glass transfer pipettes as described in section 2.2.2 of Chapter 2 (Figure 2.1).

3.2.2.2 Crude DNA Preparation And PCR Of Genomic Sequences

Crude DNA preparation: This was carried out as described in section 2.2.3.1 of Chapter 2.

3.2.2.2.1 PCR Of The P53 And BCL10 Gene

3μl of crude DNA preparations was used for PCR amplification of three genomic segments of variable sizes: p53 exon 8 (185bp), p53 exon 5 (245bp) and BCL10 coding exon 3 (460bp).The primers used for PCR of the p53 and BCL10 gene are shown in Table 2.3 of Chapter 2. PCR was performed in a thermal cycler (Hybaid, U.K.) using a "hot-start touch-down" programme with annealing temperature starting at 60°C, reducing 2°C every cycle until 55°C for 38 cycles as described in Table 2.4 of Chapter 2. PCR products were analysed by electrophoresis on 1.5% agarose gels.

3.2.2.2.2 Loss Of Heterozygosity (LOH) Analysis

Four microsatellite loci of various chromosomes, namely D3S1289, D5S406, D6S265 and D11S528, were selected and subjected to PCR with fluorescently labelled primers (Table 2.3 of Chapter 2). PCR was carried out in a thermal cycler using a "hot-start touch-down" programme as above. PCR products were first confirmed on agarose gels and then analysed on an ABI Prism 377 DNA sequencer with GeneScan software (version 3.0). Dyskaryotic and normal cells from the same case were analysed in parallel.

3.2.2.3 DNA Purification, Restriction Enzyme Digestion And PCR Based Clonality Analysis

3.2.2.3.1 DNA Purification

The crude DNA preparations were adequate for PCR amplification of genomic sequences but not for restriction enzyme digestion, which is required for many molecular investigations, including PCR based clonality analysis of the X-chromosome inactivation pattern. For such investigations, highly purified DNA samples are necessary. The crude DNA preparations from the microdissected cells were further purified as detailed in section 2.2.3.2 of Chapter 2.

3.2.2.3.2 Restriction Enzyme Digestion

4μl of purified DNA was digested with and without 12 units HpaII in a total of 10μl reaction mixture at 37°C for 16-20 hours. For each experiment, one male DNA sample was similarly digested as a control.

3.2.2.3.3 PCR Of The Androgen Receptor (AR) Gene

For clonality analysis, 5μl of HpaII digested and mock-digested DNA samples were used for PCR of the highly polymorphic CAG repeat (Figure 3.1) of the AR gene. The primers used for PCR of the AR gene are shown in Table 2.3 of Chapter 2. The amplification was carried out in a thermal cycler using a "hot-start touch-down" programme as described in Table 2.4 of Chapter 2. PCR products were separated on 7% polyacrylamide gels by electrophoresis and visualized by sliver staining (Pharmacia-Amersham, Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) as described in Section 2.2.4.3 of Chapter 2. The sample was considered monoclonal if only one allele is amplified after HpaII digestion, whereas the sample was considered as polyclonal if both alleles displayed similar intensity.

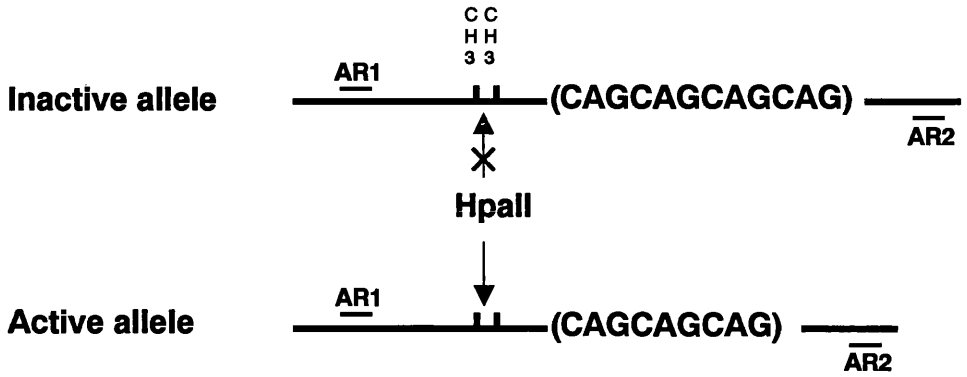

Figure 3.1: Schematic illustration of PCR based clonality analysis of the AR gene. Because the inactivated allele is heavily methylated (CH3), it will not be digested. The restriction enzyme HpaII will cut only the active allele. And only the inactivated allele will serve as a template for the PCR.

3.2.2.4 Reverse Transcription PCR Of The Glucose-6-Phosphate Dehydrogenase Gene

3.2.2.4.1 RNA Extraction

Total RNA extraction from microdissected dyskaryotic cells was carried out using an Ambion Kit (AMS Biotechnology, Oxon, UK). Cells were digested in 100 µL of digestion buffer containing 100 µg of proteinase K at 45°C for 2 hours. The digests were added with 600 µL of RNA extraction buffer and incubated at room temperature for 5 min followed by extraction with 700 µL of acid phenol: chloroform. The aqueous layer of the extracts was recovered into a fresh tube and 1 µL of linear acrylamide (5 µg/ µL) was added as a carrier. The RNA was precipitated with an

equal volume of isopropanol at -20°C for 2 hours and pelleted in a microcentrifuge at 4°C for 15 min. The RNA pellet was then washed with 70% alcohol, air-dried and dissolved in 20 μL of RNA Storage Solution™ (Ambion (Europe) Ltd., Huntingdon, Cambridgeshire, UK).

3.2.2.4.2 Reverse Transcription PCR

The extracted RNA was used for reverse transcription and subsequent cDNA amplification of the glucose-6-phosphate dehydrogenase (G6PD) gene. To eliminate amplification from contaminating genomic DNA, the sense primer was designed to span an exon-exon boundary. The sense primer was combined with three antisense primers, in order to obtain a PCR product of 67 bp, 151 bp and 242 bp, respectively (Table 2.3 of Chapter 2).

One-step reverse transcription PCR (RT-PCR) was performed using a Qiagen OneStep RT-PCR Kit (Qiagen, West Sussex, UK). G6PD primers were added to the RT-PCR mixture at a final concentration of 0.6 μM for the sense primer and 0.15 μM for each of the three antisense primers, and 5 μL of the above-extracted total RNA was used as a template for a 25-μL RT-PCR reaction. The thermal cycler was programmed to start with reverse transcription at 50°C. A final extension step at 72°C for 10 min concluded the reaction. PCR products were analysed on

10% polyacrylamide gels and visualized by ethidium bromide staining.

3.3 Results

3.3.1 Crude DNA Preparations Are Suitable For PCR Amplification Of Genomic Sequences

To test the suitability of crude DNA preparations from archival cervical smears for PCR-based molecular investigations, we first performed PCR amplification of three gene segments of various sizes. When gene fragments less than 250 bp were examined, each of the 21 cervical smears examined showed a product of expected size for both p53 exon 8 (185 bp) and exon 5 (245 bp) PCRs. When a significantly longer fragment, BCL10 coding exon 3 (460 bp), was tested, 17 of the 21 (81%) samples yielded a product of expected size (Figure 3.2A).

Similarly, PCR amplification of 4 microsatellites ranging from 100 to 260bp was successful in all 20 cases examined. LOH was detected by parallel analysis of dyskaryotic and normal epithelial cells of the same case (Figure 3.2B). Table 3.1 summarises the heterozygous rate and frequencies of LOH of these loci.

Table 3.1: summary of the heterozygous rate and frequencies of LOH of the loci studied.

Locus	CIN1		CIN2		CIN3	
	Heterozygosity	LOH	Heterozygosity	LOH	Heterozygosity	LOH
D3S1289	4/4	1/4	8/8	6/8	8/8	5/8
D5S406	3/4	0/3	7/8	3/7	6/8	2/6
D6S265	3/4	0/3	6/8	0/6	7/8	0/7
D11S528	3/4	3/3	8/8	3/8	8/8	2/8

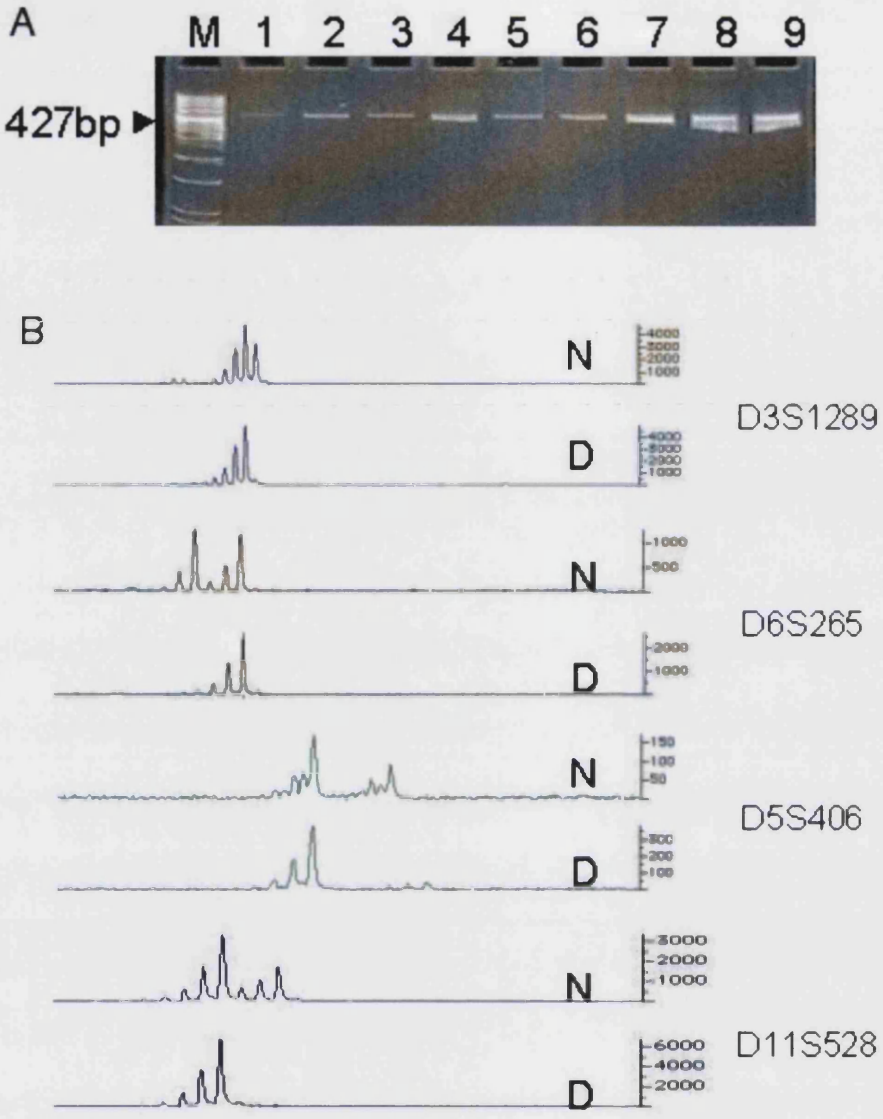

Figure 3.2 : A: Examples of PCR amplification of BCL10 coding exon 3 (460bp) from crude DNA preparations of microdissected dyskaryotic cells. M: DNA marker; 1-9: different cases.

B: Examples of LOH analysis of four microsatellite loci from crude DNA preparations of microdissected cells. Dyskaryotic cells show an allelic deletion in comparison with normal cells.

N: normal cells, D: dyskaryotic cells.

3.3.2 Purified DNA Sample Is Necessary For PCR-Based Clonality Analysis And Can Be Applied To Cervical Smears

Good quality of purified DNA was essential for complete HpaII digestion and hence clonality analysis. Of several DNA extraction protocols tested, the one detailed in the section 2.2.3.2 of Chapter 2 gave consistent results and was used for all clonality assays. To apply clonality analysis to cervical smears, we first used the formalin-fixed, paraffin-embedded cervical tissues to establish the methodology. In each experiment, HpaII-digested male DNA samples showed no PCR amplification of the AR gene, indicating complete digestion (Figure3.3). Normal epithelial cells distant from tumour lesions showed polyclonal patterns with no apparent skewing in all 12 cases examined (Figure4.1). Neoplastic cells microdissected from cervical biopsies of CIN3 and squamous cell carcinoma, displayed a monoclonal pattern in 18 of 19 heterozygous cases (Figure4.1). The remaining heterozygous case was CIN3 and exhibited a polyclonal pattern. A review of the original histology slides showed two separate foci of CIN3 lesions. Clonality analysis of these separate lesions was not performed because of the insufficient material. In each of the 6 cases of CIN3, in which both cervical smear and tissue biopsy were available, both specimens displayed the same monoclonal pattern (Figure4.1), confirming that archival cervical smears are suitable for PCR-based clonality analysis.

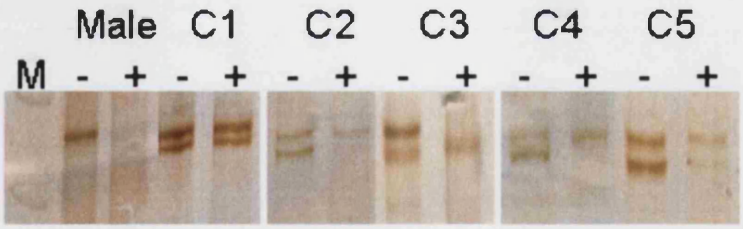

Figure 3.3: Clonality analysis of dyskaryotic cells from archival cervical smears. Purified DNA samples were prepared from microdissected dyskaryotic cells. Male DNA shows lack of amplification after HapII digestion as expected, indicating complete digestion with HapII restriction enzyme. C1 is a CIN1 and shows a polyclonal pattern. C2 and C3 are CIN2, and C4 and C5 are CIN3. They show a monoclonal pattern. M: DNA marker; -: without HpaII digestion; +: with HpaII digestion; Male: male DNA;

3.3.3 Reverse Transcription-PCR Of The G6PD Gene

To examine whether archival cervical smears are suitable for PCR-based RNA detection, we performed RT-PCR of the G6PD gene in 10 cases of CIN3. Three segments of the gene ranging from 67 bp to 242 bp were amplified in a single tube in each case. All cases showed amplification of an expected 67-bp product, and eight of the 10 cases yielded an expected 151-bp product. However, the 242 bp fragment was not amplified in any of the cases examined (Figure3.4). To ascertain further that the failure of the amplification of the 242-bp product resulted from RNA degradation rather than failed amplification in a multiplex PCR reaction, a separate RT-PCR with a single pair of primers for 242 bp fragment was performed. The single set PCR consistently showed a negative result despite amplification of an expected product from positive controls.

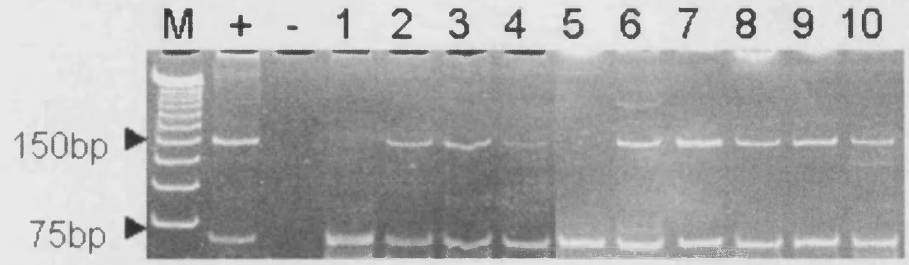

Figure 3.4: RT-PCR of the G6PD gene of dyskaryotic cells microdissected from archival smears. All cases show amplification of a 67bp product, and 8 of 10 cases display amplification of 151bp products. +: positive control, -: negative control.

3.4 Discussion

Previous studies have shown that PCR amplification of genomic DNA sequences could be achieved with DNA samples prepared from a relatively large population of mixed normal and dyskaryotic cells collected from archival cervical smears (Jackson *et al*, 1990;Roda Husman *et al*, 1995;Puranen *et al*, 1996;Chen *et al*, 1996; McGoogan *et al*, 1998). Our present study further demonstrates that PCR amplification of genomic DNA sequences can be applied to crude DNA preparation from small number of cells microdissected from archival smears. When the DNA segment to be amplified is restricted to 250 bp or less, most if not all archival smears, can be used. This means that the molecular analyses based on PCR, such as screening for gene mutation and LOH, and HPV typing, can be performed. If it is necessary to target a slightly larger fragment, archival cervical smears could still be a valuable resource for PCR up to 460 bp. Overall, the quality of DNA samples prepared from archival

cervical smears is better than those prepared from archival formalin-fixed and paraffin-embedded tissue, from which it is often difficult to amplify fragments larger than 300 bp (Jackson *et al*, 1990; Coates *et al*, 1991).

After purification, DNA samples prepared from microdissected dyskaryotic cells can be readily digested with restriction enzymes, such as HapII. They are therefore suitable for PCR-based clonality analysis of the X-chromosome inactivation pattern. By parallel analysis of cervical smears and matched cervical tissue biopsies, we demonstrated that PCR-based clonality analysis could be readily applied to archival smears with more than 10 years of storage time. In addition to clonality analysis, such purified DNA samples prepared from archival smears should be adequate for methylation specific PCR to study transcriptional suppression of TSGs such as p16, which is implicated in the pathogenesis of cervical carcinoma (Dong *et al*, 2001;Virmani *et al*, 2001).

Based on microdissected dyskaryotic cells, we also demonstrated that archival cervical smears are suitable for RT-PCR. For a successful RT-PCR, a gene-specific primer, the antisense G6PD primer, rather than an oligo (dT) primer or random hexomers, should be used for cDNA synthesis. The oligo (dT) primer targets the 3' end of RNA molecules and is not adequate for synthesis of

cDNA from degradated RNA samples, such as those prepared from archival fixed tissue/cells. The gene-specific primer targets the fragment to be amplified by RT-PCR should be restricted to less than 150 bp. With increasing data on transcriptional profiles from microarray, quantitative PCR to evaluate gene expression in a large series of cases is necessary. The RT-PCR protocol reported in the present study provides the basis to utilize such valuable archival materials.

In summary, archival cervical smears can be used for a variety of PCR-based molecular analyses at both DNA and RNA levels. In view of the huge resources of archival cervical smears and their long clinical follow-up, the potential to gain knowledge on cervical carcinoma by the molecular study of these archival materials is immense.

Chapter 4

Clonality Analysis Of Archival Cervical Smears: Monoclonality Correlates With Grade And Clinical Behaviour Of Cervical Intraepithelial Neoplasia

4.1 Introduction

Cervical smear has been used as a screening method to identify the preinvasive lesions of the cervix and has permitted a dramatic reduction in cervical carcinoma mortality (Schoell *et al*,1999). However, morphological interpretation of some cell changes, particularly in the early CIN lesions, as well as prediction of their clinical behaviour by means of cytological examination alone may be difficult. Despite the apparent morphological homogeneity within the same subgroup, CIN lesions may have variable clinical behaviour. It is estimated that, in the absence of any treatment approximately 60% of CIN1 lesions regress (Ostor, 1993;Duggan *et al*, 1998), 30% persist and 10% progress to a higher-grade lesion. For CIN2 lesions, around 40% regress while 40% and 20% persist or progress respectively (Ostor, 1993;Duggan *et al*, 1998). Similarly, 33% of CIN3 regress, 56% and 12% persist or progress respectively (Ostor, 1993;Duggan *et al*, 1998). In addition,

despite the treatment, approximately 5% of CIN lesions show disease relapse or progression (Tsukamoto; 1985;Gunasekera *et al*, 1990). Currently, there are no biomedical or molecular markers that can distinguish the lesions with potentially different clinical behaviour. Although several markers, including deletion of the p16 gene and expression of the integrated HPV oncogenes, have been associated with advanced CIN lesions (Klaes *et al*, 1999;Klaes *et al*, 2001).

