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Molecular Biology of Papillomaviruses in Pre-malignant Cervical Infection

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ABSTRACT

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MOLECULAR BIOLOGY OF PAPILLOMAVIRUSES IN PRE-MALIGNANT CERVICAL INFECTION

by Stuart Andrew Lanham

Infection with human papillomavirus (HPV) is considered a necessary prerequisite for the induction of cervical cancer. However, the majority of women infected with potentially oncogenic HPVs self-cure and only a proportion of those women who develop low grade cervical intraepithelial neoplasia (CIN) will, if untreated, develop cancer. In order to identify determinants of an adverse outcome a detailed comparative analysis of cervical samples from women with high or low grade CIN was undertaken. It was hoped that some of the factors analysed such as HPV type, load, and oncogenic transcript production, evidence of other cervical infections and/or inflammation, plus host genetic factors might have a high predictive value for pre-malignant disease.

The samples analysed were cervical scrapes taken at a colposcopy clinic. HPVs were detected in 90% of specimens with low grade lesions (CIN 1) and 100% of high grade lesions (CIN 3). HPV types commonly found in cancer (high risk types 16, 18, 31, and 45) were detected in 80% of specimens with CIN 3, but only 40% of specimens with CIN 1. Moderate risk HPV types (including types 33, 35, 39, and 56) were detected in 19% of CIN 3 and 34 % of CIN 1 and low risk HPV types (e.g. 6, 11, 42, and 66) were detected in 11% of CIN 1, but less than 1% of samples with CIN 3. No positive predictive value was found using viral load as an indicator of disease severity. Cervical infection with herpesviruses was found more often in women with progressing CIN, but there was no correlation with cervical cytokine or chemokine production and grade of CIN. However, mixed HPV infections increased in incidence with increasing lesion severity.

The most commonly isolated HPV type in all grades of CIN was HPV-16. These patients were investigated in more detail to determine if there were differences in the viral transcripts produced in high and low grade CIN. Transcripts coding for the oncogenic proteins, E6 and E7, were more commonly detected in high grade lesions than in low grade lesions.

The production of transcripts for host proteins implicated in uncontrolled cellular replication were investigated. Survivin transcripts were present in 30% of CIN samples. Interestingly, samples not expressing survivin transcripts contained higher levels of HPV-16 E6 transcripts. The cellular p53 protein interacts with HPV E6 protein and a polymorphism in an exon of the p53 gene has been reported to influence the disease course of HPV infections. We could not confirm this finding, but demonstrated that a polymorphism in intron 6 of the p53 gene was linked to the presence of CIN. However, this polymorphism only accounted for susceptibility to CIN in a minor group of women.

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Abbreviations Used

AAV	Adeno-associated virus
ATP	Adenosine triphosphate
BNA	Borderline nuclear abnormalities
bp	Base pairs
BPV	Bovine papillomavirus
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CIN	Cervical intraepithelial neoplasia
CMV	Cytomegalovirus
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
dsDNA	Double stranded deoxyribonucleic acid
E6-AP	E6-associated protein
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
EV	Epidermodysplasia verruciformis
FCS	Foetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GP	General Practitioner
IFN	Interferon
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papillomaviruses
HSV	Herpes simplex virus
hTERT	Human telomerase reverse transcriptase
hTR	Human telomerase RNA component
IL	Interleukin
kDa	Kilodaltons
LCR	Long control region
LOH	Loss of heterozygosity

MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
mRNA	Messenger ribonucleic acid
nt	Nucleotide
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PML	Promonocytic leukaemia protein
POD	Promonocytic leukaemia protein oncogenic domain
RANTES	Regulated upon activation, normal T expressed and secreted
RNA	Ribonucleic acid
RT	Reverse Transcription
STD	Sexually transmitted disease
TGF	Transforming Growth Factor
T_H	T-helper
TNF	Tumour necrosis factor
TRAP	Telomeric repeat amplification protocol
UHQ	Ultra high quality
UK	United Kingdom
VLP	Virus-like particles

Chapter 1

Introduction

1.1 Papillomavirus Infections

The papillomaviruses are a group of viruses that cause warts (papillomas) in a variety of vertebrates, including man. The infectious nature of warts was suspected for many centuries, and was shown to be caused by a virus in 1907 by Ciuffo, when transmission from person to person was established from a cell free extract of a skin wart. Warts have been recognised at many different sites in humans including skin, genital tract, and oral cavity. It was originally believed there was just one type of human papillomavirus (HPV) and the difference in morphology seen at different sites was due to the type of epithelium infected. However, with the advent of molecular techniques, it is now known there are over 100 different HPV types.

1.1.1 Skin Warts

Warts are generally rare before the age of five, the highest prevalence occurring in older children and young adults. The lower incidence in older people may represent acquired immunity and reduced exposure. Warts are transmitted by direct contact with infected material or indirectly from contaminated objects. Transmission to other sites by auto-inoculation in an infected individual is common. The majority of warts spontaneously regress after about 2 years, this is thought to be the result of a cell-mediated immune response. A high rate of transmission occurs in communally used areas where bare, wet skin is exposed to virus, such as swimming pools. Butchers, meat handlers, and slaughter house workers have a high incidence of warts on their hands. This is probably due to continued trauma to the hands during their work. The virus itself is not from animals; the warts are caused by HPV-7.

1.1.2 Epidermodysplasia Verruciformis

Epidermodysplasia verruciformis is a rare, lifelong disease, in which the patient is unable to resolve wart infections. Some of the lesions may progress to malignancy. The majority of patients have defective cell-mediated immunity. Most cases are familial, but epidermodysplasia verruciformis has been reported as a complication of HIV infection (Berger *et al.*, 1991).

This disease generally begins in childhood, with multiple wart-like lesions on the face, trunk, and extremities which tend to become confluent. Papillomavirus types that cause lesions in normal individuals, such as HPV-3 and HPV-10, are rarely detected in these lesions, but other types appear to be specific to this disease, i.e. HPV-5, 8, 9, 12, 14, 15, 17, 19, 20, and 25.

In about 30% of patients, a number of years after the onset of disease, multiple foci of malignant transformation can arise within the lesions, especially on skin exposed to sunlight. Virus particles, commonly found in the warty lesions, are scarce or absent in the malignant cells, however, multiple copies of unintegrated viral DNA are present.

1.1.3 Genital Warts

Genital warts (condylomata acuminata) are the most commonly recognised lesions of genital HPV infection. The lesions are usually florid and exophytic. In males, the condylomas occur on the penis, around the anus, on the perineum, and more rarely on the scrotum. In females, warts appear on the vulva, the vaginal introitus, the perineum, the anus, and rarely on the cervix. In an infected individual, condylomas are frequently found at more than one genital site. Many of the warts regress spontaneously or respond to treatment, however, they may reoccur during pregnancy, but regress once more after delivery. Immunosuppressed populations, such as HIV-infected homosexual men, have a high prevalence of condylomas.

The prevalence of genital papillomavirus infections is directly related to the number of lifetime sexual partners, to recent change in partners, and to age. The prevalence peaks in sexually active women aged 15-25 years and declines with increasing age, probably due to fewer recent sexual partners and the acquisition of some

immunity as a result of past infections. Smoking and the use of oral contraceptives are consistently associated with a higher prevalence of HPVs (Shah and Howley, 1996).

Most exophytic condylomas are caused by HPV types 6 or 11, which are rarely associated with cervical cancer. HPV types 6 and 11 also cause laryngeal and conjunctival papillomas, particularly in babies born to infected mothers. Many genital infections caused by HPVs are subclinical or produce flat condylomas of the cervix. Such lesions can be caused by HPV types 6 or 11 ('low risk' types), but may also be caused by HPV types 16, 18, 31, and 45 implicated in the causation of cervical cancer ('high risk' types).

Studies of serial genital specimens show that uncomplicated infections persist for several months although most individuals clear the infection completely (Hildesheim *et al.*, 1994). It is not known if infection with one type of HPV provides immunity against reinfection with the same HPV type. There are several possible outcomes of HPV infection; 1) complete elimination of the virus, 2) persistence of the virus with no cytological abnormalities, 3) transient cytological abnormalities which are completely resolved, 4) cytological abnormalities which persist, and 5) cytological abnormalities that progress to *in situ* or invasive cancer. The frequencies of these possibilities are not known, but a likely outcome is complete resolution, with cancer a rare event.

Studies on the prevalence of HPVs in males are not as extensive as those on females because it is difficult to collect adequate specimens from males and the consequences of infection are not as severe in the male as in the female. In a PCR-based study the prevalence of HPVs in males and females was similar (Barrasso *et al.*, 1987).

There is no one 'catch-all' treatment for HPV infection. A number of techniques have been used with varying success. The effectiveness of treatments is difficult to assess due to spontaneous regression and recurrence of infection.

Traditional treatments for warts have included the application of caustic agents, cryotherapy, application of inhibitors of DNA synthesis, and surgical therapy. Each treatment has varying degrees of effectiveness; response rates vary between 60 and 90%, but approximately 10-40% of patients have recurrence of the virus (Ferenczy, 1995). Similar treatments are available for cervical intraepithelial neoplasia (CIN), although the methods commonly used are liquid nitrogen, excisional biopsy (conisation), and laser therapy. Hysterectomy is the treatment that has the lowest recurrence rate, but the advantages and disadvantages of the procedure need to be fully

discussed with the patient, especially if less radical treatment would suffice in the particular situation.

1.2 Cervical Cancer

1.2.1 Introduction

Cervical cancer is the second most common cancer in women worldwide with around 500,000 new cases per year, accounting for 200,000 deaths. In 1994 in the UK there were over 1300 deaths, despite having a national screening programme. The incidence of cervical cancer increases with age; starting at around 20 years and increasing steadily with a peak incidence at 40-60 years of age. The association between cervical cancer and a sexually transmissible agent was hypothesised long before HPVs were shown to have a causative role.

In the UK, the Pap smear is used in the cervical cancer screening programme; a sample of cells is taken from the cervix with a spatula, the cells are then smeared on a glass slide, stained, and examined for abnormal cells. Although annual deaths from cervical cancer fell from 2000 in the 1980s to 1369 in 1994, it is estimated that between 5 and 30% of women with abnormalities are missed (Fricker, 1997). One problem is that around 8% of samples taken by screening clinics are not suitable for examination, and some inadequate smears maybe reported as normal. Further, the Pap test depends on human skills and is prone to error; for example, in the Kent and Canterbury Hospital misreporting of cervical smears may have contributed to eight deaths, as well as 30 hysterectomies that were necessary after re-examining slides (Committee of Public Accounts, 1998).

Women are only screened every 3 to 5 years in the UK, so any woman with undetected cervical abnormalities could develop cancer in the interval between tests. In order to prevent women 'slipping through the screening net' there is considerable political pressure to improve smear testing. However, major changes such as semi-automated examination of the smear and the introduction of testing for oncogenic HPV types remain at the research level.

The cervical screening programme in the UK targets women aged 20 to 64 years. Department of Health figures suggest of the 4.5 million smear tests taken annually,

some 3.4% show borderline abnormalities and 2.4% mild dyskaryosis. Women with moderate/severe dyskaryosis are referred directly to a colposcopy clinic. Smear tests on women with mild dyskaryosis are repeated at 6 months and if abnormalities persist the patients are referred to the colposcopy clinic.

If on colposcopic examination of women with smear abnormalities the lesion is diagnosed as CIN grade 2 or 3 (see figure 1.1), the lesion is excised by loop diathermy. A recent review suggests this conservative approach to women with CIN reduces the risk of invasive cervical cancer by 95% over an 8 year follow-up period (Soutter *et al.*, 1997).

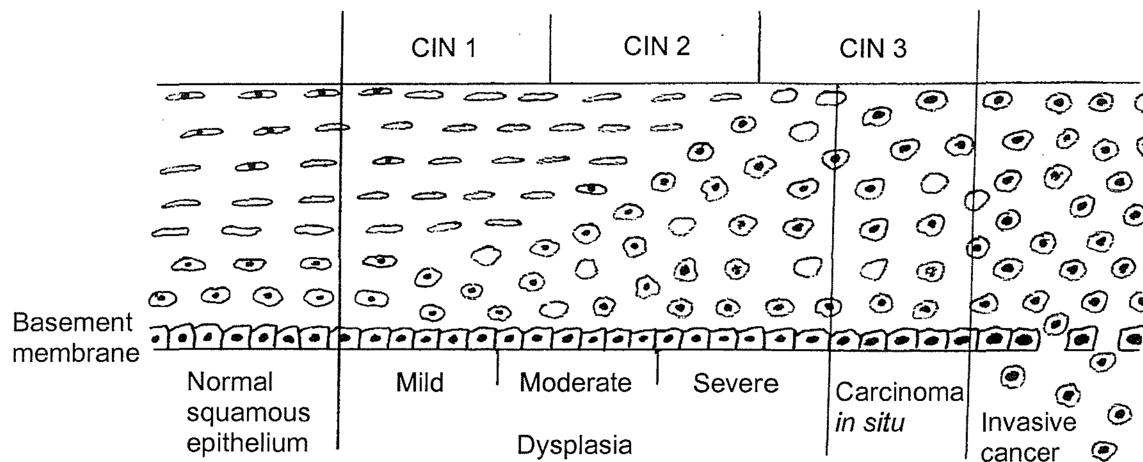


Figure 1.1. Cellular Morphology and CIN Status. The varying degrees of cervical intraepithelial neoplasia (CIN) and severity of dysplasia are shown with a schematic of the histology.

1.2.2 Pathogenesis of Cervical Cancer

1.2.2.1 Acquisition of HPV

Many epidemiological studies have been done to compare patients with cervical intraepithelial neoplasia (CIN) and cervical cancer with healthy controls. The outcomes are varied. The most common findings suggest smoking, a history of sexually

transmitted diseases, number of lifetime sexual partners, age at first sexual intercourse, and oral contraceptive use are risk factors for development of cervical cancer. Essentially these are risk factors for infection with potentially oncogenic HPVs. Further, the range of sexually transmitted diseases that appear to be indicators of risk of cervical dysplasia including herpes simplex virus type 2 (HSV-2), *Chlamydia trachomatis*, and bacterial vaginosis may be surrogate markers for the risk of acquisition of HPV infection.

1.2.2.2 Progression to Cervical Cancer

It is now agreed that infection with papillomavirus alone is necessary, but not sufficient, for carcinogenesis of the cervix.

Although papillomavirus infections are associated with cancers both in humans and other animals, only a limited number of HPV types are oncogenic. For instance, in humans with the rare skin disorder epidermodysplasia verruciformis (EV), certain types of HPV are isolated from warts, and of these only HPV-5 and HPV-8 induce carcinoma. Similarly, only specific types predominate in cervical cancer, HPV-16, HPV-18, HPV-31, and are termed high risk HPV types. In cattle, only BPV-4 is associated with cancer.

Co-factors are known to cause the progression to cancer in papillomavirus infections. Skin carcinoma in EV patients preferentially arises in lesions exposed to sunlight. In Scotland, where bracken forms part of the natural diet of cattle, this has been found to be a co-factor in the production of alimentary tract cancer with BPV-4. The role of co-factors in cervical cancer is still under investigation, however, the discovery that interleukin 1α (IL-1α) and tumour necrosis factor α (TNF-α), which are produced during inflammation of human tissue and normally suppress the growth of cervical epithelial cells, actually stimulate the growth of HPV-immortalised cervical carcinoma cells would indicate co-factors that induce inflammation may provide an enhanced growth environment for cancerous cells (Woodworth *et al.*, 1995).

Most cervical cancers originate in the 'transformation zone' where the columnar cells of the endocervix form a junction with the stratified squamous epithelium of the ectocervix/vagina. Cells of the transformation zone undergo rapid turnover and appear

to be particularly susceptible to malignant change since cancer is much less common at other sites in the lower genital tract.

Human papillomavirus infection can result in integration of the viral genome into the host cell chromosome. However, the relevance of integration to disease progression is still unclear, since episomal HPV, integrated forms of virus, and combinations of both forms, have been found in patients with cervical cancer. In one study of patients with cervical intraepithelial neoplasia where many different HPV types were investigated (Cullen *et al.*, 1991) integration of the genome, regardless of HPV type, was infrequent. In some of the carcinoma patients no evidence was found for integration of HPV-16, whereas HPV-18 was found to be integrated in all cases of cervical cancer. In most cases, integration leads to disruption of the *E1/E2* region resulting in loss of control of the E2 protein over the expression of E6 and E7 proteins. It may also be that the E6 and E7 proteins of the high risk HPV's induce integration more readily than E6 and E7 proteins from the low risk types (Kessis *et al.*, 1996). Also, if intact E2 protein is required to control viral segregation in daughter cells following cellular replication (Lehman and Botchan, 1998), and E2 function is lost as a consequence of viral integration, then cells not expressing the E2 protein may eventually lose episomal virus in subsequent replication cycles as viral segregation would be uncontrolled. Therefore, cancerous cells with integrated HPV DNA may ultimately only possess the integrated HPV form.

The increased sensitivity of the polymerase chain reaction over early studies using Southern blotting, has demonstrated a high frequency of infections with multiple papillomavirus types in cervical specimens, indeed, specimens have even been identified which contain 3 or 4 different types (Chang *et al.*, 1997). There was a suggestion of increased prevalence of mixed infections in patients with low grade cervical lesions. However, it is not know if one HPV type inhibits or enhances the replication of other types infecting adjacent cells.

The significance of smoking in the causation of cervical cancer is still uncertain. One possibility is that smoking is a marker of a social behaviour pattern which can include high risk sexual behaviour. Nevertheless, nicotine has been shown to enhance proliferation of human cervical cells, both in HPV positive and uninfected cells (Waggoner and Wang, 1994). Also, the carcinogenic tobacco-specific nitrosamine 4-(methylnitrosamino) -1- (3-pyridyl) -1- butanone (NNK) has been detected in higher

concentrations in the cervical mucus of smokers than non-smokers (Prokopczyk *et al.*, 1997).

Co-infection of the cervical epithelium with herpesviruses such as EBV or HSV-2 could lead to mutation or rearrangement of growth regulatory genes. If the cell was already infected with HPV, then the cell would survive, as the E6 protein of the HPV would prevent the cellular p53 from inducing cell death (Jones, 1995). This effect would be most pronounced with the high risk HPV types 16 and 18.

Interestingly, infection with adeno-associated virus type 2 (AAV-2) may prevent the development of cervical cancer. Su and Wu (1996) found AAV-2 reduced the growth rate of HPV containing cells. They also found AAV-2 reduced the amount of E7 protein produced in the cells, possibly by suppressing the promoters of HPVs.

The Rep78 protein of AAV-2, is required for AAV transcription activity, AAV DNA replication, and possibly for site-specific integration of AAV into human DNA. This protein appears to be able to interact with SP1, a cellular transcription factor (Hermonat, Santin, and Batchu, 1996), and inhibits the activity of SP1. Furthermore, SP1 can bind to the E6 promoter of all genital HPVs and activates transcription of HPV-16, thus Rep78 can inhibit HPV transcription by preventing SP1 binding. Adeno-associated virus has also been shown to inhibit other promoters, including c-H-ras and HIV-1, both of which contain SP1 binding motifs.

1. 3 Diagnosis

Detection of papillomavirus in a patient sample by electron microscopy relies on the presence of viral capsids, i.e. assembled L1 protein. Unfortunately, viral particles are not uniformly present within a sample and in many cases of high grade neoplasia and cervical cancer, capsids may be completely absent. Traditionally, the electron microscope is regarded as having a sensitivity of 10^6 viral particles per millilitre.

Hybridisation assays can be employed using labelled probes prepared from reference HPV clones. If these probes bind to the viral DNA of interest then the sample is considered positive. Southern hybridisation provides the size of the unknown DNA, whereas dot-blots simply show a positive or negative sample. However, these techniques are labour intensive and weak signals are difficult to interpret. Southern and dot-blot hybridisations can detect 10^5 to 10^6 copies of the HPV genome.

In *in situ* hybridisation, cells or tissue on a slide is probed in a manner similar to Southern or dot-blot hybridisation. In this test, which is often performed to detect viral RNA transcripts, the signal is seen in relation to the pathologic lesion thus demonstrating the presence of virus within abnormal cells.

Detection of HPV DNA is most commonly performed using the polymerase chain reaction (PCR). The preferred method is to use consensus primers directed against the viral L1 gene which are capable of detecting many different types of HPV. The actual HPV type is then determined by analysing the PCR product. These consensus PCR approaches are slightly less sensitive than specific primers due to areas of degeneracy within the primers or low annealing temperatures used to overcome mismatches between primers and HPV DNA. Specific primers have a sensitivity of 10 to 100 viral genome copies.

Cytological examination of cells exfoliated from the cervix using the Pap (Papanicolaou) smear has been widespread for nearly fifty years (Papanicolaou, 1928). Cells are collected from the cervix using a spatula. These cells are thinly spread on a glass slide, fixed, and sent to the laboratory where the slides are stained and examined microscopically. The clinical use of the Pap smear has expanded from the original use of detecting malignant cells, and is now used to screen for a wide variety of cellular abnormalities.

This cytological screening has caused a drop in the incidence of cervical cancer due to detection of pre-cancerous lesions which are treatable. As a screening tool the Pap smear has a number of advantages. The smear is relatively cheap, requires no patient preparation, minimal clinical training, and little specialised equipment. The specimens can be sent to a centralised laboratory for testing allowing expert interpretation away from the patient.

Of course, there are also disadvantages of the Pap smear; most importantly false-negative samples. This may be caused by a number of factors. Firstly, the lesion may not be shedding sufficient number of cells to be detected. Secondly, the location of the lesion may be such that the area is not sampled, e.g. within the endocervix, or inadequate technique. Thirdly, the cells may be on the slide, but not interpretable due to drying artefacts, excess blood, or inflammation. These sampling errors account for about 60% of false-negative cases, scarce abnormal cells may be missed by the cytologist (40% of cases), or, rarely, interpretative error where malignant cells are reported as

normal. Although a single smear has a false-negative incidence of about 20% (van der Graaf and Vooijs, 1987), a series of three negative repeat smears drops the false-negative rate to 1-2%. In England, evidence suggests that screening women every five years prevents 84% of cervical cancer, and every 3 years prevents 91% (Committee of Public Accounts, 1998). However, screening annually would only prevent 93% of cases. Many health authorities and general practitioners currently screen every 3 years, although the national policy is every 5 years.

1.4 Immune Response to Papillomaviruses

It is most likely the immune response to papillomaviruses that produces regression is a cell mediated one, as proliferation of warts occurs in patients with depressed T cell function, such as pregnancy, immunosuppressive chemotherapy, organ transplantation, or HIV infection. In the case of patients on medication, cessation of treatment often produces regression of the warts. Removal or trauma of a wart often produces regression in warts at other sites, possibly by release of antigen. Also, histological examination of regressing warts shows infiltration by mononuclear cells.

Serological examination of patients with HPV infection has been studied in many instances. However, the results in many cases have been difficult to interpret. This may in part be due to the type of method employed to detect antibodies. For instance, Western blot, which uses denatured antigens, has often produced similar results in both cervical cancer patients and apparently HPV negative controls. Enzyme-linked immunosorbent assays (ELISAs), which utilise cloned HPV antigens, have produced more consistent results; antibodies detected against HPV E6 and E7 peptides are most frequently detected in patients with invasive cervical cancer.

If the cell mediated response is the protective arm of the immune response against papillomavirus infection, then the major histocompatibility complex (MHC), also known as the human leukocyte antigen (HLA), class-I and II are also of central importance. The products of these genes are used by leukocytes to present antigen to T cells leading to leukocyte activation. Thus, a person's genetic make-up could influence their response to papillomavirus infection.

Studies have been undertaken to determine if particular MHC genes lead people to be more, or less, susceptible to CIN and/or cervical cancer. The results have been

varied; a Norwegian study has found an increased association with DQA1*0102-DQB1*0602 in CIN 2/3 and with HPV-16 presence and a reduced incidence of DQA1*0102-DQB1*0604 with HPV-16 positivity, but no association with DQB1*03 (Helland *et al.*, 1998a). A study of English women found association between DQB1*03, DRB1*04, and DRB1*11 and CIN (Odunsi *et al.*, 1996). Results from a Spanish study found increased incidence of DQB1*0301 in CIN 1/2 and increased DQB1*0402 in CIN 3/cancer, and reduced incidence of DQB1*0603 in CIN 1/2 (Montoya *et al.*, 1998). Therefore, in many cases the HLA DQB1*03 was found to be associated with an increased risk of CIN and DQB1*06 alleles had varying affects.

Two T-helper lymphocyte cell populations have been identified in respect of the cytokines they produce; T_H1 are classed based on their secretion of cytokines such as interleukin 2 (IL-2), interferon gamma (IFN-γ) and lymphotoxin, which act on effector cells to enhance cell-mediated immunity. The T_H2 cells secrete IL-4, IL-5, IL-6 and IL-10, and are associated with strong antibody response and suppression of cell-mediated immunity. Both T_H1 and T_H2 cells affect each other. For example, IL-4 and IL-10 are potent inhibitors of T_H1 cells, while IFN-γ inhibits proliferation of T_H2 cells. In a study by Clerici's group (1997) there was a T_H2 cytokine profile in peripheral blood mononuclear cells of women with HPV infection spread to other areas of the genital tract, compared to a T_H1 cytokine profile in women with HPV infection localised in the cervix. This would suggest the spread of the disease is linked with the altered cytokine profile. Other workers have found similar results; Grassegger's group (1997) found non-recurrent warts contained mRNA for T_H1 or a mixed T_H1/T_H2 profile, persistent warts had no T_H1 profile, and recurrent warts showed a T_H2 profile of cytokine production. Pao and colleagues (1995) discovered there was a reduction in IFN-γ production in the cervix of patients with CIN and cervical cancer. In addition, antibody levels against VLPs are higher in patients with high grade CIN or cervical cancer (Luxton *et al.*, 1997), supporting the evidence of a reduced T_H1 response in these patients.

1.5 Papillomavirus Assembly And Release

Natural infections occur in squamous epithelium cells. The actual epithelia is composed of 20 to 30 layers of cells with only the innermost basal layer actively

dividing. It is this layer which is initially infected by the virus via attachment to its receptor on the host cell. One group has recently identified this receptor to be the α_6 integrin (McMillan *et al.*, 1999).

Papillomaviruses are icosahedral, nonenveloped double stranded DNA viruses with a diameter of about 55 nm (Figure 1.2). The 55 kDa capsid protein designated L1, self-assembles into pentamers termed capsomers. The viral coat is composed of 72 capsomers held together by disulphide bridges (figure 1.2). The minor capsid protein, L2, has recently been located at the centre of the pentavalent capsomers (Trus *et al.*, 1997). L2 may also play a crucial role in viral assembly since the production of virus-like particles (VLPs) in baculovirus infected insect cells expressing the L1 protein is enhanced with the presence of L2. It appears that L2 localises into nuclear sub-cellular organelles, termed PODs [promonocytic leukaemia protein (PML) oncogenic domains], and in the presence of L2 the major capsid protein co-localises into the POD (Day *et al.*, 1998). The suggestion is the non-structural 48 kDa E2 protein initially binds specifically to the POD/L1/L2 complex at control regions in the viral genome, locking the newly formed papillomavirus genome into the assembling capsid. It is unclear how the E2 protein is released from the developing virion.

Recent evidence suggests both the L1 and the L2 genes have mRNA instability elements in their coding regions, for example the L2 gene destabilising sequence has 60% A+U content in the first 845 nucleotides (Sokolowski *et al.*, 1998). These workers note that the c-fos and c-myc genes also contain inhibitory sequences in their coding regions, and suggest a common cell mechanism permits c-fos production in differentiating cells and the production of L1 and L2 in HPV infected epithelial cells.

The *E4* ORF of HPV type 16 is localised in the early region of the genome (see figure 1.3), but is expressed as a late protein. The E4 protein binds to the keratin intermediate filament network causing collapse of the cytoskeleton and facilitating release of mature virions from the differentiated epithelial cell (Roberts *et al.*, 1993). However, this mechanism was not seen in HPV-1 infected cells. The fact that E4 exists in different multimeric forms which change as the cell differentiates suggests the various E4 species have different functions.