Monoclonality (clonal expansion from a single transformed cell) is the hallmark of neoplastic growth. Clonality analysis is valuable in diagnosis of neoplastic lesions, particularly at early stages, and in assessment of clonal relationship of different lesions in the same patients (Allen *et al*,1992;Gale *et al*,1994). It has been shown that most of CIN3 and invasive carcinomas as well as the majority of CIN2 lesions are monoclonal, whereas CIN1 lesions are polyclonal (Enomoto *et al*,1994; Park *et al*,1996; Ko *et al*,1997;Guo[a] *et al*, 1998). Despite such significant correlation between clonality and CIN grade, it has not been explored whether clonality can be used to predict the clinical behavior of CIN lesions.

For non-lymphoid malignancies in women including cervical carcinoma, clonality is commonly determined by PCR-based analysis of the inactivation pattern of X-chromosome genes.

The principle of the assay relies on the fact that in each cell during the process of embryogenesis in females, either the maternally derived or paternally derived X chromosome is randomly and permanently inactivated (Gale *et al*,1993;Enomoto *et al*, 1994). This inactivation pattern is stable and is inherited by its progeny cells. Thus, normal tissues in females are composed of cellular mosaics differing in which one of the two X chromosomes is inactivated. By contrast, neoplasms of females originating from a single transformed cell show an uniform inactivation pattern (Allen *et al*, 1992;Gale *et al*,1994).

Several polymorphic genes on X-chromosome including G6PD, have been explored for clonality analysis. Among these genes, androgen receptor (AR) is the most commonly used because it contains a highly polymorphic short tandem repeat, which is adjacent to a cluster of CpGs, the site of methylation (Guo[a] *et al*, 1998). The region spanning the repeat and methylation sites can be amplified by PCR since they are close to each other. With the help of methylation sensitive enzyme, such as HpaII, the two alleles can be easily differentiated (Figure 3.1, chapter 3). This PCR based clonality analysis was first applied to fresh frozen tissues and subsequently formalin fixed and paraffin embedded tissues of many carcinoma types including cervical carcinoma (Enomoto *et al*, 1997).

In the present study, we applied this PCR-based clonality assay to archival cervical smears and explored the value of clonality analysis in the prediction of CIN lesions clinical behaviour. We also correlated clonality with high risk HPV subtypes.

4.2 Materials And Methods

4.2.1 Materials

Archival cervical smears with an avarage storage time of 10 years from 33 patients (9 CIN3, 21 CIN2 and 3 CIN1) were examined. These cervical smears were selected because they contained abundant dyskaryotic cells. In each case, the cytological diagnosis was confirmed by histological examination of the cervical biopsy. The morphology of the cervical smears was reviewed by Dr G. Kocjan. Formalin-fixed, paraffin-embedded tonsils from 2 male patients were used as controls.

4.2.2 Methods

4.2.2.1 Microdissection And DNA Extraction

Normal and dyskaryotic cells from cervical smears were microdissected as described in Section 2.2.2 of Chapter 2. Highly purified DNA samples were prepared from the microdissected cells as detailed in Section 2.2.3.2 of

Chapter 2. Highly purified DNA samples were also prepared from two tonsils of male patients.

4.2.2.2 Restriction Enzyme Digestion

Purified DNA (4μL) was digested with and without 12 units HpaII as detailed in Section 3.2.2.3.2 of Chapter 3. For each experiment, a male DNA sample was similarly digested as a control.

4.2.2.3 Polymerase Chain Reaction

HpaII-digested and mock-digested DNA samples were used for PCR of the highly polymorphic CAG repeat of AR gene as described in Section 2.2.4.2 of Chapter 2 (The primers used for PCR of the AR gene and high risk HPV subtypes are shown in Table 2.3 of Chapter 2). The PCR products were separated on 7% polyacrylamide gels by electrophoresis and visualized by silver staining as detailed in Section 2.2.5.3 of Chapter 2.

4.2.2.4 Interpretation Of Clonality Data

Cases were considered as heterozygous (informative) if the PCR products from the undigested DNA from samples of normal cells showed two bands of similar intensity. Cases with only one major band were homozygous for the AR gene

and thus uninformative. In informative cases, clonality was judged by visual comparison of the relative intensity of two alleles in presence and absence of restriction enzyme digestion. The sample was considered monoclonal if only one allele is amplified after HpaII digestion, whereas the sample was considered as polyclonal if both alleles displayed similar intensity.

4.2.2.5 HPV PCR

High-risk HPV subtypes 16 and 18 and 33 were detected by PCR using primers established in previous studies as described in Table 2.3 of Chapter 2 (Miller *et al*,1994;Ostwald *et al*,1994;Karlsen *et al*, 1996). PCR products were analyzed on 7% of polyacrylamide gels.

4.3 Results

4.3.1 Correlation Of Clonality With Grade And Clinical Behavior Of CIN

Dyskaryotic cells from cervical smears showed monoclonal patterns in 9/9 CIN3 and 15/21 CIN2. The remaining 6 CIN2 and 3 CIN1 showed polyclonal patterns (Figure 4.1). All patients with monoclonal CIN lesions, including 9 CIN3 and 15 CIN2, were shown to have had either persistence or progression of the disease during follow-up, despite treatment. In 6 CIN2 cases (nos.4,9,10,13-15) in which

sufficient dyskaryotic cells were available for analysis from subsequent CIN lesions, both the original CIN2 and subsequent lesions (CIN3 in 3 cases and CIN2 in 3) showed an identical monoclonal pattern (Figure 4.1), suggesting a clonal link. In contrast, patients with polyclonal CIN lesions, including 3 CIN1 and 6 CIN2, became negative after treatment and remained disease free during follow-up. Clonality therefore appears to have had a strong clinical predictive value, particularly in the case of CIN2 lesions. There was no apparent difference in treatment modality or subtypes of HPV infection between monoclonal and polyclonal CIN2 lesions (Table 4.1).

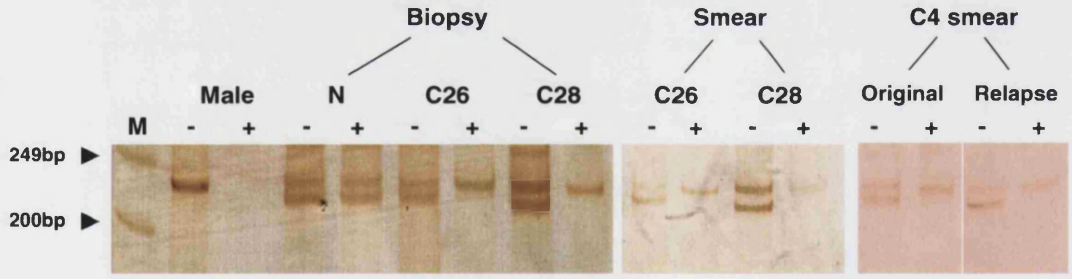

Figure 4.1: Clonality analysis of microdissected dyskaryotic cells from cervical smears and cervical biopsies. M: DNA marker; -: without HpaII digestion; +: with HpaII digestion; Male: male DNA; N: normal cervical epithelial cells; Dyskaryotic cells from cervical smears (CIN3) in cases 26 and 28 show identical monoclonal patterns to their corresponding tumour cells from cervical biopsies. The original CIN2 and subsequent CIN3 lesion in case 4 showed an identical monoclonal pattern, suggesting a clonal link.

Table 4.1: Summary of clinical and laboratory studies.

Case No.	CIN grade	Clonality	High risk HPV	Treatment	Treatment outcome*	Total follow-up (month)
1	CIN1	P	- ve	No	DF	36
2	CIN1	P	- ve	No	DF	60
3	CIN1	P	- ve	No	DF	120
4	CIN2	M	HPV16	CB	CIN3 (8)	48
5	CIN2	M	HPV16	NA	CIN3 (9)	72
6	CIN2	M	HPV33+16	CB	CIN3 (13)	12
7	CIN2	M	HPV18	NA	CIN3 (21)	120
8	CIN2	M	HVP33+16	CB	CIN3 (68)	132
9	CIN2	M	HPV16	CB & LE	CIN3 (23)	60
10	CIN2	M	HPV16	CB	CIN3 (13)	13
11	CIN2	M	HPV16	CB & LE	CIN2 (5)	36
12	CIN2	M	HPV16	CB	CIN2 (6)	36
13	CIN2	M	HPV16	NA	CIN2 (21)	84
14	CIN2	M	HPV33+16	CB	CIN2 (10)	24
15	CIN2	M	HPV33	NA	CIN2 (14)	24
16	CIN2	M	HPV18	NA	CIN1 (26)	72
17	CIN2	M	HPV16	NA	CIN1 (1)	24
18	CIN2	M	HPV16	NA	NA	
19	CIN2	P	HPV33	CB	DF	60
20	CIN2	P	HPV16	LE	DF	12
21	CIN2	P	HPV16	LE	DF	36
22	CIN2	P	HPV33	CB & LE	DF	108
23	CIN2	P	- ve	CB	DF	72
24	CIN2	P	- ve	NA	DF	108
25	CIN3	M	HPV16	CB & LE	CIN3 (9)	60
26	CIN3	M	HPV18	CB	CIN3 (39)	120
27	CIN3	M	HPV18	CB	CIN3 (23)	120
28	CIN3	M	HPV16	NA	CIN3 (6)	84
29	CIN3	M	HPV33	CB	CIN2 (8)	84
30	CIN3	M	HPV33	CB & LE	CIN1 (11)	24
31	CIN3	M	HPV16	CB	CIN1 (17)	48
32	CIN3	M	HPV33	CB	CIN1 (67)	120
33	CIN3	M	HPV16	CB	NA	

CB: cone biopsy; LE: loop excision; DF: disease free; NA: not available.
*number in brackets indicates the time (months) of the recurrent disease.

4.3.2 Correlation Between Clonality And HPV Subtypes

High-risk HPV subtypes(16,18 and 33) were detected in all monoclonal CIN lesions, including 9 CIN3 and 15 CIN2 and 4 of the 6 polyclonal CIN2 (Table 4.1). HPV 16 accounted for 62% of the positive cases. In 3 CIN2, both HPV 16 and 33 were present. High-risk HPV subtypes were not detected in CIN1 lesions.

4.4 Discussion

CIN lesions show variable clinical behaviour, with approximately 5% of CIN2 lesions progressing to CIN3 or invasive lesions despite treatment (Tsukamoto, 1985;Gunasekera *et al*, 1990). One of the major difficulties in the clinical management of patients with CIN lesions is to identify those that will progress or persist. To date, there are no reliable clinical or molecular markers for CIN progression. We investigated the value of clonality as a prognostic marker retrospectively using archival cervical smears, of which the clinical follow-up was well documented.

By parallel analysis of cervical smears and matched cervical biopsies, we demonstrated in Chapter 2 that PCR-based clonality analysis could be readily applied to archival smears with >10 years of storage time. Our results from

cervical smears are in line with previous findings from studies based on biopsies of cervical carcinoma:

CIN3 and the majority of CIN2 lesions are monoclonal, whereas CIN1 lesions are polyclonal (Enomoto *et al*,1994;Park *et al*,1996; Ko *et al*,1997;Enomoto *et al*,1997 ;Guo[a] *et al*,1998).

We focused on CIN2 lesions since they show a variable clonal pattern. Our results demonstrate that patients with monoclonal CIN2 lesions have either disease persistence or progression despite treatment, while those with polyclonal CIN2 lesions became negative and remained disease free after treatment. There was no apparent difference in the treatment modality between monoclonal and polyclonal CIN2. However, a proportion of polyclonal CIN2 lesions were negative for high risk HPV. It is possible that the different clinical behaviour between the two groups reflects their intrinsic genetic makeup as well as the HPV status.

The incidence of disease relapse in this series was relatively high. This may reflect our selection bias toward those lesions that contained abundant dyskaryotic cells. However, our results indicate that clonality analysis of cervical smears is potentially valuable in the detection of true neoplastic lesions and prediction of the clinical behaviour of preneoplastic lesions in particular CIN2.

In each of the 6 cases examined, both the original CIN2 and subsequent lesions (CIN2 in 3 cases and CIN3 in 3) showed an identical monoclonal pattern, suggesting a clonal link. Thus, it is likely that the recurrent lesions in these cases were due to the relapse of the original CIN rather than emergence of a new neoplastic clone. It remains to be investigated whether tumour relapse results from incomplete excision of a CIN lesion or from the growth of occult neoplastic cells in other part of the cervix. Enomoto et al examined the clonal composition of multiple CIN and carcinoma in situ lesions in 6 cases and demonstrated in each case that the multiple lesions originated from a single clone (Enomoto *et al* ,1997).

Similarly, Guo et al studied the clonal relationship between a synchronous CIN lesion and invasive carcinoma that were topographically well separated and showed that the preinvasive lesion was clonally related to the invasive carcinoma in the majority of cases (Guo *et al*,2000). Nonetheless, unrelated CIN lesions in different parts of the same cervix have been reported (Enomoto *et al*,1997). The possibility that the recurrent CIN lesion results from a clone different from the original CIN exists.

The finding that monoclonal CIN2 lesions tend to relapse despite treatment suggests that they should be treated more "aggressively". Currently, CIN2 lesions are treated by variable approaches, such as laser ablation, cone biopsy and loop excision at colposcopy. For successful treatment, it is critical to accurately estimate the extent of the lesion in the cervix. A systematic examination of CIN lesions, particularly those showing monoclonality, at both the histological and molecular levels would yield critical information to help gynecologists to manage this disease appropriately and avoid over treatment.

In line with previous reports, high-risk subtypes of HPV were significantly associated with CIN3 and CIN2 lesions (Park *et al*,1996;Kaufman *et al*,1999). However, the presence of high-risk HPV subtypes does not distinguish monoclonal CIN2 from polyclonal CIN2, although their incidence is much higher in monoclonal CIN2 than in polyclonal CIN2. Clonality analysis of cervical smears is potentially useful as an additional factor in the prognosis of CIN2.

The significant correlation between clonality and clinical behaviour of CIN lesions strongly suggests that genetic markers can be used in prognosis of CIN. However, application of the clonality assay in routine practice is

hindered by its requirement of a highly purified DNA sample and a relative large number of cells, as well as by the lack of a high throughput approach. Thus, research for other genetic markers are desired.

CHAPTER 5
CIN PROGNOSIS BY COMBINED LOH ANALYSIS OF MULTIPLE LOCI

5.1 Introduction

As discussed in the general introduction and Chapter 4, CIN lesions show markedly variable clinical behaviour despite their apparent morphological homogeneity within the same group (Cirisano, 1999). This presents a challenge for clinical management of patients with CIN i.e. to identify those likely to persist and progress. Using PCR based clonality analysis of the X-chromosome linked AR gene, we showed (Chapter 4) a significant correlation between clonality and clinical behaviour, in particular of CIN2 lesions. However, the application of the clonality assay in routine practice is hindered by its requirement for the highly purified DNA samples and a relative large number of cells, as well as by lack of a high throughput approach. Thus, other molecular prognostic markers are needed.

The prognostic value of HPV typing has been extensively studied. Despite that high risk HPV infection is strongly implicated in the genesis of cervical carcinoma, the role of HPV typing at single points alone in CIN prognosis is limited. This is mainly due to frequent presence of high risk HPV in normal cervical smears (2-10%) (Lorincz *et al*, 1992; Kjaer *et al*, 2002), borderline (10-

50%) and CIN1 (20-80%) lesions (Lorincz *et al*, 1992;Kjaer *et al*, 2002). In addition, HPV infection may be transient (Nobbenhuis *et al*, 1999). On the other hand, persistent infection of high risk HPVs has been shown to be associated with persistence and progression of CIN lesions (Nobbenhuis *et al*, 1999). Monitoring HPV infection every 6 months may help to identify CIN lesions having a high risk of progression.

There is strong evidence that HPV infection alone is insufficient to cause malignant transformation. Growing evidence indicates that genetic changes play a critical role in the transformation of HPV infected cells and may account for the irreversible progression of CIN. A number of chromosomal regions including 3p14.1-p22, 4p16, 4q21-35, 5p13-15, 6p213-22, 6q21-25, 11p15, 11q23, 13q12.3-q13, 17p13.3 and 18q12.2-22 show LOH in cervical carcinoma (Lazo, 1999). LOH at 3p14.2, 3p21-22, 6p21 and 11q23 is frequent in cervical carcinoma (Kersemaekers[a] *et al*, 1998; Kersemaekers[a] *et al*,1998; Skomedal *et al*,1999; Kersemaekers *et al*,1999; Chung *et al*, 2000; ; Pulido *et al*, 2000 Guo[b] *et al*,2000;Chuaqui *et al*, 2001; Harima *et al*,2001; Chatterjee *et al*, 2001;Acevedo *et al*, 2002;Senchenko *et al*,2003) and occurs more often in invasive carcinoma, CIN3 and CIN2 than CIN1 (Guo[b] *et al*, 2000;Chatterjee *et al*, 2001). LOH at 3p14.2 has been reported to be significantly higher in recurrent CIN lesions (Lin[b] *et al*, 2000). In addition, LOH at 3p21 positively correlates with mitotic activity of tumour cells

(Kersemaekers[a] *et al*, 1998). Deletion at 6p21 is significantly associated with poor overall and disease-free survival in patients with cervical carcinoma (Harima *et al*, 2001).

Functional studies have shown that chromosome 11 carries genes that suppress tumourigenic properties of human cervical carcinoma cell lines (Saxon *et al*, 1986). LOH analysis of cervical carcinoma and study of in vitro immortalisation of human keratinocytes by HPV16 indicate that the TSGs are located at 11q23 (Steenbergen *et al*, 1996;Pulido *et al*, 2000). A correlation between LOH at 11q 23.3 with extensive lymphovascular space invasion in radical hysterectomy specimens have been shown by Huettner et al. (Huettner *et al*, 1998). Additionally a significant association between tumours with LOH at 11q21.2, 18q22 and short survival has been reported (Kersemaekers[a] *et al*, 1998;Harima *et al*, 2001).

In view of the above evidence, we hypothesized that allelic deletion at 3p14.2, 3p21-22, 6p21 and 11q23 is critical for development of CIN lesions and may influence their clinical behaviour and thus have prognostic value. To test this, we screened 12 microsatellite markers including 10 from the above chromosomal regions for LOH in 164 cases of CIN and evaluated their value as prognostic markers.