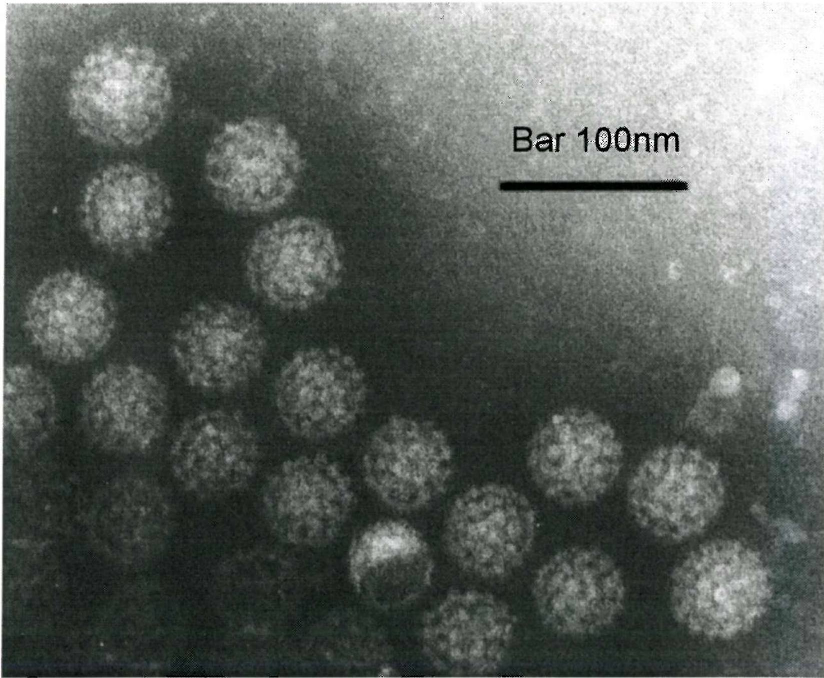
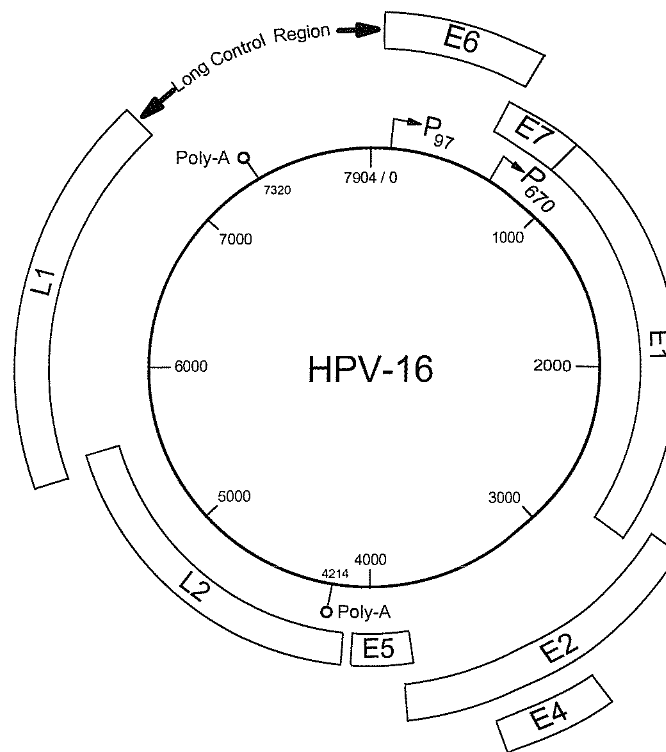


Figure 1.2. Electron Micrograph of Human Papillomavirus Particles. The icosahedral nature of the virions and surface protrusions of the 72 capsomeres that form the capsid are clearly evident. The capsomeres are formed from the L1 protein. The location of the L2 protein is at the centre of the capsomeres. (Lanham, S. 1995).

1.6 Viral Replication

From DNA sequence analysis, all human papillomaviruses have the same genomic organisation. They have a circular double stranded DNA genome of about 8,000 bases in length, which codes for 8 genes. The early region has 6 open reading frames (ORFs), whereas the late region codes for just 2 (see figure 1.3).

Due to the technical difficulties of growing the virus *in vitro*, little is known about virion entry and uncoating. Since the basal cell is the only cell of the squamous epithelium capable of dividing, the virus must infect this cell in order to produce a persistent infection. Early gene expression occurs in these cells. In contrast, late gene expression, vegetative DNA synthesis, capsid production, and virion assembly, only occur in terminally differentiated squamous epithelial cells.



Protein	Function
E6	Binds p53, prevents apoptosis
E7	Binds Retinoblastoma protein, causes cell cycling
E1	Initiates replication
E2	Controls transcription/replication
E4	Causes collapse of cytoskeleton
E5	Undetermined
L1	Major capsid protein
L2	Minor capsid protein

Figure 1.3. Genomic Map of HPV-16 and Protein Function. The genome is double stranded and transcription occurs in a clockwise direction. The open reading frames E1-E7, L1, and L2 are shown. The long control region (LCR) contains transcriptional and replicational regulatory elements. The polyadenylation sites for the early and late genes are located at bases 4214 and 7320 respectively. Two promoters for the early genes are shown (P_{97} and P_{670}); no separate promoter site for the late genes has been identified. The functions of the proteins are shown in the table.

Early transcripts initiate upstream of the E6 protein; the P_{97} promoter in HPV-16, under transcriptional regulation from the long control region (LCR), which contains binding sites for many cellular factors. Factors that regulate HPV early gene expression include AP-1, NF-1, Oct-1, YY-1, TEF-1, SP1 and TATA box binding protein (TBP). The HPV early transcripts encode E6, E7, E1^E4, E5, or truncated forms of E6. The functions of the truncated forms of E6 are uncertain, but probably encode for E7.

The *E1* open reading frame (ORF) is the largest ORF in the papillomavirus genome, and has a relatively well conserved sequence throughout the papillomaviruses. The 68 kDa E1 protein functions include ATPase, helicase, and nucleotide-binding activities (Clertant and Seif, 1984). The E1 protein interacts with the p180 subunit of the cellular polymerase α-primase, and thereby may recruit the cellular DNA replication initiation machinery to the viral replication origin (Park *et al.*, 1994). By itself, the E1 protein binds weakly to the papillomavirus origin of replication, but with the additional binding of E2 protein to adjacent sites, the affinity of E1 protein binding is increased (Mohr *et al.*, 1990). This origin bound E1/E2 complex constitutes the replication initiation complex. The E1 protein binds to the origin as an oligomeric structure recognising A/T rich sequences and requires the presence of ATP.

The 48 kDa E2 protein has two domains; the first 200 amino acids at the amino end has transactivation activity, the carboxyl-terminal 100 amino acids have both sequence specific DNA binding and E2 dimerisation functions; E2 binds the DNA consensus sequence $ACCN_6GGT$ (where N is any base) as a dimer. The two domains are joined by a hinge region.

Both the amino and carboxyl domains have relatively conserved sequences between papillomaviruses, but the hinge region shows variable sequence and variability in length.

A major role of E2 is to target E1 to the replication origin and therefore enable viral DNA replication to occur. Also, the E2 protein can display both activation and repression of transcription of the viral DNA. This appears to depend on the proximity of an E2 binding sequence to an SP1 site (a promoter element) or the TATA box of the promoter of interest. For instance, in BPV, there is a cluster of four E2 binding sites several hundred bases upstream from the P_{89} promoter. E2 is found to activate this promoter. By contrast, the P_{97} promoter of HPV-16 and the equivalent P_{105} of HPV-18, are repressed by E2 protein. In these two viruses there are two E2 binding sites

overlapping a SP1 site and are in close proximity to the TATA box of the promoter. Thus, E2 protein represses transcription of E6 and E7. Repression by the E2 protein probably results from interfering with assembly of the transcriptional initiation complex (see figure 1.4).

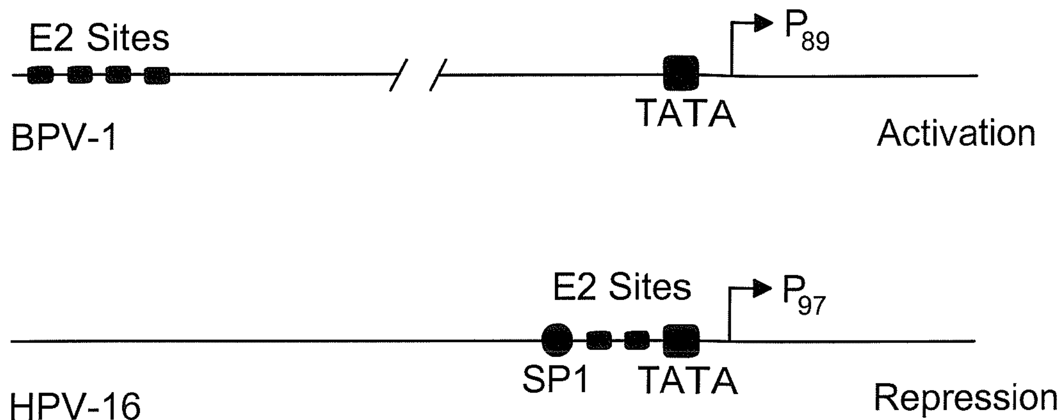


Figure 1.4. Influence of E2 Binding Site on Transcriptional Regulation. Transcription of papillomavirus genes appears to be influenced by the proximity of E2 binding sites to SP1 sites and the TATA box of the promoter. In BPV-1, the E2 binding sites are several hundred bases from the early promoter P_{89}. This promoter is activated by E2 protein. In contrast, the same promoter in HPV-16, P_{97}, has E2 binding sites near the SP1 site and the TATA box. When E2 protein bind to these E2 sites it displaces SP1 from the promoter and interferes with transcription pre-initiation complex formation.

However, Bouvard's group (1994) suggest it is the length of the E2 protein that determines the effect on transcription of E6 and E7. The investigators found the full length E2 protein of HPV-16 activated P_{97}, whereas a short form, lacking the N-terminal end, repressed P_{97}. The shorter protein repressed the activity of the full length form and could also displace the full length protein from an oligonucleotide. This may, therefore, represent a method of gene regulation, as mRNA of both forms of the protein, and others, have been found *in vivo*. Work by Kovelman and colleagues (1996) suggests the E2 proteins from different papillomaviruses have different potentials to activate transcription, for instance protein from HPV-16 and HPV-18 are effective activators, whereas HPV-6 and HPV-11 proteins are weak activators of transcription.

For the induction of late viral gene expression, i.e. production of L1 and L2 proteins, not only must the cells be terminally differentiated, but it appears the viral genome must be maintained as extra-chromosomal elements (Frattini, Lim, and Laimins, 1996). For BPV-1, phosphorylation of the E2 protein at the time of nuclear membrane breakdown appears to be necessary for viral segregation and nuclear retention in daughter cells (Lehman and Botchan, 1998). Lack of this phosphorylation leads to loss of viral DNA from the cells in subsequent cell replication cycles. Induction of the promoter that regulates late gene expression is largely unknown, but appears to be activated upon epithelial differentiation.

Thus, after infecting the basal cell transcription of the E6 and E7 genes is initiated to maintain cell cycling. Following as yet unknown signals, transcription of the E1 and E2 genes occurs; this represses the E6 and E7 genes and allows viral DNA replication to proceed; increasing the viral copy number to between 20 and 100 per basal cell. Following cell differentiation, induction of the late genes occurs, producing the capsid proteins L1 and L2, which assemble into viral capsids in which the viral DNA, now at thousands of copies per cell, is packaged.

In cervical cancer, over production of E6 and E7 leads to uncontrolled cell growth and replication. In some tumours, integration of the viral DNA into the host cell chromosome can occur. Integration usually occurs within the E2 gene, thus the circular genome is linearised with each portion of the E2 gene 'coding' in opposite directions and therefore *E2* can no longer be transcribed.

1.7 Cellular Transformation

The E6 and E7 proteins of high risk HPV types are found to interact with 2 cellular proteins, p53 and retinoblastoma protein, more efficiently than the same proteins from low risk HPV types.

Loss of function of the cellular retinoblastoma protein (pRB) is associated with a significant risk in the development of cancer; this protein plays an important role in cell cycle regulation. The pRB protein has two phosphorylation phases within the cell cycle, controlled by the cyclin-dependent kinase family (cdks). The hypophosphorylated form binds and inactivates E2F-1 in early G1. The transcription factor E2F-1 regulates genes associated with progression of the cell cycle into S phase. Activation of the cdks during

the cell cycle results in the phosphorylation of pRB with the subsequent release of active E2F-1 and entry into S phase.

Mutation of the tumour suppressor protein p53 is a common abnormality in cancer cells. In normal cells, activation of *p53* produces increased levels of active protein which induces cell cycle arrest or apoptosis (Hupp, Sparks, and Lane, 1995).

1.7.1 HPV E7 Protein

The E7 protein of HPV is a small nuclear protein composed of around 100 amino acids. It contains regions with similar functions to portions of the adenovirus E1A protein and SV40 large T antigen; these regions bind cellular proteins including pRB. Five residues C-terminal to the pRB binding site are two casein kinase II phosphorylation sites, which are not required for pRB binding, but appear necessary for efficient transformation by E7. A zinc binding motif contributes to the stability and dimerisation of the protein (see figure 1.5).

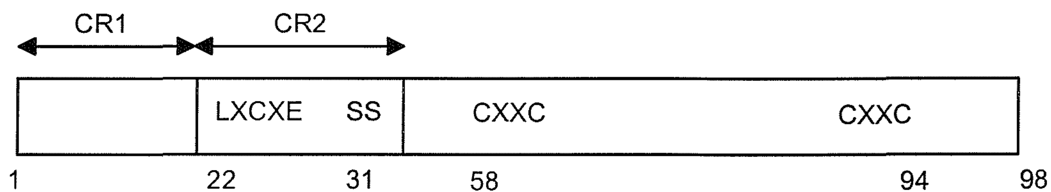


Figure 1.5. Structure of HPV-16 E7 Protein. The protein has two conserved regions (CR1, CR2) at the 38 amino acids of the amino terminal, that share sequence homology with adenovirus E1A protein and SV40 large T antigen. The CR2 region contains the pRB binding site (LXCXE) and two casein kinase II phosphorylation sites (SS). The two CXXC motifs form a zinc binding domain. The amino acid positions are shown.

The E7 proteins from low risk HPVs such as types 6 and 11 bind to pRB with around tenfold less affinity than from high risk HPV types 16 and 18 (Munger *et al.*, 1989), possibly explaining the greater transforming ability of high risk HPV E7 proteins.

The HPV protein E7 interacts with the pRB/E2F-1 complex causing the release of E2F-1 and entry of the cell into S phase (see figure 1.6). However, this abnormal

proliferation can result in apoptosis of the cell due to induction of increased p53 levels. The HPV E6 protein causes degradation of p53 via the ubiquitin mediated pathway (see figure 1.8). Thus, apoptosis of the proliferating cells is prevented.

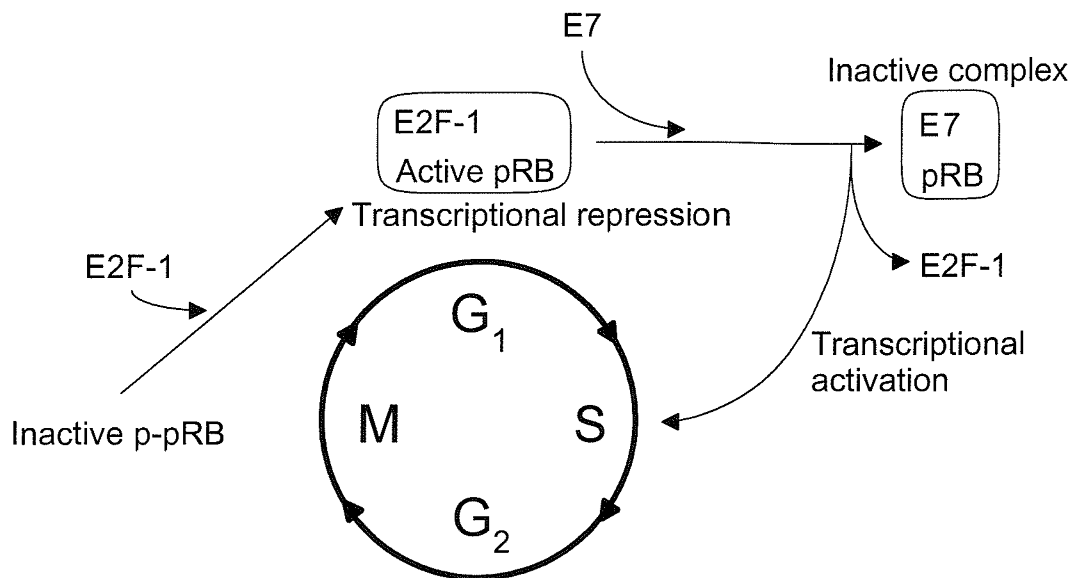


Figure 1.6. Interaction of E7 and pRB. During the cell cycle, pRB is preferentially phosphorylated (p-pRB). The active hypophosphorylated form is only detected in the G_0/G_1 phase and acts as a negative regulator of cell cycle progression. The transcription factor E2F-1 binds to the hypophosphorylated pRB and cannot therefore activate transcription. Binding of E7 to the E2F-1/pRB complex results in release of E2F-1 allowing transcription to be activated and cell progression to the S phase.

In addition to binding pRB, E7 protein of HPV-16 has been found to interact with AP1 transcription factors, including c-Jun, JunB, JunD and c-Fos proteins (Antinore *et al.*, 1996). This interaction is abolished by mutation in the zinc binding motif. This interaction may be an additional requirement for immortalisation of cells, as E7 protein deficient in pRB binding can still induce immortalisation of primary human keratinocytes (Jewers *et al.*, 1992).

It has also been shown by Massimi and Banks (1997) that the E7 protein, of at least HPV-6, 11, 16, and 18, can inhibit the transcriptional activity of p53 in a similar manner to that of the E1a protein of adenovirus. This activity even occurs in mutant E7 proteins that do not bind pRB. The p53 binding appears to require the casein kinase II

(CKII) recognition site of the protein. E7 does not bind directly to p53, but the authors suggest that the binding may be through an E7-TATA box binding protein interaction.

1.7.2 HPV E6 Protein

The E6 protein of HPVs are approximately 150 amino acids in length and contains two zinc binding domains (figure 1.7).

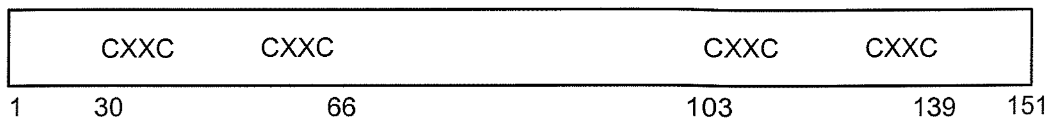


Figure 1.7. Structure of HPV-16 E6 Protein. The protein contains four CXXC motifs which form two zinc binding domains; from amino acids 30-66, and 103-139. The amino acid positions are shown.

Although the protein shows no sequence homology with adenovirus E1B protein of SV40 large T antigen, it does share the ability to bind p53. However, unlike E1B and large T, where inactivation of p53 is caused by the binding, E6 together with E6-associated protein (E6-AP) leads to complete degradation of p53. This occurs through the ubiquitin proteolysis system, a major pathway for intracellular degradation that is highly conserved among eukaryotes. The pathway involves the covalent linkage of the carboxy terminus of ubiquitin protein to the amino group of lysine side chains. Additional ubiquitin moieties can be linked together through a lysine residue at amino acid 48 of ubiquitin. Multi-ubiquitinated proteins can then be recognised and degraded by the 26S proteasome.

E6-AP, after being labelled with ubiquitin by a ubiquitin-conjugating enzyme, can act with E6 as a ubiquitin ligase (Scheffner *et al.*, 1993). The E6/E6-AP complex binds p53 and then transfers the ubiquitin molecule from E6-AP to p53. Subsequently, additional ubiquitin molecules are added to p53 which is then degraded by the proteasome (see figure 1.8).

Recent evidence suggests two common polymorphic variants of p53 have different susceptibilities to HPV E6 proteins. E6 proteins from high and low risk HPV

types target the arginine variant more efficiently than the proline form for ubiquitin-mediated degradation (Storey *et al.*, 1998), thus people homozygous for the proline form may be less susceptible to CIN or cervical cancer.

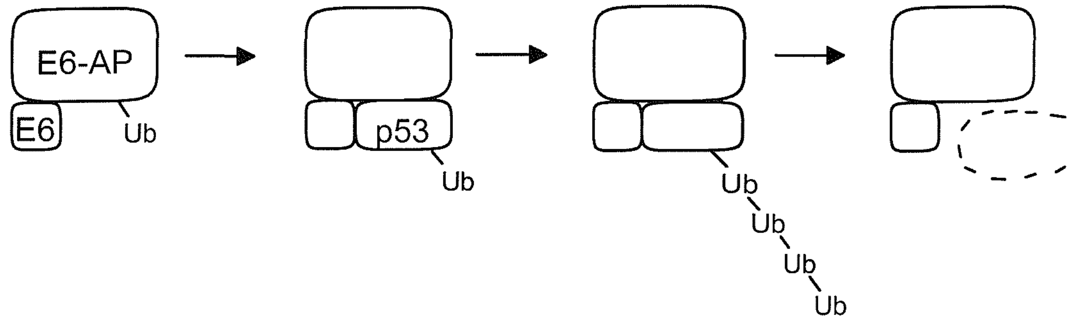


Figure 1.8. Degradation of p53 by E6. The E6 protein binds to the cellular protein E6-AP, which has been labelled with ubiquitin, and this complex then binds p53. The E6/E6-AP complex acts as an E3 (ubiquitin protein ligase) resulting in the transfer of the ubiquitin from E6-AP to p53 and then subsequent multi-ubiquitination and degradation of the p53 protein by the 26S proteasome.

The E6 protein may have another function, in that it may activate telomerase (Klingelhutz, Foster, and McDougall, 1996). Telomeres are 5-8 base pair sequence repeats at the ends of chromosomes. During DNA replication, the extreme 3' end of the chromosome cannot be replicated. These telomeres act as sacrificial 3' ends, allowing the DNA to be replicated without loss of functionality of the DNA. Once the telomeres are lost the chromosome itself is soon lost. By activating telomerase, which is normally absent from somatic tissue, the E6 protein may be able to extend the replicative life of the cell. However, HarleBachor and Boukamp (1996) state that the basal layer of epidermis of normal skin naturally produces telomerase and this property may be essential to produce 'new skin' throughout life. Of course, this does not rule out the possibility that papillomavirus E6 protein may increase the production of telomerase or may induce more cells, both within the basal layer and other layers, to produce telomerase. However, Gorham and colleagues (1997) only found 1 cervical sample in 22 samples with CIN 2-3 to have any telomerase activity, but 6 of 6 cervical carcinoma samples were telomerase positive, although no HPV detection was performed.

1.8 Aims of this Project

The objective of this study was to identify markers which predict an adverse outcome in women infected with potentially oncogenic HPVs.

The initial plan was to undertake a longitudinal study of women with minor abnormalities detected on cervical cytology. The fact that many patients are referred back to their GP for follow-up or attend colposcopy clinics run by different clinicians meant it was not possible to obtain significant numbers of follow-up specimens. The study design was modified to seek differences between women with severe clinical abnormalities (CIN 2/3) and women with minor changes (CIN 0/CIN 1). A full profile of each patient required detailed analysis of the infecting HPV, the search for co-factors such as other cervical infections, and the identification of patient risk factors.

Studies on the infecting HPV. The study sought to establish: 1). If all women with cervical abnormalities are infected with HPV and whether the broad division into low, moderate, and high risk types holds for this population. 2). The significance of mixed HPV infections. 3). If the number of copies of HPV genome shed is a function of lesion severity. 4). If viral transcript expression and quantity for HPV-16 infections vary with grade of CIN and if this can be used to determine the outcome of a high risk HPV infection.

Co-factors. In the Southampton population I sought to establish the frequency of cervical infections with agents such as chlamydia and HSV-2 which have been linked to cervical carcinoma. Secondly, I postulated that co-infections with herpesvirus types 6, 7, 8, EBV, or CMV which produce cytokine and growth factor analogues, and have been implicated in other cancers, might be significant determinants of pre-malignant change.

Host-factors. There is evidence to suggest that genetic factors enhance susceptibility to HPV infection (e.g. HLA type) and variant tumour suppressor genes may increase risk of development of cervical cancer. In these studies I examined the hypothesis that polymorphisms in the p53 gene, and haplotypes, are important in the pathogenesis of cervical cancer. Finally, two cellular proteins which have been shown to be absent in normal tissue, but active in different types of cancer are telomerase and survivin. These proteins appear to be specifically expressed in other cancers and were examined to determine if they play a role in the development of cervical cancer.

Chapter 2

Methods

2.1 Collection and Processing of Cervical Samples

Full Ethical Committee approval was given for the collection of cervical samples from women attending the colposcopy clinic at Princess Anne Hospital, Southampton or a routine cervical smear clinic at a Southampton Health Centre. Women referred for colposcopy following an abnormal smear result were asked to give written consent to take part in the study. The study population comprised women with moderate/severe dyskaryosis who were referred to the clinic after one abnormal smear. In patients with mild dyskaryosis, the smear was repeated after six months, and the women referred for colposcopy if the test remained abnormal. If the repeat smear was normal, a further smear was taken after a year; if this was abnormal then the patient was referred to the clinic.

Specimens were taken during colposcopic examination using a plastic 'Rolon Jordan' spatula (Cellpath) to sample the ectocervix including the transformation zone, and a cytobrush (Cellpath) to take an endocervical sample. Both sampling devices were placed into 2.5 ml RPMI medium + 10% FCS (both Sigma) containing 0.05% sodium azide as preservative. The containers were processed in the laboratory within a few hours of collection.

Cells and virus were detached from the sampling devices by vortex mixing. A 0.5 ml aliquot was centrifuged at 60,000 x g for 30 minutes at 20 °C in a Beckman Avanti 30 centrifuge; this high speed centrifugation step was designed to pellet free HPV virions as well as infected cells. The pellet was stored frozen at -20 °C. A 1 ml aliquot from the remaining sample was centrifuged at 1,000 x g for 5 minutes to pellet the cells. The supernatants was stored at -80 °C for cytokine analysis.

To enable transcripts to be studied in colposcopy patients, samples were collected as before but placed into 2.5 ml RNAlater solution (Ambion). Samples were stored at -20 °C until processed; the mRNA remaining intact in the RNAlater at this temperature for at least 6 months. Processing of these samples required the addition of 0.5 ml PBS to each 0.5 ml aliquot of RNAlater to pellet the cells. For DNA extraction, a

PBS wash of the cells was also necessary. Only DNA and mRNA could be analysed from these specimens.

Samples taken at the health clinic were from women with no history of cervical abnormalities. The same procedure for sample collection as described previously was used. However, to maintain nucleic acid viability during transportation, specimens were placed into 2.5 ml RNAlater solution.

2.2 Extraction of DNA from Stored, Frozen Samples

The DNA was extracted by alkali lysis of the frozen cell pellet; 200 µl 2M ammonium hydroxide (Applied Biosystems) was added to the pellet, which was resuspended by vortex mixing. The sealed tubes were placed in a waterbath at 90 °C (±1 °C) for 10 minutes to denature any viruses. Next, the screw caps were removed and the tubes reheated in the waterbath for a further 70 minutes to evaporate all the ammonia.

The extracted DNA was centrifuged at 2,000 x g for 5 minutes at room temperature to pellet debris, and aliquots from each specimen were added into 200 µl PCR tubes (Strip-ease-12, Genetic Research Instrumentation Ltd.), capped (MicroAmp, Applied Biosystems) and stored at -80 °C until used for PCR.

2.3 Detection of Papillomavirus DNA by Polymerase Chain Reaction

In order to obtain the maximum chance of detecting HPVs in the cervical samples, a series of different PCR reactions were performed. Initially each sample was screened using the GP5+/GP6+ consensus primer set (de Roda Husman *et al.*, 1995) with FAM labelled GP5+ primer (GP5F). Negative samples were re-tested using the CPI/CPIIG consensus primer set (Tieben *et al.*, 1993). Any remaining negative samples were screened using the MY09/MY11 primer set (Manos *et al.*, 1989). Finally, two specific primer pairs were designed, using the Gene Runner programme (Hastings Software), to exclude HPV-16 and HPV-18 infection of specimens still HPV negative. All the primers used in this study were made on an Expedite Nucleic Acid synthesiser (Millipore). The HPV primers are presented in table 2.1.

PCR Reactions. For the GP5F/GP6+ PCR, the reaction mixture consisted of 2 mM $MgCl_2$, PCR buffer (Promega), deoxynucleotide triphosphates (Promega), each at

300 μM, primers at 1 mM, and 1.5 units of *Taq* DNA polymerase (Promega). Five microlitres of extracted DNA (approx. 10-100 ng) was used in a total PCR volume of 25 μl. The PCR was performed on a MJ Research PTC-225 machine using 40 cycles of denaturing at 94 °C for 1 minute, annealing at 40 °C for 2 minutes, followed by extension at 72 °C for 1 minute 30 seconds. The PCR product size was 139-148 bp. For the CPI/CPIIG and MY09/MY11 PCRs the reaction mixture was the same as used for the GP5F/GP6+ PCR. The cycle conditions were 40 cycles of denaturing at 94 °C for 30 seconds, annealing at 55 °C for 50 seconds, followed by extension at 72 °C for 20 seconds. The PCR specific for HPV-16 used the same PCR reaction mixture as GP5F/GP6+. The reaction conditions were 40 cycles of 94 °C for 30 seconds, 60 °C for 50 seconds, and 72 °C for 20 seconds. The PCR specific for HPV-18 used the same reaction mixture as GP5F/GP6+ except 2.5 mM $MgCl_2$ was used. The reaction conditions were 40 cycles of 94 °C for 30 seconds, 62 °C for 50 seconds, and 72 °C for 20 seconds.

Genescan Analysis. The fluorescein labelled GP5F/GP6+ PCR products were detected using Genescan on an ABI 373A DNA sequencer with a 6% polyacrylamide denaturing gel. This method is more sensitive than agarose, accurately sizes the product, and is quantitative.

The gel for Genescan analysis was made using Sequagel 6 (Flowgen) and 1.3x TBE buffer (Applied Biosystems), and set using 0.05% TEMED (Kodak) and 0.5 mg/ml freshly reconstituted ammonium persulphate (Kodak). The gel was allowed to set for at least 2 hours.

The loading dye for Genescan was made using de-ionised formamide (Kodak), 8.3 mM EDTA pH 8.0 (Kodak) and 0.1% Orange G solution (Merck). The PCR product was mixed with the loading dye and ROX-2500 DNA standard (Applied Biosystems) and heated at 90 °C for 2 minutes to denature the DNA strands then immediately placed on ice. Once cool the samples were loaded on the gel. The data were collected using Applied Biosystems Genescan 672 software.

The software calculates the size in base pairs of the fluorescent signals detected on the gel. These signals correspond to DNA containing the fluorescein molecule, i.e. PCR products. The software also calculates the intensity of the fluorescence.

Table 2.1. PCR Primers Used for HPV Detection.

Primer Name	Sequence 5' → 3' [a]	Binding Site	Region	Product size
GP5+	TTTGTTACTGTGGTAGATACTAC	6624 - 6646 [b]	L1	142 bp
GP6+	GAAAAATAAACTGTAAATCATATTC	6765 - 6741		
MY11	GCMCAGGGWCATAAYAATGG	6582 - 6601 [b]	L1	450 bp
MY09	CGTCCMARRGGAWACTGATC	7033 - 7014		
CPIIG	ATGTTAATWSAGCCWCCAAAATT	1776 - 1798 [b]	E1	188 bp
CPI	TTATCWTATGCCCAYTGTACCAT	1963 - 1941		
HPV16For	GTCAAAAGCCACTGTGTCCT	420 - 439 [b]	E7	499 bp
HPV16Rev	CCATCCATTACATCCCGTAC	918 - 899		
HPV18For	ACAATCCTCCATTTTGCTGTG	7435 - 7455 [c]	LCR	384 bp
HPV18Rev	ATAAACTATGTCTGCACAGCTTA	7818 - 7796		
Primers used with GP5+/GP6+ PCR product:				
HPV6	ATCCGTAACTACATCTTCCACATACACCAA	6815 - 6844 [d]	L1	89 bp
HPV11	ATCTGTGTCTAAATCTGCTACATACACTAA	6799 - 6828 [e]	L1	89 bp
HPV16	GTCATTATGTGCTGCCATATCTACTTCAGA	6662 - 6691 [b]	L1	104 bp
HPV18	TGCTTCTACACAGTCTCCTGTACCTGGGCA	6647 - 6676 [c]	L1	98 bp
HPV31	TGTTTGTGCTGCAATTGCAAACAGTGATAC	6583 - 6612 [f]	L1	101 bp
HPV33	TTTATGCACACAAGTAACTAGTGACAGTAC	6622 - 6651 [g]	L1	98 bp

[a] - M is A or C, W is A or T, Y is T or C, R is G or A, and S is G or C.

Numbering based on Genebank accession numbers: [b] K02718 (HPV-16, complete genome), [c] X05015 (HPV-18, complete genome), [d] L41216 (HPV-6, complete genome), [e] M14119 (HPV-11, complete genome), [f] J04353 (HPV-31, complete genome), [g] M12732 (HPV-33, complete genome).

2.4 Typing of Papillomavirus from PCR Product

Papillomaviruses detected by PCR were typed by sequencing the PCR product and comparing the sequence with the same segment of known HPV types.

PCR products were purified from the primers using the Promega PCR Wizard kit following the manufacturer's instructions. The purified PCR product was stored at -20 °C until used for DNA sequencing.

Where the PCR produced multiple bands indicative of infection with more than one HPV type, the bands of interest were cut from agarose and frozen at -20 °C for 1 hour to break-up the agarose. After thawing, gel fragments were placed into a Promega

Wizard minicolumn, centrifuged at 14,000 x g for 1 minute and the eluent collected. Next, 25 μl of UHQ water was added to the column, after 30 minutes the column was re-centrifuged for 1 minute at 14,000 x g. To precipitate the DNA, 200 μl of absolute ethanol and 11 μl of 2M sodium acetate were added to the pooled eluents and the mixture held at -20 °C for 1 hour. The solution was centrifuged at 17,000 x g for 10 minutes, the supernatant removed and the remaining pellet vacuum dried. Finally, 10 μl of UHQ water was added and the DNA solution stored at -20 °C.

PCR product DNA sequences were determined using a fluorescently labelled di-deoxy terminator kit (Amersham Life Sciences). The sequencing mixture consisted of kit reaction mix, 5 μM of one of the PCR primers, and purified PCR product. The sequencing reaction had the following conditions on a MJ Research PTC-225 machine, 96 °C for 10 seconds, 50 °C for 15 seconds, and 60 °C for 1 minute, for 60 cycles. Sequencing reaction products were precipitated with 750 mM ammonium acetate (Amersham Life Sciences) and 65% ethanol, and pelleted by centrifuging for 15 minutes at 17,000 x g.

The sequencing products were detected on an Applied Biosystems 373A DNA Sequencer using a 6% polyacrylamide gel (as used for Genescan).

The pellets were resuspended in loading dye supplied in the sequencing kit, heated at 90 °C for 2 minutes to denature double stranded products and then placed on ice. The entire sample was loaded on the sequencing gel. The sequence data were analysed using Applied Biosystems 373 DNA sequencing software. The sequences generated were then compared to human papillomavirus sequences at Genebank using the FastA program.

2.5 Quantification of Papillomavirus

2.5.1 Production of Internal Quantification Standards

In order to assess if the number of HPV genome copies recovered from the cervix was an indicator of disease severity we utilised the Applied Biosystems 373A DNA sequencer in Genescan mode. The software calculates the peak height of the PCR product permitting direct comparison with internal standards of a different size. The strategy for the design of DNA standards is shown in figure 2.1.

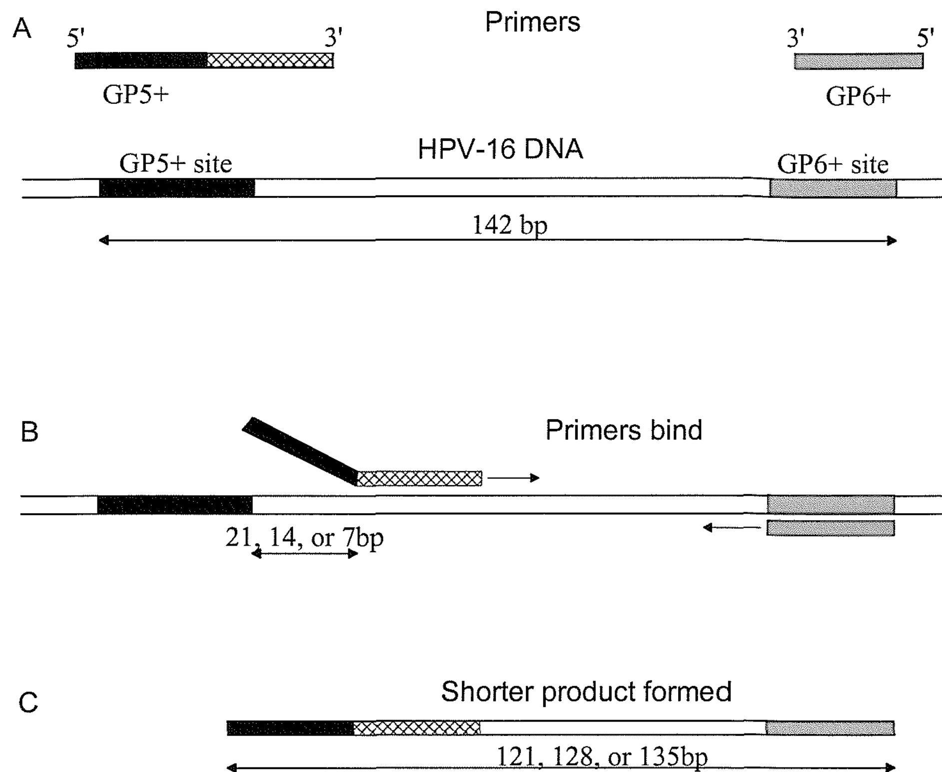


Figure 2.1. Production of Quantitative PCR Standards. (A) Oligonucleotides containing the normal GP5+ primer binding sequence of HPV-16 at the 5' end and a sequence homologous to a portion of the DNA 7, 14, or 21 bases further along from the GP5+' site at the 3' end (GP5135, GP5128, and GP5121 respectively) were made. (B) These primers, together with GP6+, were used in a PCR reaction where the annealing temperature was such that only the 3' non-GP5+ portion of the oligonucleotide would bind wild-type HPV-16 DNA. (C) The separate PCR reactions yielded products of 121 bp, 128 bp, and 135 bp instead of the normal 142 bp with the wild-type DNA. However, these shorter products had GP5+ and GP6+ binding sites at either end, with wild-type DNA between.

PCR Reaction. Each primer (1 mM) was used in a PCR reaction mixture consisting of 2 mM $MgCl_2$, PCR buffer (Promega), deoxynucleotide triphosphates (Promega), each at 300 μM, GP6+ primer at 1 mM, and 1.5 units of *Taq* DNA polymerase (Promega). HPV-16 genome (in pBR322 plasmid) was used as the target. The PCR was performed on a MJ Research PTC-225 machine using 40 cycles of denaturing at 94 °C for 30 seconds, annealing at 40 °C for 50 seconds, followed by extension at 72 °C for 20 seconds. The PCR product sizes were 121 bp for GP5121, 128 bp for GP5128, and 135 bp for GP5135.

The PCR products were purified using the Promega PCR Wizard kit which does not purify products greater than 3,500 base pairs; the papillomavirus plasmid is about 12,000 base pairs. The DNA pellet from each reaction was reconstituted in UHQ water. The concentration of the DNA was determined by measuring the OD_{260} of the solution. The solutions were diluted in UHQ water to give an estimated 75,000 copies per microlitre for GP5121 product, 50,000 copies per microlitre for GP5128 product, and 25,000 copies per microlitre for GP5135 product.

2.5.2 Quantification of Patient Samples

Patient's samples were tested using the PCR reaction mixture containing 2 mM $MgCl_2$, PCR buffer (Promega), deoxynucleotide triphosphates (Promega), each at 300 μM, GP5F and GP6+ primers each at 1 mM, and 1.5 units of *Taq* DNA polymerase (Promega), 300,000 copies of GP5121 standard, 200,000 copies of GP5128 standard, and 100,000 copies of GP5135 standard. Initially, patient DNA was screened at 1/40 dilution. The PCR was performed on a MJ Research PTC-225 machine using 22 cycles of denaturing at 94 °C for 1 minute, annealing at 40 °C for 2 minutes, followed by extension at 72 °C for 1 minute 30 seconds. This was found to give accurate quantification between 100,000 and 250,000 HPV genome copies per sample.

As controls, CaSki and HeLa cells were used. CaSki cells contain between 400 and 800 copies of HPV-16 per cell, and HeLa cells contain between 10 and 50 copies of HPV-18 per cell. The PCR products were mixed with Orange G Loading dye and ROX-2500 DNA standard (Applied Biosystems). The products from these reactions were then run on a 6% sequencing gel. The data were collected using Applied Biosystems Genescan 672 software.

The software calculates the size in base pairs of the fluorescent signals and records peak intensity of the bands.

2.6 Detection of Mixed Papillomavirus Infections

In order to test for mixed HPV types within a patient's sample, primers specific for 6 different HPV types (-6, -11, -16, -18, -31, and -33) were designed (see table 2.1).

These primers bind to sequences within the GP5+/GP6+ PCR product producing PCR products characteristic of the specific HPV types present in a mixed infection.

PCR products from the GP5F/GP6+ reaction with evidence of mixed papillomavirus infection, i.e. two or more bands of the correct sizes on the Genescan, or ambiguous sequencing data, were retested. The PCR reaction mixture consisted of PCR buffer (Promega), deoxynucleotide triphosphates (Promega), each at 300 μM, specific primer at 1 mM, and 1.5 units of *Taq* DNA polymerase (Promega) and 2 μl of GP5F/GP6+ PCR reaction. The PCR was performed on a MJ Research PTC-225 machine using 60 cycles of denaturing at 94 °C for 30 seconds, annealing at 64 °C for 50 seconds, followed by extension at 72 °C for 20 seconds. The PCR product from this reaction was run on a 4% Metaphor gel (Flowgen) and stained with ethidium bromide. Any reaction that showed a product of 89-104 bp was considered positive for that papillomavirus type.

2.7 Detection of Other Infections

Evidence of cervical infections with those STD pathogens and different herpesviruses possibly implicated with causation of cancer were sought.

For all pathogens the PCR reaction mix containing PCR buffer (Promega), deoxynucleotide triphosphates (Promega), each at 300 μM, appropriate primers at 1 mM, and 1.5 units of *Taq* DNA polymerase (Promega). The $MgCl_2$ concentration used for each PCR is shown in table 2.2. Five microlitres of extracted DNA (approx. 10-100 ng) was used in a total PCR volume of 25 μl. The PCR was performed on a MJ Research PTC-225 machine using 40 cycles of denaturing at 94 °C for 30 seconds, annealing at appropriate temperature for 50 seconds, followed by extension at 72 °C for 20 seconds.

The HS6AE/HS6AF primers were used to distinguish between human herpesvirus type 6 variants A and B. Variant B produced products of 235 bp and 516 bp in size when digested with *Hin*d III, variant A is not cut with this enzyme.

The PCR products were run on 2.6-3% agarose gel (SeaKem, Flowgen) depending on the size of the product, then stained for 1 hour with Sybr Gold (Molecular Probes) diluted 1/10,000 in TBE buffer. The gel was viewed on a Storm 860 system (Molecular Dynamics) using the blue fluorescence filter.

Table 2.2. Variables for Other Pathogens PCRs.

Pathogen	Primer	Sequence 5' → 3' [a]	Binding Site	PCR Product Size	Reference [m]	$MgCl_2$ / mM	Anneal / °C
Neisseria	HO1	GCTACGCATACCCGCGTTGC	3143 - 3162 [b]	392 bp	Mahony *et al.*,	2	55
gonorrhoeae	HO3	CGAAGACCTTCGAGCAGACA	3515 - 3534		1995		(Multiplex
Chlamydia	KL1	TCCGGAGCGAGTTACGAAGA	1381 - 1400 [c]	236 bp	Mahony *et al.*,	2	PCR)
trachomatis	KL2	AATCAATGCCCGGGATTGGT	1597 - 1616		1995		
Herpes simplex	HSVBR	CGGAGCCGCCGACGCCACC	55699 - 55717 [d]	334 bp (HSV-1)		2	65 to 50
virus	HSVBA	CCSGACTGCAGCCGCCCGACCTCCGAAG	56005 - 56032	310 bp (HSV-2)			(Reduce by
Cytomegalovirus	CMV2	TCCAGAGGTGGTGGGTTCYTCA	171054 - 171075 [e]	118 bp		2	0.4°C per
	CMV1	GGGTGCTCAGGAGGAGCRGG	171152 - 171171				cycle)
Epstein-Barr virus	EBV1	CTCTGGTAGTGATTTGGACCCG	13995 - 14016 [f]	240 bp	Jiwa *et al.*, 1993	2	(Multiplex
	EBV2	GTGAAGTCACAAACAAGCCCACT	14212 - 14234		(modified)		PCR)
Human	HHV61	CTTTGTGTAGGTGGTCGAATGCGAC	864 - 888 [g]	494 bp	Sada *et al.*, 1996	2.5	60
herpesvirus type 6	HHV62	ACAGCGCAGCAACATGTTTCAGAGC	1333 - 1357		(modified)		
	HS6AE	CGGCCATTTAACGGAACCCTAG	45444 - 45465	751 bp	Dewhurst *et al.*,	2.5	61
	HS6AF	TCCAGAGAAAGGGTGTTGCG	46175 - 46194		1993		
Human	HHV71	ATCCAGAAATGATAGACAGATGTTGG	391 - 416 [h]	133 bp	Sada *et al.*, 1996	3	61
herpesvirus type 7	HHV72	GGTAGCACTAGATTTTTTGAAAAAGATTTAATAAC	489 - 523		(modified)		
Human	KS3-5	CCCTTCTAGCGTTGGCTAGTC	612 - 632 [j]	608 bp	O'Neill *et al.*,	2	60
herpesvirus type 8	KS1-3	TCCGTGTTGTCTACGTCCA	1201 - 1219		1996		
Adeno-associated	AAV1	GCGGAGGCCATAGCCC	1350 - 1365 [k]	218 bp	Han *et al.*, 1996	1.5	64
virus	AAV2	ACGGGAGTCGGGTCTATCTG	1548 - 1567				

[a] - M is A or C, W is A or T, Y is T or C, R is G or A, and S is G or C.

Numbering based on Genebank accession numbers: [b] M10316 (gonococcal plasmid), [c] X06707 (*C. trachomatis* plasmid), [d] X14112, [e] X17403, [f] V01555, [g] X83413, [h] U65005, [j] U18551, [k] J01901.

[m] - where no reference is given, the primers were designed using the Gene Runner programme (Hastings Software).

2.8 Extraction of mRNA from Clinical Samples and Production of cDNA

Cell samples from RNAlater were placed into Trizol reagent (Life Technologies) and RNA extraction carried out following the manufacturer's instructions. Finally, the pellet was vacuum dried for 30 seconds then the RNA dissolved in UHQ water by heating at 55 °C for 10 minutes. Any DNA contamination of the RNA was removed by incubating with 1 unit of amplification grade DNAse I (Life Technologies) at room temperature for 15 minutes. The mixture was heated to 65 °C for 10 minutes to denature the enzyme and stored at -80 °C until used for RT-PCR. A sample was stored at -20 °C as a control to test for DNA contamination.

Reverse Transcription. The RNA was mixed with 1.5 pmoles each of primers 880/3357Rev, 880/2708Rev, 880/2582Rev, 226/3357Rev, 226/2708Rev, E1Rev, L1Rev, L2Rev, GAPDHRev, SurvRev, hTRev, and hTERTRev then incubated at 70 °C for 10 minutes, allowed to cool and the RT mixture consisting of Superscript buffer (Life Technologies), DL-Dithiothreitol 10 mM (Life Technologies), dNTPs (each 500 μM) (Promega), 400 ng acetylated BSA (Promega), 40 units RNAse inhibitor (Promega), and 200 units Superscript reverse transcriptase (Life Technologies) added. The mixture was then incubated at 37 °C for 1 hour to allow the RT step to complete, followed by 95 °C for 5 minutes to denature the reverse transcriptase. The cDNA solutions were then stored at -20 °C until used.

2.9 Detection of HPV-16 cDNA in Cervical Intraepithelial Neoplasia

In order to test for the presence of the transcripts, PCR forward primers were designed upstream of position 226 and within the region 226-409, as well as spanning the 226/409 and 226/526 regions. Reverse primers were designed to span the 880/3357, 880/2709, 880/2582, and 226/3357 regions. The sequences of the primers are shown in table 2.3.

For all transcripts the PCR reaction mix contained PCR buffer (Promega), 2 mM $MgCl_2$ (Promega), deoxynucleotide triphosphates (Promega), each at 300 μM, appropriate primers at 900 nM, and 1.5 units of *Taq* DNA polymerase (Promega). Five microlitres of cDNA solution (approx. 10-100 pg) was used in a total PCR volume of 25 μl. The PCR was performed on a MJ Research PTC-225 machine using 40 cycles of

denaturing at 94 °C for 30 seconds, annealing at 60 °C (68 °C for 226/3357 primer) for 50 seconds, followed by extension at 72 °C for 30 seconds.

The PCR product was run on 2.8% agarose gel (SeaKem, Flowgen) and stained with ethidium bromide.

Table 2.3. PCR Primers Used for HPV cDNA Detection.

Primer Name	Sequence 5'→3'	Binding Site*
E6For151	CACAGAGCTGCAAACAACTATACATG	151 - 176
E6For	CAAACCGTTGTGTGATTTGTTAATTA	382 - 407
E6*I	TACTGCGACGTGAGGTGTATTAAC	213 - 418
E6*II	TACTGCGACGTGAGATCATCA	213 - 532
E6Rev	GCTTTTTGTCCAGATGTCTTTGC	470 - 448
E6*Common	CAGTTGTCTCTGGTTGCAAATC	622 - 601
880/2582Rev	GCCACCTAGAACTGCAGGAT	2592 - 872
880/2708Rev	GACCACGTCTGCAGGAT	2716 - 872
880/3357Rev	CGTTGCTGCTGCAGGAT	3364 - 872
226/3357Rev	GTTGCTGCTCACGTCGC	3363 - 217
226/2708Rev	GACCACGTCTCACGTCGC	2716 - 217
E1For	GCGTAGTACAGCAGCAGCATTAT	1799 - 1821
E1Rev	GCCCATTGTACCATCTGTGATAAT	1954 - 1931
L2For	CGCACAACACAACAGGTTAAAG	4922 - 4943
L2Rev	CAAAAAGTCAGGATCTGGAGCTATATT	5086 - 5060

* - Numbering based on Genebank accession number K02718 (HPV-16, complete genome).

2.9.1 Quantification of cDNA by Fluorogenic Probe Assay

The E1, E6, E7, and L1 gene sequences of HPV-16 from Genebank accession number K02718 as well as the sequences of the human GAPDH, telomerase, and survivin genes were used with the Primer Express program (Applied Biosystems) to

determine suitable regions for a fluorogenic probe assay for each gene. The assay is based on PCR with a probe located internally of the two primers.

The probe was selected under the following criteria; no G on the 5' end of the probe, the amount of Gs and Cs in the probe was 20-80%, no runs of more than 3 consecutive Gs, the strand selected with more Cs than Gs, and a Primer Express T_m of 68-70 °C. The primers were selected under the following criteria; forward and reverse primers were as close as possible to the probe without overlapping the probe, the amount of Gs and Cs in the primers was 20-80%, no runs of more than 3 consecutive Gs, Primer Express T_m of 58-60 °C, and the five nucleotides at the 3' end had only 1-2 Gs or Cs.

The primers/probe selected for the E1 gene were:

Forward primer: E1For (see table 2.3)

Reverse primer: E1Rev

Probe: $^{\text{nt 1885}}$ 5' TGTATCCATTCTGGCGTGTCTCCATACACTT 3' $^{\text{nt 1855}}$

The primers/probe selected for the E6 gene were:

Forward primer: E6For

Reverse primer: E6Rev

Probe: $^{\text{nt 410}}$ 5' TGTATTAACTGTCAAAAGCCACTGTGTCCTGAAGAA 3' $^{\text{nt 445}}$

The primers/probe selected for the E7 gene were:

Forward primer: $^{\text{nt 699}}$ 5' ACCGGACAGAGCCCATTACA 3' $^{\text{nt 718}}$

Reverse primer: $^{\text{nt 816}}$ 5' GCCCATTAACAGGTCTTCCAAA 3' $^{\text{nt 795}}$

Probe: $^{\text{nt 721}}$ 5' ATTGTAACCTTTTGTTGCAAGTGTGACTCTACGCTT 3' $^{\text{nt 756}}$

The primers/probe selected for the L1 gene were:

Forward primer: $^{\text{nt 6794}}$ 5' CGTTATGACATACATACATTCTATGAATTCC 3' $^{\text{nt 6824}}$

Reverse primer: $^{\text{nt 6904}}$ 5' TGGGTTACAAACCTATAAGTATCTTCTAGTGT 3' $^{\text{nt 6873}}$

Probe: $^{\text{nt 6839}}$ 5' CTGGAATTTTGGTCTACAACCTCCCCCAG 3' $^{\text{nt 6867}}$

The primers/probe selected for the E6*I transcript were:

Forward primer: E6*I

Reverse primer: E6*Common

Probe: $^{\text{nt 574}}$ 5' TATCTCCATGCATGATTACAGCTGGGTTTCTCTAC 3' $^{\text{nt 540}}$

The primers/probe selected for the E6*II transcript were:

Forward primer: E6*II

Reverse primer: E6*Common

Probe: As for E6*I

All numbering is based on Genebank accession number K02718 (HPV-16 genome).

The primers/probe selected for GAPDH were:

Forward primer: $^{\text{nt 37}}$ 5' GGGAAGGTGAAGGTCGGAGT 3' $^{\text{nt 56}}$

Reverse primer: $^{\text{nt 266}}$ 5' TGGAAGATGGTGATGGGATTTC 3' $^{\text{nt 245}}$

Probe: $^{\text{nt 145}}$ 5' ATTGACCTCAACTACATGGTTTACATGTTCCAATATGA 3' $^{\text{nt 182}}$

Numbering based on Genebank accession number M17851 (Human GAPDH mRNA).

The primers/probe selected for Telomerase RNA component were:

Forward primer: $^{\text{nt 121}}$ 5' GGTGGTGGCCATTTTTTGTC 3' $^{\text{nt 140}}$

Reverse primer: $^{\text{nt 257}}$ 5' CTAGAATGAACGGTGGAAGGC 3' $^{\text{nt 237}}$

Probe: $^{\text{nt 186}}$ 5' CGCGCTGTTTTTCTCGCTGACTTTC 3' $^{\text{nt 210}}$

Numbering based on Genebank accession number U86046 (Human telomerase RNA).

The primers/probe selected for Telomerase reverse transcriptase were:

Forward primer: $^{\text{nt 1784}}$ 5' CGGAAGAGTGTCTGGAGCAA 3' $^{\text{nt 1803}}$

Reverse primer: $^{\text{nt 1973}}$ 5' CGACGTAGTCCATGTTCACAATC 3' $^{\text{nt 1951}}$

Probe: $^{\text{nt 1837}}$ 5' CTTCAAGTGCTGTCTGATTCCAATGCTTTGC 3' $^{\text{nt 1807}}$

Numbering based on Genebank accession number AF015950 (Human telomerase reverse transcriptase mRNA).

The primers/probe selected for survivin were:

Forward primer: $^{nt\ 5187}$ 5' GCGCTTTCCTTTCTGTCAAGA 3' $^{nt\ 5207}$

Reverse primer: $^{nt\ 12016}$ 5' GCACGGCGCACTTTCTT 3' $^{nt\ 12000}$

Probe: $^{nt\ 11991}$ 5' CCTCAAATTCTTTCTTCTTATTGTTGGTTTCCTTTGCC 3' $^{nt\ 11954}$

Numbering based on Genebank accession number U75285 (Human survivin gene).

The probes were manufactured by Scandinavian Gene Synthesis AB or Eurogentec with 7=6-FAM at the 5' end and T=TAMRA at the 3' end.