5.2 Materials And Methods

5.2.1 Patient Material

164 cases of CIN (54 CIN1, 59 CIN2 and 51 CIN3) were retrieved from the Department of Histopathology, University College London Hospital (UCLH). They were chosen according to the availability of diagnostic specimens and clinical follow-up data except in the case of cervical smears where those with abundant clumps of dyskaryotic cells were selected in preference for microdissection. The clinical follow-up period ranged between 6 to 387 months, with an average of 34.8 months. The diagnosis of CIN was based on histological tissue biopsy. All patients were treated according to the same protocol at UCLH.

5.2.2 Microdissection And DNA Preparation

The morphology of cervical smears and biopsies in all cases was reviewed before microdissection. Normal and dyskaryotic cells from cervical smears and cervical biopsies were microdissected as described in Section 2.2.2 of Chapter 2. Crude DNA samples were prepared from the microdissected cells as detailed in Section 2.2.3.1 of Chapter 2.

5.2.3 Detection Of LOH By PCR

Twelve microsatellite markers including D3S1566 (3p13), D3S1285 (3p14.1), D3S1300 (3p14.2), D3S1289 (3p21.1),

D3S1260 (3p22.2), D3S1611 (3p22.3), D5S406 (5p15.32), D6S105 (6p21.1), D6S265 (6p21.1), D6S277 (6p24.3), D11S35 (11q22.1) and D11S528 (11q23.3), at which LOH frequently occurs in cervical carcinoma (Kersemaekers[a] *et al*, 1998; Kersemaekers[a] *et al*,1998; Skomedal *et al*,1999; Kersemaekers *et al*,1999; Chung *et al*, 2000; Pulido *et al*, 2000 Guo[b] *et al*,2000;Chuaqui *et al*, 2001; Harima *et al*,2001; Chatterjee *et al*, 2001;Acevedo *et al*, 2002;Senchenko *et al*,2003) were studied. The primers used for PCR of the microsatellite markers are detailed in Table 2.3 of Chapter 2.

Primers were designed to immediately flank the tandem repeats, thus the fragment to be amplified was small, allowing amplification from DNA samples prepared from archival fixed specimens. One of the paired primers was fluorescently labelled and PCR was carried out in a thermal cycler using a "hot-start touch-down" programme as described in Table 2.4 of Chapter 2. The specificity of PCR products was verified on agarose gels and products analyzed on an ABI377 DNA sequencer using the GeneScan software (Version 3.0) as detailed in Section 2.3 of Chapter 2. Dyskaryotic/neoplastic and normal cells from the same cases were analyzed in parallel. Allelic loss was identified by a computer programme when the peak height ratio of tumour to normal alleles was 0.25 or less as described previously (Lakhani *et al*, 1996), then confirmed by visual inspection in each case.

The molecular data were generated without the knowledge of clinical follow-up details.

5.2.4 Detection Of High-Risk HPV

High-risk HPV including 16, 18, 33, 45 and 56 were detected by PCR separately using primers established in previous studies (The primers used for PCR of HPV are shown in Table 2.3 of Chapter 2) (Miller *et al*, 1994;Ostwald *et al*, 1994; Karlsen *et al*, 1996). DNA samples prepared from microdissected dyskaryotic or neoplastic cells were used for HPV detection in each case. Strict laboratory procedures as detailed in Section 2.2.4.2 of Chapter 2, including separate set-up areas for DNA preparation, PCR and post-PCR analysis were followed and serial controls, at various stages of DNA preparations and PCR set-up, were included in each set of experiments to avoid and detect potential cross contamination.

5.2.5 Statistical Analysis

Stepwise analysis of the 12 markers was carried out using two way student t-test to obtain group of markers that show difference between disease free and disease progression. This was done by progressively analysing the 12 markers in stepwise manner until a group of markers of $p < 0.05$ was obtained.
Student t-test on the group of markers showing statistical significance was used for comparison of age and follow-up time

between CIN lesions showing different clinical behaviour. $\chi 2$ was used to analyse the relationship between LOH and clinical outcome, HPV status and treatment methods.

5.3 Results

5.3.1 Identification of Loci at which LOH is potentially valuable in CIN prognosis

Primers for microsatellite analysis were designed to amplify the minimum fragment containing the tandem repeat, thus suitable for amplification of DNA samples prepared from archival specimens. PCR was successful for all DNA samples prepared from cervical smears and 95% of those from tissue biopsies. For cases that were failed for PCR in the first instance, repeat PCR with various amounts of template DNA were carried out and those without successful PCR amplification were excluded from the analysis.

To identify the loci at which LOH was associated with disease behaviour and may thus have prognostic value, we first screened 12 microsatellite markers and correlated LOH with clinical outcome in 71 cases of CIN (15 CIN1, 22 CIN2 and 34 CIN3) using diagnostic cervical smears. Patients were divided into two groups: those that became disease free (DF) within 6 month after the first treatment and those that showed disease persistence (DP) at the same or a higher CIN grade 6 months after the initial treatment. The average time showing the disease recurrence was

26 months, ranging from 6 to 90 months. As resection margin could not be reliably evaluated in each case, no attempt was made to correlate the extent of tumour involvement with the outcome of treatments.

As the number of cases examined from each CIN group was relatively small, we correlated LOH with clinical outcome irrespective of CIN grade. Among the 12 markers investigated, D3S1300 (3p14.2), D3S1260 (3p22.2) and D11S528 (11q23.3) showed LOH at significantly higher frequencies in the DP than the DF group ($p<0.01$, Figure 5.1A). D11S35 (11q22.1) exhibited the next highest statistical difference between the two groups although not at a significant level. Using a stepwise statistical analysis, various combination of the 12 markers were tested and the above four markers collectively gave the highest statistical significance ($p<0.02$) between the two groups and were selected for further analysis. As the value of using these loci alone as prognostic markers was limited, we tested the 4 makers together (Figure 5.1B). By stepwise examination of the sensitivity and specificity of the combined 4 LOH markers, i.e. 1 or 2 or 3 or all of the 4 loci showing LOH, the best cut-off point was when 2 of the 4 loci showing LOH was applied. At this threshold, around 45-58% of CIN lesions in the DP group but none in the DF group were identified.

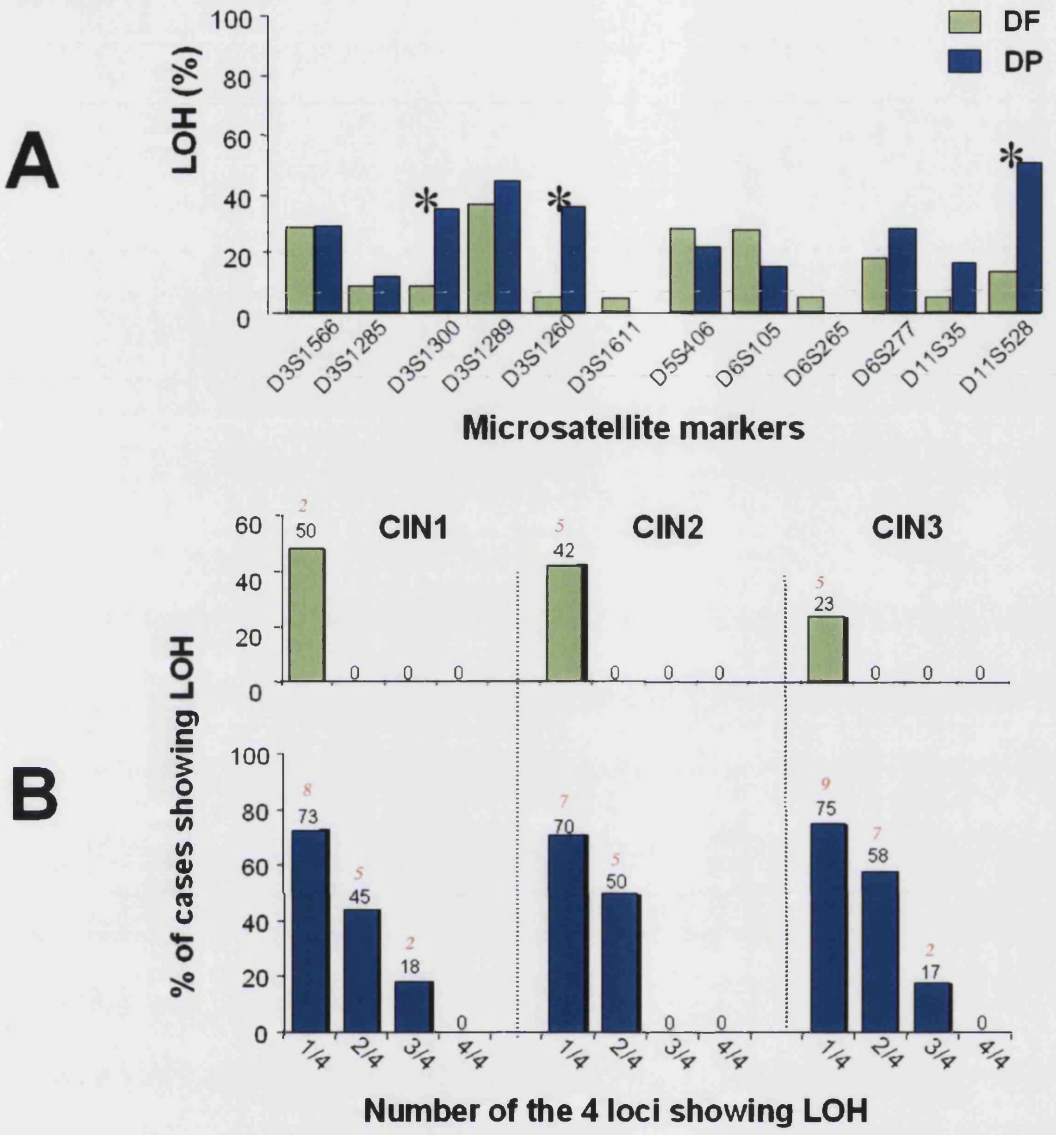

Figure 5.1: Identification of LOH loci that are potentially valuable in CIN prognosis. (Values in black represent percentage LOH and in red represent number of cases)

A: Frequencies of LOH at 12 microsatellite markers between CIN lesions showing disease free (DF) or disease persistence (DP) after initial treatment. *The incidence of LOH at D3S1300 (3p14.2), D3S1260 (3p22.2) and D11S528 (11q23.3) is significantly higher in the DP than the DF group ($p<0.01$ in each). D11S35 (11q22.1) exhibited the next highest statistical difference between the two groups, although not at a significant level. Using a stepwise statistical analysis testing various combination of the 12 markers, the above four markers collectively gave the highest statistical significance ($p<0.02$) between the two groups and were further analysed as detailed in Figure 1B.

B: Prognostic value of combined LOH analysis at the above 4 loci. By stepwise testing the sensitivity and specificity of the combined 4 LOH markers in CIN prognosis, i.e. 1 or 2 or 3 or 4 of the 4 loci showing LOH, the best cut-off point is LOH at 2 of the 4 loci. At this threshold, between 45-58% of CIN lesions in the DP group but none in the DF group show LOH.

5.3.2 Prognostic Value Of LOH At D3S1300 (3p14.2), D3S1260 (3p22.2), D11S35 (11q22.1) And D11S528 (11q23.3) In CIN

To further confirm the prognostic value of LOH at the above 4 markers in CIN, we examined a further 93 cases using diagnostic tissue biopsies since it was easier to microdissect neoplastic cells from biopsies than cervical smears. LOH was correlated with clinical follow-up as above. The data from these additional cases were compatible to those obtained from the cervical smears in the pilot study although the LOH frequency in the DP group was higher in the cervical smears than tissue biopsies particularly for the CIN1 and CIN2 lesions (Figure 5.1B and Figure 5.2C). This most likely reflected the selection of cervical smears with abundant clumps of dyskaryotic cells, which may bias selection of those patients with extensive disease, and the number of the cases examined in the pilot study was small. No statistical differences were found between the age, HPV status, treatment modalities and follow-up time between cervical smears and tissue biopsies.

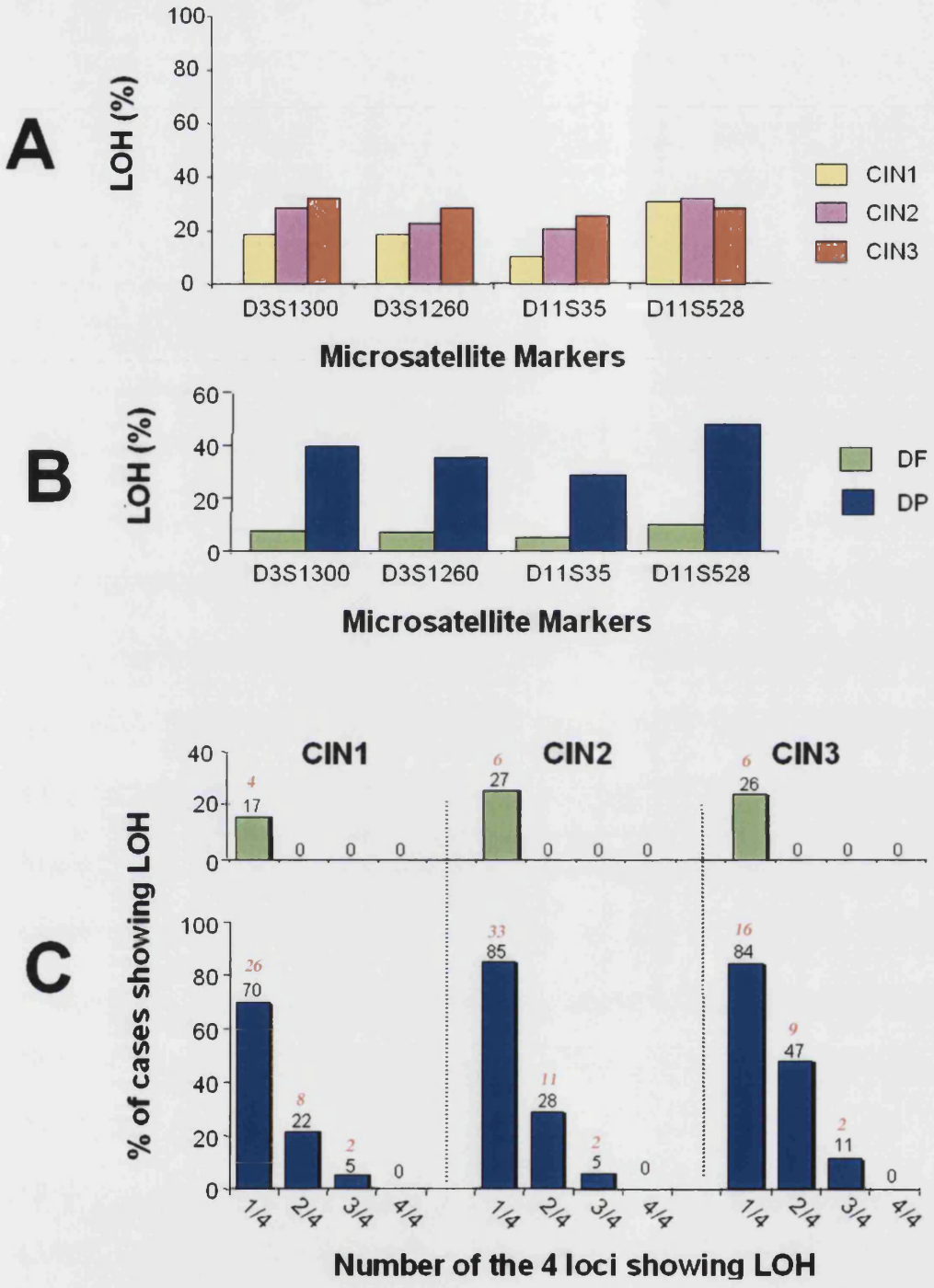

Figure 5.2: Prognostic value of LOH at D3S1300 (3p14.2), D3S1260 (3p22.2), D11S35 (11q22.1) and D11S528 (11q23.3). (Values in black represent percentage LOH and in red represent number of cases).

A: Correlation of LOH with CIN grade. LOH at D3S1300, D3S1260 and D11S35 correlates positively with the grade, while the incidence of LOH at D11S528 is similar among different CIN lesions.
B: Comparison of LOH between CIN lesions showing disease free (DF) or disease persistence (DP).
C: By stepwise testing the sensitivity and specificity of the combined 4 LOH markers in CIN prognosis, i.e. 1 or 2 or 3 or 4 of the 4 loci showing LOH, the best cut-off point is LOH at 2 of the 4 loci. At this threshold, between 22-47% CIN lesions of the DP groups can be identified with 100% specificity.

In total, we examined 164 cases of CIN (54 CIN1, 59 CIN2 and 51 CIN3). The frequency of LOH at D3S1300, D3S1260 and D11S35 positively correlated with CIN grade, while the incidence of LOH at D11S528 was similar among different CIN groups (Figure 5.2A). As expected, the frequency of LOH at these loci was significantly higher in the DP than the DF group ($p<0.0005$, Figure 5.2B). By combining the 4 markers together as above, 22% of CIN1, 28% of CIN2 and 47% of CIN3 of the DP group but none of the DF group showed LOH at 2 or more of the 4 loci examined (Figure 5.2C). Thus, LOH analysis at these loci could identify 22-47% of CIN lesions that showed disease persistence despite treatment.

The percentage of infection of high risk HPV was significantly higher in the DP (90%) than in the DF group (43%) ($p<0.0005$) (Table 5.1A). Among different high risk HPV, HPV16 infection was significantly associated with the DP group ($p<0.001$) despite the fact that we may have underestimated its real incidence, particularly in samples prepared from paraffin-embedded tissue specimens, since the fragment to be amplified is relatively large (398bp). Interestingly, only HPV16 was significantly associated with LOH at each of the four loci examined ($p<0.01$), particularly D3S1300 ($p<0.0005$) (Table 5.1B). Treatment information was available in 102 cases including 47 and 55 from the DF and DP group respectively. Laser ablation was more frequently used in the DF than the DP group ($p<0.02$), whereas large loop excision

of the transformation zone (LLETZ) was applied more often to the DP (62%) than the DF group (21%) ($p<0.005$) (Table5.1A). There were no significant differences in other treatment modalities, age and follow-up time between the two groups.

Table 5.1A: Clinical features of CIN lesions between the DF and the DP group.

	DF		DP	
Number of cases	75		89	
Age (year)				
Mean	44		36	
Range	31-66		23-56	
High risk HPV infection				
Number of cases with high risk HPV*	32	43%	80	90%
HPV16*	1	1%	26	29%
HPV18	9	12%	18	20%
HPV33	15	20%	18	20%
HPV45	5	7%	9	10%
HPV56	1	1%	2	2%
Double virus	1	1%	7	8%
Treatment				
Number of cases with available data	47		55	
Cold Coagulation	1	2%	0	-
Laser ablation*	15	32%	3	6%
Cone biopsy	21	45%	18	32%
LLETZ *	10	21%	34	62%
Follow up				
Mean	34		36	
Range	16-376		6-387	

* Significant difference between DF and DP groups.
LLETZ: Large loop excision of the transformation zone

Table 5.1B: Correlation of HPV16 infection with the four markers.

Marker		LOH +ve	LOH -ve	p-value
D11S528	HPV +ve	24	24	0.002152
	HPV -ve	36	87	
		LOH +ve	LOH -ve	p-value
D11S35	HPV +ve	20	30	0.002326
	HPV -ve	28	106	
		LOH +ve	LOH -ve	p-value
D3S1300	HPV +ve	27	24	0.000653 *
	HPV -ve	32	102	
		LOH +ve	LOH -ve	p-value
D3S1260	HPV +ve	23	29	0.004165
	HPV -ve	26	100	

* Marker with the most significant association between HPV and LOH.
The analysis is carried out using χ^2 (Chi-squared) between LOH and HPV for each marker.