The reaction mixture for the fluorogenic probe assays consisted of 3.5 mM $MgCl_2$, reaction buffer (Applied Biosystems), 200 μM dATP, dCTP, dGTP, 400 μM dUTP (all Applied Biosystems), forward and reverse primers at 900 nM, probe at 200 nM, 0.25 units Amperase Uracil N-Glycosylase (Applied Biosystems), 0.625 units AmpliTaq Gold DNA polymerase (Applied Biosystems). Five microlitres of the cDNA mixture was used in a total volume of 25 μl on an Applied Biosystems Prism 7700 Sequence Detector.

The cycle conditions consisted of 50 °C for 2 minutes to allow the Uracil N-Glycosylase to digest any product containing dUTP, then 95 °C for 10 minutes to inactivate the Uracil N-Glycosylase and activate the DNA polymerase. Followed by 50 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds.

Following the PCR reaction, the results were analysed using the ABI Prism 7700 Sequence Detection Software (Applied Biosystems).

2.10 Quantification of Host Cell Number

The PicoGreen stain (Molecular Probes) has a high affinity for double stranded DNA and a large fluorescence enhancement upon binding. This property was used in order to give a simple method to quantify the dsDNA content in patient's samples.

In order to produce a calibration curve of DNA content against PicoGreen fluorescence, lymphocytes were removed from fresh blood using Lymphoprep (Nycomed), washed twice in phosphate buffered saline, and counted in a Coulter automated cell counter. The cells were then diluted to give different numbers of cells per sample. The DNA from the samples was then extracted using the method described previously in 'Extraction of DNA from stored, frozen samples.' This DNA was then used to produce a calibration curve.

The PicoGreen stock solution (Molecular Probes) was diluted 1/400 in TE buffer (10 mM TRIS, 1 mM EDTA, pH 7.5). The extracted DNA solution (representing 1/20 of the total number of cells in the sample) was diluted 1/10 in picogreen/TE in a black fluorometer plate (Labsystems). The wells were read in a Fluostar fluorometer (SLT) with an excitation wavelength of 485 nm and emission wavelength of 538 nm.

The values recorded were then used to produce a curve, shown in figure 2.2. This calibration curve was subsequently used to determine the total number of cells in a clinical sample. From the results shown in figure 2.2, it was important the fluorescence value given for a sample was less than 3 Arbitrary Units, thus any sample that produced a value greater than this was diluted so the value fell within the correct range.

To test that the calibration curve worked with epithelial cells, the DNA from 18200, 8000, and 150 CaSki cells was quantified and the graph used to determine the number of cells present. The values recorded were 19000, 8500, and 300.

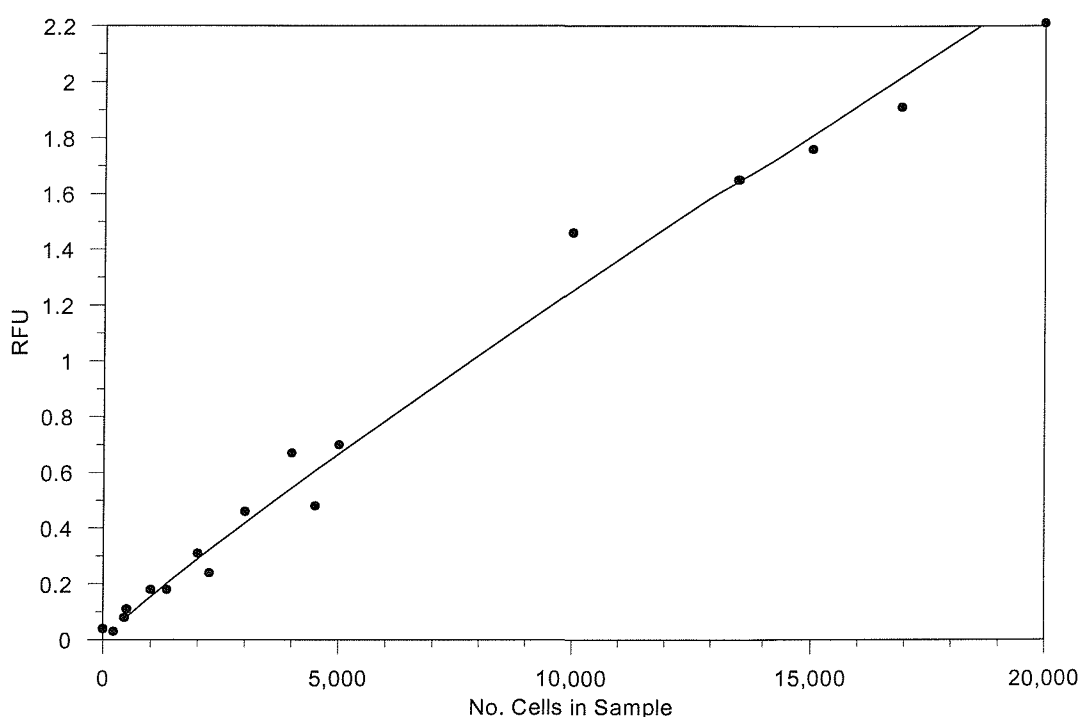


Figure 2.2. Quantification of Cell Number Using PicoGreen. Lymphocytes were counted with a Coulter automated Cell Counter, and diluted to give a range of values. The cells were then treated as for a colposcopy specimen, and the resulting DNA stained with PicoGreen. The amount of fluorescence (RFU) produced from the stain was determined using a fluorometer.

Chapter 3

Detection of Papillomavirus in Cervical Samples

3.1 Introduction

Around 100 different human papillomaviruses have been identified so far. The polymerase chain reaction is the most sensitive method for detection of these viruses. However, due to marked variability in gene sequences between HPV types, the design of PCR primers able to detect all HPV types with equal efficiency has proved problematic. Further, during the course of infection, the HPV genome can integrate into the host cell chromosome, possibly resulting in the loss of certain genes. Only the long control region, E6, and E7 genes appear to be necessary for malignant transformation and the non-essential E1, E2, L1, and L2 genes are lost from the HPV genome in some cervical cancers.

In order to ensure the detection of all known HPV types in population surveys it would be necessary to have primers for the different HPV types and even different genes within those types. This is impractical, so most researchers use consensus primers designed to detect different HPV types using a single primer set. These consensus primers are possible for HPV L1 and E1 genes, other genes are too variable in sequence.

The purpose of this study of women referred for colposcopy was to establish if HPV infection could be detected in every patient with a cervical abnormality (CIN 1,2, and 3).

3.2 Methods

To compare the ability of different primer sets to detect HPV types, 40 randomly chosen cervical samples were analysed with two commonly used consensus primer sets; GP5+/GP6+ and MY09/MY11. All the PCR products obtained were re-analysed using the HPV-6, -11, -16, -18, -31, and -33 specific primers to test for multiple HPV infections (see table 2.1).

3.3 Results

Of the specimens tested using consensus primer sets; GP5+/GP6+ primers detected HPV in 35 of the 40 samples and MY09/MY11 primers detected HPV in 33 of

the 40 samples. The 2 isolates not detected using the MY09/MY11 set were typed as HPV-30 and HPV-CP6108.

The GP primer set detected mixed infections in 6 of the 40 samples; high risk viruses detected were HPV-16 (1) and HPV-18 (2). Using the MY primers a further 7 mixed infections were detected (table 3.1).

Table 3.1. Comparison of MY and GP+ Primer Sets for Mixed HPV Detection.

Specimen	Consensus Primer Set Used	
	MY09/MY11	GP5+/GP6+
1	51+X	51+X
2	35+X	35+X
3	33+X	33+X
4	18+X	18+X
5	18+X	18+X
6	X+Y	X+Y
7	45+33	45
8	40+6	40
9	39+33	39
10	16+33	16
11	16+33	16
12	16+33	16
13	16+31	16

Forty samples were tested for the presence of HPV, using the MY and GP+ primer sets. The PCR product obtained was sequenced and also tested with a specific primer for 6 different HPV types to produce a second, smaller PCR product. Results for specimens with mixed HPV infections are shown. X or Y indicates the presence of unidentified HPV types.

Thus, overall, the MY primers were better at detecting HPV-33 isolates than the GP primers, however, the GP primers were better at detecting a wide range of HPV types. No ideal PCR primer set was found to detect all HPVs. Three different consensus primer sets were used to screen for human papillomavirus DNA in the clinical samples. These primers were directed at two different genes within the papillomavirus genome; GP5+/GP6+ (de Roda Husman *et al.*, 1995) and MY09/MY11 (Manos *et al.*, 1989) target the L1 gene and CPI/CPIIG (Tieben *et al.*, 1993) detect part of the E1 gene. An important advantage is that some HPV infections appear only to be detected with one primer type (Karlsen *et al.*, 1996). In addition, primer sets specific against HPV-16 E7

gene and HPV-18 LCR were used to detect possible integrated forms of HPV-16 and HPV-18.

Unlike the MY09/MY11 and CPI/CPII PCR products, the fragments produced by GP5+/GP6+ PCR reactions differ in size according to the HPV type (table 3.2). To facilitate accurate size analysis GP5+/GP6+ reactions were run using a fluorescent primer (GP5F) and analysed on an Applied Biosystems 373A DNA sequencer run in Genescan mode. The potential advantages were first, an increased ability to detect mixed infections together with a preliminary indication of the HPV type(s) involved, second, quantification of the cervical HPV load, and finally, increased sensitivity.

Table 3.2. Differences in PCR Product Sizes and HPV Type Using GP5+/GP6+ Primers.

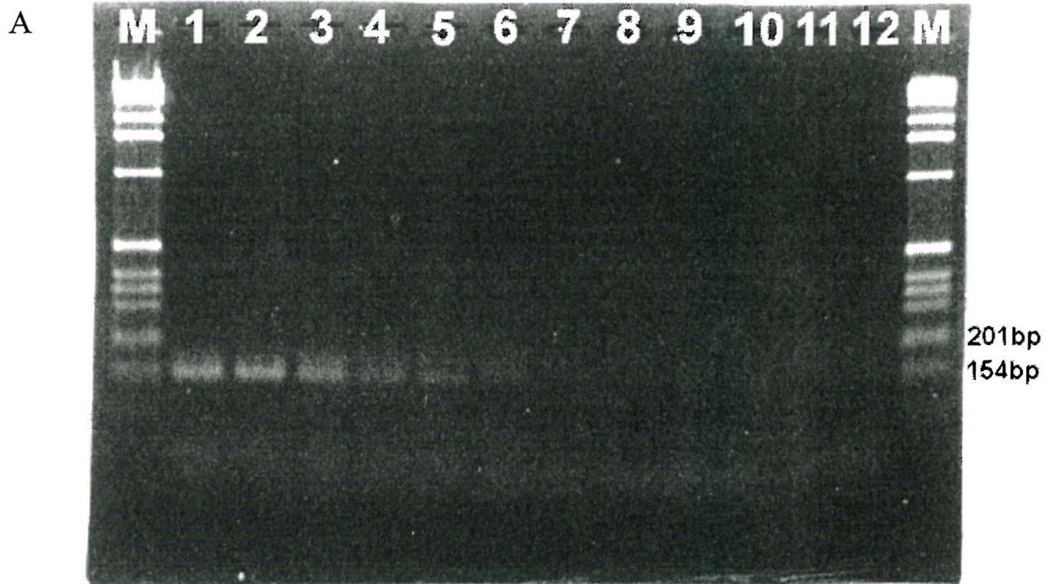
GP5+/GP6+ product size / bp	HPV types
139	1, 2, 3, 6, 11, 28, 30, 32, **33**, 41, 42, **52**, 53, 54, **56**, 57, **58**, 62, 66, **67**, **CP6108**, **CP8304**, **han831** **mm8**
142	**16**, 22, 23, 27, **31**, **35**, **51**, 61, 63, 72
145	7, 9, 10, 13, 15, 17, **18**, 26, 29, 37, 38, **39**, 40, 43, 44, **45**, 49, 55, **59**, 68, 69, 70, 74, 77, 80
148	4, 34, 64, 65, 73, IS324, IS601
151 - 169	5, 8, 12, 14, 19, 20, 21, 24, 25, 36, 47, 48, 50, 60

The known high and moderate risk genital types are shown in bold. The DNA sequences of all HPV types in the EMBL database at Daresbury Laboratory were searched for sequences most closely matching the GP5+ and GP6+ primer sequences. From the location of the two sites of the HPV genome, the expected size of the PCR product could be determined.

The sensitivities of 3% agarose stained with ethidium bromide and analysis using the Genescan system were compared by making dilutions of the GP5135 standard (see section 2.5.1) to give known amounts of starting copies. The GP5F/GP6+ PCR was then performed. Ten microlitres of the PCR products was run on an agarose gel, and 2μl run on Genescan, the results are shown in figure 3.1.

From figure 3.1, the limit of sensitivity of detection using agarose was 3,000-5,000 starting copies, whereas with Genescan the sensitivity was 30-50 starting copies. These data confirm Genescan analysis was around 100 times more sensitive than agarose despite using 5 times less sample. The peak heights (figure 3.1B) show a linear relationship between starting copy number of the L1 gene up to 250 copies.

A



B

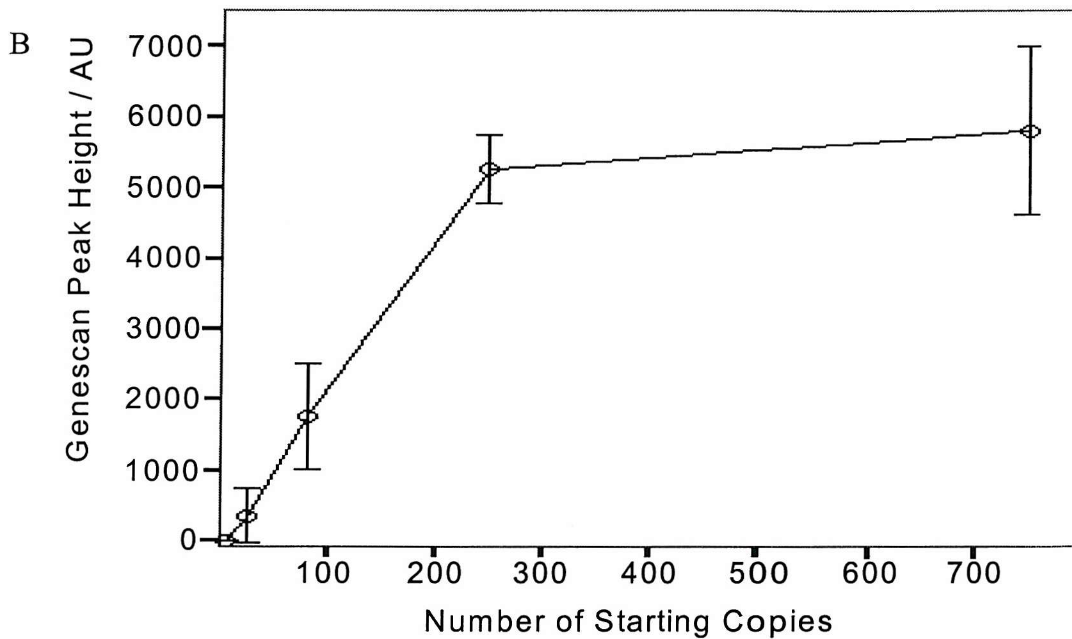


Figure 3.1. Sensitivity of Agarose Gel and Genescan. Figure 3.1A shows the agarose gel. M, 1 kb molecular weight marker. Lane 1 is 60,000 starting copies of GP5135 PCR product. Lane 2 is 30,000 starting copies. Lane 3 is 20,000 starting copies. Lane 4 is 10,000 starting copies. Lane 5 is 5,000 starting copies. Lane 6 is 3,000 starting copies. Lane 7 is 750 starting copies. Lane 8 is 250 starting copies. Lane 9 is 83 starting copies. Lane 10 is 28 starting copies. Lane 11 is 9 starting copies. Lane 12 is 0 starting copies. Figure 3.1B shows the Genescan results for the same samples (only the lowest 6 samples are shown). The number of starting copies was plotted against the height of the GP5135 PCR product. The error bars show 95% confidence limits. The maximum possible Genescan peak height was 6500 AU. The cut-off for detection on Genescan was set at a peak height of 500 AU.

3.3.1 Analysis of Cervical Samples

Initially each sample was screened using the GP5+/GP6+ consensus primer set. Negative samples were re-tested using the CPI/CPIIG primers. All remaining negative samples were screened using the MY09/MY11 primer set, the HPV-16 E7 and the HPV-18 LCR primers.

Four hundred and sixteen samples were tested for the presence of papillomavirus DNA. Initially, 119 specimens were negative using the GP5F/GP6+ primer set, of these, 23 were found to be positive using the CPI/CPIIG primers. Of the remaining 96, 20 gave a positive result with the MY09/MY11 PCR. All of the 76 remaining negative samples were screened with a PCR that detects the E7 gene of HPV type 16. Of those tested, a further 10 gave positive results. Four more samples were positive using the HPV-18 LCR primers. Finally, the 62 samples which gave negative PCR results with the papillomavirus primers, were tested for the presence of the human globin gene by PCR, in order to determine if the specimen was inhibitory for PCR. All of the samples tested gave positive results with these primers. Thus, none of the samples were significantly inhibitory for PCR. Overall, of 416 specimens, 354 contained detectable papillomavirus DNA, this is a positive detection rate of 85.1% or 92.7% of women referred for colposcopy.

The grade of CIN was determined by Dr. Amanda Herbert from a biopsy taken at colposcopy at the same time as the cervical sample. The proportion of samples with detectable HPV are shown in table 3.3. CIN 0 was defined as patients referred to the colposcopy clinic with abnormal smear subsequently found to have a normal cervical appearance and biopsy.

Table 3.3. Percentage of Samples Positive for HPV Using Different Primer Sets.

CIN Status	GP Primers Only	GP+CP Primers	GP+CP+MY Primers	GP+CP+MY+E7+LCR Primers
Normal	18	26	39	42
CIN 0	61	70	75	79
CIN 1	74	80	86	90
CIN 2	93	93	95	99
CIN 3	92	93	97	100

Specimens with different grades of CIN were tested with GP5+/GP6+ primers only, any negative samples were then tested using the CPI/CPIIG primers. Samples still negative for HPV were next tested with MY09/MY11 primers. Any remaining negative samples were tested with HPV-16 E7 and HPV-18 LCR specific primers. Figures are given as the percentage of samples positive for HPV at each stage. CIN 0 was defined as colposcopy patients found to have a normal biopsy.

Thirty-five different HPV types were detected in the samples. Of the types detected only HPV-10 is not normally associated with genital lesions; HPV-10 normally causes flat skin warts. This could be a contaminant from cutaneous surfaces when the sample was taken, or could be a HPV type present in the cervix of some healthy individuals. Most of the HPV types have been grouped according to the risk association with cervical cancer (Shah and Howley, 1996), as shown in table 3.4. Although there is no official classification for HPV type han831; based on the occurrence of this HPV type in both CIN 0/1 and CIN 2/3 in the population studied we classified han831 a moderate risk type for subsequent analysis.

The specimens analysed were categorised by the age of the patient and the histopathology result. Normals were women whose smear tests show no significant abnormality. The results are shown in figure 3.2.

Figure 3.2 shows the 26-35 age group had the highest incidence of CIN 3 and the lowest incidence of CIN 0. The highest incidence of Normal samples occurred in the oldest age group. With increasing age there were small fluctuations in the incidence of CIN 1 and CIN 2, whereas the incidence of CIN 3 initially increased in the 26-35 age range, but then continually reduced to the 46-55 group.

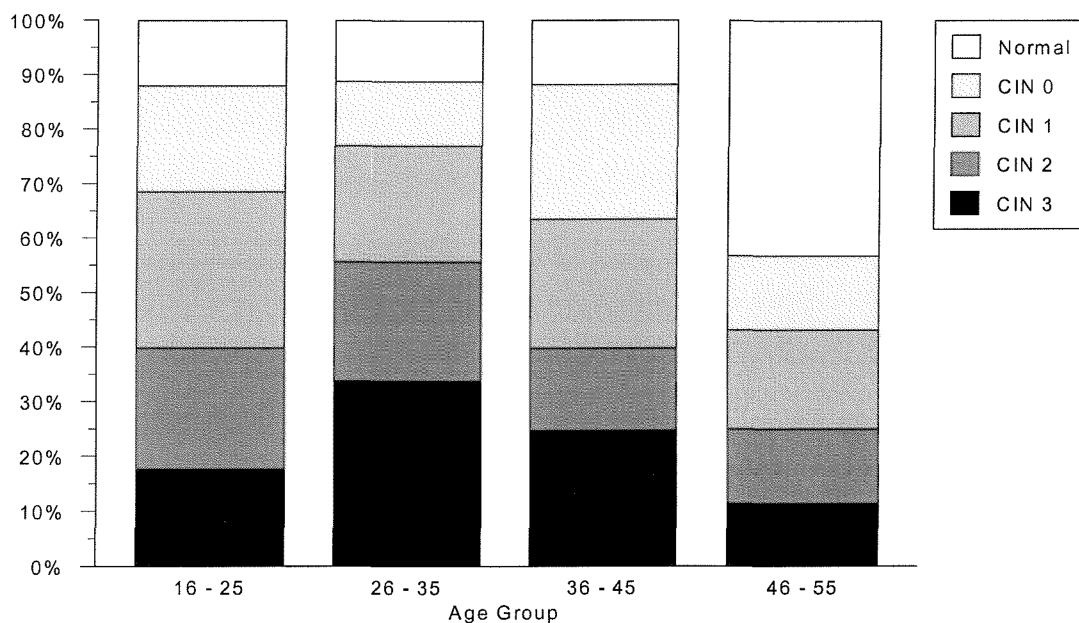


Figure 3.2. Patient Age Range and CIN Status. The histopathology result from the biopsy taken at the same time as the HPV sample was matched with the age of the patient. Two patients are excluded for ages above 55. CIN 0 was defined as colposcopy patients found to have a normal biopsy. The number of specimens for age groups 16-25, 26-35, 36-45, and 46-55, was 108, 169, 93, and 44 respectively.

Table 3.4. Summary of HPV types Detected in Cervical Specimens.

Risk Type*	HPV Type	Number of Specimens Detected	% HPV Positive Samples with:		
			Normal	CIN 0/1	CIN 2/3
High	16	106	8.3	18.2	42.2
	18	18	4.2	7	3.8
	31	25	0	5.6	9.2
	45	10	4.2	2.8	2.7
Moderate	33	25	4.2	6.3	8.1
	35	8	0	2.8	2.2
	39	3	0	1.4	0.5
	51	10	0	7	0
	52	10	12.5	3.5	1.1
	56	9	0	4.9	1.1
	58	5	8.3	0	1.6
	59	3	0	0.7	1.1
	67	3	0	1.4	0.5
	68	1	0	0.7	0
	han831	7	0	2.8	1.6
	ME180	3	12.5	0	0
Low	6	8	0	4.9	0.5
	10	2	8.3	0	0
	11	2	0	1.4	0
	30	1	0	0.7	0
	40	1	0	0.7	0
	42	4	4.2	1.4	0.5
	43	5	4.2	1.4	1.1
	53	2	4.2	0.7	0
	54	5	8.3	1.4	0.5
	66	4	0	1.4	1.1
Undetermined	61	1	4.2	0	0
	70	2	4.2	0.7	0
	73	1	0	0	0.5
	77	1	0	0.7	0
	CP6108	2	0	1.4	0
	CP8304	1	0	0.7	0
	IS324	1	0	0.7	0
	IS601	1	0	0.7	0
	mm8	2	0	1.4	0
Mixed Types		61	8.3	14.7	20

* The risk type is that defined by Shah and Howley (1996) based on the incidence of HPV types in cervical cancers.

Figure 3.3 shows the HPV risk type distribution against patient age. For χ^2 analysis purposes pooled data for the age range 16 to 35 and 36 to 55 were compared. There were more cases of high risk HPV infection in the lower age range (p-value 0.007), and fewer HPV negative cases (p-value 0.001). There were no differences in the occurrence of moderate or low risk types with age.

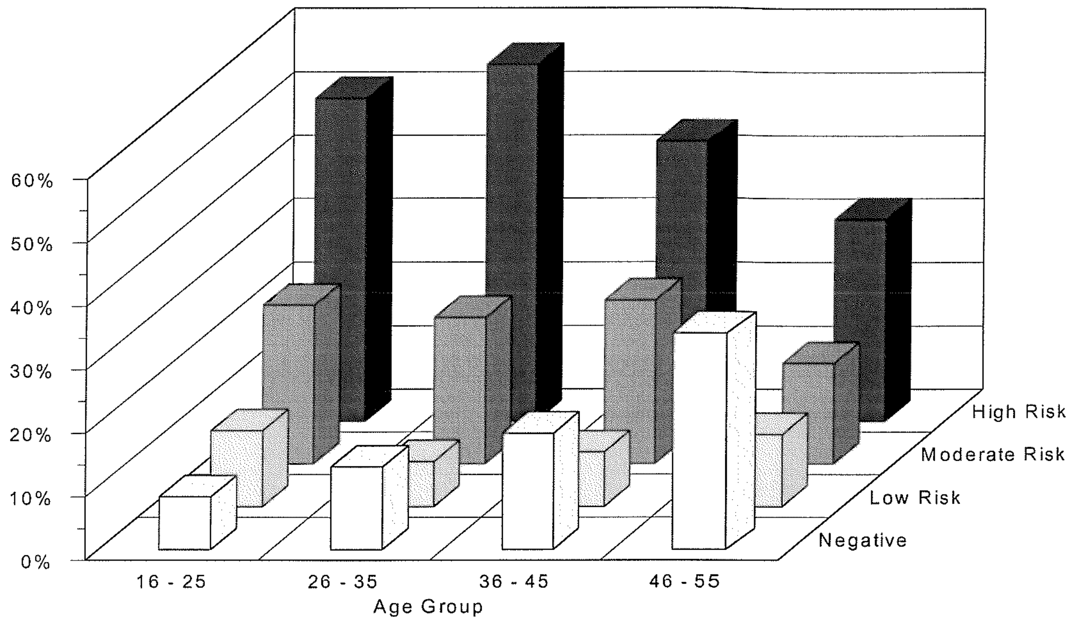


Figure 3.3. Patient Age and HPV Risk Type Detected. The risk assigned for each HPV detected is as in table 3.4. Specimens that contained mixed HPV infections were grouped according to the highest risk type present. For age groups 16-25, 26-35, 36-45, and 46-55, the number of specimens was 108, 169, 93, and 44 respectively. Results do not include 2 samples excluded by age. Results for samples containing HPV with undetermined risk were excluded.

The incidence of the high, moderate, and low risk types detected were compared with the CIN status of the biopsy taken with the specimen at colposcopy shown in figure 3.4. CIN 0 was defined as patients referred to the colposcopy clinic with abnormal smear subsequently found to have a normal biopsy.

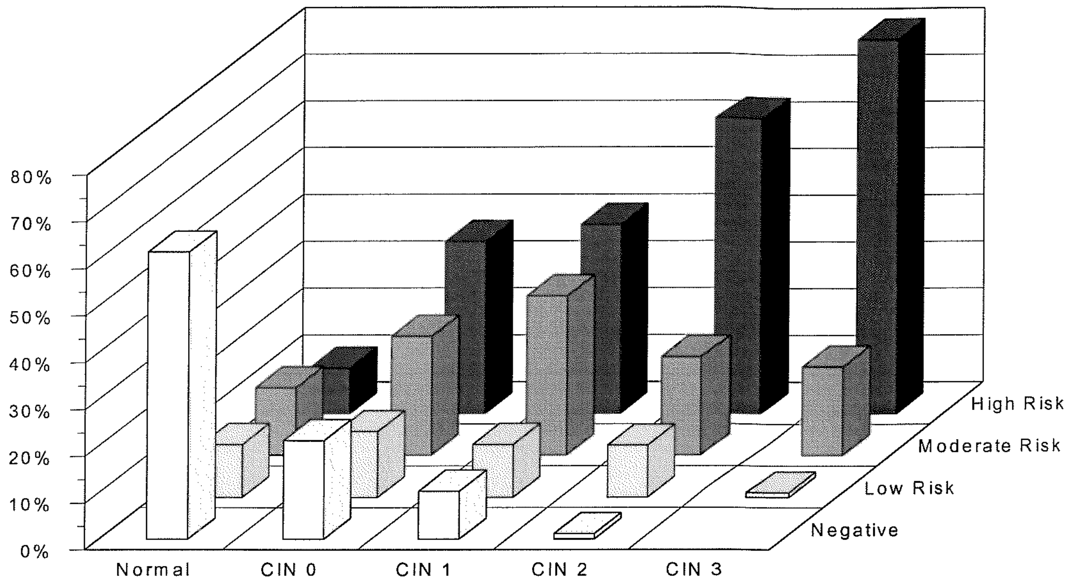


Figure 3.4. CIN Status and HPV Risk Type Detected. The CIN status was determined from the histopathology of a biopsy taken at the same time as the HPV specimen. The risk assigned for each HPV detected is shown in table 3.4. Specimens that contained mixed HPV infections were grouped according to the highest risk type present. For Normal, CIN 0, CIN 1, CIN 2, and CIN 3, the number of specimens was 62, 71, 97, 81, and 105 respectively. Results for samples containing HPV with undetermined risk were excluded.