To further evaluate the association of LOH with clinical outcome of CIN, we examined both diagnostic and follow-up biopsies in 22 cases from the DP group. Of these cases, 5 (numbers 1-5) became disease free after the first follow-up biopsy (7-15 months) and none of them showed accumulation of LOH in the follow-up biopsy (Table 5.2). The remaining 17 cases showed disease persistence at the same (8 cases) or higher CIN grade (9 cases) during follow-up, and 13 cases (numbers 9-21) displayed LOH at additional loci (Figure 5.3). The average time to gain LOH at an additional locus was 30 months, ranging from 5 to 93 months. Except case 22, the LOH observed in the diagnostic sample was always seen in the follow-up biopsy (Table 5.2). In

case 22, LOH was seen at D3S1260 and D11S35 in the diagnostic biopsy but not in the follow-up biopsy three years later, suggesting that the two CIN lesions may not be clonally related.

In 6 cases (numbers 12-14, 16-18), the diagnostic biopsy showed no LOH or LOH at only 1 of the 4 loci, but the follow-up biopsy displayed LOH at 2 or more of the 4 loci, reaching the threshold of prognostic significance as detailed above. Interestingly, two of these cases (numbers 17 and 18) also showed infection by an additional high-risk HPV.

Table 5.2A: Comparison of LOH and HPV status between diagnostic and followup specimens.

	Diagnosis	Time (year-month)	Treatment	HPV	D3S1300	D3S1260	D11S35	D11S528
1	CIN3	1995-6	Cone biopsy	33	+	H	-	H
	CIN3	1996-3	Cone biopsy	33	+	H	-	H
2	CIN2	1999-5	LLETZ	45	-	H	-	-
	CIN2	1999-12	NA	45	-	H	-	-
3	CIN1	1998-9	No	18	-	-	-	+
	CIN1	1999-12	NA	18	-	-	-	+
4	CIN1	1989-8	No	33	-	+	-	-
	CIN2	1990-6	LLETZ	33	-	+	-	-
5	CIN1	1997-10	LLETZ	33	-	-	H	-
	CIN1	1998-6	LLETZ	NA	-	-	H	-
6	CIN2	1994-11	LLETZ	33	-	-	+	-
	CIN2	1995-5	NA	33	-	-	+	-
7	CIN2	1998-11	No	16	-	+	-	-
	CIN3	2000-2	LLETZ	16	-	+	-	-
8	CIN1	1994-7	LLETZ	18	+	-	H	-
	CIN1	1995-3	NA	18	+	-	H	-
9	CIN2	1992-2	Cone biopsy	18	-	H	-	-
	CIN2	1998-11	NA	18	-	H	-	+
10	CIN1	1997-9	NA	18	-	-	H	-
	CIN2	1999-4	NA	18	-	-	H	+
11	CIN1	1997-4	LLETZ	33	-	H	-	-
	CIN3	2000-9	Cone biopsy	33	-	H	-	+
12	CIN1	1991-10	No	16	-	-	+	-
	CIN3	1995-1	LLETZ	16	+	-	+	-
13	CIN2	1995-7	No	16	+	-	-	-
	CIN3	1998-4	LLETZ	16	+	-	-	+
14	CIN1	1995-3	No	NA	-	-	-	-
	CIN1	1996-10	No	NA	-	+	+	+
15	CIN1	1989-4	No	16	+	-	-	+
	CIN3	1992-8	LLETZ	33	+	-	+	+
16	CIN2	1988-11	Cone biopsy	16	-	-	-	+
	CIN3	1992-2	Cone biopsy	16	-	+	+	+
17	CIN3	1991-5	Cone biopsy	18	-	-	+	-
	CIN3	1994-11	Cone biopsy	18+16	-	+	+	-
18	CIN2	1991-10	Cone biopsy	16	+	H	-	-
	CIN3	1997-10	LLETZ	16+45	+	H	+	-
19	CIN3	1989-11	Cone biopsy	16	+	-	+	-
	CIN3	1991-4	NA	16+18	+	+	+	-
20	CIN1	1989-6	NA	18	+	-	-	+
	CIN1	1989-12	LLETZ	18	+	-	+	+
21	CIN1	1990-4	NA	16	+	-	+	+
	CIN2	1994-4	NA	16	+	-	+	+
	CIN3	1994-9	NA	16	+	+	+	+
22	CIN3	1990-1	NA	33	-	+	+	-
	CIN3	1993-1	NA	33	NA	-	-	-

Cases 1-5 became disease free after the first follow-up biopsy; Cases 7-21 continually showed disease persistence or progression after the first follow-up biopsy (data shown in table 5.2B). NA: not available; +: LOH positive; -: LOH negative; H: homozygous.

Table 5.2B: Cases with "worst recurrence" after second biopsy.

Case No.	Diagnosis	Time (year)	Treatment
7	CIN2	1998	No
	CIN3	2000	LLETZ
	CIN3	2001	
8	CIN1	1994	LLETZ
	CIN1	1995	NA
	CIN1	1995	
9	CIN2	1992	Cone biopsy
	CIN2	1998	NA
	CIN1	2000	
10	CIN1	1997	NA
	CIN2	1999	NA
	CIN2	1999	
11	CIN1	1997	LLETZ
	CIN3	2000	Cone biopsy
	CIN3	2001	
12	CIN1	1991	No
	CIN3	1995	LLETZ
	CIN3	1996	
13	CIN2	1995	No
	CIN3	1998	LLETZ
	CIN1	2000	Cervical biopsy
14	CIN1	1995	NA
	CIN1	1996	NA
	CIN1	1997	
15	CIN1	1989	No
	CIN3	1992	LLETZ
	CIN2	1993	
16	CIN2	1988	Cone biopsy
	CIN3	1992	Cone biopsy
	CIN3	1993	
17	CIN3	1991	Cone biopsy
	CIN3	1994	Cone biopsy
	CIN3	1995	
18	CIN2	1991	Cone biopsy
	CIN3	1997	LLETZ
	CIN2	1998	
19	CIN3	1989	Cone biopsy
	CIN3	1991	NA
	CIN3	1991	
20	CIN1	1989	NA
	CIN1	1989	LLETZ
	CIN1	1991	
21	CIN1	1990	NA
	CIN2	1994	NA
	CIN2	1994	LLETZ
	CIN3	1995	

Table 5.3: Worst outcome of cases with an original diagnosis of CIN1, CIN2 and CIN3.

Original Diagnosis	Followup Outcome		
	CIN1	CIN2	CIN3
CIN1	15	15	5
CIN2	0	9	26
CIN3	0	0	13

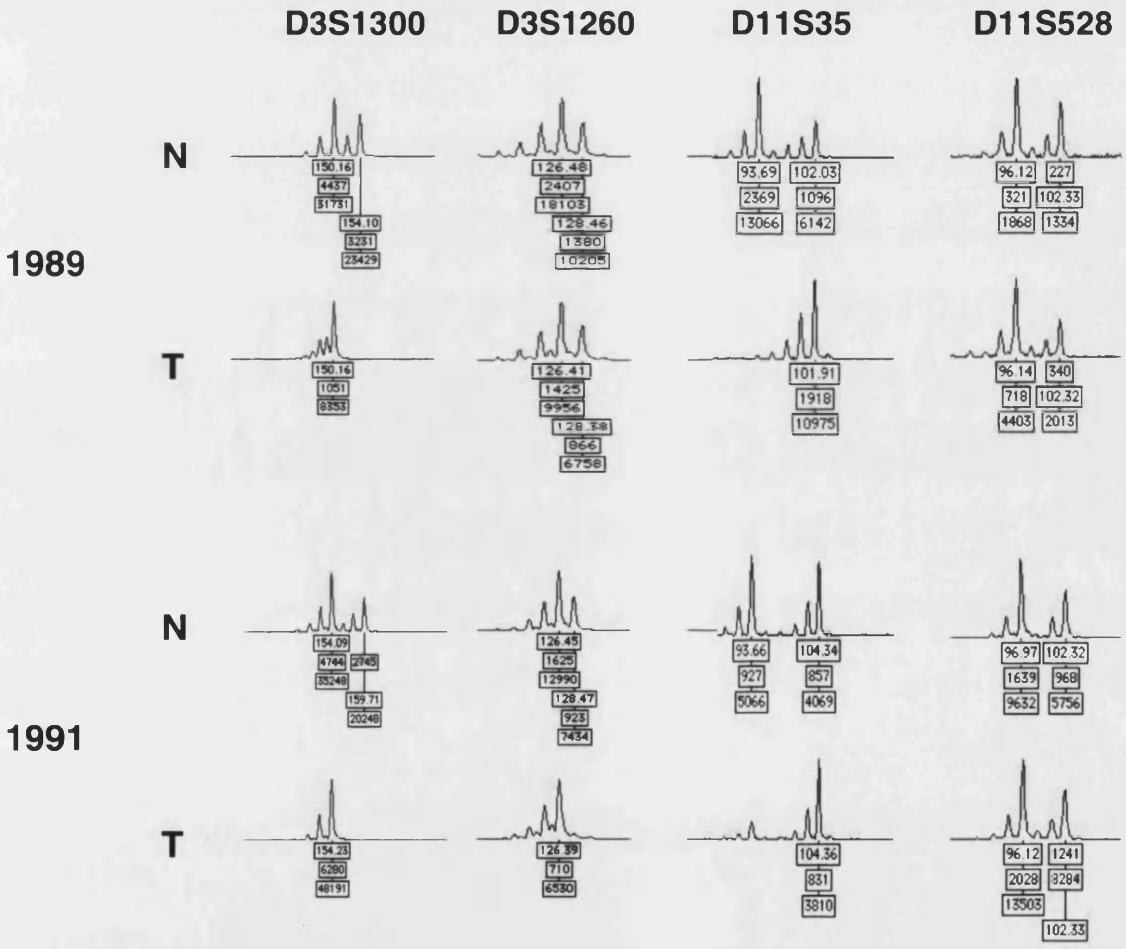

Figure 5.3: Example of LOH analysis. The diagnostic biopsy (CIN3) of case 19 shows LOH at D3S1300 and D11S35. Despite treatment, the patient continually presented with CIN3. The follow-up biopsy 17 months after diagnosis displays additional LOH at D3S1260. N: normal cells; T: tumour cells.

5.3.3 Putative Tumour Suppressor Genes At D3S1300 (3p14.2), D3S1260 (3p22.2), D11S35 (11q22.1) And D11S528 (11q23.3)

Bioinformatic analysis of the genomic regions at D3S1300, D3S1260, D11S35 and D11S528 identified putative genes that may be target of the deletions. LOC152071 (an EST clone), FHIT (fragile histidine triad gene) and NPCR (nasopharyngeal carcinoma related protein) are at or in the vicinity of D3S1300. Since FHIT is frequently deleted in a variety of human carcinomas including cervical carcinoma (Yoshino *et al*, 2000;Butler *et al*, 2000;Helland *et al*, 2000), it is a likely target of the deletion. Among the genes in the vicinity of D3S1260 including ORCTL (organic cationic transporter-like 4) and XYLB (Xylukokinase homolog), DLEC1 (deleted in lung and esophageal carcinoma 1) may be relevant as it is associated with carcinoma. AD031, PR (progesterone receptor) and TRPC6 (transient receptor potential cation channel, superfamily C, member 6) are at or in the vicinity of D11S35. PR appears to be the most relevant given its role in inhibition of human endometrial carcinoma. Finally, TRIM29 (tripartite motif-containing 29) and several hypothetical genes are around D11S528. TRIM29 has multiple zinc finger motifs and a leucine zipper motif and may act as a transcriptional regulatory factor.

5.4 Discussion

Allelic deletions at several chromosomal loci including 3p14.2, 3p21-22, 6p21 and 11q23 are significantly associated with high grade CINs and invasive cervical carcinomas (Kersemaekers[a] *et al*, 1998; Kersemaekers[a] *et al*,1998; Skomedal *et al*,1999; Kersemaekers *et al*,1999; Chung *et al*, 2000; ; Pulido *et al*, 2000 Guo[b] *et al*,2000;Chuaqui *et al*, 2001; Harima *et al*,2001; Chatterjee *et al*, 2001;Acevedo *et al*, 2002;Senchenko *et al*,2003) However, whether LOH at these loci can be used to determine the prognosis of CIN is largely unknown. We investigated retrospectively the prognostic value of LOH markers in CIN lesions based on archival cell and tissue material. The molecular analyses were performed without the knowledge of clinical data and LOH was recorded by a computer programme established in previous studies (Lakhani *et al*, 1996), thus avoiding subjective bias. All cases were retrieved from a single teaching hospital and were managed clinically according to the standard treatment protocol. There was no significant difference in the age, nature of the specimen (cervical smear or tissue biopsy) and follow-up time between the two groups.

To identify the loci at which LOH may have prognostic value in CIN, we first screened 12 microsatellite markers and correlated LOH with clinical behaviour of CIN lesions in a pilot study.

D3S1300, D3S1260, D11S35 and D11S528 showed potential prognostic value and were further investigated in a larger cohort. Although independently LOH at these loci did not allow a clear separation between the DF and the DP groups, combined analysis of the four LOH markers permitted identification of a significant proportion of the DP group with high specificity. The best cut-off point was 2 of 4 loci showing LOH. At this threshold, 22-47% of CIN lesions in the DP group could be identified with 100% specificity.

The association of LOH at these loci with clinical behaviour of CIN was further reinforced by analysis of follow-up biopsies in cases from the DP group. None of the 5 cases that became disease free after the first follow-up biopsy exhibited accumulation of LOH in the follow-up specimens. In contrast, 13 of the 17 cases that had disease persistence or progression showed, accumulation of LOH at additional loci in the follow-up specimens and in 6 cases the prognostic value of LOH reached the threshold of significance. The results strongly indicate that LOH analysis of follow-up specimens could further segregate CIN lesions with different clinical outcome. It remains to be determined when is the best interval to implement such analysis although our study showed that an average of 30 months was required for acquisition of an additional LOH at the 4 loci examined.

Infection of high-risk HPV is significantly associated with increased risk of cervical carcinoma development. In support of this, our results showed that the percentage of infection of high risk HPVs, particularly HPV16 and double viruses, was higher in the DP than the DF group. Furthermore, HPV16 infection significantly correlated with LOH at each of the four markers examined, particularly D3S1300 as shown in table 5.1B. HPV16 integrations in cervical carcinomas preferentially target common fragile sites including FRA3B (3p14.2) where the FHIT gene and D3S1300 are located, and are accompanied by deletion of cellular genes (Wilke *et al*, 1996;Thorland *et al*, 2003). Thus, LOH at these loci may be directly attributed to HPV16 infection in some cases.

The significant association of LOH at D3S1300 (3p14.2), D3S1260 (3p22.2), D11S35 (11q22.1) and D11S528 (11q23.3) with poor prognosis of CIN strongly suggests that these markers may be at or in the vicinity of tumour suppresser genes. Bioinformatic analysis of the genomic region at these loci identified several genes including two known genes FHIT and PR, which could be the target of deletions.

FHIT, spanning the FRA3B fragile site and the breakpoint of t(3;8) of familial renal carcinoma, has been proposed as a tumour

suppresser gene (Ohta *et al*, 1996). Deletion and reduced or absent expression of the FHIT gene occur in a wide range of human carcinomas including cervical carcinoma (Birrer *et al*, 1999;Yoshino *et al*, 2000;Butler *et al*, 2000;Helland *et al*, 2000). In CIN lesions, LOH and reduced or absent FHIT expression positively correlate with the histological grade. Moreover, LOH is associated with reduced FHIT expression (Birrer *et al*, 1999; Connolly *et al*, 2000;Butler *et al*, 2002). Our results reinforce the association of FHIT gene deletion with progression of CIN and further indicate that deletion of the gene has prognostic value.

PR, encodes two isoforms, PR-A and PR-B, functioning as ligand activated transcriptional factors. PR-A and PR-B have different transcription activation properties and play distinctive roles in different tissues (Connolly *et al*, 2000). PR-B acts as a transcriptional activator and is important for normal proliferative response of mammary glands to progesterone, while PR-A functions as a transcriptional repressor and is critical for progesterone dependent reproductive responses in uterus and ovary (Connolly *et al*, 2000). PR-A represses the activity of PR-B and other steroid receptors including oestrogen receptor alpha (Connolly *et al*, 2000), which may underlie the mechanisms of progesterone mediated antiproliferative effect in endometrial carcinoma. Deletion of the PR gene and loss of its expression are

associated with aggressive endometrial carcinoma and epithelial ovarian tumours. In cervical carcinoma, reduced PR expression has been found in cervical carcinoma in comparison with normal cervix. Taken these data together, PR is likely to be the target of deletion at D11S35. This is in line with the suggestion by both epidemiological and laboratory studies that steroid hormones play a role in development of squamous carcinoma of the cervix (Moodley *et al*, 2003).

In summary, we have identified four markers at which LOH is significantly associated with the clinical behaviour of CIN lesions. Combined analysis of LOH at these loci could identify at least a third of CIN lesions that persist or progress despite treatment. In view of the enhanced power of combined LOH analysis of multiple loci, addition of other molecular markers of prognostic significance should further improve the sensitivity of the current assay and eventually allow development of a prognostic test for CIN lesions, Leading to improved patients management.

Hence the prognostic markers will only be effective if used at critical times of the evolution of the lesion. It is yet to be determined when is the best interval to implement such analysis. Although our study showed that average time for the lesion to show recurrence was 26 months (6-90 months). Whereas average time to gain LOH is 30 months (5-93 months). So for a lesion to

progress many genetic changes should occur; LOH being one of them according to our results.

It is interesting to note that LOH was seen in a proportion of CIN1 lesions, suggesting that these CIN1 lesions maybe monoclonal. This appears to be different from the observation of clonality analysis with the X-linked androgen receptor gene where polyclonal pattern was shown in 3 of the 3 CIN1 lesions examined. The discrepancy is most likely due to: 1) the number of cases studied by clonality analysis is small, and 2) LOH analysis is more sensitive than clonality analysis in detection of monoclonal cells.

Chapter 6

General Discussion

The overall objective of this thesis was to find a prognostic marker for CIN. The specific aims were threefold: 1) to investigate the suitability of archival cervical smears for PCR-based molecular investigations; 2) to examine the prognostic value of PCR-based clonality analysis of the X-linked androgen receptor gene in CIN; and 3) to screen a series of microsatellite markers, which show high frequencies of LOH in cervical carcinoma, in a large cohort of CIN, identify those associated with CIN persistence and/or progression and examine their combined value in prognosis of CIN.

Currently, there is no biochemical or molecular marker, which can distinguish same grade CIN with different clinical behaviour.

The prognostic value of HPV typing has been extensively studied. Despite that high risk HPV infection is strongly implicated in the genesis of cervical carcinoma, the role of HPV typing alone in CIN prognosis is limited. This is mainly due to frequent presence of high risk HPV in normal cervical smears (Lorincz *et al*, 1992;Kjaer *et al*, 2002), and borderline lesions (Lorincz *et al*, 1992;Kjaer *et al*, 2002). In addition, HPV infection may be frequently transient (Nobbenhuis *et al*, 1999).