The CIN 0/1 and CIN 2/3 results in figure 3.4 were combined for χ^2 statistical analysis. Commercially available HPV detection kits divide HPV types into 2 groups; low risk and moderate/high risk. The data presented here clearly show an association between the presence of high risk HPV types and CIN 2/3 (p-value $3x10^{-17}$), whereas moderate risk type was not significantly linked with CIN 2/3 (p-value 0.14). Therefore detection of a moderate risk type is not necessarily predictive of a pre-cancerous lesion, whereas detection of a high risk type is predictive. Linking these two risk types in the same pool in a HPV detection kit may give misleading results. Also, low risk HPVs occur more often in Normal/CIN 0/1 samples (p-value 0.017) and absence of HPV is most common in samples without CIN (p-value $3x10^{-21}$).

These finding are similar to those found in 2 other studies (Wheeler, 1996); one involving 1030 predominantly white women in Portland, Oregon, USA, the other was 325 Hispanic and non-Hispanic white women in Albuquerque, New Mexico, USA. In both studies only the MY09/11 primer set was used. Both groups found an increase in incidence of high risk HPV types, and a decrease in low risk types with increasing CIN status, and fluctuations in the amount of moderate risk types with CIN.

3.3.1.1 Analysis of Mixed HPV Infection in Cervical Samples

All cervical samples where the PCR product showed more than one band in the range 139-169 base pairs on the Genescan analysis, or sequence analysis indicated different bases at the same point, were tested for the presence of more than one papillomavirus type. This was done using the GP5F/GP6+ PCR product and primers for HPV-6, 11, 16, 18, 31, or 33 (see table 2.1), the commonest high and moderate risk HPV types detected in the clinic population.

Overall, of 416 specimens tested for the presence of HPV, 61 contained mixed HPV infections. This is a mixed infection rate of 14.7% overall, or 17.2% of HPV positive samples. Of these 61 samples, 73.8% contained high risk HPV types, of the remainder; 13.1% contained moderate risk types, 6.6% contained low risk types, and 6.6% contained unidentified HPV types. These figures are based on the highest risk type detected in the sample.

Figure 3.5 shows the proportion of patients with mixed infections and the histopathology grade of the specimen. It shows the proportion of samples containing more than one HPV type increased with the severity of CIN, from around 3% in Normal samples up to 23% in CIN 3. A χ^2 analysis performed on these data showed a significant difference (p-value 0.007) between specimens from Normal cervixes with mixed HPV infection and women with any grade of CIN, and this increased in significance with CIN, i.e. p-value 0.002 for Normal versus CIN 2/3. Thus, mixed infections increased in frequency with increasing CIN.

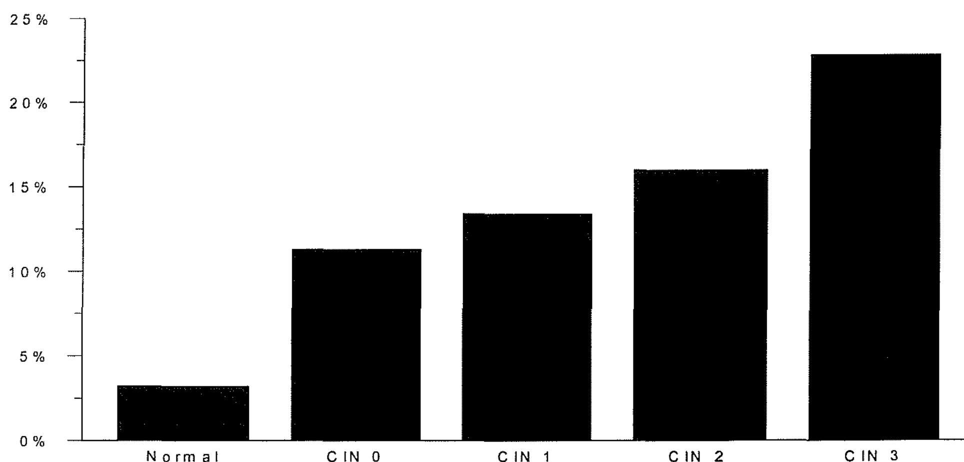


Figure 3.5. Variation in Proportion of Mixed HPV Infections with CIN Status. The percentage of specimens with 2 or more HPV types detected in the same sample was plotted against the histopathology result of the biopsy taken at the same time as the sample. The total number of samples for the groups were 62 Normal, 71 in CIN 0, 97 in CIN 1, 81 in CIN 2, and 105 in CIN 3.

When the age of the patients with mixed infections were analysed, see figure 3.6, there was a reduction in number from 21% with mixed HPV types in the age range 16-25, to less than 3% in the range 46-55 years.

There was a statistically significant fall in mixed infection rate when patients aged 36-55 were compared to those aged 16-35 (p-value 0.0001 using χ^2 analysis).

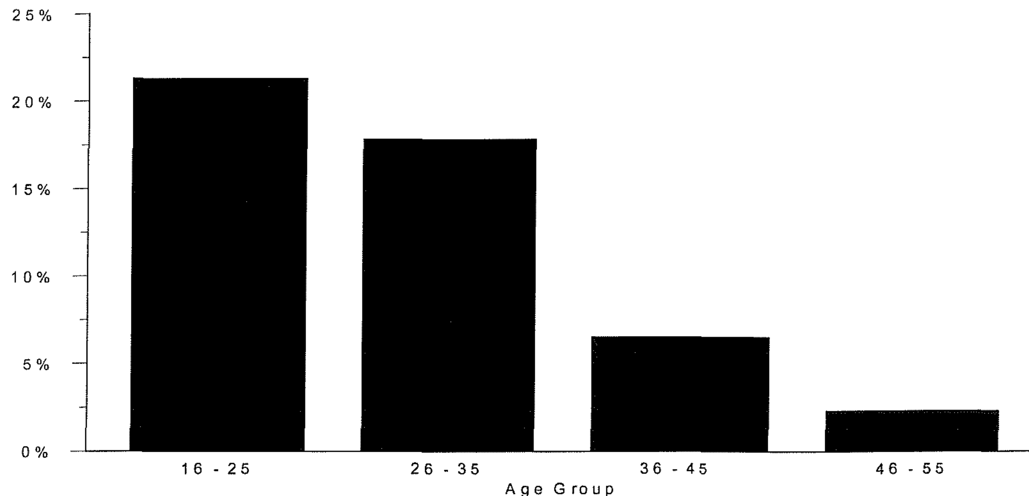


Figure 3.6. Variation in Occurrence of Mixed HPV Infections with Patient Age. Samples with more than one HPV type detected in the specimen were plotted against the age of the patient. The total number of samples for the groups was 108 in 16-25, 169 and 93 in 26-35 and 36-45 respectively, and 44 in 46-55. Two patients were excluded as their ages were above 55.

3.3.1.2 Assessment of Cervical Disease Status

The referral smear can be compared to subsequent colposcopy clinic findings giving some assessment of disease progression. Errors in referral smear classification were excluded by re-examination (Dr. Amanda Herbert) of the original referral smear for samples where there was a change between the reported smear grade and colposcopy clinic findings. From the two results, the disease course was classified as regressing if the colposcopy result was less severe than the referral result, unchanged if the results were comparable, or progressing if the colposcopy result was more severe than the referral smear suggested. A comparison was made of the HPV risk type and the disease course. As specimens with a histopathology grade of CIN 0 cannot have progression of disease using the referral and histopathology results, the data for disease courses of unchanged and progression were merged. The results are shown in figure 3.7.

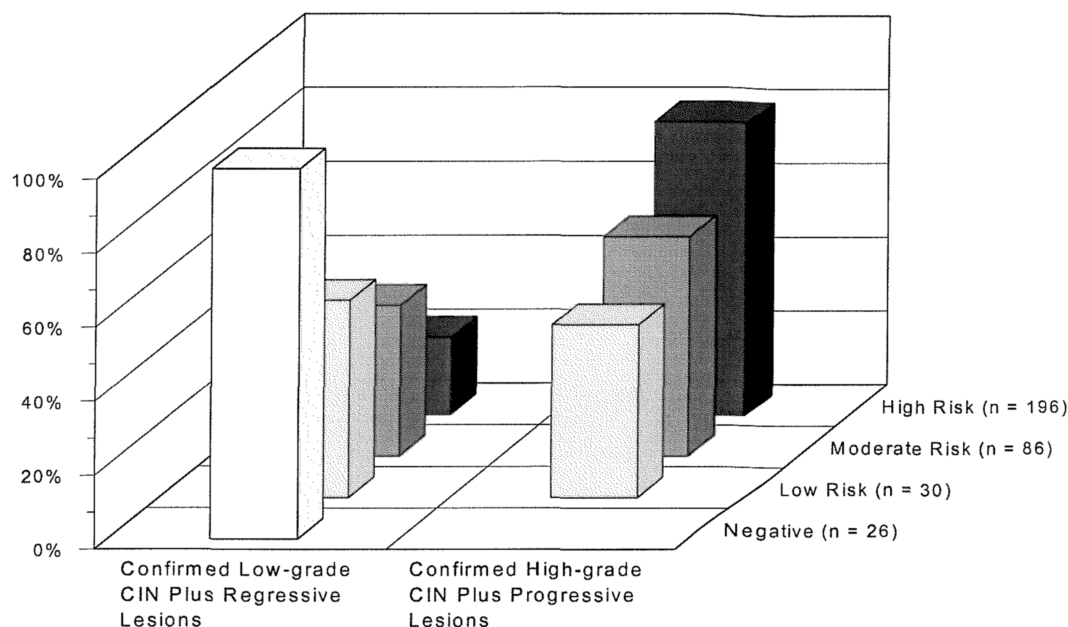


Figure 3.7. Variation of Disease Severity with HPV Type Detected. The HPV type detected was assigned its risk type as in table 3.4. The disease course of the patient was determined from the results of the referral smear and the biopsy taken at colposcopy. Regressive lesions were classified as the referral result being more severe or progressive lesions if the colposcopy result was more severe. Disease was classified as low grade CIN if the patient's histopathology result remained as CIN 0 or CIN 1, and high grade CIN if the result remained as CIN 2 or CIN 3. The number of specimens (n) in each group are shown. Nine specimens with HPV of undetermined risk were excluded, together with 7 samples where the disease course could not be assigned.

The results in figure 3.7 indicate that with increasing HPV-risk type the proportion of samples with regressing disease or low grade CIN reduced and with progressing disease or high grade CIN increased. Although no cases of frank cervical cancer were detected. One sample with CIN 2 did not contain detectable HPV, however, the disease severity of this sample was found to be regressing so was not included in the confirmed high grade CIN results.

3.4 Conclusions

A comparison of the results of this study and some previous studies using a variety of PCR primer sets is shown in table 3.5. With all of the studies shown there is a very high incidence of HPV in the CIN 2/3 groups, regardless of the method of detection used. Also illustrated is the potential increase in detection rate using multiple primer sets. The short-fragment PCR assay developed by Kleter *et al.* (1998) using a

cocktail of 4 forward and 2 reverse primers shows the highest incidence rates in grades of CIN for a single PCR. The amplicon is only 65 bp in length, and not all HPV types can be identified by sequencing the PCR product. Also, the authors made no comment on the identification of mixed HPV infections with the SPF primers. According to the authors data, the SPF primers may be the best single PCR screening method found to date. The SPF primers were tested on the HPV negative samples obtained in this study, and no further samples were found to be HPV positive.

Table 3.5. Percentage of CIN Types with Detectable HPV in Different Studies.

Study	Primers	Grade of Lesion				
		Normal	CIN 0†	CIN 1	CIN 2	CIN 3
This Study	GP/CP/MY/16-E7/18-LCR	42	79	90	99	100
Becker, 1994	MY	42			94^{+}	
Burger, 1995	MY	35		44	69	86
Chang, 1997	6/11/16/18	58		84*	72^{+}	
Cromme, 1993	GP			68	91	100
Kalantari, 1997	MY		69	71	81	84
Kleter, 1998	SPF	23		97		99
Lungu, 1995	High risk E6	30		45*	91^{+}	
Schiffman, 1993	MY	18		92*	90^{+}	

* - defined as low grade squamous intraepithelial lesions.

$^{+}$ - defined as high grade squamous intraepithelial lesions.

† - patients referred for colposcopy found to have no subsequent pathology.

The primer sets used were GP (consensus primers GP5+/GP6+, detect the L1 region), MY (consensus primers MY09/MY11, also detect part of the L1 region), CP (consensus primers CPI/CPIIG, against E1 region), 16-E7 (HPV type 16 specific primers targeting the E7 gene), High risk E6 (consensus primers for the E6 gene of high and moderate risk types), 6/11/16/18 (specific primers for the 4 HPV types stated, the target was the LCR/E6 region of the genomes), and SPF consisting of 4 forward and 2 reverse primers located in the L1 region.

A method was devised for the sensitive detection and sizing of PCR products using the GP5+/GP6+ consensus HPV primer set. The results obtained here demonstrate an increased incidence of high risk HPV types with increasing grade of CIN and a corresponding reduction in the presence of HPV negative samples. Also, the importance of using multiple HPV consensus primers for maximum HPV detection was shown.

Chapter 4

Relationship of Cervical HPV Load and Lesion Severity

4.1 Introduction

Initially, PCR was viewed as lacking quantitative power because the ability to amplify very small quantities of nucleic acid was thought to be complex and variable.

However, the amplification process follows the simple equation: $Y_n=(1+R)n$, where Y_n is the amplification factor after n cycles and R is the efficiency of amplification at each cycle. Thus, if amplification occurs at 100% efficiency (R=1) then the number of amplicons doubles at each cycle. In most PCRs the overall efficiency is less than 100%, typically running at a constant efficiency of 70-80% from the 15th to 30th cycle (Ferre, 1992). However, the increase in the number of amplicons remains exponential for a limited number of cycles, after which amplification plateaus. The cycle number this plateau occurs at within a PCR run depends on a number of factors, including substrate saturation of enzyme, product strand reannealing, and incomplete product strand separation. These factors must be taken into account in order to establish a linear range in which the amount of amplified product produced is directly proportional to the amount of starting material.

Quantitative PCR also demands that losses of target genome during sample processing and the carry-over of PCR inhibitors, which will vary between samples, can be corrected for. To define the amount of starting material requires standards of known copy numbers of the target genome. Ideally a cell line containing a known amount of viral genome per cell is used to produce a standard curve from which subsequent reactions are quantified. The quantity of viral DNA compared to the amount of cellular DNA can be determined by co-amplifying both a viral and a cellular target and quantifying the results obtained with the appropriate standard curves. This method takes into account loss of efficiency of the PCR reaction, because both the control DNA (cellular target) and the unknown (viral DNA) will be affected. However, both sets of primers must have equal efficiency of amplification for this to work.

An alternative is to use internal standards for quantification, where the standard uses the same primers as the sample target. The commonly used system is to generate standards that gives a different sized PCR product to the genome of interest. This

permits control DNA and the unknown to be present at comparable concentrations, ensuring each product has similar reaction kinetics. One other complication is the sequence of the control DNA, if this differs greatly from that of the target DNA, then secondary structure of the DNA can produce different reaction characteristics. A way of getting over this problem is to modify wild-type DNA to produce the standards.

The method chosen was to use 3 different sized standards in each reaction. Each of the standards was derived directly from the wild-type gene; the differences in sizes being produced by removal of 7, 14, or 21 bases from the sequence between the primer binding sites (see section 2.5.1). Thus, each standard had wild-type primer binding sites and a segment of normal wild-type DNA between these sites (figure 4.1).

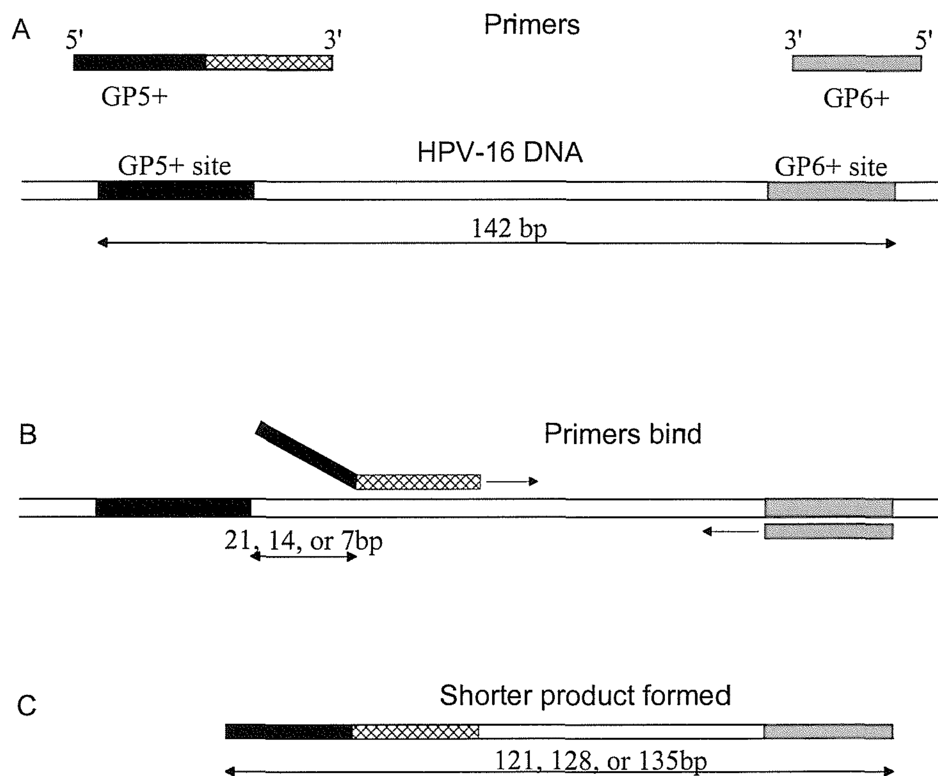


Figure 4.1. Production of Quantitative PCR Standards. (A) Oligonucleotides containing the normal GP5+ primer binding sequence of HPV-16 at the 5' end and a sequence homologous to a portion of the DNA 7, 14, or 21 bases further along from the GP5+' site at the 3' end were made. (B) These primers, together with GP6+, were used in a PCR reaction where the annealing temperature was such that only the 3' non-GP5+ portion of the oligonucleotide would bind wild-type HPV-16 DNA. (C) The separate PCR reactions yielded products of 121 bp, 128 bp, and 135 bp instead of the normal 142 bp with the wild-type DNA. However, these shorter products had GP5+ and GP6+ binding sites at either end separated by wild-type DNA.

Detection of the PCR products was achieved by labelling the GP5+ primers with fluorescein. PCR products were run on a 6% denaturing polyacrylamide gel analysed using an ABI 373A DNA sequencer with the fluorescent products detected by laser using Genescan software (Applied Biosystems). The number of starting copies of each standard and the number of cycles for the PCR reaction were determined, and these data used to evaluate the amount of HPV genomes present in the specimen.

4.2 Results

PCR reactions containing different amounts of each standard were run for 20, 22, 25 or 30 cycles. The peak height for each standard was plotted against the number of starting copies for that standard. The results in figure 4.2 show at 22 cycles and below there was linearity of quantification, i.e. as the number of starting copies of standard increased so the corresponding fluorescence increased (indicated by peak height) to give a linear graph with fluorescence signal plotted on a logarithmic scale. Thus, the amplification reaction was in exponential phase. Above 22 cycles, the system reached a plateau whereby the fluorescent signal saturated the detection system.

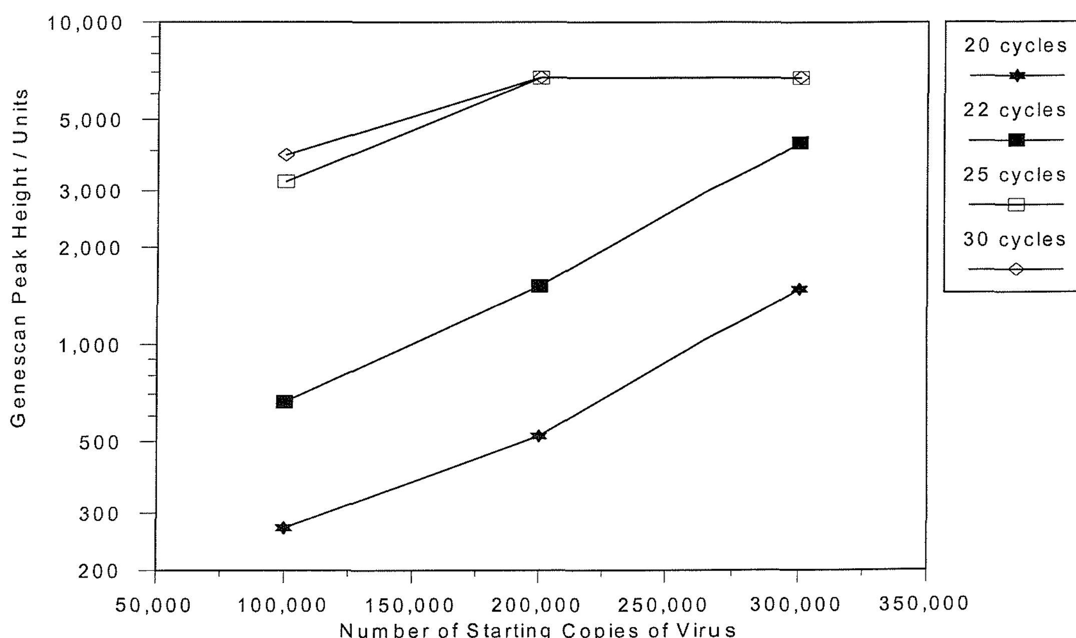


Figure 4.2. Determination of Cycle Number Required for Quantitative PCR. Separate PCR reactions were run using the same number of starting copies of each DNA standard. Each reaction had a different number of cycles. The products of the PCR were tested by Genescan and the height of the fluorescent peaks for each standard determined. The results show the variation of the peak heights with the number of cycles.

For maximum sensitivity within the system parameters, 22 cycles was used for quantification of clinical samples. Two hundred and thirty-four specimens from colposcopy clinic patients were analysed using the quantitative PCR method. Figure 4.3 shows the result of plotting the number of HPV genomes detected against the colposcopy clinic findings.

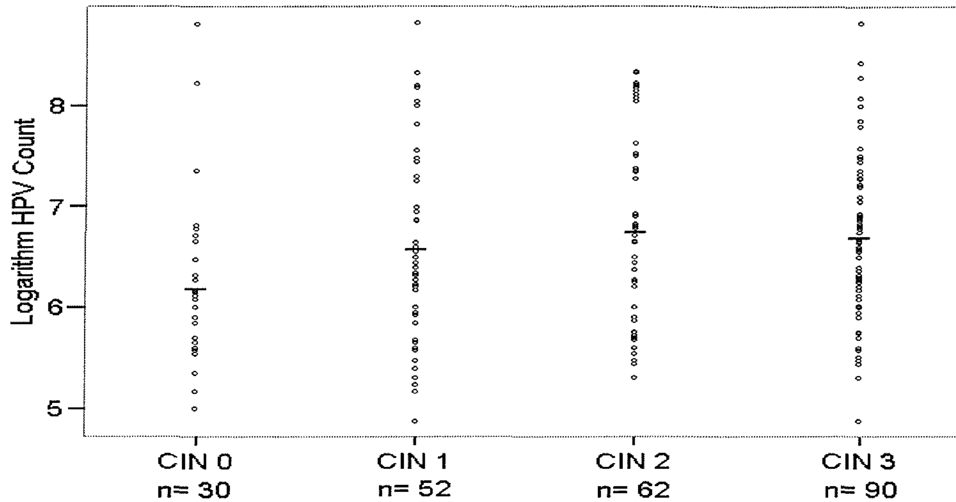


Figure 4.3. Variation in Amount of Virus Present with Histopathology. The number of viruses present per PCR sample was determined by quantitative PCR for specimens with HPV present. The logarithm of this value was plotted against the histopathology result for the specimen. The graph shows the scatter of the individual results together with the mean. The number of specimens (n) in each category are shown. Results are for samples containing a single HPV type.

A Student t-test was performed on the data shown in figure 4.3 in order to determine if there were significant differences between the mean viral load and the grade of histopathology. Significantly more viruses were found in all CIN compared to CIN 0 (p-value 0.004). These results were for any HPV viral types detected.

Figure 4.4 shows the result of plotting the number of HPV-16 genomes against the colposcopy clinic finding. There was no significant difference between the mean viral count for HPV-16 between the different grades of CIN.

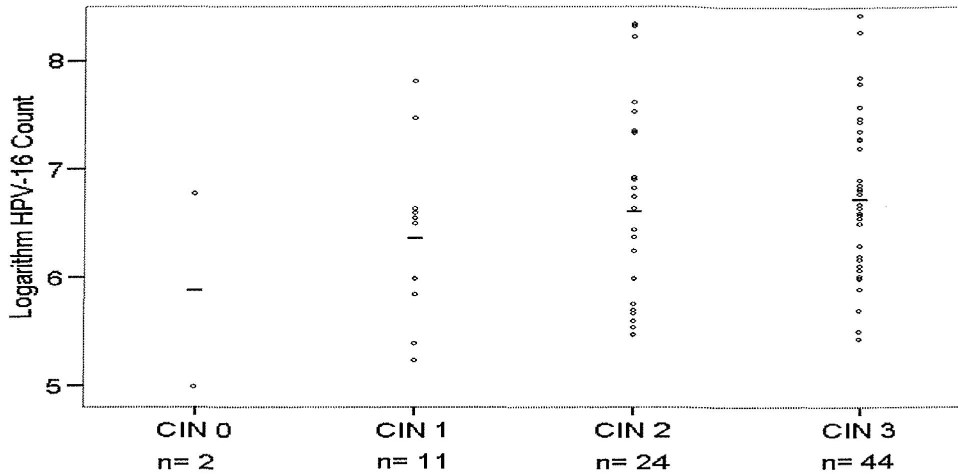


Figure 4.4. Variation in Amount of HPV-16 Present with Histopathology. The number of HPV-16 genomes present per PCR sample was determined by quantitative PCR. The logarithm of this value was plotted against the histopathology result for the specimen. The graph shows the scatter of the individual results together with the mean. The number of specimens (n) in each category are shown.

Figure 4.5 shows the results for the viral genome copy number per sample against the age of the patient.

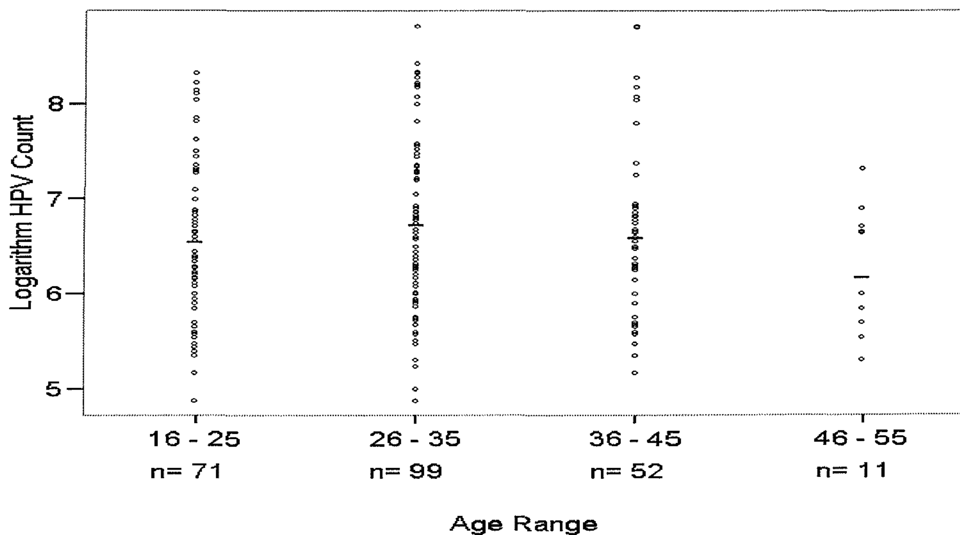


Figure 4.5. Variation in Amount of Virus Present with Patient Age. The number of viruses present per PCR sample was determined by quantitative PCR for specimens with HPV present. The logarithm of this value was plotted against the age of the patient. The graph shows the scatter of the individual results together with the mean. The number of specimens (n) in each category are shown. Results do not include 1 sample excluded by age. Results are for samples containing a single HPV type.