To identify potential molecular markers that may serve as a prognostic marker for CIN, we have carried out a series of studies based on archival cervical smears containing CIN lesions, for which appropriate clinical follow-up data were available. First, we have established and optimized several molecular methods on archival cervical smears, which provided a basis for subsequent investigations.

By systematically testing different DNA extraction methods, we have demonstrated that PCR amplification of genomic DNA sequences can be applied to crude DNA preparation from minute cells microdissected from archival smears. When the DNA segment to be amplified is restricted to 250 bp or less, most if not all archival smears, are suitable for PCR. If it is necessary to target a slightly larger fragment, archival cervical smears are still a valuable resource because majority of cases can be used for PCR up to 460 bp. In fact, the quality of DNA samples prepared from archival cervical smears is much better than those prepared from archival formalin-fixed and paraffin-embedded tissues, from which it is often difficult to amplify fragments larger than 300 bp (Jackson *et al*, 1990;Coates *et al*, 1991). Thus, crude DNA preparation from minute microdissected cells should meet the need of most common PCR-based molecular analyses, such as screening for gene mutation, LOH, and HPV typing.

After purification, DNA samples prepared from microdissected dyskaryotic cells can be readily digested with restriction enzymes, such as HapII, are therefore suitable for PCR-based clonality analysis of the X-chromosome inactivation pattern. By parallel analysis of cervical smears and matched cervical biopsies, we have shown that PCR-based clonality analysis can be readily applied to archival smears with more than 10 years of storage time (el Hamidi *et al*, 2003). Furthermore, we have shown that microdissected dyskaryotic cells from archival cervical smears are suitable for RT-PCR. However, the fragment to be amplified by RT-PCR should be restricted to less than 150 bp (Hamidi *et al*, 2002;Liu *et al*, 2002).

Having established the methods for molecular investigations of archival cervical smears, we have retrospectively examined the prognostic value of clonality in archival cervical smears. Our results from cervical smears are in line with previous findings from studies based on cervical carcinoma biopsies: CIN3 and the majority of CIN2 lesions are monoclonal, whereas CIN1 lesions are polyclonal (Enomoto *et al*, 1994; Park *et al*, 1996; Enomoto *et al*, 1997;Ko *et al*, 1997;Guo[b], et al. 1998).

Importantly, we have shown that clonality may be valuable in prognosis of CIN2 lesions. All patients with monoclonal CIN2 lesions showed either persistence or progression of the disease during follow-up, despite treatment. In contrast, patients with

polyclonal CIN2 lesions became negative after treatment and remained disease free during follow-up.

It is interesting to note that the presence of high-risk HPV subtypes did not distinguish monoclonal CIN2 from polyclonal CIN2, although their incidence is much higher in monoclonal CIN2 than in polyclonal CIN2.

The significant correlation between clonality and clinical behaviour of CIN2 lesions strongly indicates that genetic markers can be used for CIN prognosis. However, application of the clonality assay in routine practice is hindered by its need for highly purified DNA samples, a relatively large number of cells, and a lack of high throughput approach. Thus, other genetic markers are desired. We therefore retrospectively investigated the prognostic value of LOH markers in CIN.

To identify the loci at which LOH may be of prognostic value in CIN, we reviewed the literature and identified the chromosomal regions and microsatellite markers that show high incidences of LOH in cervical carcinoma, and where possible in CIN lesions. We have selected 12 microsatellite markers including 10 from 3p14.2, 3p21-22, 6p21 and 11q23, which show high frequency LOH in cervical carcinoma, and screened them for LOH in various CIN lesions (Kersemaekers[a] *et al*, 1998; Kersemaekers[a] *et al*,1998; Skomedal *et al*,1999; Kersemaekers *et al*,1999; Chung *et al*, 2000; ; Pulido *et al*, 2000 Guo[b] *et al*,2000;Chuaqui *et al*, 2001; Harima *et al*,2001; Chatterjee *et al*, 2001;Acevedo *et al*,

2002;Senchenko *et al*,2003). The initial screening was based on 71 cases of CIN (15 CIN1, 22 CIN2 and 34 CIN3) using cervical smears. The frequency of LOH at various microsatellite markers was compared between CIN that showed disease free and those that showed disease persistence or progression after treatment. The aim of the pilot study was to identify microsatellite markers that may have potential prognostic value for CIN.

Among the 12 markers examined, D3S1300 (3p14.2), D3S1260 (3p22.2) and D11S528 (11q23.3) showed LOH at significantly higher frequencies in the DP than DF group ($p<0.01$), (Figure 5.1A). D11S35 (11q22.1) exhibited the next highest statistical difference between the two groups although not at a significant level.

Using a stepwise statistical analysis testing various combination of the 12 markers, the above four markers collectively gave the highest statistical significance ($p<0.02$) between the two groups and were selected for further analysis. Given that the value of using these loci alone as prognostic markers was limited, we tested the four markers together (Figure 5.1B). By stepwise examination of the sensitivity and specificity of the combined 4 LOH markers, i.e. 1 or 2 or 3 or all of the 4 loci showing LOH, the best cut-off point was when 2 of the 4 loci showing LOH was applied. At this threshold, around 45-58% of CIN lesions in the DP group but none in the DF group were identified.

Having identified the four microsatellite markers that are potentially valuable in CIN prognosis, we have further validated the prognostic value of these markers in additional 93 cases of CIN. To make the experiment easier, we have used diagnostic biopsies rather than cervical smears since it was much easier to microdissect neoplastic cells from the former. The results obtained from biopsies were compatible to those from the cervical smears in the pilot study.

In total, we have screened 164 cases of CIN including 54 CIN1, 59 CIN2 and 51 CIN3 for LOH at D3S1300, D3S1260, D11S35, and D11S528. The frequency of LOH at D3S1300, D3S1260, and D11S35 positively correlated with CIN grade, while the incidence of LOH at D11S528 was similar among different CIN groups (Figure 5.2A). As expected, the frequency of LOH at these loci was significantly higher in the DP group ($p<0.0005$, Figure 5.2B). By combining the 4 markers together, 22% of CIN1, 28% Of CIN2 and 47% of CIN3 of the DP group but none of the DF group showed LOH at 2 or more of the 4 loci examined (Figure 5.2C). Thus, LOH analysis at these loci could identify 22-47% of CIN lesions that showed disease persistence despite treatment.

To further evaluate the association of LOH with clinical outcome of CIN, we have examined both diagnostic and follow-up biopsies in 22 cases from the DP group. None of the 5 cases that became disease free after the first follow-up biopsy exhibited

accumulation of LOH in the follow-up specimens. In contrast, 13 of the 17 cases that had disease persistence or progression showed accumulation of LOH at additional loci in the follow-up specimens. In 6 cases, the prognostic value of LOH was not significant in the diagnostic specimens but reached the threshold of significance in the follow-up biopsies (Table 5.2). The results strongly indicate that LOH analysis of follow-up specimens could further segregate CIN lesions with different clinical outcome. However, it remains to be determined when is the best interval to implement such analysis. Our preliminary study showed that an average of 30 months was required for acquisition of an additional LOH at the 4 loci examined.

The finding of a significant association of LOH at D3S1300 (3p14.2), D3S1260 (3p22.2), D11S35 (11q22.1) and D11S528 (11q23.3) with poor prognosis of CIN strongly suggests that these markers may be at or in the vicinity of TSGs. We have performed bioinformatic analysis of the genomic region at these loci attempting to identify the potential genes targeted by deletion. We have found two known genes FHIT and *PR*, which could be the target of deletions as shown by LOH at D3S1300 and D11S35 respectively.

FHIT has been proposed as a TSGs (Ohta *et al*, 1996). Deletion of the gene and reduced or absent of its protein expression have been found in a wide range of human carcinomas including

cervical carcinoma (Birrer *et al*, 1999;Yoshino *et al*, 2000;Butler *et al*, 2000; Helland *et al*, 2000;Baykal, 2003). In CIN, LOH and reduced or absent FHIT protein expression positively correlate with the histological grade. Moreover, LOH is associated with reduced FHIT protein expression (Birrer *et al*, 1999; Connolly *et al*, 2000;Butler *et al*, 2002). Interestingly, HPV16 integrations in cervical carcinomas preferentially target common fragile sites including FRA3B (3p14.2) where the FHIT gene and D3S1300 are located, and are accompanied by deletion of cellular genes (Wilke *et al*, 1996;Thorland *et al*, 2003). We have found that HPV16 infection significantly correlates with LOH at D3S1300. Taken together, our results reinforce the association of FHIT gene deletion with progression of CIN and further indicate that deletion of this gene has a prognostic value.

Deletion of the *PR* gene and loss of its expression are associated with aggressive endometrial carcinoma and epithelial ovarian tumours. Reduced PR expression has been found in cervical carcinoma in comparison with normal cervix .Taken these data together, PR is likely to be the target of deletion at D11S35.

In summary, 1) We have established and optimized several PCR based molecular methods on archival cervical smears, which provides a basis for molecular investigations of archival pathological specimens; 2) We evaluated the prognostic value of clonality in archival cervical smears, and found a significant

correlation between clonality and clinical behavior of CIN2 lesions; 3) We identified four microsatellite markers at which LOH is significantly associated with the clinical behaviour of CIN.

6.1 Future work

Using molecular markers in large cohort of CIN lesions may identify new groups of CIN lesions with different clinical behaviour. This may help in updating the morphological grading system and making it more clinically relevant. Investigating the D6S277 LOH marker by carrying out LOH analysis in similar manner to the 4 LOH markers done in this thesis. Testing the combined prognostic values of the LOH markers by developing a robust high throughput assay.

Also, performing extensive bioinformatics study to identify the genetic network that links the genes associated with the LOH markers, once the network is identified, a pathway would be identified leading to better understanding of CIN molecular mechanism.

References

Abdul-Karim FW, Fu YS Reagan JW Wentz WB. (1982). Morphometric study of intraepithelial neoplasia of the uterine cervix. Obstet Gynecol. 60(2):210-4. 60(2), 210-214. 8-8-1982.

Acevedo,C.M., M.Henriquez, M.R.Emmert-Buck and R.F.Chuaqui. (2002). Loss of heterozygosity on chromosome arms 3p and 6q in microdissected adenocarcinomas of the uterine cervix and adenocarcinoma in situ. Cancer 94:793-802.

Allen,R.C., H.Y.Zoghbi, A.B.Moseley, H.M.Rosenblatt and J.W.Belmont. (1992). Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. Am J Hum Genet 51:1229-1239.

Anttila,T., P.Saikku, P.Koskela, A.Bloigu, J.Dillner, I.Ikaheimo, E.Jellum, M.Lehtinen, P.Lenner, T.Hakulinen, A.Narvanen, E.Pukkala, S.Thoresen, L.Youngman and J.Paavonen. (2001). Serotypes of Chlamydia trachomatis and risk for development of cervical squamous cell carcinoma. JAMA 285:47-51.

Arbeit,J.M., K.Munger, P.M.Howley and D.Hanahan. (1994). Progressive squamous epithelial neoplasia in K14-human papillomavirus type 16 transgenic mice. J Virol 68:4358-4368.

Arends,M.J., C.H.Buckley and M.Wells. (1998). Aetiology, pathogenesis, and pathology of cervical neoplasia. J Clin Pathol 51:96-103.

Arias-Pulido,H., G.Narayan, H.Vargas, M.Mansukhani and V.V.Murty. (2002). Mapping common deleted regions on 5p15 in cervical carcinoma and their occurrence in precancerous lesions. Mol Cancer 1:3.

Arnold,N., L.Hagele, L.Walz, W.Schempp, J.Pfisterer, T.Bauknecht and M.Kiechle. (1996). Overrepresentation of 3q and 8q material and loss of 18q material are recurrent findings in advanced human ovarian cancer. Genes Chromosomes Cancer 16:46-54.

Atkin, N. B. Prognostic value of cytogenetic studies of tumors of female genital tract. Koss LG, Coleman D (1984). Advances in clinical cytology, vol. 2. New York: Masson,;123-134.

Baisch,H. and J.Gerdes. (1987). Simultaneous staining of exponentially growing versus plateau phase cells with the proliferation-associated antibody Ki-67 and propidium iodide: analysis by flow cytometry. Cell Tissue Kinet 20:387-391.

Baldwin,P., R.Laskey and N.Coleman. (2003). Translational approaches to improving cervical screening. Nat Rev Cancer 3:217-226.

Barbacid,M. (1987). ras genes. Annu Rev Biochem 56:779-827.:779-827.

Barbosa,M.S., W.C.Vass, D.R.Lowy and J.T.Schiller. (1991). In vitro biological activities of the E6 and E7 genes vary among human papillomaviruses of different oncogenic potential. J Virol 65:292-298.

Barron BA & Richart RM (1970). Statistical model of the natural history of cervical carcinoma:II estimates of the transition time from dysplasia to carcinoma in situ. J.Natl.Cancer Inst. 1025.

Benedet,J.L., D.M.Miller, K.G.Nickerson and G.H.Anderson. (1987). The results of cryosurgical treatment of cervical intraepithelial neoplasia at one, five, and ten years. Am J Obstet Gynecol 157:268-273.

Bigrigg,A., D.K.Haffenden, A.L.Sheehan, B.W.Codling and M.D.Read. (1994). Efficacy and safety of large-loop excision of the transformation zone. Lancet 343:32-34.

Bigrigg,M.A., B.W.Codling, P.Pearson, M.D.Read and G.R.Swingler. (1990). Colposcopic diagnosis and treatment of cervical dysplasia at a single clinic visit. Experience of low-voltage diathermy loop in 1000 patients. Lancet 336:229-231.

Birrer,M.J., D.Hendricks, J.Farley, M.J.Sundborg, T.Bonome, M.J.Walts and J.Geradts. (1999). Abnormal Fhit expression in malignant and premalignant lesions of the cervix. Cancer Res 59:5270-5274.

Bistoletti,P., A.Zellbi, J.Moreno-Lopez and A.Hjerpe.(1988). Genital papillomavirus infection after treatment for cervical intraepithelial neoplasia (CIN) III. Cancer 62:2056-2059.

Bosch,F.X., M.M.Manos, N.Munoz, M.Sherman, A.M.Jansen, J.Peto, M.H.Schiffman, V.Moreno, R.Kurman and K.V.Shah. (1995). Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. J Natl Cancer Inst 87:796-802.

Bosch[a], F.X., A.Lorincz, N.Munoz, C.J.Meijer and K.V.Shah. (2002). The causal relation between human papillomavirus and cervical cancer. J Clin Pathol 55:244-265.

Bosch[b],F.X. and N.Munoz. (2002). The viral etiology of cervical cancer. Virus Res 89:183-190.

Bourhis,J., M.G.Le, M.Barrois, A.Gerbaulet, D.Jeannel, P.Duvillard, D.Le, V, D.Chassagne and G.Riou. (1990). Prognostic value of c-myc proto-oncogene overexpression in early invasive carcinoma of the cervix. J Clin Oncol 8:1789-1796.

Brass,N., I.Ukena, K.Remberger, U.Mack, G.W.Sybrecht and E.U.Meese. (1996). DNA amplification on chromosome 3q26.1-q26.3 in squamous cell carcinoma of the lung detected by reverse chromosome painting. Eur J Cancer 32A:1205-1208.

Broccoli,D., J.W.Young and T.de Lange. (1995). Telomerase activity in normal and malignant hematopoietic cells. Proc Natl Acad Sci U S A 92:9082-9086.

Butler,D., C.Collins, M.Mabruk, W.C.Barry, M.B.Leader and E.W.Kay. (2000). Deletion of the FHIT gene in neoplastic and invasive cervical lesions is related to high-risk HPV infection but is independent of histopathological features. J Pathol 192:502-510.

Butler,D., C.Collins, M.Mabruk, M.B.Leader and E.W.Kay. (2002). Loss of Fhit expression as a potential marker of malignant progression in preinvasive squamous cervical cancer. Gynecol Oncol 86:144-149.

Castellsague,X., F.X.Bosch and N.Munoz. (2002). Environmental co-factors in HPV carcinogenesis. Virus Res 89:191-199.

Chan,K.S., C.W.Kwok, K.M.Yu, S.Y.Sin and L.C.Tang. (1997). A three-year review of treatment of cervical intraepithelial neoplasia with large loop excision of the transformation zone. Hong Kong Med J 3:21-26.

Chan,P.K., A.R.Chang, J.L.Cheung, D.P.Chan, L.Y.Xu, N.L.Tang and A.F.Cheng. (2002). Determinants of cervical human papillomavirus infection: differences between high- and low-oncogenic risk types. J Infect Dis 185:28-35.

Chanen,W. and R.M.Rome. (1983). Electrocoagulation diathermy for cervical dysplasia and carcinoma in situ: a 15-year survey. Obstet Gynecol 61:673-679.

Chatterjee,A., H.A.Pulido, S.Koul, N.Beleno, A.Perilla, H.Posso, M.Manusukhani and V.V.Murty. (2001). Mapping the sites of putative tumor suppressor genes at 6p25 and 6p21.3 in cervical carcinoma: occurrence of allelic deletions in precancerous lesions. Cancer Res 61:2119-2123.

Chen,J.T., M.A.Lane and D.P.Clark. (1996). Inhibitors of the polymerase chain reaction in Papanicolaou stain. Removal with a simple destaining procedure. Acta Cytol 40:873-877.

Cherpes,T.L., L.A.Meyn, M.A.Krohn and S.L.Hillier. (2003). Risk factors for infection with herpes simplex virus type 2: role of smoking, douching, uncircumcised males, and vaginal flora. Sex Transm Dis 30:405-410.

Choo,K.B., C.C.Pan and S.H.Han. (1987). Integration of human papillomavirus type 16 into cellular DNA of cervical carcinoma: preferential deletion of the E2 gene and invariable retention of the long control region and the E6/E7 open reading frames. Virology 161:259-261.

Chuaqui,R., M.Silva and M.Emmert-Buck. (2001). Allelic deletion mapping on chromosome 6q and X chromosome inactivation clonality patterns in cervical intraepithelial neoplasia and invasive carcinoma. Gynecol Oncol 80:364-371.

Chung,T.K., T.H.Cheung, W.K.Lo, M.Y.Yu, G.M.Hampton, H.K.Wong and Y.F.Wong. (2000). Loss of heterozygosity at the short arm of chromosome 3 in microdissected cervical intraepithelial neoplasia. Cancer Lett 154:189-194.

Cirisano,F.D. (1999). Management of pre-invasive disease of the cervix. Semin Surg Oncol 16:222-227.

Coates,P.J., A.J.d'Ardenne, G.Khan, H.O.Kangro and G.Slavin. (1991). Simplified procedures for applying the polymerase chain reaction to routinely fixed paraffin wax sections. J Clin Pathol 44:115-118.

Connolly,D.C., D.L.Greenspan, R.Wu, X.Ren, R.L.Dunn, K.V.Shah, R.W.Jones, F.X.Bosch, N.Munoz and K.R.Cho. (2000). Loss of fhit expression in invasive cervical carcinomas and intraepithelial lesions associated with invasive disease. Clin Cancer Res 6:3505-3510.

Corden,S.A., L.J.Sant-Cassia, A.J.Easton and A.G.Morris. (1999). The integration of HPV-18 DNA in cervical carcinoma. Mol Pathol 52:275-282.

Counter,C.M., A.A.Avilion, C.E.LeFeuvre, N.G.Stewart, C.W.Greider, C.B.Harley and S.Bacchetti. (1992). Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J 11:1921-1929.

Counter,C.M., H.W.Hirte, S.Bacchetti and C.B.Harley. (1994). Telomerase activity in human ovarian carcinoma. Proc Natl Acad Sci U S A 91:2900-2904.

Coussens,L.M., D.Hanahan and J.M.Arbeit. (1996). Genetic predisposition and parameters of malignant progression in K14-HPV16 transgenic mice. Am J Pathol 149:1899-1917.

Couturier,J., X.Sastre-Garau, S.Schneider-Maunoury, A.Labib and G.Orth. (1991). Integration of papillomavirus DNA near myc genes in genital carcinomas and its consequences for proto-oncogene expression. J Virol 65:4534-4538.

Cramer,D.W. and S.J.Cutler. (1974). Incidence and histopathology of malignancies of the female genital organs in the United States. Am J Obstet Gynecol 118:443-460.

Creasman,W.T., W.M.Hinshaw and D.L.Clarke-Pearson. (1984). Cryosurgery in the management of cervical intraepithelial neoplasia. Obstet Gynecol 63:145-149.

Crusius,K., E.Auvinen and A.Alonso. (1997). Enhancement of EGF- and PMA-mediated MAP kinase activation in cells expressing the human papillomavirus type 16 E5 protein. Oncogene 15:1437-1444.

Cuzick,J. and P.Sasieni. (1999). Cervical screening in the United Kingdom. Hong Kong Med J 5:269-271.

Dellas,A., J.Torhorst, F.Jiang, J.Proffitt, E.Schultheiss, W.Holzgreve, G.Sauter, M.J.Mihatsch and H.Moch. (1999). Prognostic value of genomic alterations in invasive cervical squamous cell carcinoma of clinical stage IB detected by comparative genomic hybridization. Cancer Res 59:3475-3479.

Demeter,L.M., M.H.Stoler, T.R.Broker and L.T.Chow. (1994). Induction of proliferating cell nuclear antigen in differentiated keratinocytes of human papillomavirus-infected lesions. Hum Pathol 25:343-348.

Desaintes,C., C.Demeret, S.Goyat, M.Yaniv and F.Thierry. (1997). Expression of the papillomavirus E2 protein in HeLa cells leads to apoptosis. EMBO J 16:504-514.

Dey,P., A.Gibbs, D.F.Arnold, N.Saleh, P.J.Hirsch and C.B.Woodman.(2002). Loop diathermy excision compared with cervical laser vaporisation for the treatment of intraepithelial neoplasia: a randomised controlled trial. BJOG 109:381-385.

Dillner,J., M.Lehtinen, T.Bjorge, T.Luostarinen, L.Youngman, E.Jellum, P.Koskela, R.E.Gislefoss, G.Hallmans, J.Paavonen, M.Sapp, J.T.Schiller, T.Hakulinen, S.Thoresen and M.Hakama. (1997). Prospective seroepidemiologic study of human papillomavirus infection as a risk factor for invasive cervical cancer. J Natl Cancer Inst 89:1293-1299.

Dipaolo,J.A., C.D.Woodworth, N.C.Popescu, V.Notario and J.Doniger. (1989). Induction of human cervical squamous cell carcinoma by sequential transfection with human papillomavirus 16 DNA and viral Harvey ras. Oncogene 4:395-399.

Dollard,S.C., J.L.Wilson, L.M.Demeter, W.Bonnez, R.C.Reichman, T.R.Broker and L.T.Chow. (1992). Production of human papillomavirus and modulation of the infectious program in epithelial raft cultures. OFF. Genes Dev 6:1131-1142.

Donato,D.M. (1999). Surgical management of stage IB-IIA cervical carcinoma. Semin Surg Oncol 16:232-235.

Dong,S.M., H.S.Kim, S.H.Rha and D.Sidransky.(2001). Promoter hypermethylation of multiple genes in carcinoma of the uterine cervix. Clin Cancer Res 7:1982-1986.

Donzelli,M., R.Bernardi, C.Negri, E.Prosperi, L.Padovan, C.Lavialle, O.Brison and A.I.Scovassi. (1999). Apoptosis-prone phenotype of human colon carcinoma cells with a high level amplification of the c-myc gene. Oncogene 18:439-448.

Dowhanick,J.J., A.A.McBride and P.M.Howley. (1995). Suppression of cellular proliferation by the papillomavirus E2 protein. J Virol 69:7791-7799.

Drain,P.K., K.K.Holmes, J.P.Hughes and L.A.Koutsky. (2002). Determinants of cervical cancer rates in developing countries. Int J Cancer 100:199-205.

Dudzinski,M.R., S.J.Haskill, W.C.Fowler, J.L.Currie and L.A.Walton. (1987). DNA content in cervical neoplasia and its relationship to prognosis. Obstet Gynecol 69:373-377.

Duggan,M.A., S.E.McGregor, G.C.Stuart, S.Morris, V.Chang-Poon, A.Schepansky and L.Honore. (1998). The natural history of CIN I lesions. Eur J Gynaecol Oncol 19:338-344.

Durst,M., C.M.Croce, L.Gissmann, E.Schwarz and K.Huebner. (1987). Papillomavirus sequences integrate near cellular oncogenes in some cervical carcinomas. Proc Natl Acad Sci U S A 84:1070-1074.

Dyson,N., P.M.Howley, K.Munger and E.Harlow. (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 243:934-937.

El Hamidi,A., G.Kocjan and M.Q.Du. (2003). Clonality analysis of archival cervical smears. Correlation of monoclonality with grade and clinical behavior of cervical intraepithelial neoplasia. Acta Cytol 47:117-123.

Elfgren,K., P.Bistoletti, L.Dillner, J.M.Walboomers, C.J.Meijer and J.Dillner. (1996). Conization for cervical intraepithelial neoplasia is followed by disappearance of human papillomavirus deoxyribonucleic acid and a decline in serum and cervical mucus antibodies against human papillomavirus antigens. Am J Obstet Gynecol 174:937-942.

Enomoto,T., M.Fujita, M.Inoue, O.Tanizawa, T.Nomura and K.R.Shroyer.(1994). Analysis of clonality by amplification of short tandem repeats. Carcinomas of the female reproductive tract. Diagn Mol Pathol 3:292-297.

Enomoto,T., T.Haba, M.Fujita, T.Hamada, K.Yoshino, R.Nakashima, H.Wada, H.Kurachi, K.Wakasa, M.Sakurai, Y.Murata and K.R.Shroyer. (1997). Clonal analysis of high-grade squamous intra-epithelial lesions of the uterine cervix. Int J Cancer 73:339-344.

Facchini,L.M. and L.Z.Penn. (1998). The molecular role of Myc in growth and transformation: recent discoveries lead to new insights. FASEB J 12:633-651.

Franceschi,S., L.Dal Maso, S.Arniani, P.Crosignani, M.Vercelli, L.Simonato, F.Falcini, R.Zanetti, A.Barchielli, D.Serraino and G.Rezza. (1998). Risk of cancer other than Kaposi's sarcoma and non-Hodgkin's lymphoma in persons with AIDS in Italy. Cancer and AIDS Registry Linkage Study. Br J Cancer 78:966-970.

Freeman,A., L.S.Morris, A.D.Mills, K.Stoeber, R.A.Laskey, G.H.Williams and N.Coleman. (1999). Minichromosome maintenance proteins as biological markers of dysplasia and malignancy. Clin Cancer Res 5:2121-2132.

Fu,Y.S., J.W.Reagan and R.M.Richart. (1981). Definition of precursors. Gynecol Oncol 12:S220-S231.

Galaktionov,K., X.Chen and D.Beach. (1996). Cdc25 cell-cycle phosphatase as a target of c-myc. Nature 382:511-517.

Gale,R.E. and J.S.Wainscoat. (1993). Clonal analysis using X-linked DNA polymorphisms. Br J Haematol 85:2-8.

Gale,R.E., H.Wheadon, P.Boulos and D.C.Linch. (1994). Tissue specificity of X-chromosome inactivation patterns. Blood 83:2899-2905.

Golijow,C.D., M.C.Abba, S.A.Mouron, M.A.Gomez and F.N.Dulout. (2001). c-myc gene amplification detected in preinvasive intraepithelial cervical lesions. Int J Gynecol Cancer 11:462-465.

Goodwin,E.C., L.K.Naeger, D.E.Breiding, E.J.Androphy and D.DiMaio. (1998). Transactivation-competent bovine papillomavirus E2 protein is specifically required for efficient repression of human papillomavirus oncogene expression and for acute growth inhibition of cervical carcinoma cell lines. J Virol 72:3925-3934.

Goodwin,E.C. and D.DiMaio. (2000). Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes the orderly reactivation of dormant tumor suppressor pathways. Proc Natl Acad Sci U S A 97:12513-12518.

Gorham,H., K.Yoshida, T.Sugino, G.Marsh, S.Manek, M.Charnock, D.Tarin and S.Goodison. (1997). Telomerase activity in human gynaecological malignancies. J Clin Pathol 50:501-504.

Green,J.A., J.M.Kirwan, J.F.Tierney, P.Symonds, L.Fresco, M.Collingwood and C.J.Williams. (2001). Survival and recurrence after concomitant chemotherapy and radiotherapy for cancer of the uterine cervix: a systematic review and meta-analysis. Lancet 358:781-786.

Greenspan,D.L., D.C.Connolly, R.Wu, R.Y.Lei, J.T.Vogelstein, Y.T.Kim, J.E.Mok, N.Munoz, F.X.Bosch, K.Shah and K.R.Cho. (1997). Loss of FHIT expression in cervical carcinoma cell lines and primary tumors. Cancer Res 57:4692-4698.

Grendys,E.C., Jr., W.A.Barnes, J.Weitzel, J.Sparkowski and R.Schlegel. (1997). Identification of H, K, and N-ras point mutations in stage IB cervical carcinoma. Gynecol Oncol 65:343-347.

Gunasekera,P.C., J.H.Phipps and B.V.Lewis. (1990). Large loop excision of the transformation zone (LLETZ) compared to carbon dioxide laser in the treatment of CIN: a superior mode of treatment. Br J Obstet Gynaecol 97:995-998.

Guo[a],Z., U.Thunberg, J.Sallstrom, E.Wilander and J.Ponten. (1998). Clonality analysis of cervical cancer on microdissected archival materials by PCR-based X-chromosome inactivation approach. Int J Oncol 12:1327-1332.

Guo[b],Z., E.Wilander, J.Sallstrom and J.Ponten. (1998). Deletion of chromosome 3p is an early event in malignant progression of cervical cancer. Anticancer Res 18:707-712.

Guo[a],Z., F.Ponten, E.Wilander and J.Ponten. (2000). Clonality of precursors of cervical cancer and their genetical links to invasive cancer. Mod Pathol 13:606-613.

Guo[b],Z., X.Hu, G.Afink, F.Ponten, E.Wilander and J.Ponten. (2000). Comparison of chromosome 3p deletions between cervical precancers synchronous with and without invasive cancer. Int J Cancer 86:518-523.

Hamidi,A.E., H.Liu, Y.Zhang, R.Hamoudi, G.Kocjan and M.Q.Du. (2002). Archival cervical smears: a versatile resource for molecular investigations. Cytopathology 13:291-299.

Hamoudi,R.A., S.Johnston, G.Hutchinson and J.D'Errico. (2002). High Throughput Methods for Gene Identification, Cloning and Functional Genomics Using the GeneTAC G3 Robotics Workstation. Journal of the Association for Laboratory Automation 7:53-59.

Hanselaar,A.G., G.P.Vooijs, P.S.Oud, M.M.Pahlplatz and J.L.Beck. (1988). DNA ploidy patterns in cervical intraepithelial neoplasia grade III, with and without synchronous invasive squamous cell carcinoma. Measurements in nuclei isolated from paraffin-embedded tissue. Cancer 62:2537-2545.

Harima,Y., K.Harima, S.Sawada, Y.Tanaka, S.Arita and T.Ohnishi. (2000). Loss of heterozygosity on chromosome 6p21.2 as a potential marker for recurrence after radiotherapy of human cervical cancer. Clin Cancer Res 6:1079-1085.

Harima,Y., S.Sawada, K.Nagata, M.Sougawa and T.Ohnishi. (2001). Chromosome 6p21.2, 18q21.2 and human papilloma virus (HPV) DNA can predict prognosis of cervical cancer after radiotherapy. Int J Cancer %20;96:286-296.

Harper,J.W., G.R.Adami, N.Wei, K.Keyomarsi and S.J.Elledge. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell %19;75:805-816.

Hawley-Nelson,P., K.H.Vousden, N.L.Hubbert, D.R.Lowy and J.T.Schiller. (1989). HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. EMBO J 8:3905-3910.

Helland,A., S.M.Kraggerud, G.B.Kristensen, R.Holm, V.M.Abeler, K.Huebner, A.L.Borresen-Dale and R.A.Lothe. (2000). Primary cervical carcinomas show 2 common regions of deletion at 3P, 1 within the FHIT gene: evaluation of allelic imbalance at FHIT, RB1 and TP53 in relation to survival. Int J Cancer 88:217-222.

Herbert,A. (1997). Is cervical screening working? A cytopathologist's view from the United Kingdom. Hum Pathol 28:120-126.

Heselmeyer,K., E.Schrock, M.S.du, H.Blegen, K.Shah, R.Steinbeck, G.Auer and T.Ried. (1996). Gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma of the uterine cervix. Proc Natl Acad Sci U S A 93:479-484.

Hickman,E.S., M.C.Moroni and K.Helin. (2002). The role of p53 and pRB in apoptosis and cancer. Curr Opin Genet Dev 12:60-66.

Hildesheim,A., R.Herrero, P.E.Castle, S.Wacholder, M.C.Bratti, M.E.Sherman, A.T.Lorincz, R.D.Burk, J.Morales, A.C.Rodriguez, K.Helgesen, M.Alfaro, M.Hutchinson, I.Balmaceda, M.Greenberg and M.Schiffman. (2001). HPV co-factors related to the development of cervical cancer: results from a population-based study in Costa Rica. Br J Cancer 84:1219-1226.

Hiyama,K., Y.Hirai, S.Kyoizumi, M.Akiyama, E.Hiyama, M.A.Piatyszek, J.W.Shay, S.Ishioka and M.Yamakido. (1995). Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. J Immunol 155:3711-3715.

Hubbert,N.L., S.A.Sedman and J.T.Schiller. (1992). Human papillomavirus type 16 E6 increases the degradation rate of p53 in human keratinocytes. J Virol 66:6237-6241.

Huettner,P.C., D.S.Gerhard, L.Li, D.J.Gersell, K.Dunnigan, T.Kamarasova and J.S.Rader. (1998). Loss of heterozygosity in clinical stage IB cervical carcinoma: relationship with clinical and histopathologic features. Hum Pathol 29:364-370.

Hwang,E.S., D.J.Riese, J.Settleman, L.A.Nilson, J.Honig, S.Flynn and D.DiMaio. (1993). Inhibition of cervical carcinoma cell line proliferation by the introduction of a bovine papillomavirus regulatory gene. J Virol 67:3720-3729.

Hwang,E.S., T.Nottoli and D.DiMaio. (1995). The HPV16 E5 protein: expression, detection, and stable complex formation with transmembrane proteins in COS cells. Virology 211:227-233.

Iwasaka,T., M.Yokoyama, M.Oh-uchida, N.Matsuo, K.Hara, K.Fukuyama, T.Hachisuga, K.Fukuda and H.Sugimori. (1992). Detection of human papillomavirus genome and analysis of expression of c-myc and Ha-ras oncogenes in invasive cervical carcinomas. Gynecol Oncol 46:298-303.

Jackson,D.P., J.Payne, S.Bell, F.A.Lewis, G.R.Taylor, K.R.Peel, J.Sutton and P.Quirke. (1990). Extraction of DNA from exfoliative cytology specimens and its suitability for analysis by the polymerase chain reaction. Cytopathology 1:87-96.

Jastreboff,A.M. and T.Cymet. (2002). Role of the human papilloma virus in the development of cervical intraepithelial neoplasia and malignancy. Postgrad Med J 78:225-228.

Johnson J and J. Patnick (2000). Achievable standards, benchmarks for reporting, and criteria for evaluating cervical cytopathology. NHSCSP Publication No. 1.

Jussawalla,D.J. and B.B.Yeole. (1984). Epidemiology of cancer of the cervix in greater Bombay. J Surg Oncol 26:53-62.

Karlsen,F., P.H.Rabbitts, V.Sundresan and B.Hagmar. (1994). PCR-RFLP studies on chromosome 3p in formaldehyde-fixed, paraffin-embedded cervical cancer tissues. Int J Cancer 58:787-792.

Karlsen,F., M.Kalantari, A.Jenkins, E.Pettersen, G.Kristensen, R.Holm, B.Johansson and B.Hagmar. (1996). Use of multiple PCR primer sets for optimal detection of human papillomavirus. J Clin Microbiol 34:2095-2100.

Kaufman,R.H. and E.Adam. (1999). Is human papillomavirus testing of value in clinical practice? Am J Obstet Gynecol 180:1049-1053.

Kawai,K., Y.Yaginuma, H.Tsuruoka, M.Griffin, H.Hayashi and M.Ishikawa. (1998). Telomerase activity and human papillomavirus (HPV) infection in human uterine cervical cancers and cervical smears. Eur J Cancer 34:2082-2086.

Keating,P.J., F.V.Cromme, M.Duggan-Keen, P.J.Snijders, J.M.Walboomers, R.D.Hunter, P.A.Dyer and P.L.Stern. (1995). Frequency of down-regulation of individual HLA-A and -B alleles in cervical carcinomas in relation to TAP-1 expression. Br J Cancer 72:405-411.

Kersemaekers[a],A.M., J.Hermans, G.J.Fleuren and M.J.van de Vijver. (1998). Loss of heterozygosity for defined regions on chromosomes 3, 11 and 17 in carcinomas of the uterine cervix. Br J Cancer 77:192-200.

Kersemaekers[b],A.M., G.G.Kenter, J.Hermans, G.J.Fleuren and M.J.van de Vijver. (1998). Allelic loss and prognosis in carcinoma of the uterine cervix. Int J Cancer 79:411-417.

Kersemaekers,A.M., M.J.van de Vijver, G.G.Kenter and G.J.Fleuren. (1999). Genetic alterations during the progression of squamous cell carcinomas of the uterine cervix. Genes Chromosomes Cancer 26:346-354.

Kersemaekers,A.M., M.J.van de Vijver and G.J.Fleuren. (2000). Comparison of the genetic alterations in two epithelial collision tumors of the uterine cervix. A report of two cases. Int J Gynecol Pathol 19:225-230.