There was a slightly lower mean level of virus in patients aged 46 to 55 compared to the other age ranges (p-value 0.08 using Student t-test analysis), however, none of the lower age groups showed any differences in the mean viral count. These results were for samples infected with a single HPV type.

Table 4.1 demonstrates it was not possible to use a cut-off value for HPV load in order to predict CIN 2/3 for any HPV type. Although a sample load of $5x10^6$ HPV genomes shows a significant difference between high and low grade CIN (p-value 0.024 using χ^2 analysis) this difference does not continue above or below these values. HPV type 16 was the most common HPV type detected in the samples. Although table 4.2 shows there were similar results for the cut-off values compared to table 4.1, the low number of patients with CIN 0/1 meant statistical significance was not achieved.

Table 4.1. Correlation of Cervical HPV Load with Grade of CIN.

Colposcopy Findings	Percentage of women with HPV load exceeding the cut-off value									
	$5x10^4$	$1x10^5$	$5x10^5$	$1x10^6$	$5x10^6$	$1x10^7$	$5x10^7$	$1x10^8$	$5x10^8$	$1x10^9$
CIN 0/1 (n=64)	100	98.4	79.7	71.9	35.9	26.6	15.6	12.5	3.1	0
CIN 2/3 (n=122)	100	100	90.2	82	53.3*	30.3	14.8	13.1	0.8	0

Data shown are only for women with detectable HPV.

* p-value 0.024 between CIN 0/1 and CIN 2/3 values using χ^2 analysis.

Table 4.2. Correlation of Cervical HPV-16 Load with Grade of CIN.

Colposcopy Findings	Percentage of women with HPV-16 load exceeding the cut-off value									
	$5x10^4$	$1x10^5$	$5x10^5$	$1x10^6$	$5x10^6$	$1x10^7$	$5x10^7$	$1x10^8$	$5x10^8$	$1x10^9$
CIN 0/1 (n=13)	100	92.3	76.9	53.8	23.1	15.4	7.7	0		
CIN 2/3 (n=68)	100	100	85.3	77.9	48.5	27.9	10.3	7.4	0	

Data shown are only for women with detectable HPV-16.

Any relationship in the change of disease status and cervical HPV load was analysed by comparing the referral smear result and the colposcopy findings, i.e. if the referral result was more severe than the colposcopy result then the disease status was classified as regression, if the results were the same it was termed unchanged, and if the

colposcopy result was more severe than the referral result the disease was classed as progression. The samples could be divided into 2 groups; those that presented with a referral smear of persistent mild, and those with a referral smear of moderate or severe. The results are shown in figure 4.6.1 and 4.6.2. Using Student t-test analysis the only statistically significant result was lower viral loads in regressing lesions compared to progressing lesions for referral smears of mild (p-value 0.04). However, there was less than 1 log difference between the two mean values, and hence the statistical significance may be coincidental.

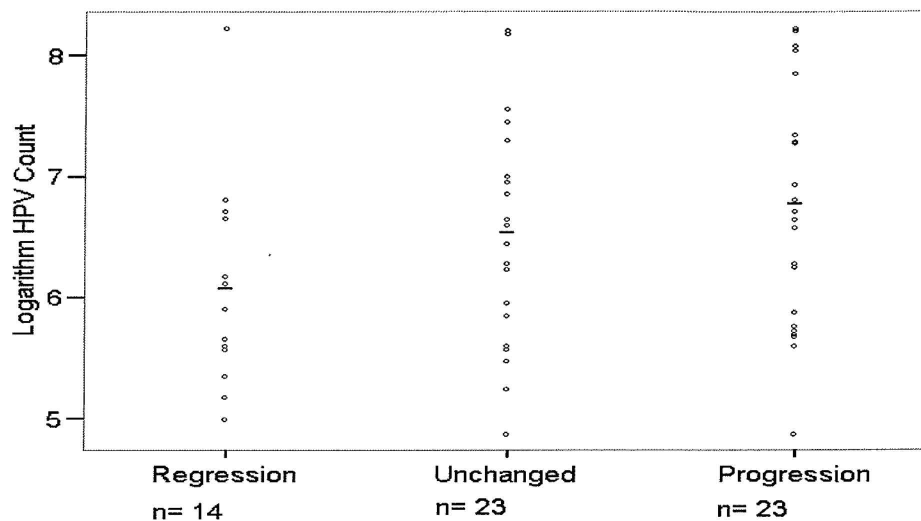


Figure 4.6.1. Variation of Viral Load with 'Mild' Referral Smear and Disease Status. The number of viruses present per PCR sample was determined by quantitative PCR for specimens with HPV present. The logarithm of this value was plotted against the histopathology result for the specimen. The results also show the disease status of the sample determined by the change in severity of the lesion between the referral smear and the colposcopy result. The graph shows the scatter of the individual results together with the mean. The number of specimens (n) in each category are shown. Results are for samples containing a single HPV type.

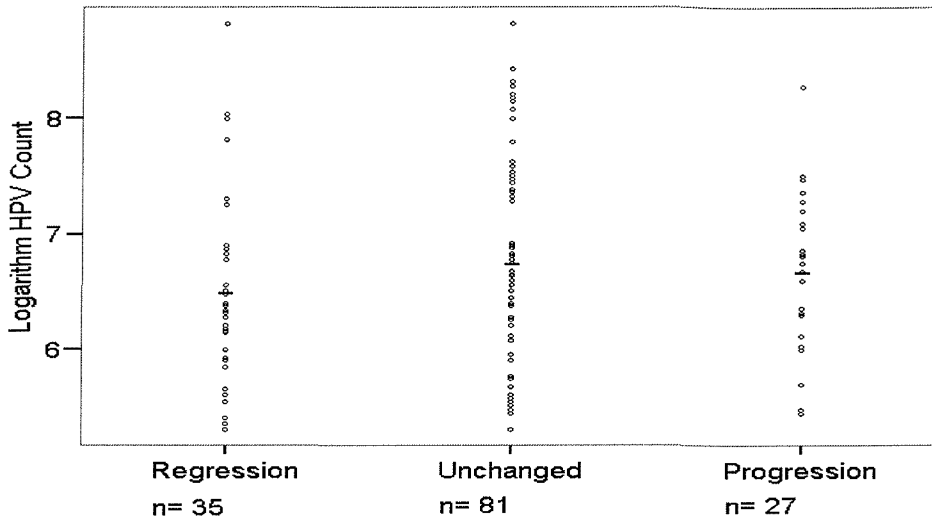


Figure 4.6.2 Variation of Viral Load with 'Moderate/Severe' Referral Smear and Disease Status. The number of viruses present per PCR sample was determined by quantitative PCR for specimens with HPV present. The logarithm of this value was plotted against the histopathology result for the specimen. The results also show the disease status of the sample determined by the change in severity of the lesion between the referral smear and the colposcopy result. The graph shows the scatter of the individual results together with the mean. The number of specimens (n) in each category are shown. Results are for samples containing a single HPV type.

4.3 Conclusions

After initial infection HPV genomes are stably maintained within basal cells at a copy number of about 50-200 copies per cell. During productive infection this increases to 1,000-10,000 copies per cell as the epithelial cell differentiates (Bedell *et al.*, 1991). Both free HPV virions and infected uppermost cells are removed by the sampling devices used in this study. Thus in women with high grade lesions undifferentiated cells containing episomal and integrated HPV genomes will be sampled. A number of cervical cancer cell lines have been analysed in order to determine the HPV genome copy number; SiHa cells each contained around 5 copies of HPV-16, HeLa cells had approximately 40 copies of HPV-18 per cell, and each CaSki cell contained about 800 copies of HPV-16.

It would appear likely that the HPV load in CIN 2/3 patients will be a function of the HPV genome copy number per abnormal cell and the lesions size which determine the number of infected cells analysed. By contrast, it would seem possible

that in women with a clinically normal cervix, but detectable HPV infection, the virus is undergoing a normal replication cycle resulting in samples containing 1,000-10,000 HPV copies. Such a model would explain the considerable overlap in HPV load in women with CIN 0/1 and CIN 2/3. In order to explore this model further a quantitative assay for HPV-16 was developed (see chapter 7). This permitted accurate analysis of cell-associated HPV genome and cell-free HPV virions.

Chapter 5

Detection of Cervical Infections in Colposcopy Clinic Patients

5.1 Introduction

Human papillomaviruses play an essential part in the development of cervical cancer, but the role of other infectious agents is not clear. A history of multiple genital infections is more common in women with cervical cancer, suggesting these agents increase the risk of developing cancer. However, genital infections may simply be indicators of sexual activity and hence surrogate markers of oncogenic HPV infection.

Some pathogens are reputed to act as co-factors in oncogenesis, these include: *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, herpes simplex virus, cytomegalovirus, Epstein-Barr virus, human herpesvirus types 6, 7, and 8, and adeno-associated virus.

Both *Neisseria gonorrhoeae* and *Chlamydia trachomatis* cause the production of inflammatory cytokines, such as TNF-α and IL-1β, by host cells during infection (Ingalls *et al.*, 1995). These cytokines inhibit the growth of normal, uninfected endocervical and ectocervical cells, but stimulate proliferation of cervical cells immortalised with high risk HPVs inducing a selective growth advantage to these cells and therefore a risk factor for progression to high grade CIN in women with HPV infection (Woodworth *et al.*, 1995).

Herpes simplex virus (HSV) infection can induce cell cycle transformation with the gene products from two sites within the HSV type 2 genome; mtrII and mtrIII (Jones, 1995). Also, infection itself, induces cell cycle progression and increases mutagenesis. If these cells were infected with a high risk HPV type then the p53 protein would not cause apoptosis and the cell could continue to grow abnormally.

Efficient cytomegalovirus (CMV) replication relies on host cell stimulation and the virus can induce many cellular proteins. This activation may have consequences on the activation of other viruses in co-infected cells. Like HSV, CMV produces proteins with transforming potential; mtrI, mtrII, and mtrIII (Mocarski, 1996). Any role CMV may play in cervical cancer is still not fully understood.

Epstein-Barr virus (EBV) is known to cause Burkitt's lymphoma and nasopharyngeal carcinoma (Rickinson and Kieff, 1996). EBV normally shows B cell tropism, however Sixbey *et al.*, (1983) showed EBV could infect cultured ectocervical

cells and produce late viral antigens and EBV DNA in the exfoliated cells, but not the attached monolayer, suggesting an association with viral replication and epithelial cell differentiation. In addition, infectious EBV has been isolated from the cervical washings of women recovering from infectious mononucleosis and seropositive women with no evidence of acute infection (Sixbey, Lemon, and Pagano, 1986). Landers and co-workers (1993) detected EBV DNA in a significant proportion of CIN 2/3 and cervical cancer specimens, but not in CIN 1 or Normal cervixes. Hence the cervical epithelium may be a site of EBV replication. Also, EBV produces an IL-10 homologue (Hsu *et al.*, 1990) that causes a T_H2 immune response, which would not prevent spread of HPV infection.

Human herpesvirus type 6 (HHV-6) was first discovered in the blood of a patient with AIDS related lymphomas (Salahuddin *et al.*, 1986). The most substantial growth of the virus occurs in CD4+ cells, although it can infect epithelial cells and produce a latent infection (Chen *et al.*, 1994). Also, HHV-6 contains transactivators that can stimulate the E6 and E7 genes of HPV-16 and 18. Of the two variants of HHV-6 discovered, A and B, only variant B has been associated with disease, namely exanthem subitum of young children and severe infections in immunocompromised adults. However, HHV-6A has been isolated from a number of disease states. Serological data indicate that most people become infected with HHV-6 by the age of 2 years.

Human herpesvirus type 7 (HHV-7) can be detected in the blood of healthy individuals and was discovered in 1990 (Frenkel *et al.*). Infection appears to occur slightly later than that for HHV-6 at around 3 years of age. There is serological cross-reaction between HHV-7 and HHV-6A, and T-cell clones reacting with HHV-6 also respond to HHV-7. The host cell range of HHV-7 is more limited than for HHV-6; growing best in blood lymphocytes. HHV-7 can apparently reactivate HHV-6 from latency, particularly HHV-6B.

Human herpesvirus type 8 (HHV-8) is the most recently discovered of the herpesviruses (Chang *et al.,* 1994). HHV-8 has been found in many Kaposi's sarcoma lesions and is therefore also known as Kaposi's sarcoma herpes virus (KSHV).

Adeno-associated Virus (AAV) is a Dependovirus, which have many characteristics of Parvoviruses, but rely on co-infections with other viruses to replicate (Berns, 1996). AAV has not been associated with any disease in humans despite the ability to integrate into the cell genome as a rescuable provirus. The Rep78 protein of

AAV is required for AAV transcription activity, AAV DNA replication, and possibly for site-specific integration of AAV into human DNA. This protein appears to be able to interact with SP1, a cellular transcription factor (Hermonat, Santin, and Batchu, 1996), and inhibits the activity of SP1. Furthermore, SP1 can bind to the E6 promoter of all genital HPVs and activates transcription of HPV-16, thus Rep78 can inhibit HPV transcription by preventing SP1 binding. AAV has also been shown to inhibit other promoters, including c-H-ras and HIV-1, both of which contain SP1 binding motifs. Therefore, infection with AAV may reduce the risk of developing cervical cancer.

5.2 Detection Methods

The primers used to detect *Neisseria gonorrhoeae* target part of the cryptic plasmid carried by this bacterium (Mahony *et al.*, 1995). The plasmid occurs at a frequency of about 10 per bacterium, enhancing the chance for detection of the gonococci by PCR. The primers used to detect *Chlamydia trachomatis* also target a plasmid carried by the organism (Mahony *et al.*, 1995).

The glycoprotein B gene of herpes simplex virus types 1 and 2 was used as the source for diagnostic primers. The sequence of the gene in both viruses is known (Hammerschmidt *et al.*, 1988, Stuve *et al.*, 1987), and from the 6,000 bp coding for the glycoprotein, a region of approximately 300 bp was selected. The consensus primers chosen produce an amplicon with 20 bp missing in HSV-2, compared to HSV-1. This enables the virus to be typed directly with one primer pair.

The primers used to detect cytomegalovirus are directed against the Immediate Early gene (Stenberg, 1984). For the detection of Epstein-Barr virus, the primers were those used by Jiwa *et al.* (1993) with slight modifications as the primer sequences were printed incorrectly when compared to the confirmed DNA sequence of the virus.

Human herpesvirus types 6 and 7 were detected using the inner primer sets of Sada *et al.* (1996), with slight modifications in order to give all primers similar T_m values for annealing temperature. The primers of Dewhurst and co-workers (1993) were used in order to distinguish between HHV-6 types A and B. Human herpesvirus 8 was detected using two of the primers published by O'Neill and colleagues (1996).

The primers used to detect adeno-associated virus were those published by Han and co-workers (1996), again with slight modifications for annealing temperature. These primers target the Rep78 gene of AAV types 2 and 3, but not type 5.

Details of the primer sequences and PCR conditions used are given in section 2.7.

5.3 Results

Two hundred and seventy-five cervical samples were screened for all the organisms described. The findings were compared to the histopathology result of the patient, these data are shown in figure 5.1. There appears to be no obvious link with CIN status and detection of HSV, CMV, EBV, HHV-6, HHV-8, or chlamydia. For AAV there was a significant increase in incidence from Normal specimens compared to CIN samples using χ^2 analysis (all p-values < 0.04). With HHV-7, the rate of incidence is around 18% for Normal specimens and increases to about 45% for CIN 0, CIN 1, and CIN 2, but this rate increases with CIN 3 to almost 65%. Using χ^2 analysis there was a significant difference between the occurrence of HHV-7 in Normal samples compared to samples with CIN (all p-values < 0.007).

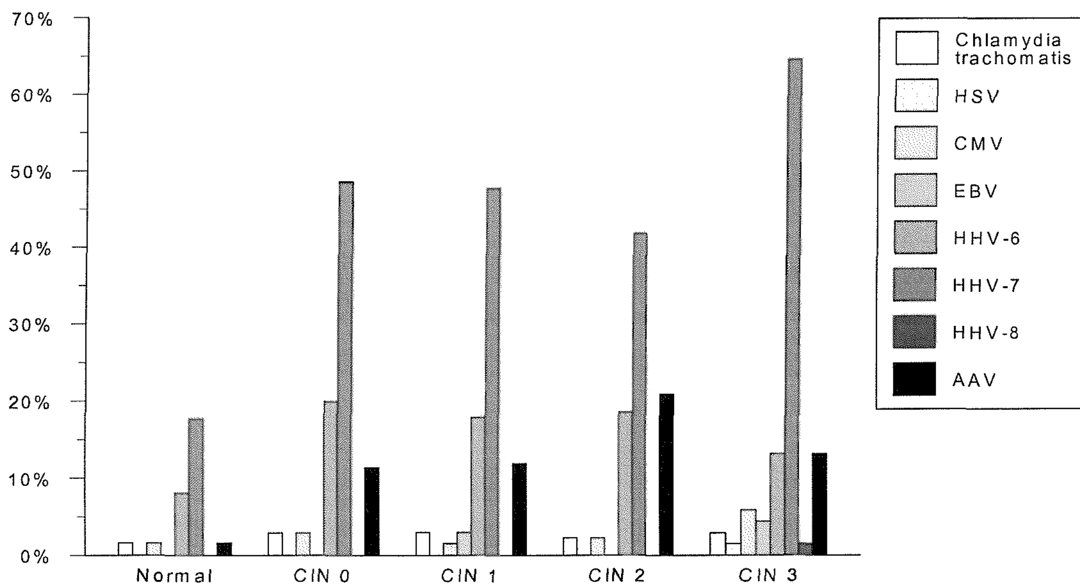


Figure 5.1. Detection of Other Infections and CIN Status. Specimens were tested for the organisms shown and the results were linked with the histopathology result from the patient taken at the same time as the specimen. The total number of samples for the groups were 62 in Normal, 35 in CIN 0, 67 and 43 in CIN 1 and CIN 2 respectively, and 68 in CIN 3.

The incidence of the organisms compared to patient age are shown in figure 5.2. The most important finding was that chlamydia were detected in the age range 16-25 at a frequency of about 7% falling to around 1% over this age (p-value 0.005 using χ^2 analysis). For AAV there were similar levels of detection in all age groups (p-value 0.4 for age ranges 16-35 versus 36-55). One case of HSV-1 was found in the age range 26-35. Cytomegalovirus was found in all the age ranges at low levels. Epstein-Barr virus was only found in the range 16-35. The frequency of HHV-6 remained at around 15% in all age ranges. The incidence of HHV-7 fluctuated throughout the age ranges. One case of HHV-8 was detected in the 26-35 year age range.

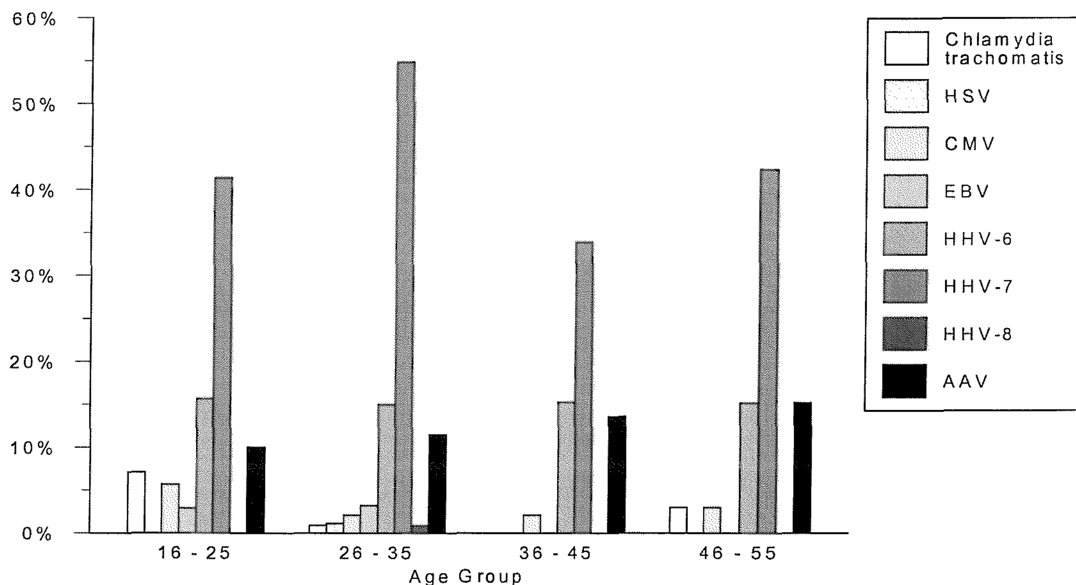


Figure 5.2. Distribution of Other Infections with Age. The samples positive for the presence of the organisms shown were matched with the age of the patient. For 16-25, 26-35, 36-45, and 46-55, the number of specimens was 70, 113, 59, and 33 respectively.

Only AAV, HHV-6, and HHV-7 positive specimens were present in sufficiently high numbers to enable further analysis of their distribution. Figure 5.3 shows the distribution of the presence of the 3 viruses with the HPV risk type detected in the specimen. For HHV-7 positive samples there was a significant difference between those containing high risk HPV and patients with less severe risk types or HPV negative (p-value 0.008 using χ^2 analysis). There were no differences in the occurrence of HHV-6 or AAV.

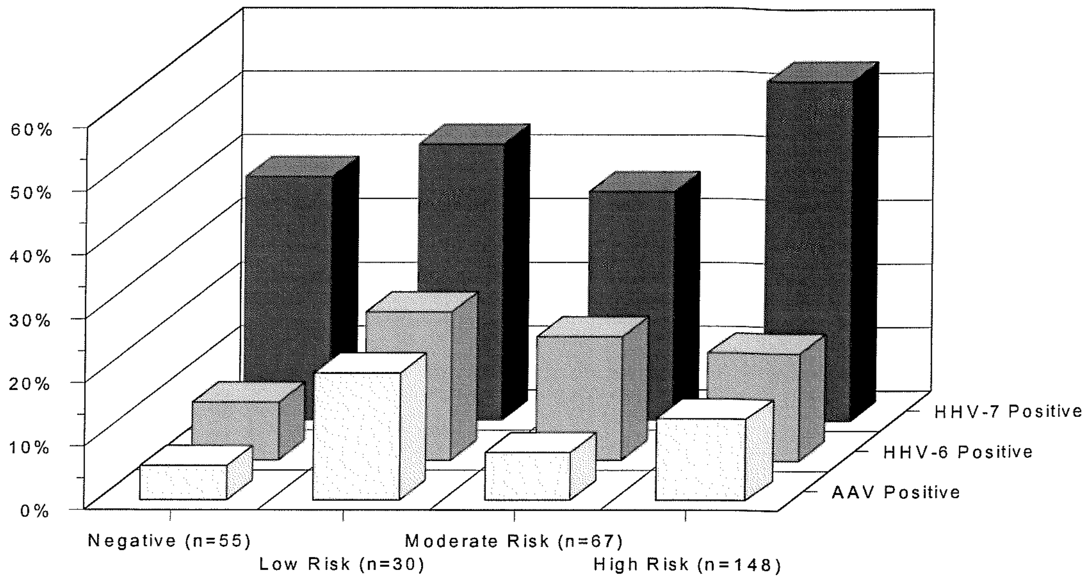


Figure 5.3. Presence of Other Infections with HPV Risk Type. Samples positive for the viruses shown were compared to the HPV risk type detected in the specimen. The number of specimens (n) in each risk type are shown.

The occurrence of the viruses with the referral smear result and the corresponding disease status are shown in figure 5.4. The disease course of the patient was determined from the results of the referral smear and the biopsy taken at colposcopy. Regressive lesions were classified as the referral result being more severe or progressive lesions if the colposcopy result was more severe. Disease was classified as low grade CIN if patients histopathology result remained as CIN 0 or CIN 1, and high grade CIN if the result remained as CIN 2 or CIN 3. Errors in referral smear classification were excluded by re-examination (Dr. Amanda Herbert) of the original referral smear for samples where there was a change between the reported smear grade and colposcopy clinic findings. No significant differences were found between the proportion of specimens with low grade/regressing lesions or high grade/progressing lesions and the presence or absence of the viruses.

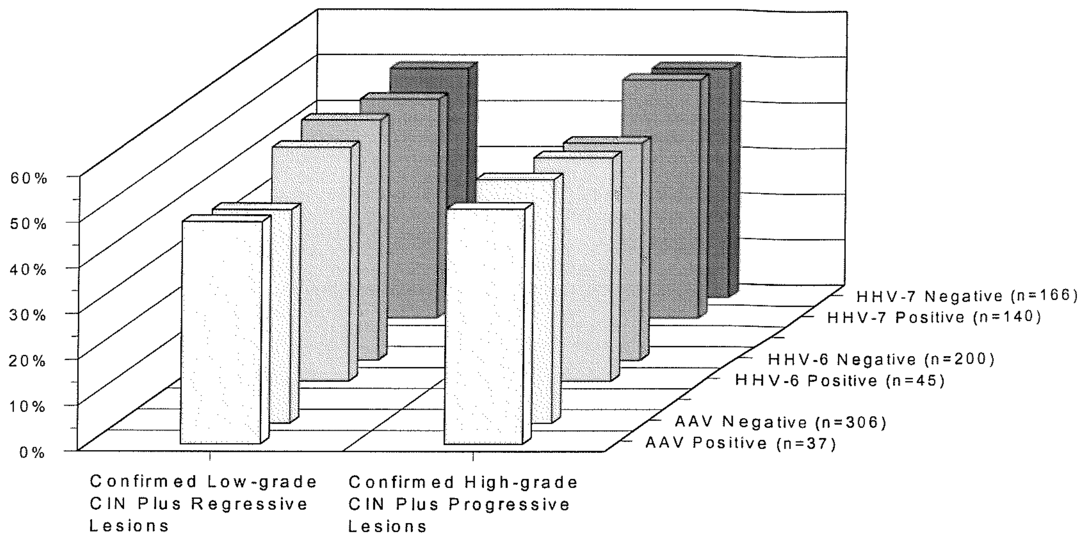


Figure 5.4. Effect of Presence of Different Organisms on Cervical Disease Progression. Samples were grouped according to the presence or absence of the infection shown. The disease course of the patient was determined from the results of the referral smear and the biopsy taken at colposcopy. Regressive lesions were classified as the referral result being more severe or progressive lesions if the colposcopy result was more severe. Disease was classified as low grade CIN if patients histopathology result remained as CIN 0 or CIN 1, and high grade CIN if the result remained as CIN 2 or CIN 3. The number of specimens (n) in each group are shown.

To test the hypothesis that the production of growth factors by any herpesvirus would affect the disease course, data from patients infected with HSV, CMV, EBV, HHV-6 or HHV-7 were pooled. The results are shown in figure 5.5. Significantly more samples with high grade or progressing lesions were found to be infected with any herpesvirus compared to samples not containing any of these viruses (p-value 0.017 using χ^2 analysis).