Kirchhoff,M., H.Rose, B.L.Petersen, J.Maahr, T.Gerdes, C.Lundsteen, T.Bryndorf, N.Kryger-Baggesen, L.Christensen, S.A.Engelholm and J.Philip. (1999). Comparative genomic hybridization reveals a recurrent pattern of chromosomal aberrations in severe dysplasia/carcinoma in situ of the cervix and in advanced-stage cervical carcinoma. Genes Chromosomes Cancer 24:144-150.

Kjaer,S.K., A.J.Van Den Brule, G.Paull, E.I.Svare, M.E.Sherman, B.L.Thomsen, M.Suntum, J.E.Bock, P.A.Poll and C.J.Meijer. (2002). Type specific persistence of high risk human papillomavirus (HPV) as indicator of high grade cervical squamous intraepithelial lesions in young women: population based prospective follow up study. BMJ 325:572.

Klaes,R., S.M.Woerner, R.Ridder, N.Wentzensen, M.Duerst, A.Schneider, B.Lotz, P.Melsheimer and D.M.von Knebel. (1999). Detection of high-risk cervical intraepithelial neoplasia and cervical cancer by amplification of transcripts derived from integrated papillomavirus oncogenes. Cancer Res 59:6132-6136.

Klaes,R., T.Friedrich, D.Spitkovsky, R.Ridder, W.Rudy, U.Petry, G.Dallenbach-Hellweg, D.Schmidt and D.M.von Knebel. (2001). Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. Int J Cancer 92:276-284.

Klingelhutz,A.J., S.A.Foster and J.K.McDougall. (1996). Telomerase activation by the E6 gene product of human papillomavirus type 16. Nature 380:79-82.

Ko,H.M., C.Choi, C.S.Park and S.W.Juhng. (1997). Analysis of clonality by X chromosome inactivation in uterine cervix cancer. J Korean Med Sci 12:322-326.

Kohno,T., H.Takayama, M.Hamaguchi, H.Takano, N.Yamaguchi, H.Tsuda, S.Hirohashi, H.Vissing, M.Shimizu, M.Oshimura and . (1993). Deletion mapping of chromosome 3p in human uterine cervical cancer. Oncogene 8:1825-1832.

Konishi,I., S.Fujii, H.Nonogaki, Y.Nanbu, T.Iwai and T.Mori. (1991). Immunohistochemical analysis of estrogen receptors, progesterone receptors, Ki-67 antigen, and human papillomavirus DNA in normal and neoplastic epithelium of the uterine cervix. Cancer 68:1340-1350.

Koskinen,P.J. and K.Alitalo. (1993). Role of myc amplification and overexpression in cell growth, differentiation and death. Semin Cancer Biol 4:3-12.

Koutsky,L.A., K.K.Holmes, C.W.Critchlow, C.E.Stevens, J.Paavonen, A.M.Beckmann, T.A.DeRouen, D.A.Galloway, D.Vernon and N.B.Kiviat. (1992). A cohort study of the risk of cervical intraepithelial neoplasia grade 2 or 3 in relation to papillomavirus infection. N Engl J Med 327:1272-1278.

Koutsky,L.A., K.A.Ault, C.M.Wheeler, D.R.Brown, E.Barr, F.B.Alvarez, L.M.Chiacchierini and K.U.Jansen. (2002). A controlled trial of a human papillomavirus type 16 vaccine. N Engl J Med 347:1645-1651.

Kruse,A.J., J.P.Baak, P.C.de Bruin, M.Jiwa, W.P.Snijders, P.J.Boodt, G.Fons, P.W.Houben and H.S.The. (2001). Ki-67 immunoquantitation in cervical intraepithelial neoplasia (CIN): a sensitive marker for grading. J Pathol 193:48-54.

Kruse,A.J., J.P.Baak, P.C.de Bruin, F.R.van de Goot and N.Kurten. (2001). Relationship between the presence of oncogenic HPV DNA assessed by polymerase chain reaction and Ki-67 immunoquantitative features in cervical intraepithelial neoplasia. J Pathol 195:557-562.

Kyo,S., M.Takakura, H.Ishikawa, T.Sasagawa, S.Satake, M.Tateno and M.Inoue. (1997). Application of telomerase assay for the screening of cervical lesions. Cancer Res 57:1863-1867.

La Vecchia,C., S.Franceschi, A.Decarli, M.Fasoli, A.Gentile and G.Tognoni. (1986). Cigarette smoking and the risk of cervical neoplasia. Am J Epidemiol 123:22-29.

Lakhani,S.R., D.N.Slack, R.A.Hamoudi, N.Collins, M.R.Stratton and J.P.Sloane. (1996). Detection of allelic imbalance indicates that a proportion of mammary hyperplasia of usual type are clonal, neoplastic proliferations. Journal of Laboratory Investigation 74:129-135.

Larson[a],A.A., S.Kern, S.Curtiss, R.Gordon, W.K.Cavenee and G.M.Hampton. (1997). High resolution analysis of chromosome 3p alterations in cervical carcinoma. Cancer Res 57:4082-4090.

Larson[b],A.A., S.Y.Liao, E.J.Stanbridge, W.K.Cavenee and G.M.Hampton. (1997). Genetic alterations accumulate during cervical tumorigenesis and indicate a common origin for multifocal lesions. Cancer Res 57:4171-4176.

Lazo,P.A. (1999). The molecular genetics of cervical carcinoma. Br J Cancer 80:2008-2018.

Lee,J.H., S.K.Lee, M.H.Yang, M.M.Ahmed, M.Mohiuddin and E.Y.Lee. (1996). Expression and mutation of H-ras in uterine cervical cancer. Gynecol Oncol 62:49-54.

Lehtinen,M., P.Koskela, E.Jellum, A.Bloigu, T.Anttila, G.Hallmans, T.Luukkaala, S.Thoresen, L.Youngman, J.Dillner and M.Hakama. (2002). Herpes simplex virus and risk of cervical cancer: a longitudinal, nested case-control study in the nordic countries. Am J Epidemiol 156:687-692.

Lei,M. and B.K.Tye. (2001). Initiating DNA synthesis: from recruiting to activating the MCM complex. J Cell Sci 114:1447-1454.

Levi,F., F.Lucchini, E.Negri, S.Franceschi and C.La Vecchia. (2000). Cervical cancer mortality in young women in Europe: patterns and trends. Eur J Cancer 36:2266-2271.

Lin,W.M., E.A.Michalopulos, N.Dhurander, P.C.Cheng, W.Robinson, R.Ashfaq, R.L.Coleman and C.Y.Muller. (2000). Allelic loss and microsatellite alterations of chromosome 3p14.2 are more frequent in recurrent cervical dysplasias. Clin Cancer Res 6:1410-1414.

Lin,W.M., R.Ashfaq, E.A.Michalopulos, A.Maitra, A.F.Gazdar and C.Y.Muller. (2000). Molecular Papanicolaou tests in the twenty-first century: molecular analyses with fluid-based Papanicolaou technology. Am J Obstet Gynecol 183:39-45.

Liu,H., X.Huang, Y.Zhang, H.Ye, A.E.Hamidi, G.Kocjan, A.Dogan, P.G.Isaacson and M.Q.Du. (2002). Archival fixed histologic and cytologic specimens including stained and unstained materials are amenable to rt-PCR. Diagn Mol Pathol 11:222-227.

Lorincz,A.T., R.Reid, A.B.Jenson, M.D.Greenberg, W.Lancaster and R.J.Kurman. (1992). Human papillomavirus infection of the cervix: relative risk associations of 15 common anogenital types. Obstet Gynecol 79:328-337.

Luesley,D.M., A.McCrum, P.B.Terry, T.Wade-Evans, H.O.Nicholson, M.J.Mylotte, J.M.Emens and J.A.Jordan. (1985). Complications of cone biopsy related to the dimensions of the cone and the influence of prior colposcopic assessment. Br J Obstet Gynaecol 92:158-164.

Luesley,D.M., J.Cullimore, C.W.Redman, F.G.Lawton, J.M.Emens, T.P.Rollason, D.R.Williams and E.J.Buxton. (1990). Loop diathermy excision of the cervical transformation zone in patients with abnormal cervical smears. BMJ 300:1690-1693.

Lusky,M. and E.Fontane. (1991). Formation of the complex of bovine papillomavirus E1 and E2 proteins is modulated by E2 phosphorylation and depends upon sequences within the carboxyl terminus of E1. Proc Natl Acad Sci U S A 88:6363-6367.

Ma,Y.Y., S.J.Wei, Y.C.Lin, J.C.Lung, T.C.Chang, J.Whang-Peng, J.M.Liu, D.M.Yang, W.K.Yang and C.Y.Shen. (2000). PIK3CA as an oncogene in cervical cancer. Oncogene 19:2739-2744.

Manolaraki,M.M., D.A.Arvanitis, G.Sourvinos, S.Sifakis, E.Koumantakis and D.A.Spandidos. (2002). Frequent loss of heterozygosity in chromosomal region 9pter-p13 in tumor biopsies and cytological material of uterine cervical cancer. Cancer Lett 176:175-181.

McGoogan,E., A.L.Seagar and H.A.Cubie. (1998). Detection of high-risk human papillomavirus nucleic acid in archival cervical smears. Acta Cytol 42:1079-1083.

McGrath,J.P., D.J.Capon, D.V.Goeddel and A.D.Levinson. (1984). Comparative biochemical properties of normal and activated human ras p21 protein. Nature 310:644-649.

McIndoe,W.A., M.R.McLean, R.W.Jones and P.R.Mullins. (1984). The invasive potential of carcinoma in situ of the cervix. Obstet Gynecol 64:451-458.

Meijer,C.J. and J.M.Walboomers. (2000). Cervical cytology after 2000: where to go? J Clin Pathol 53:41-43.

Meyerson,M., C.M.Counter, E.N.Eaton, L.W.Ellisen, P.Steiner, S.D.Caddle, L.Ziaugra, R.L.Beijersbergen, M.J.Davidoff, Q.Liu, S.Bacchetti, D.A.Haber and R.A.Weinberg. (1997). hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. Cell 90:785-795.

Michael,D. and M.Oren. (2002). The p53 and Mdm2 families in cancer. Curr Opin Genet Dev 12:53-59.

Miller,C.S., M.S.Zeuss and D.K.White. (1994). Detection of HPV DNA in oral carcinoma using polymerase chain reaction together with in situ hybridization. Oral Surg Oral Med Oral Pathol 77:480-486.

Mitra, A.B., V.V.Murty, R.G.Li, M.Pratap, U.K.Luthra and R.S.Chaganti. (1994). Allelotype analysis of cervical carcinoma. Cancer Res 54:4481-4487.

Mitra,A.B., V.V.Murty, V.Singh, R.G.Li, M.Pratap, P.Sodhani, U.K.Luthra and R.S.Chaganti. (1995). Genetic alterations at 5p15: a potential marker for progression of precancerous lesions of the uterine cervix. J Natl Cancer Inst 87:742-745.

Mitra,A.B. (1999). Genetic deletion and human papillomavirus infection in cervical cancer: loss of heterozygosity sites at 3p and 5p are important genetic events. Int J Cancer 82:322-324.

Mittal,R., K.Tsutsumi, A.Pater and M.M.Pater. (1993). Human papillomavirus type 16 expression in cervical keratinocytes: role of progesterone and glucocorticoid hormones. Obstet Gynecol 81:5-12.

Miyashita,T., S.Krajewski, M.Krajewska, H.G.Wang, H.K.Lin, D.A.Liebermann, B.Hoffman and J.C.Reed. (1994). Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene 9:1799-1805.

Miyashita,T. and J.C.Reed. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80:293-299.

Monsonego,J., P.Valensi, L.Zerat, C.Clavel and P.Birembaut. (1997). Simultaneous effects of aneuploidy and oncogenic human papillomavirus on histological grade of cervical intraepithelial neoplasia. Br J Obstet Gynaecol 104:723-727.

Moodley,M., J.Moodley, R.Chetty and C.S.Herrington.(2003). The role of steroid contraceptive hormones in the pathogenesis of invasive cervical cancer: a review. Int J Gynecol Cancer 13:103-110.

Morelli,A.E., C.Sananes, G.Di Paola, A.Paredes and L.Fainboim. (1993). Relationship between types of human papillomavirus and Langerhans' cells in cervical condyloma and intraepithelial neoplasia. Am J Clin Pathol 99:200-206.

Moreno,V., F.X.Bosch, N.Munoz, C.J.Meijer, K.V.Shah, J.M.Walboomers, R.Herrero and S.Franceschi. (2002). Effect of oral contraceptives on risk of cervical cancer in women with human papillomavirus infection: the IARC multicentric case-control study. Lancet 359:1085-1092.

Morin,D.M., J.S.Mandel, M.S.Linet, E.Ron, J.H.Lubin, J.D.Boice, Jr. and J.F.Fraumeni, Jr.(2000). Mortality among Catholic nuns certified as radiologic technologists. Am J Ind Med 37:339-348.

Moroni,M.C., E.S.Hickman, E.L.Denchi, G.Caprara, E.Colli, F.Cecconi, H.Muller and K.Helin. (2001). Apaf-1 is a transcriptional target for E2F and p53. Nat Cell Biol 3:552-558.

Muller,C.Y., J.D.O'Boyle, K.M.Fong, I.I.Wistuba, E.Biesterveld, M.Ahmadian, D.S.Miller, A.F.Gazdar and J.D.Minna. (1998). Abnormalities of fragile histidine triad genomic and complementary DNAs in cervical cancer: association with human papillomavirus type. J Natl Cancer Inst 90:433-439.

Mullokandov,M.R., N.G.Kholodilov, N.B.Atkin, R.D.Burk, A.B.Johnson and H.P.Klinger. (1996). Genomic alterations in cervical carcinoma: losses of chromosome heterozygosity and human papilloma virus tumor status. Cancer Res 56:197-205.

Munoz,N., S.Franceschi, C.Bosetti, V.Moreno, R.Herrero, J.S.Smith, K.V.Shah, C.J.Meijer and F.X.Bosch. (2002). Role of parity and human papillomavirus in cervical cancer: the IARC multicentric case-control study. Lancet 359:1093-1101.

Murphy,N., M.Ring, A.G.Killalea, V.Uhlmann, M.O'Donovan, F.Mulcahy, M.Turner, E.McGuinness, M.Griffin, C.Martin, O.Sheils and J.J.O'Leary. (2003). p16INK4A as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and ThinPrep smears. J Clin Pathol 56:56-63.

Nakamura,T.M., G.B.Morin, K.B.Chapman, S.L.Weinrich, W.H.Andrews, J.Lingner, C.B.Harley and T.R.Cech. (1997). Telomerase catalytic subunit homologs from fission yeast and human. Science 277:955-959.

Nakano,K., E.Watney and J.K.McDougall. (1998). Telomerase activity and expression of telomerase RNA component and telomerase catalytic subunit gene in cervical cancer. Am J Pathol 153:857-864.

Nakano,K. and K.H.Vousden. (2001). PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 7:683-694.

Nguyen,H.N. and H.E.Averette. (1999). Biology of cervical carcinoma. Semin Surg Oncol 16:212-216.

NHS. Achievable standards, benchmarks for reporting, and criteria for evaluating cervical cytopathology, 2 nd edn. NHS Cancer Screening Programmes, 2000 (NHSCSP Publication No. 1).

Ning,Y., J.L.Weber, A.M.Killary, D.H.Ledbetter, J.R.Smith and O.M.Pereira-Smith. (1991). Genetic analysis of indefinite division in human cells: evidence for a cell senescence-related gene(s) on human chromosome 4. Proc Natl Acad Sci U S A 88:5635-5639.

Nobbenhuis,M.A., J.M.Walboomers, T.J.Helmerhorst, L.Rozendaal, A.J.Remmink, E.K.Risse, H.C.van der Linden, F.J.Voorhorst, P.Kenemans and C.J.Meijer. (1999). Relation of human papillomavirus status to cervical lesions and consequences for cervical-cancer screening: a prospective study. Lancet 354:20-25.

Nobbenhuis,M.A., T.J.Helmerhorst, A.J.Van Den Brule, L.Rozendaal, F.J.Voorhorst, P.D.Bezemer, R.H.Verheijen and C.J.Meijer. (2001). Cytological regression and clearance of high-risk human papillomavirus in women with an abnormal cervical smear. Lancet 358:1782-1783.

Oda[a],E., R.Ohki, H.Murasawa, J.Nemoto, T.Shibue, T.Yamashita, T.Tokino, T.Taniguchi and N.Tanaka. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science 288:1053-1058.

Oda[b],K., H.Arakawa, T.Tanaka, K.Matsuda, C.Tanikawa, T.Mori, H.Nishimori, K.Tamai, T.Tokino, Y.Nakamura and Y.Taya. (2000). p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell 102:849-862.

Ohta,M., H.Inoue, M.G.Cotticelli, K.Kastury, R.Baffa, J.Palazzo, Z.Siprashvili, M.Mori, P.McCue, T.Druck and . (1996). The FHIT gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. Cell 84:587-597.

Ostor,A.G. (1993). Natural history of cervical intraepithelial neoplasia: a critical review. Int J Gynecol Pathol 12:186-192.

Ostwald,C., P.Muller, M.Barten, K.Rutsatz, M.Sonnenburg, K.Milde-Langosch and T.Loning. (1994). Human papillomavirus DNA in oral squamous cell carcinomas and normal mucosa. J Oral Pathol Med 23:220-225.

Owen-Schaub,L.B., W.Zhang, J.C.Cusack, L.S.Angelo, S.M.Santee, T.Fujiwara, J.A.Roth, A.B.Deisseroth, W.W.Zhang, E.Kruzel and . (1995). Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. Mol Cell Biol 15:3032-3040.

Pan,L.X., T.C.Diss, H.Z.Peng and P.G.Isaacson. (1994). Clonality analysis of defined B-cell populations in archival tissue sections using microdissection and the polymerase chain reaction. Histopathology 24:323-327.

Pao,C.C., C.J.Tseng, C.Y.Lin, F.P.Yang, J.J.Hor, D.S.Yao and S.Hsueh. (1997). Differential expression of telomerase activity in human cervical cancer and cervical intraepithelial neoplasia lesions. J Clin Oncol 15:1932-1937.

Paraskevaidis,E., G.Koliopoulos, V.Malamou-Mitsi, K.Zikopoulos, M.Paschopoulos, L.Pappa, N.J.Agnantis and D.E.Loli. (2001). Large loop excision of the transformation zone for treating cervical intraepithelial neoplasia: a 12-year experience. Anticancer Res 21:3097-3099.

Park,T.W., R.M.Richart, X.W.Sun and T.C.Wright, Jr. (1996). Association between human papillomavirus type and clonal status of cervical squamous intraepithelial lesions. J Natl Cancer Inst %20;88:355-358.

Patnick,J. (2000). Cervical cancer screening in England. Eur J Cancer 36:2205-2208.

Pisani,P., D.M.Parkin, F.Bray and J.Ferlay. (1999). Estimates of the worldwide mortality from 25 cancers in 1990. Int J Cancer 83:18-29.

Prives,C. (1998). Signaling to p53: breaking the MDM2-p53 circuit. Cell 95:5-8.

Pulido,H.A., M.J.Fakruddin, A.Chatterjee, E.D.Esplin, N.Beleno, G.Martinez, H.Posso, G.A.Evans and V.V.Murty. (2000). Identification of a 6-cM minimal deletion at 11q23.1-23.2 and exclusion of PPP2R1B gene as a deletion target in cervical cancer. Cancer Res 60:6677-6682.

Puranen,M., S.Saarikoski, K.Syrjanen and S.Syrjanen. (1996). Polymerase chain reaction amplification of human papillomavirus DNA from archival, Papanicolaou-stained cervical smears. Acta Cytol 40:391-395.

Quinn,M., P.Babb, J.Jones and E.Allen. (1999). Effect of screening on incidence of and mortality from cancer of cervix in England: evaluation based on routinely collected statistics. BMJ 318:904-908.

Rader,J.S., D.S.Gerhard, M.J.O'Sullivan, Y.Li, L.Li, H.Liapis and P.C.Huettner. (1998). Cervical intraepithelial neoplasia III shows frequent allelic loss in 3p and 6p. Genes Chromosomes Cancer 22:57-65.

Reesink-Peters,N., M.N.Helder, G.B.Wisman, A.J.Knol, S.Koopmans, H.M.Boezen, E.Schuuring, H.Hollema, E.G.de Vries, S.de Jong and A.G.van der Zee. (2003). Detection of telomerase, its components, and human papillomavirus in cervical scrapings as a tool for triage in women with cervical dysplasia. J Clin Pathol 56:31-35.

Rhyu,M.S. (1995). Telomeres, telomerase, and immortality. J Natl Cancer Inst 87:884-894.

Riou,G., M.Barrois, M.G.Le, M.George, D.Le, V and C.Haie. (1987). C-myc proto-oncogene expression and prognosis in early carcinoma of the uterine cervix. Lancet 1:761-763.

Rock,C.L., C.W.Michael, R.K.Reynolds and M.T.Ruffin. (2000). Prevention of cervix cancer. Crit Rev Oncol Hematol 33:169-185.

Roda Husman,A.M., P.J.Snijders, H.V.Stel, A.J.Van Den Brule, C.J.Meijer and J.M.Walboomers. (1995). Processing of long-stored archival cervical smears for human papillomavirus detection by the polymerase chain reaction. Br J Cancer 72:412-417.

Rome,R.M., W.Chanen and R.Pagano. (1987). The natural history of human papillomavirus (HPV) atypia of the cervix. Aust N Z J Obstet Gynaecol 27:287-290.

Rose,P.G. (2002). Chemoradiotherapy for cervical cancer. Eur J Cancer 38:270-278.

Rowlands,D.C., H.E.Brown, P.C.Barber and E.L.Jones. (1991). The effect of tissue fixation on immunostaining for proliferating cell nuclear antigen with the monoclonal antibody PC10. J Pathol 165:356-357.

Sadan,O., E.Schejter, S.Ginath, R.Bachar, M.Boaz, J.Menczer and M.Glezerman. (2003). Premalignant lesions of the uterine cervix in a large cohort of Israeli Jewish women. Arch Gynecol Obstet ..

Sagae,S., R.Kudo, N.Kuzumaki, T.Hisada, Y.Mugikura, T.Nihei, T.Takeda and M.Hashimoto. (1990). Ras oncogene expression and progression in intraepithelial neoplasia of the uterine cervix. Cancer 66:295-301.

Sano,T., N.Masuda, T.Oyama and T.Nakajima. (2002). Overexpression of p16 and p14ARF is associated with human papillomavirus infection in cervical squamous cell carcinoma and dysplasia. Pathol Int 52:375-383.

Saxon,P.J., E.S.Srivatsan and E.J.Stanbridge. (1986). Introduction of human chromosome 11 via microcell transfer controls tumorigenic expression of HeLa cells. EMBO J %20;5:3461-3466.

Scheffner,M., J.M.Huibregtse, R.D.Vierstra and P.M.Howley. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell 75:495-505.

Schiffman,M.H., H.M.Bauer, R.N.Hoover, A.G.Glass, D.M.Cadell, B.B.Rush, D.R.Scott, M.E.Sherman, R.J.Kurman, S.Wacholder and . (1993). Epidemiologic evidence showing that human papillomavirus infection causes most cervical intraepithelial neoplasia. J Natl Cancer Inst 85:958-964.

Schoell,W.M., M.F.Janicek and R.Mirhashemi. (1999). Epidemiology and biology of cervical cancer. Semin Surg Oncol 16:203-211.

Schwarz,E., U.K.Freese, L.Gissmann, W.Mayer, B.Roggenbuck, A.Stremlau and H.H.zur. (1985). Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature 314:111-114.

Selik,R.M. and C.S.Rabkin. (1998). Cancer death rates associated with human immunodeficiency virus infection in the United States. J Natl Cancer Inst 90:1300-1302.

Senchenko,V., J.Liu, E.Braga, N.Mazurenko, W.Loginov, Y.Seryogin, I.Bazov, A.Protopopov, F.L.Kisseljov, V.Kashuba, M.I.Lerman, G.Klein and E.R.Zabarovsky.(2003). Deletion mapping using quantitative real-time PCR identifies two distinct 3p21.3 regions affected in most cervical carcinomas. Oncogene 22:2984-2992.

Serraino,D., P.Carrieri, C.Pradier, E.Bidoli, M.Dorrucci, E.Ghetti, A.Schiesari, R.Zucconi, P.Pezzotti, P.Dellamonica, S.Franceschi and G.Rezza.

(1999). Risk of invasive cervical cancer among women with, or at risk for, HIV infection. Int J Cancer 82:334-337.

Shanta,V., S.Krishnamurthi, C.K.Gajalakshmi, R.Swaminathan and K.Ravichandran. (2000). Epidemiology of cancer of the cervix: global and national perspective. J Indian Med Assoc 98:49-52.

Shayesteh,L., Y.Lu, W.L.Kuo, R.Baldocchi, T.Godfrey, C.Collins, D.Pinkel, B.Powell, G.B.Mills and J.W.Gray. (1999). PIK3CA is implicated as an oncogene in ovarian cancer. Nat Genet 21:99-102.

Sherwood,J.B., N.Shivapurkar, W.M.Lin, R.Ashfaq, D.S.Miller, A.F.Gazdar and C.Y.Muller.(2000). Chromosome 4 deletions are frequent in invasive cervical cancer and differ between histologic variants. Gynecol Oncol 79:90-96.

Shurbaji,M.S., S.K.Brooks and T.S.Thurmond. (1993). Proliferating cell nuclear antigen immunoreactivity in cervical intraepithelial neoplasia and benign cervical epithelium. Am J Clin Pathol 100:22-26.

Skomedal,H., G.B.Kristensen, J.M.Nesland, A.L.Borresen-Dale, C.Trope and R.Holm. (1999). TP53 alterations in relation to the cell cycle-associated proteins p21, cyclin D1, CDK4, RB, MDM2, and EGFR in cancers of the uterine corpus. J Pathol 187:556-562.

Smith,J.S., N.Munoz, R.Herrero, J.Eluf-Neto, C.Ngelangel, S.Franceschi, F.X.Bosch, J.M.Walboomers and R.W.Peeling. (2002). Evidence for Chlamydia trachomatis as a human papillomavirus cofactor in the etiology of invasive cervical cancer in Brazil and the Philippines. J Infect Dis 185:324-331.

Snijders,P.J., M.van Duin, J.M.Walboomers, R.D.Steenbergen, E.K.Risse, T.J.Helmerhorst, R.H.Verheijen and C.J.Meijer. (1998). Telomerase activity exclusively in cervical carcinomas and a subset of cervical intraepithelial neoplasia grade III lesions: strong association with elevated messenger RNA levels of its catalytic subunit and high-risk human papillomavirus DNA. Cancer Res 58:3812-3818.

Soder,A.I., S.F.Hoare, S.Muir, J.J.Going, E.K.Parkinson and W.N.Keith. (1997). Amplification, increased dosage and in situ expression of the telomerase RNA gene in human cancer. Oncogene 14:1013-1021.

Soler,M.E., L.Gaffikin and P.D.Blumenthal. (2000). Cervical cancer screening in developing countries. Prim Care Update Ob Gyns 7:118-123.

Sonnex,C. (1998). Human papillomavirus infection with particular reference to genital disease. J Clin Pathol 51:643-648.

Southern,S.A. and C.S.Herrington. (1998). Molecular events in uterine cervical cancer. Sex Transm Infect 74:101-109.

Speicher,M.R., C.Howe, P.Crotty, M.S.du, J.Costa and D.C.Ward. (1995). Comparative genomic hybridization detects novel deletions and amplifications in head and neck squamous cell carcinomas. Cancer Res 55:1010-1013.

Srivatsan,E.S., R.Chakrabarti, K.Zainabadi, S.D.Pack, P.Benyamini, M.S.Mendonca, P.K.Yang, K.Kang, D.Motamedi, M.P.Sawicki, Z.Zhuang, R.A.Jesudasan, U.Bengtsson, C.Sun, B.A.Roe, E.J.Stanbridge, S.P.Wilczynski and J.L.Redpath. (2002). Localization of deletion to a 300 Kb interval of chromosome 11q13 in cervical cancer. Oncogene 21:5631-5642.

Stanley,M.A. (2001). Human papillomavirus and cervical carcinogenesis. Best Pract Res Clin Obstet Gynaecol 15:663-676.

Steele,C., L.M.Cowsert and E.J.Shillitoe. (1993). Effects of human papillomavirus type 18-specific antisense oligonucleotides on the transformed phenotype of human carcinoma cell lines. Cancer Res 53:2330-2337.

Steenbergen,R.D., J.M.Walboomers, C.J.Meijer, van der Raaij-Helmer EM, J.N.Parker, L.T.Chow, T.R.Broker and P.J.Snijders. (1996). Transition of human papillomavirus type 16 and 18 transfected human foreskin keratinocytes towards immortality: activation of telomerase and allele losses at 3p, 10p, 11q and/or 18q. Oncogene %19;13:1249-1257.

Stewart,A.C., A.M.Eriksson, M.M.Manos, N.Munoz, F.X.Bosch, J.Peto and C.M.Wheeler. (1996). Intratype variation in 12 human papillomavirus types: a worldwide perspective. J Virol 70:3127-3136.

Stockton,D., P.Cooper and R.N.Lonsdale. (1997). Changing incidence of invasive adenocarcinoma of the uterine cervix in East Anglia. J Med Screen 4:40-43.

Sugita,M., N.Tanaka, S.Davidson, S.Sekiya, M.Varella-Garcia, J.West, H.A.Drabkin and R.M.Gemmill. (2000). Molecular definition of a small amplification domain within 3q26 in tumors of cervix, ovary, and lung. Cancer Genet Cytogenet 117:9-18.

Symonds,R.P., T.Habeshaw, J.Paul, D.J.Kerr, A.Darling, R.A.Burnett, F.Sotsiou, S.Linardopoulos and D.A.Spandidos. (1992). No correlation between ras, c-myc and c-jun proto-oncogene expression and prognosis in advanced carcinoma of cervix. Eur J Cancer 28A:1615-1617.

Syrjanen,K., R.Mantyjarvi, M.Vayrynen, S.Syrjanen, S.Parkkinen, M.Yliskoski, S.Saarikoski and O.Castren. (1987). Human papillomavirus (HPV) infections involved in the neoplastic process of the uterine cervix as established by prospective follow-up of 513 women for two years. Eur J Gynaecol Oncol 8:5-16.

Takakura,M., S.Kyo, T.Kanaya, M.Tanaka and M.Inoue. (1998). Expression of human telomerase subunits and correlation with telomerase activity in cervical cancer. Cancer Res 58:1558-1561.

Talis,A.L., J.M.Huibregtse and P.M.Howley. (1998). The role of E6AP in the regulation of p53 protein levels in human papillomavirus (HPV)-positive and HPV-negative cells. J Biol Chem 273:6439-6445.

Thorland,E.C., S.L.Myers, B.S.Gostout and D.I.Smith. (2003). Common fragile sites are preferential targets for HPV16 integrations in cervical tumors. Oncogene 22:1225-1237.

Tran-Thanh,D., D.Provencher, A.Koushik, E.Duarte-Franco, A.Kessous, P.Drouin, C.M.Wheeler, J.Dubuc-Lissoir, P.Gauthier, G.Allaire, R.Vauclair, J.A.Dipaolo, P.Gravitt, E.Franco and F.Coutlee. (2003). Herpes simplex virus type II is not a cofactor to human papillomavirus in cancer of the uterine cervix. Am J Obstet Gynecol 188:129-134.

Tsukamoto,N. (1985). Treatment of cervical intraepithelial neoplasia with the carbon dioxide laser. Gynecol Oncol 21:331-336.

Umayahara,K., F.Numa, Y.Suehiro, A.Sakata, S.Nawata, H.Ogata, Y.Suminami, M.Sakamoto, K.Sasaki and H.Kato. (2002). Comparative genomic hybridization detects genetic alterations during early stages of cervical cancer progression. Genes Chromosomes Cancer 33:98-102.

van Driel,W.J., M.Y.Tjiong, C.G.Hilders, B.J.Trimbos and G.J.Fleuren. (1996). Association of allele-specific HLA expression and histopathologic progression of cervical carcinoma. Gynecol Oncol 62:33-41.

Van Le,L., J.Stoerker, C.A.Rinehart and W.C.Fowler. (1993). H-ras codon 12 mutation in cervical dysplasia. Gynecol Oncol 49:181-184.

Verheijen,R., H.J.Kuijpers, R.van Driel, J.L.Beck, J.H.van Dierendonck, G.J.Brakenhoff and F.C.Ramaekers. (1989). Ki-67 detects a nuclear matrix-associated proliferation-related antigen. II. Localization in mitotic cells and association with chromosomes. J Cell Sci 92:531-540.

Virmani,A.K., C.Muller, A.Rathi, S.Zoechbauer-Mueller, M.Mathis and A.F.Gazdar. (2001). Aberrant methylation during cervical carcinogenesis. Clin Cancer Res 7:584-589.

Waggoner,S.E. and X.Wang. (1994). Effect of nicotine on proliferation of normal, malignant, and human papillomavirus-transformed human cervical cells. Gynecol Oncol 55:91-95.

Waggoner,S.E. (2003). Cervical cancer. Lancet 361:2217-2225.

Walboomers,J.M., M.V.Jacobs, M.M.Manos, F.X.Bosch, J.A.Kummer, K.V.Shah, P.J.Snijders, J.Peto, C.J.Meijer and N.Munoz. (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 189:12-19.

Wallin,K.L., F.Wiklund, T.Angstrom, F.Bergman, U.Stendahl, G.Wadell, G.Hallmans and J.Dillner. (1999). Type-specific persistence of human papillomavirus DNA before the development of invasive cervical cancer. N Engl J Med 341:1633-1638.

Wallin,K.L., F.Wiklund, T.Luostarinen, T.Angstrom, T.Anttila, F.Bergman, G.Hallmans, I.Ikaheimo, P.Koskela, M.Lehtinen, U.Stendahl, J.Paavonen and J.Dillner. (2002). A population-based prospective study of Chlamydia trachomatis infection and cervical carcinoma. Int J Cancer 101:371-374.

Werness,B.A., A.J.Levine and P.M.Howley. (1990). Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science 248:76-79.

Wilke,C.M., B.K.Hall, A.Hoge, W.Paradee, D.I.Smith and T.W.Glover. (1996). FRA3B extends over a broad region and contains a spontaneous HPV16 integration site: direct evidence for the coincidence of viral integration sites and fragile sites. Hum Mol Genet 5:187-195.

Williams,G.H., P.Romanowski, L.Morris, M.Madine, A.D.Mills, K.Stoeber, J.Marr, R.A.Laskey and N.Coleman. (1998). Improved cervical smear assessment using antibodies against proteins that regulate DNA replication. Proc Natl Acad Sci U S A 95:14932-14937.

Willis,G., B.Jennings, R.Y.Ball, N.E.New and I.Gibson. (1993). Analysis of ras point mutations and human papillomavirus 16 and 18 in cervical carcinomata and their metastases. Gynecol Oncol 49:359-364.

Winkelstein,W., Jr. (1990). Smoking and cervical cancer--current status: a review. Am J Epidemiol 131:945-957.

Wisman,G.B., H.Hollema, S.de Jong, J.ter Schegget, A.H.S.Tjong, M.H.Ruiters, M.Krans, E.G.de Vries and A.G.van der Zee. (1998). Telomerase activity as a biomarker for (pre)neoplastic cervical disease in scrapings and frozen sections from patients with abnormal cervical smear. J Clin Oncol 16:2238-2245.

Wisman,G.B., A.J.Knol, M.N.Helder, M.Krans, E.G.de Vries, H.Hollema, S.de Jong and A.G.van der Zee. (2001). Telomerase in relation to clinicopathologic prognostic factors and survival in cervical cancer. Int J Cancer 91:658-664.

Wistuba,I.I., F.D.Montellano, S.Milchgrub, A.K.Virmani, C.Behrens, H.Chen, M.Ahmadian, J.A.Nowak, C.Muller, J.D.Minna and A.F.Gazdar. (1997). Deletions of chromosome 3p are frequent and early events in the pathogenesis of uterine cervical carcinoma. Cancer Res 57:3154-3158.

Wong,Y.F., T.K.Chung, T.H.Cheung, S.K.Lam, Y.G.Xu and A.M.Chang. (1995). Frequent ras gene mutations in squamous cell cervical cancer. Cancer Lett 95:29-32.

Woodworth,C.D., J.Doniger and J.A.Dipaolo. (1989). Immortalization of human foreskin keratinocytes by various human papillomavirus DNAs corresponds to their association with cervical carcinoma. J Virol 63:159-164.

Yashima,K., R.Ashfaq, J.Nowak, G.Von, V, S.Milchgrub, A.Rathi, J.Albores-Saavedra, J.W.Shay and A.F.Gazdar. (1998). Telomerase activity and expression of its RNA component in cervical lesions. Cancer 82:1319-1327.

Yoshino,K., T.Enomoto, T.Nakamura, R.Nakashima, H.Wada, J.Saitoh, K.Noda and Y.Murata. (1998). Aberrant FHIT transcripts in squamous cell carcinoma of the uterine cervix. Int J Cancer 76:176-181.

Zhang,A., C.Zheng, M.Hou, C.Lindvall, K.L.Wallin, T.Angstrom, X.Yang, A.C.Hellstrom, E.Blennow, M.Bjorkholm, A.Zetterberg, A.Gruber and D.Xu. (2002). Amplification of the telomerase reverse transcriptase (hTERT) gene in cervical carcinomas. Genes Chromosomes Cancer 34:269-275.

Zhang,B., D.F.Spandau and A.Roman. (2002). E5 protein of human papillomavirus type 16 protects human foreskin keratinocytes from UV B-irradiation-induced apoptosis. J Virol 76:220-231.

Zielinski,G.D., P.J.Snijders, L.Rozendaal, F.J.Voorhorst, H.C.van der Linden, A.P.Runsink, F.A.de Schipper and C.J.Meijer. (2001). HPV presence precedes abnormal cytology in women developing cervical cancer and signals false negative smears. Br J Cancer 85:398-404.

zur Hausen H. (1999). Immortalization of human cells and their malignant conversion by high risk human papillomavirus genotypes. Semin Cancer Biol 9:405-411.

zur Hausen Hausen H. (2002). Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer 2:342-350.