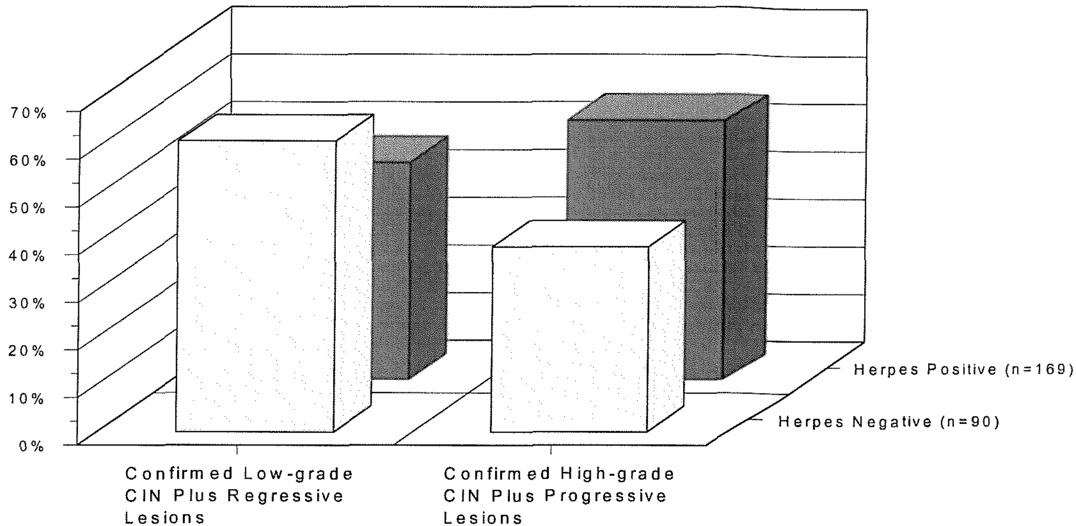


Figure 5.5. Variation of Disease Progression with Presence of Any Herpesvirus. Samples were grouped according to the presence of HSV, CMV, EBV, HHV-6, or HHV-7, or absence of all herpesviruses. The disease course of the patient was determined from the results of the referral smear and the biopsy taken at colposcopy. Regressive lesions were classified as the referral result being more severe or progressive lesions if the colposcopy result was more severe. Disease was classified as low grade CIN if patients histopathology result remained as CIN 0 or CIN 1, and high grade CIN if the result remained as CIN 2 or CIN 3. The number of specimens (n) in each group are shown.

5.4 Conclusions

Endocervical and ectocervical specimens obtained from a colposcopy clinic were tested for the presence of DNA from herpes simplex virus, cytomegalovirus, Epstein-Barr virus, human herpesvirus types 6, 7, and 8, adeno-associated virus, *Chlamydia trachomatis*, and *Neisseria gonorrhoea*.

The most common infections detected were HHV-7 (45%), HHV-6 (15%), and AAV (12%). The remaining pathogens were detected at a rate of 3% or less; gonorrhoea was not detected. Significantly more chlamydia was detected in patients less than 26 years old (7%) compared to older women (1%) attending the colposcopy clinic. This raises the question as to whether women attending a colposcopy clinic, especially if young, should be routinely screened for chlamydia infection. It is agreed that the number of new sexual partners, and the non-use of condoms increases the likelihood of HPV DNA being present in the cervix. However, the incidence of previous sexually

transmitted diseases was reported as very low by the colposcopy clinic patients, suggesting that other STDs in the past were not important in the development of CIN.

When related to CIN status of the patient only the increased incidence of AAV and HHV-7 with increasing CIN were found to be statistically significant. In addition, HHV-7 was detected more often in samples containing high risk HPV types. The high incidence of HHV-6 and HHV-7 in cervical samples suggests these viruses are sexually transmissible. A significantly higher proportion of samples containing any herpesvirus were found to have high grade CIN or lesions with progressing disease than samples without any herpesvirus detected.

Chapter 6

Analysis of Cytokines and Chemokines in Cervical Intraepithelial Neoplasia

6.1 Introduction

In the majority of women with cervical HPV infections the virus is cleared and the infection heals. The mucosal immune response to HPV determines the outcome of infection; all components required for a protective response occur in the normal cervix (Parr and Parr, 1994). Immunosuppressed women, for example women with AIDS, have an increased susceptibility to cervical neoplasia. *In situ* hybridisation studies on cervical biopsies of HIV+ women showed an increase in T-helper type 2 (T_H2) cytokines (IL-4, IL-5 and IL-10) and a reduction in T_H1 cytokines (IFN-γ) suggesting that a dominant T_H2 response to HPV may enhance the risk of cervical cancer (Olaitan *et al.*, 1998). However, why a small percentage of otherwise healthy women fail to clear HPV infection and go on to develop cervical cancer is not understood.

Malignant cells respond to a wide range of growth factors, cytokines, and chemokines; the role of these agents in tumour progression is the subject of a detailed recent review (Ardestani *et al.*, 1999).

Chemokines are a superfamily of small (8-10 kDa) inducible, secreted, pro-inflammatory peptides with a conserved four-cysteine motif. In the C-X-C subfamily the first 2 cysteines are separated by another amino acid. The C-X-C chemokines, including IL-8, Gro-α, β, γ, and ENA-78, are chemoattractant for and activators of neutrophils. These chemokines play an important role in tumour angiogenesis and some tumours including melanoma, cervical, and skin cancers produce IL-8. The C-C subfamily of chemokines lack the spacer amino acid; important examples are: RANTES, MIP-1α, MIP-1β, and MCP-1, 2, and 3. The C-C chemokines are chemoattractant and activators of monocytes thus stimulating macrophage attack on the tumour.

Cytokines are a more diverse group of secreted peptides whose main function is to orchestrate the proliferation and activation of T and B lymphocytes. However some cytokines, particularly IL-1α and β, IL-6, TNF-α and β, and TGF-β, have wider biological effects and are produced by various cells in response to a variety of signals.

IL-1 production by monocytes and epithelial cells shows a dramatic increase in response to infection. IL-1 was originally termed tumour inhibitory factor-2, but may play a role in stimulating angiogenesis. IL-6 is a multi-functional cytokine that plays a key role in host defence, acute phase reactions, and immune responses. IL-6 production by macrophages, fibroblasts, epithelial, and endothelial cells increases in response to infections or antigenic stimuli. IL-6 is produced by a wide range of carcinomas, sarcomas, melanomas, myelomas, etc., and can stimulate both tumour growth and angiogenesis. TNF-α is predominately produced by macrophages in response to antigenic and infectious stimuli. Originally considered a selective inhibitor of transformed cells, TNF-α has now been shown to have mitogenic effects on some cells, e.g. fibroblasts, and stimulates the growth of some cancers, e.g. ovarian.

Transforming growth factor beta (TGF-β) modulates the growth of many cell types, mediates cellular matrix formation, and is a potent stimulator of angiogenesis. TGF-α is an unrelated protein belonging to the epidermal growth factor family. Inappropriate expression of TGF-α and other growth factors is involved in the production and/or progression of some neoplasias.

The role of these different factors in tumour cell growth and migration, and in the neo-vascularisation of cancers is the subject of much current research. Studies on cervical cancer cell lines have shown the production of both IL-6 (Iglesias, Plowman, and Woodworth, 1995) and IL-8 (Schulte *et al.*, 1996) and that both TNF-α and IL-1α stimulate the growth of HPV transformed cells, but not normal epithelial cells (Woodworth *et al.*, 1995). The objectives of my study were to 1) determine if IL-6 production by HPV transformed cells in women with CIN 2/3 resulted in raised levels of this cytokine in cervical secretions, 2) establish if chemokines/cytokines which promote tumour growth and angiogenesis are present in raised concentrations in women with CIN 2/3, and 3) determine if cervical infections with either bacteria or viruses induce the production of these cytokines.

6.2 Methods

Cell-free supernatants were tested for the presence of cytokines with ELISA as follows. To coat the polystyrene surface of 96-well FluoroNunc Maxisorp plates, capture antibodies at the manufacturer's recommended concentration (R&D Systems) were applied in carbonate buffer, pH 9.6 (35 mM sodium hydrogen carbonate, 15 mM

sodium carbonate and 0.05% (w/v) sodium azide). The plates were then sealed, incubated overnight at room temperature, and subsequently aspirated prior to the addition of 250 µl/well blocking solution (PBS with 1% (w/v) bovine serum albumin (RIA grade; Sigma) and 5% (w/v) sucrose (BDH)). Following a one hour incubation at 37 °C the wells were washed three times with 0.05% (v/v) Tween 20 (Sigma) and 150 mM sodium chloride immediately before transfer of the samples and standards to the plates.

Aliquots of supernatants were diluted with assay buffer (25 mM Tris-HCl, pH 7.8 with 150 mM sodium chloride, 0.1% (v/v) polyoxyethylene ether W-1 (Sigma), 0.023% (w/v) bovine -globulins (Cohn fraction II; NBS Biologicals), 10 µM diethylenetriaminepentaacetic acid (Sigma), 0.025% (w/v) sodium azide and 0.25% (w/v) BSA (Sigma)), which was used as the diluent throughout the remainder of the protocol. A 2.5-fold dilution series of the recombinant cytokine standard (R&D Systems) was then prepared for each protein assayed. 'Blanks' consisted of assay buffer only. The samples and standards (100 µl/well) were incubated at 37 °C for 2 hours on the plate following which the wells were washed four times.

Subsequently, biotinylated detector antibodies at the manufacturer's recommended concentration (R&D Systems) were added to the wells, and the plate was again incubated at 37 °C for 2 hours. Four washes were conducted before the Europium-labelled streptavidin (Wallac, 100 ng/ml) was applied to the plate and incubated at 37 °C for 1 hour. Following a series of five washes, Delfia Enhancement Solution (Wallac) was dispensed into the wells, after which the plate was agitated at 37 °C for 10 minutes on a Titertek shaker (Flow Laboratories). Fluorescence was measured on a Wallac 1234 Delfia fluorometer.

6.3 Results

6.3.1 Production of Cytokines by HPV-16 Immortalised Cells

SiHa cells were grown in DMEM medium supplemented with 5% foetal calf serum at 37 °C in 5% CO_2. A 0.5 ml aliquot of supernatant was removed after 3 and 4 days cell growth. The supernatants was tested for the presence of IFN-γ, IL-1β, IL-6, IL-8, TGF-β, and TNF-α by ELISA. The DMEM medium used did not contain detectable amounts of these cytokines. IL-6 was produced at a concentration of 1.6 ng/ml after 3 days and 5 ng/ml after 4 days. IL-8 was produced at 300 pg/ml after 3 days

and 120 pg/ml after 4 days. None of the other cytokines were detected in the supernatant from SiHa cells. On the basis of this finding we decided to focus on those cytokines which stimulated growth of HPV immortalised epithelial cells (IL-1β and TNF-α) and those produced by these cells (IL-6 and IL-8).

6.3.2 Production of Cytokines in Patient Samples

Cell-free supernatants from colposcopy samples were tested for the presence of cytokines which stimulated growth of HPV immortalised epithelial cells (IL-1β and TNF-α) and those produced by SiHa cells (IL-6 and IL-8). Only IL-1β (figure 6.1), IL-8 (figure 6.2), and IL-6 (figure 6.3) were present in detectable amounts.

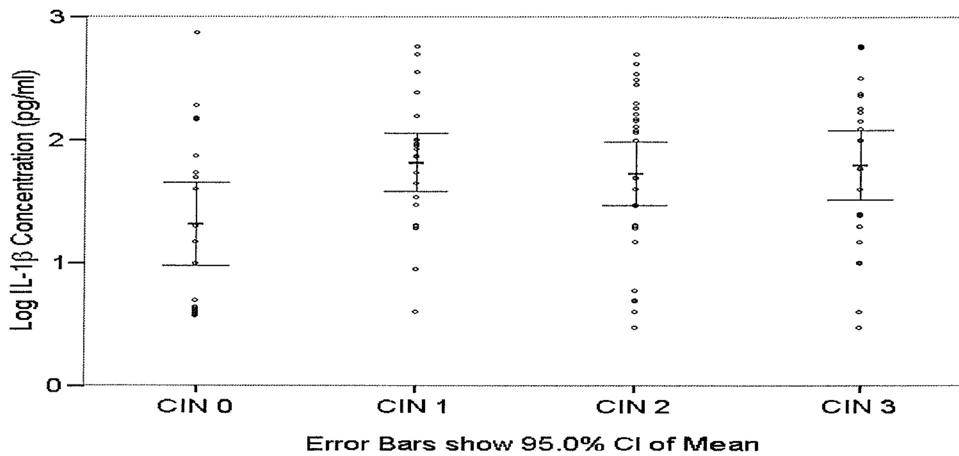


Figure 6.1. Concentration of IL-1β in Different Grades of CIN. The graphs show the 95% confidence limits together with the mean for each grade of CIN. The number of specimens in each group is 21 in CIN 0, 22 in CIN 1, 28 in CIN 2, and 24 in CIN 3.

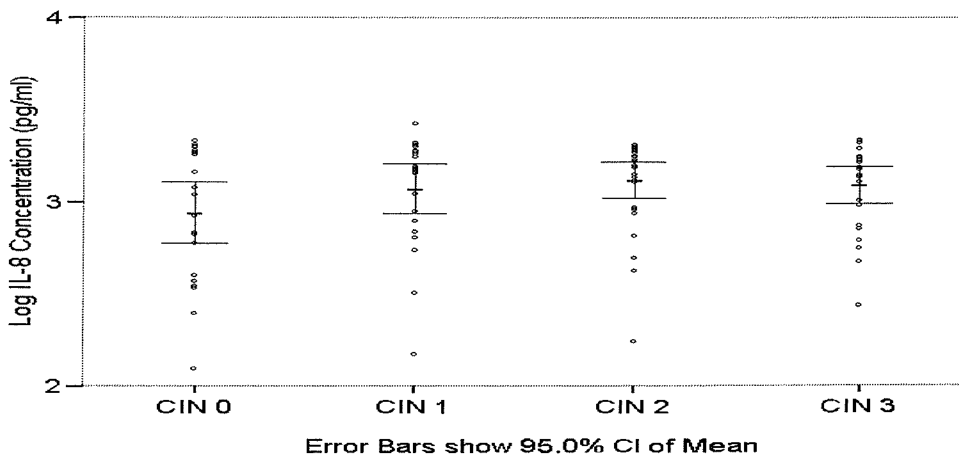


Figure 6.2. Concentration of IL-8 in Different Grades of CIN. The graphs show the 95% confidence limits together with the mean for each grade of CIN. The number of specimens in each group is 21 in CIN 0, 22 in CIN 1, 28 in CIN 2, and 24 in CIN 3.

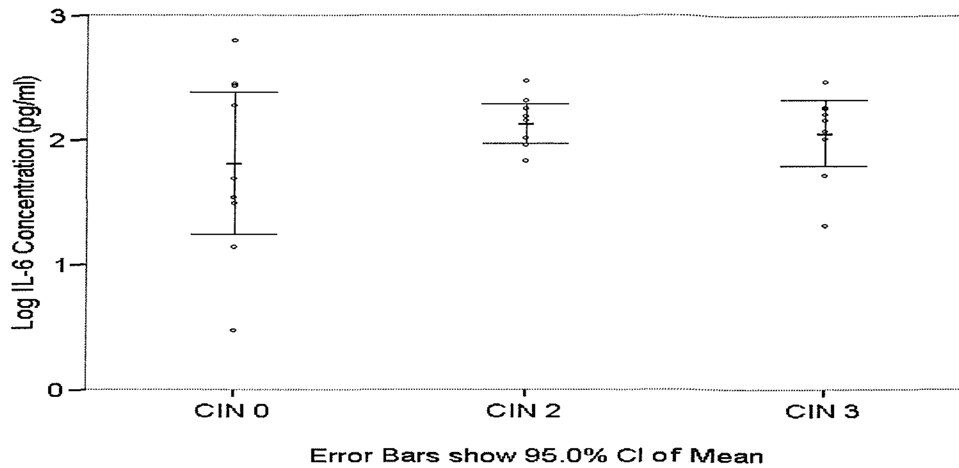


Figure 6.3. Concentration of IL-6 in Different Grades of CIN. The graphs show the 95% confidence limits together with the mean for each grade of CIN. The number of specimens in each group is 9 in CIN 0, 9 in CIN 2, and 9 in CIN 3.

For IL-1β (figure 6.1) there was a statistically significant difference between the mean level of the cytokine in CIN 0 patients compared to those with higher grades of CIN (p-value 0.014 using Student t-test analysis). Analysis of the results for IL-8 (figure 6.2) were close to significance for CIN 0 compared to CIN 1/2/3 (p-value 0.08 using Student t-test). No significant results were found for IL-6 (figure 6.3), however fewer number of samples were available for IL-6 testing.

To test if the presence of other organisms affected the production of different cytokines, the concentration of the cytokine was analysed against presence of each organism as tested in chapter 5. Only adeno-associated virus (AAV) and human herpesvirus types 6 and 7 (HHV-6 and HHV-7) were present in sufficient number to enable analysis. Of these only HHV-7 with IL-8 gave any significant results, shown in figure 6.4. For samples with CIN 0 there was a significantly higher mean level of IL-8 in samples containing HHV-7 compared to those without the virus (p-value 0.03 using Student t-test analysis).

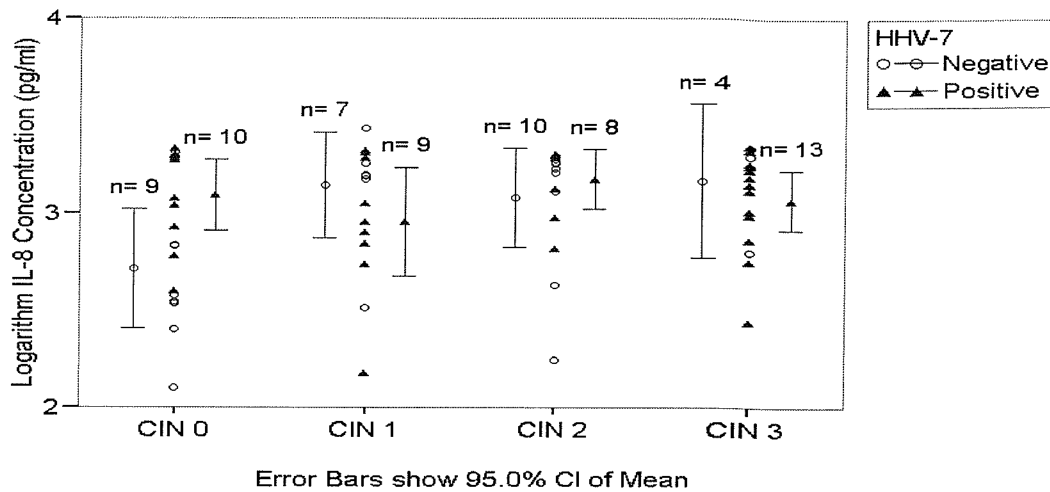


Figure 6.4. Influence of HHV-7 on Levels of IL-8 in Different Grades of CIN. The logarithm of the IL-8 level detected in the specimens was plotted against the histopathological CIN grade of the specimen together with the presence or absence of HHV-7. The graph shows the 95% confidence limits together with the mean for each grade of CIN. The number of specimens in each group (n) are also shown.

Figure 6.5 shows the results for IL-1β and the presence of HHV-7. There was no significant differences found in the amount of the cytokine produced in the different grades of CIN and the presence of HHV-7.

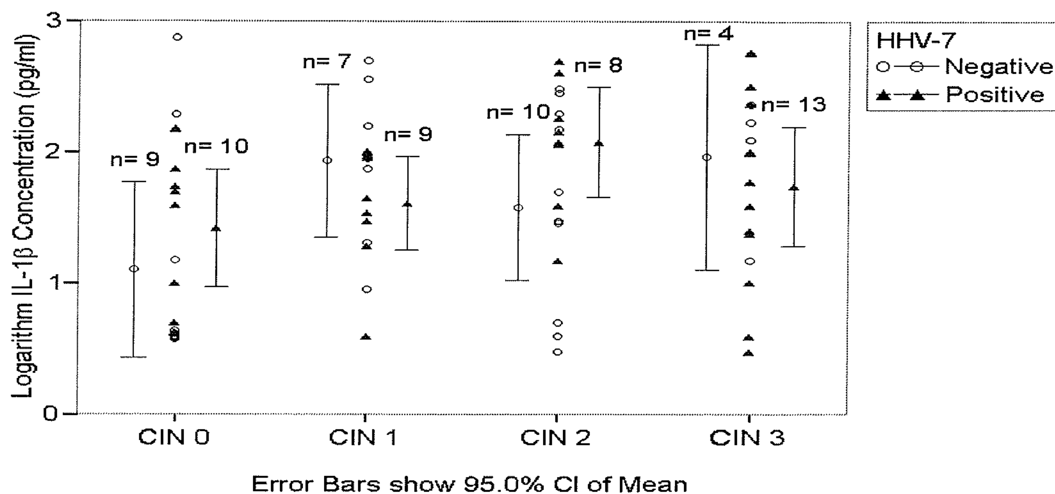


Figure 6.5. Influence of HHV-7 on Levels of IL-1β in Different Grades of CIN. The logarithm of the IL-1β level detected in the specimens was plotted against the histopathological CIN grade of the specimen together with the presence or absence of HHV-7. The graph shows the 95% confidence limits together with the mean for each grade of CIN. The number of specimens in each group (n) are also shown.

6.4 Conclusions

In a continuous cervical cancer cell line (SiHa) IL-8 was detected at a concentration of 300 pg/ml after 3 days and 120 pg/ml after 4 days culture and IL-6 was detected at 5 ng/ml after 4 days, IL-1β was not detected.

In patient samples there was significantly higher mean levels of IL-1β in CIN 1-3 (60 pg/ml) compared to CIN 0 (20 pg/ml). A similar pattern was found for mean levels of IL-8 which were 870 pg/ml in CIN 0 and 1240 pg/ml in CIN 1-3, however these results were not statistically significant. No difference was found in the mean levels of IL-6 which were around 100 pg/ml in CIN 0 and CIN 2/3.

Also, the absence of HHV-7 in CIN 0 samples gave lower levels of IL-8 than samples with HHV-7 present.

Chapter 7

Transcript Detection Using Fluorogenic Probe Assays

7.1 Introduction

The kinetics of PCR were investigated by Higuchi *et al.* (1993) using a thermal cycler designed to illuminate the samples with ultraviolet light, and detect the fluorescence produced by the intercalation of ethidium bromide into the increasing amounts of double stranded DNA produced by the reaction. A plot of the increase in fluorescence versus cycle number gave a more accurate picture of the amplification reaction than analysis of product accumulation after a fixed number of cycles.

The main drawback of the intercalating dye technique is that both specific and non-specific products, e.g. primer dimers, will be detected. The development of fluorogenic probes exploiting the 5' exonuclease activity of *Taq* DNA polymerase enabled the detection of specific product in real-time, without the need for post-PCR processing. An outline of the fluorogenic probe PCR assay is shown in figure 7.1. When the target sequence is present, the probe will bind downstream to one of the primers. The 5' exonuclease activity of the *Taq* DNA polymerase cleaves the probe, separating the reporter dye from the quencher dye thus generating a fluorescent signal. 5' exonuclease cleavage removes the entire probe which therefore has no affect on the PCR reaction itself. Additional probe molecules are removed at each cycle resulting in an increase of fluorescence which is proportional to the amount of specific product produced. In principle the PCR reaction is monitored in real-time and the cycle number when a significant increase in fluorescent signal is detected, rather than the number of amplicons produced after a set number of cycles, is used to quantify the reaction.

The variable C_T is defined as the fractional cycle number at which the change in fluorescence generated by the cleaved reporter passes a fixed threshold level above baseline. Higuchi's group (1993) showed that plotting the log of the starting copy number of the target against the corresponding C_T value produced a straight line. By determining the C_T values of unknowns, their starting copy number could be calculated from this standard curve.

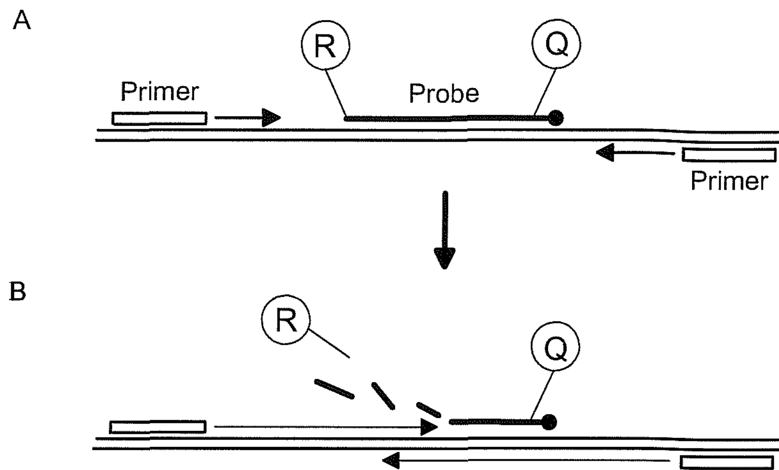
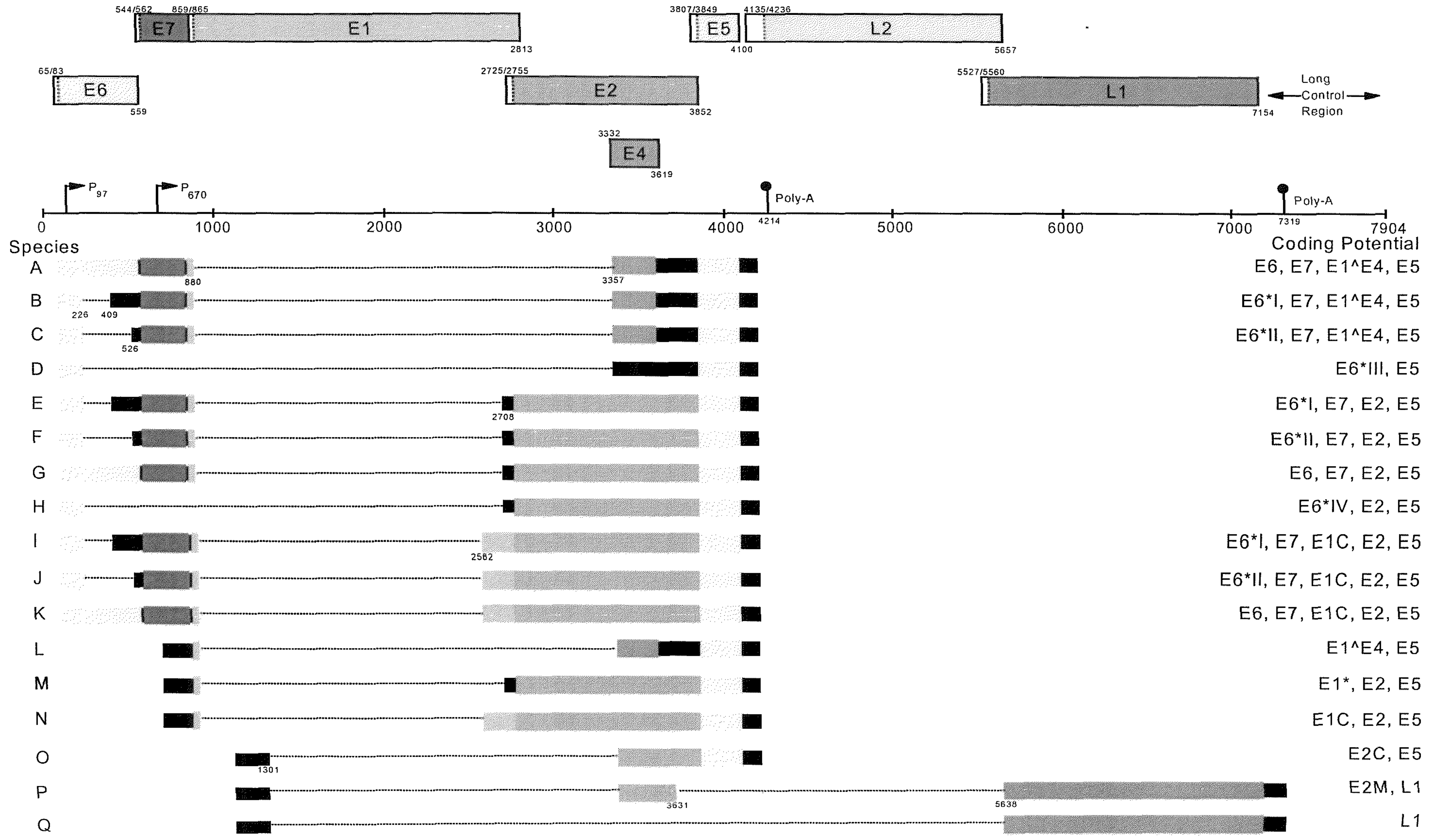


Figure 7.1. Outline of Fluorogenic Probe Assay. (A) A portion of the selected gene is amplified with specific primers, in addition the fluorogenic probe binds between the primers. The probe has a reporter (R) and a quencher (Q) dye and is blocked at its 3' end to prevent extension by the DNA polymerase. (B) During DNA amplification, the 5'→3' exonuclease activity of *Taq* DNA polymerase degrades any probe bound to the template between the primers, releasing the reporter dye from the effect of the quencher dye and thus when excited produces fluorescence. The intensity of the fluorescence is proportional to the amount of specific product.

Detection of the C_T value occurs when the reaction components are not limiting, and amplicon production is still in the exponential phase. Endpoint measurements of accumulated product are normally taken at a time when the reactants are rate limiting and the reaction is no longer exponential. Side reactions such as primer dimers can consume reagents faster than the specific product, thus samples with a high starting copy number can produce less specific product than expected. Accurate quantification of gene copy number using real-time analysis of the 5' exonuclease reaction requires a perfect match between the probe and target DNA. Given the sequence hypervariability of all HPV genes between different types, accurate analysis was only possible within a single type. HPV-16 was chosen for detailed study because it belongs to the high risk group and is the most common HPV type in the population studied.

Previous studies have shown HPV type 16 expresses a large variety of mRNAs, produced by alternative splicing, encoding both early and late gene products. The known transcripts are shown in figure 7.2. All splices conform to the consensus sequence of GT and AG at the ends of the intron (Mount, 1996). Functional E6 protein can only be produced by the unspliced transcripts, i.e. species A, G, or K. However, E7 protein could be translated from many different transcripts; A, B, C, E, F, G, I, J, and K. The functional significance of these different transcripts are unknown.



Transcript Map for Human Papillomavirus Type 16
E7
E1
E5
L2
E6
E2
L1
E4
Long Control Region
P97
P670
Poly-A
Poly-A
0
1000
2000
3000
4000
5000
6000
7000
7904
Species
Coding Potential
A E6, E7, E1^E4, E5
B E6*I, E7, E1^E4, E5
C E6*II, E7, E1^E4, E5
D E6*III, E5
E E6*I, E7, E2, E5
F E6*II, E7, E2, E5
G E6, E7, E2, E5
H E6*IV, E2, E5
I E6*I, E7, E1C, E2, E5
J E6*II, E7, E1C, E2, E5
K E6, E7, E1C, E2, E5
L E1^E4, E5
M E1*, E2, E5
N E1C, E2, E5
O E2C, E5
P E2M, L1
Q L1

The E2 protein of HPV-16 is also transcribed from a number of different splice variants. Transcripts E, H, and I from figure 7.2 were studied by Alloul and Sherman (1999) and all were shown to produce E2 protein; the protein was translated more efficiently from transcript H compared to transcripts E and I. Transcript H was found to mainly code for E2 whereas transcripts E and I mainly coded for E7 protein.

Many malignant cervical lesions possess the HPV genome integrated into the host chromosome. An important consequence of this integration event is the disruption of the viral E1 and E2 genes. The E1 and E2 proteins control viral transcription and replication, reduction of the protein levels results in abnormal expression of the E6 and E7 proteins, which are consistently over-expressed in cervical cancers. Expression of E6 and E7 extends the lifespan and results in immortalisation of epithelial cells.

Traditional methods (e.g. Northern blotting) have been used to analyse HPV-16 gene expression in cervical cancer specimens, However, these methodologies require large amounts of starting material as the techniques are relatively insensitive. The fluorogenic probe assay provides an opportunity to measure gene expression using small amounts of sample such as that from a cervical scrape. Using this quantitative PCR method it was planned to measure the relative amounts of transcripts from the E6 and E7 genes, as well as the E1 and L1 genes in HPV-16 infected samples. In addition, the presence of polycistronic transcripts would be analysed. These data may give an insight into whether there is normal or abnormal gene expression from the virus, and therefore if the sample is likely to become malignant.

As well as measuring HPV-16 transcripts, it was decided to investigate abnormal production of factors which affect epithelial cell growth. Two cellular proteins which have been shown to be absent in normal tissue, but active in different types of cancer are telomerase and survivin. These proteins appear to be specifically expressed in cancers and therefore may have a role in the development of cancer. Analysis of these two potentially oncogenic proteins would determine if they are involved in premalignant cervical intraepithelial lesions.

Telomeres are specialised structures at the ends of chromosomes (Griffith *et al.*, 1999). In humans they consist of 1000-3000 repeats of (TTAGGG) and specific telomere bound proteins. Telomeres have several functions, including protecting the ends of chromosomes, preventing chromosomes from fusing, and segregating chromosomes. Because of the mechanism by which DNA can only be replicated in the

5'→3' direction, 50-100 base pairs at the end of each chromosome are lost after each DNA replication cycle. Thus, telomeres act as non-coding sacrificial DNA. However, when telomere shortening reaches a critical level it triggers the cell to stop dividing. Therefore, cells can only replicate a finite number of times, although cells have the potential to activate an enzyme complex called telomerase which adds back telomeres (McKenzie, Umbricht, and Sukumar, 1999). Telomerase consists of several proteins together with a RNA component. This RNA strand (hTR) contains a domain complementary to one of the TTAGGG telomere repeats. One of the proteins in the complex (hTERT) has reverse transcriptase activity (Meyerson *et al.*, 1997). Thus, telomerase binds to the 3' end of DNA and adds telomere repeats using the RNA template. Telomerase activity appears to be turned off in most tissues as they differentiate. However, most human cancers show telomerase activity making it the most prevalent biochemical cancer marker (Kim *et al.*, 1994). In some cases, the presence or absence of telomerase activity is sufficient for cancer diagnosis, but in others quantitative analysis is necessary, as the tissue contains many normal cells with telomerase activity, although the relative levels of telomerase activity are higher in cancer cells.

Survivin is a member of the inhibitor of apoptosis protein (IAP) family and is expressed in proliferating cells, but is undetectable in quiescent tissues (Ambrosini, Adida, and Altieri, 1997). Survivin is expressed in the G_2/M phase of the cell cycle and rapidly down-regulated following cell cycle arrest (Li *et al.*, 1998). At the beginning of mitosis, the survivin associates with the mitotic spindle. Disruption of this interaction causes apoptosis. Thus, survivin counteracts a default induction of apoptosis in the G_2/M phase. Over-expression of survivin in cancer cells would overcome this checkpoint and allow progression of transformed cells through mitosis.

Quantitative assays for a range of HPV-16 mRNAs plus host cell GAPDH, hTERT, hTR and survivin mRNA were developed using fluorogenic probe assay technology. To establish the validity of this methodology preliminary analyses were performed on cervical cancer derived cell lines containing HPV-16 and two HPV-16 positive surgically removed cervical tumours; one was a fast growing carcinoma from a 34 year old women, and the other a slow growing carcinoma from a women of 78. In addition, the two cell lines had different growth rates.

7.2 HPV-16 Transcripts

7.2.1 Quantification of HPV-16 Transcripts

The E6 and E7 proteins of HPV-16 are the major oncoproteins expressed by the virus, and are known to cause transformation of cells. However, E6 and E7 can be produced from a number of different polycistronic transcripts.

Transcription of the E6/E7 ORFs give rise to three different splice products, using a common splice donor site at nt 226, and two different splice acceptor sites at nt 409 and 526. The full length unspliced transcript codes for functional E6 protein, whereas the E6*I (nt 409 splice) and E6*II (nt 526 splice) most likely code for the E7 protein.

The E6 assays used are outlined in figure 7.3. The location of the primers and probes allowed the identification and quantification of full length E6, E6*I, and E6*II transcripts. Fluorogenic probe assays were also used to detected cDNA between nt 699-816, nt 1799-1954, and nt 6794-6904. These would quantify transcripts which produce E7, E1 and L1 proteins respectively.

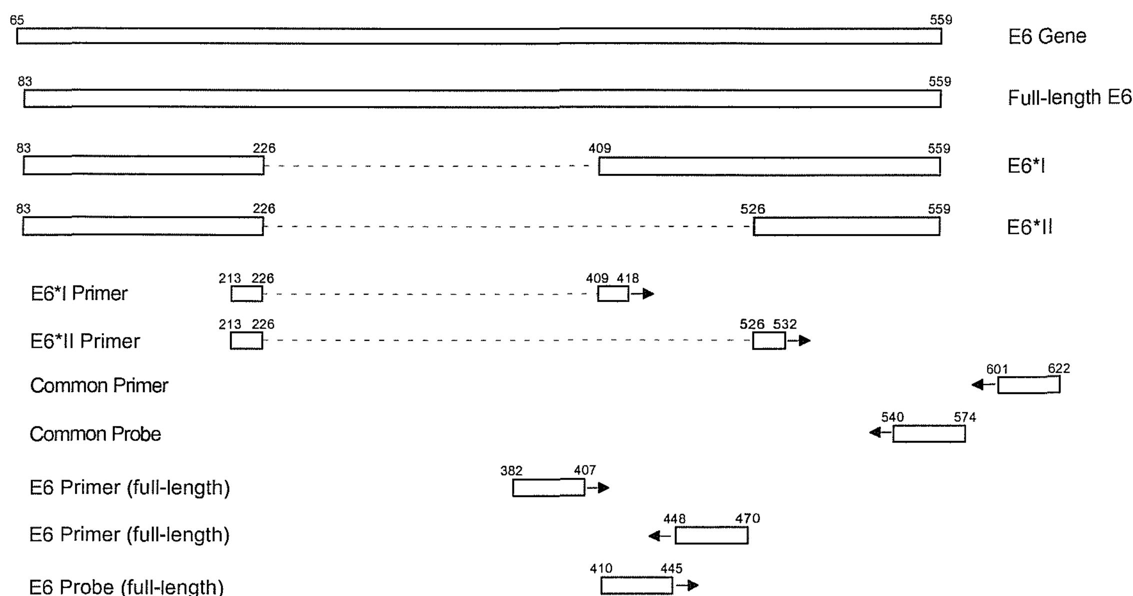


Figure 7.3. Detection of Splice Variants of the HPV-16 E6 Gene. The HPV-16 E6 gene codes for three splice products; full length functional E6, and two splice variants E6*I and E6*II. The location of the primers and fluorogenic probe used to detect the splice variants are shown. The E6*I and E6*II primers span the spliced portion of the relevant cDNA. The arrows indicate the 5'→3' orientation of the oligonucleotides.

7.2.2 Detection of Polycistronic HPV-16 mRNA

The cDNA from CaSki and SiHa cell lines was tested by PCR for the presence of transcripts A-K shown in figure 7.2. Primers were designed to overlap the splice junctions so the 11 different transcripts could be detected with only 9 individual primers; 4 forward (E6For151, E6For, E6*I and E6*II) and 5 reverse primers (880/3357Rev, 880/2708Rev, 880/2582Rev, 226/3357Rev, and 226/2708Rev). All primer sequences are shown in table 2.3 or in section 2.9.1.

The primers were designed to meet fluorogenic probe assay criteria. However, the PCR products of approximately 500 bp produced were found to be too large to produce positive results using fluorogenic probe assay PCR conditions, thus conventional PCR using agarose gels was used.

In addition, a forward primer located between the P_{670} site and the 880 splice site, (E7 Forward, section 2.9.1), plus a combination of the 3 reverse primers detecting the 880/3357, 880/2708, and 880/2582 sites (table 2.3), was used to detect production of transcripts L, M, or N. However, this assay would only confirm transcripts L, M, and N in the absence of transcripts containing the full-length E7 gene.

The expected PCR product sizes for the different transcripts are shown in table 7.1.

Table 7.1. Primer Combinations Used for HPV-16 Transcript Detection.

Transcript (see fig. 7.2)	Forward Primer	Reverse Primer	PCR Product Size /bp
E6, E7, E1^E4, E5	E6For	880/3357Rev	507
E6*I, E7, E1^E4, E5	E6*I	880/3357Rev	494
E6*II, E7, E1^E4, E5	E6*II	880/3357Rev	377
E6*III, E5	E6For151	226/3357Rev	84
E6*I, E7, E2, E5	E6*I	880/2708Rev	495
E6*II, E7, E2, E5	E6*II	880/2708Rev	378
E6, E7, E2, E5	E6For	880/2708Rev	508
E6*IV, E2, E5	E6For151	226/2708Rev	85
E6*I, E7, E1C, E2, E5	E6*I	880/2582Rev	497
E6*II, E7, E1C, E2, E5	E6*II	880/2582Rev	380
E6, E7, E1C, E2, E5	E6For	880/2582Rev	510
E1^E4, E5	E7For	880/3357Rev	190
E2, E5	E7For	880/2708Rev	190
E1C, E2, E5	E7For	880/2582Rev	191

7.2.3 Development of Fluorogenic Probe Assays

The PCR reactions were tested with DNA from CaSki cells (containing HPV-16) and HeLa cells (containing HPV-18) with $MgCl_2$ concentrations of 1.5 mM, 3.5 mM, 6.0 mM, and 9.0 mM.

A single band was produced with CaSki cells for all 6 PCRs with $MgCl_2$ concentrations of 3.5 mM and above. No bands were produced with 1.5 mM $MgCl_2$. For HeLa cells, non-specific bands were produced in the E6 PCR with 9 mM $MgCl_2$, in the E7 PCR with 6 mM and 9 mM $MgCl_2$, and in the L1 PCR with 9 mM $MgCl_2$. Multiple bands were also produced from CaSki cDNA with the E6* PCRs above 3.5 mM $MgCl_2$. Therefore 3.5 mM $MgCl_2$ was used in all 6 PCRs.

Once the magnesium chloride concentration had been determined, the optimisation of the primer concentrations was finalised using the fluorogenic probe. The fluorogenic PCR was run using both forward and reverse primer concentrations of 50 nM, 300 nM, and 900 nM therefore giving 9 different primer combinations.

The amount of fluorescence produced with PCR cycle number for the E6 probe is shown in figure 7.4. The other reactions had similar results to E6. DNA from CaSki cells was used as the target.

The results shown in figure 7.4 indicate the forward primer concentration is rate limiting with primer concentrations of 50 nM (plots 1-3). In contrast, the reverse primer concentration is rate limiting at 50 nM when higher concentrations of forward primer are used (plots 4 and 7). The forward primer also limits the reaction at 300 nM (plots 5 and 6), as does the reverse primer, slightly, at 300 nM (plot 8). Therefore, the primer combinations with the greatest increase in fluorescence is 900 nM for both forward and reverse primers. The same distribution of plots was seen with the other reactions. Thus, forward and reverse primers were used at a final concentration of 900 nM for all 6 reactions.

The specificity of the assays was tested by using DNA from HPV types -6, -11. -16, -18, -31, -33, -45, and -51. The amplification plot for the E6 assay is shown in figure 7.5. The results of the E1, E6*, E7, and L1 reactions were similar to that shown. The results indicate the assays would only detect HPV-16 and not other common HPV types.

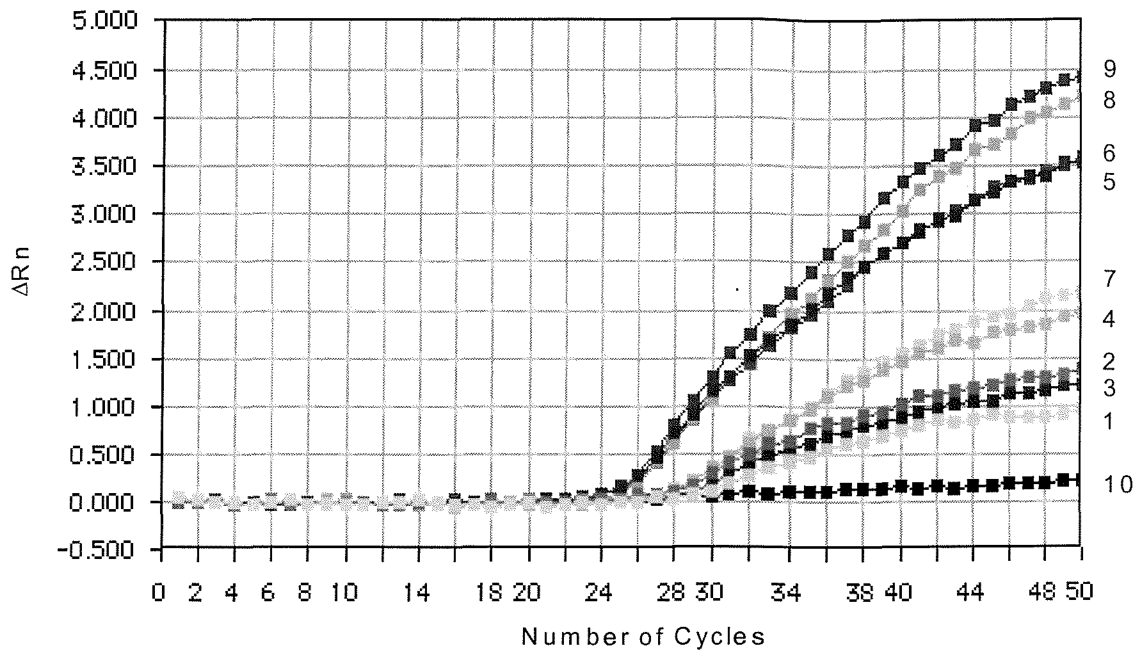


Figure 7.4. Variation of Fluorescence Signal with Primer Concentration. The Y-axis represents the change in relative fluorescence and the X-axis shows the number of cycles completed in the PCR reaction. The primer concentrations used were varied in each reaction. Plots 1-3; forward primer concentration of 50 nM, plots 4-6; forward primer concentration 300 nM, plots 7-9; forward primer concentration 900 nM, plots 1,4,7; reverse primer concentration 50 nM, plots 2,5,8; reverse primer concentration 300 nM, plots 3,6,9; reverse primer concentration 900 nM. Plot 10; forward primer 300 nM, reverse primer 300 nM and no target DNA added. For all the plots, the probe concentration was 200 nM. The graph shows the accumulation of fluorescence with the number of PCR cycles performed, which is proportional to the amount of PCR product formed. The graph shows the results for the E6 assay, the E1, E6*, E7, and L1 assays gave similar results. DNA from CaSki cells was used as the target.

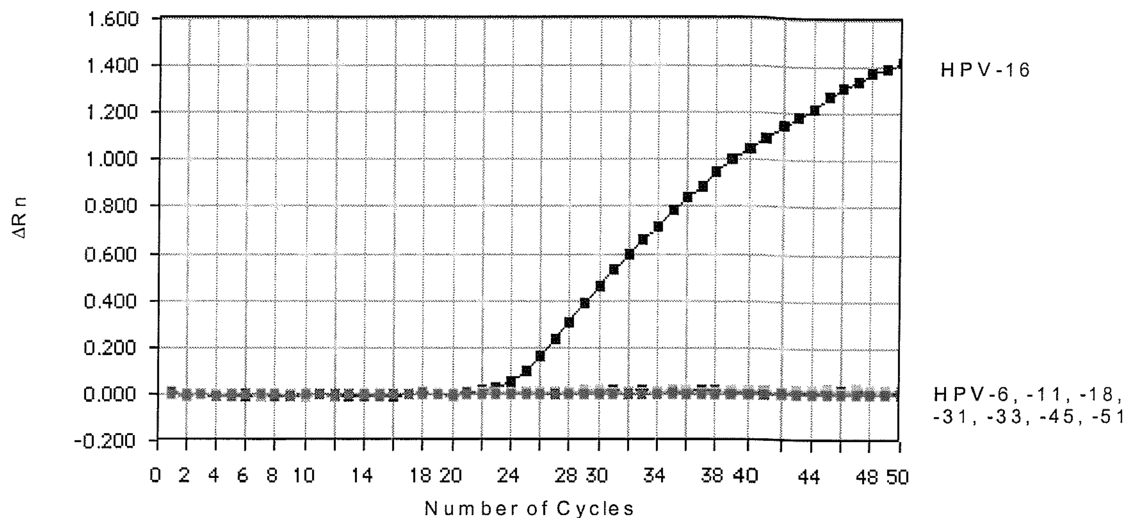


Figure 7.5. Specificity of Fluorogenic Probe Assay for HPV-16. The Y-axis represents the change in relative fluorescence and the X-axis shows the number of cycles completed in the PCR reaction. The fluorogenic probe assay was run using DNA from HPV types -6, -11. -16, -18, -31, -33, -45, and -51. The assay shows fluorescence was only produced with HPV-16 DNA. The graph shows the results for the E6 assay, the E1, E6*, E7, and L1 assays gave similar results.

The sensitivity of the E1, E6, E7, and L1 assays was checked using cloned HPV-16 DNA in pBR322 plasmid. The plasmid containing HPV-16 genome was extracted from *E. coli* using Wizard *Plus* Minipreps (Promega) following the manufacturer's instructions. The E6* assays were tested by using a known quantity of specific PCR product produced from CaSki cDNA.

The DNA was diluted to give a range of starting copies. These dilutions were then run on each assay to determine the sensitivity. The C_T value is defined as the fractional cycle number at which the change in fluorescence signal passes a fixed threshold above baseline. The threshold was set at a level equivalent to 10 standard deviations of the average baseline level of the reporter signal. The C_T was determined by plotting the change in fluorescence signal on a log scale against the cycle number as shown in figure 7.6. Thus the C_T values for the different dilutions of the HPV-16 plasmid were determined.

The initial starting copy number of HPV-16 genomes was plotted against the C_T value for that dilution, for all 6 assays. The results are shown in figure 7.7.

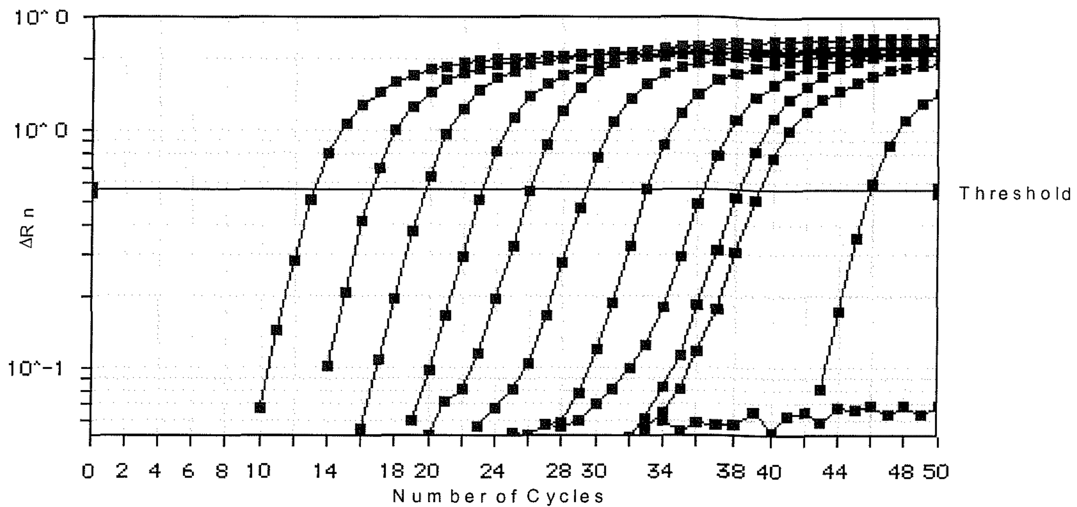


Figure 7.6. Sensitivity of Fluorogenic Probe Assay. The Y-axis represents the log of the change in relative fluorescence and the X-axis shows the number of cycles completed in the PCR reaction. The results shown are for a dilution series of HPV-16 containing plasmid. The dilution increases from left to right. The C_T value for each dilution was taken as the cycle value where the plot crossed the threshold value, which was set at a level equivalent to 10 standard deviations of the average baseline level of the reporter signal. The results shown are for the E6 assay. Similar results were obtained for the E1, E6* (using known numbers of PCR products derived from CaSki cDNA), E7 and L1 assays.

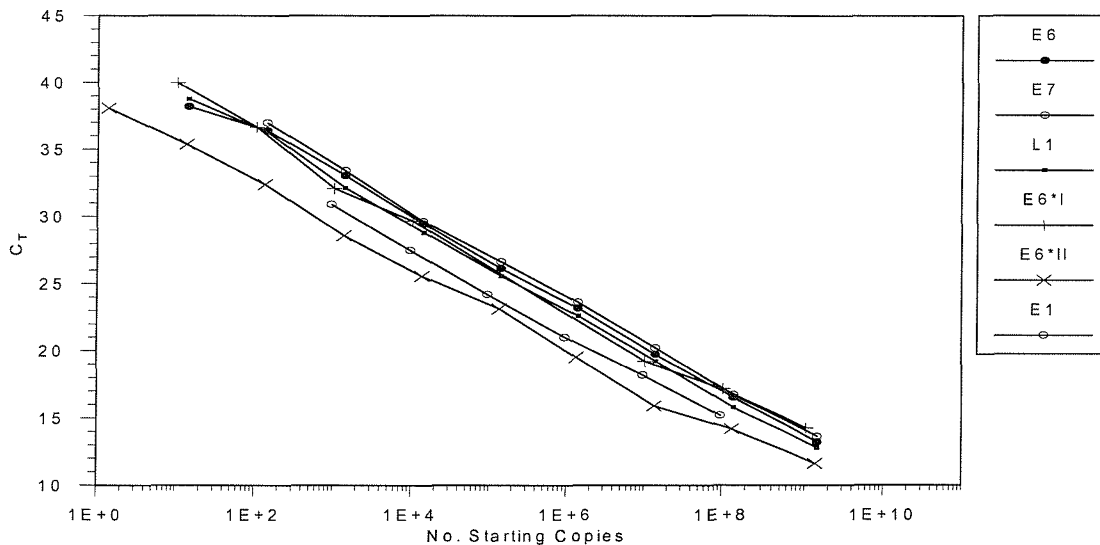


Figure 7.7. Standard Curve for Initial Starting Copy Number for HPV-16 E1, E6 E6*I, E6*II, E7, and L1 Assays. The graphs represent the change in the calculated C_T value with variation in the initial starting copy number of HPV-16 genomes. The results indicate four of the assays produced similar results when the same number of starting copies are used. However, the E1 and E6*II assays gave lower C_T values than the other assays for the same number of starting copies. The E6*II assay gave linear results with single figure starting copies, the E6, E6*I, and L1 assays produced linear results above 10 starting copies, whereas the E7 and E1 assays had linearity above 100 and 1000 starting copies respectively.

From figure 7.7 the sensitivity of the E6*II assay was around 1 starting copy, for the E6, E6*I, and L1 assays was 10 starting copies, for the E7 assay was 100 starting copies, and the E1 assay detected down to 1000 starting copies.

To test the reproducibility of the assay, plasmid containing HPV-16 and viral DNA from a clinical sample were diluted to give a range of starting copy numbers. These dilutions were tested with the E6 fluorogenic assay. Figure 7.8 shows the results of replicate samples. When the number of starting copies is below 1000 the accuracy of the assay is reduced in comparison with higher starting copy numbers of HPV-16 genome. The starting copy number of the specimen was calculated using the calibration curve, whereas the starting copy number of the plasmid was calculated from the DNA concentration. Figure 7.8 therefore also shows the consistency of results between different samples.

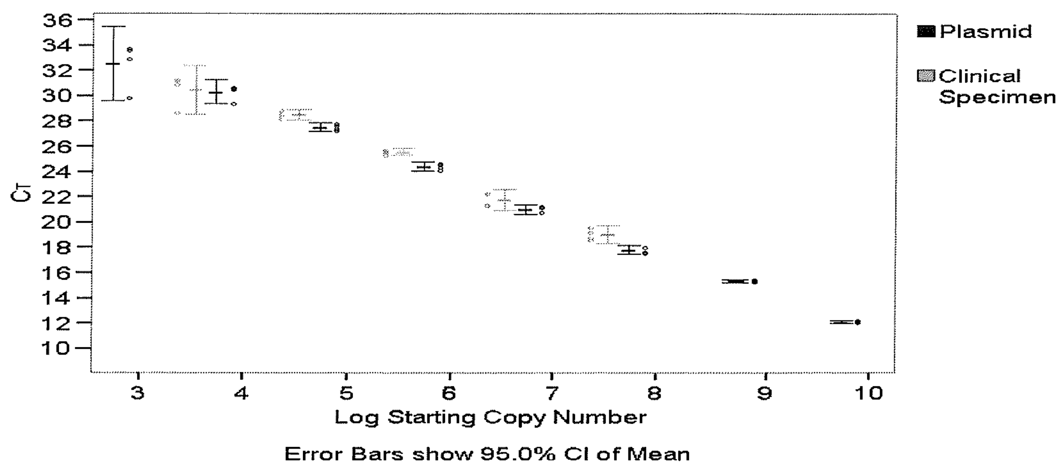


Figure 7.8. Reproducibility of Fluorogenic Probe Assay. Plasmid containing HPV-16 genome and a HPV-16 containing clinical specimen were diluted to give a range of genome starting copy number. Each sample was tested 4 times. The C_T was plotted against the starting copy number. Plasmid copy number was determined by DNA concentration, the clinical sample was quantified using the calibration curve shown in figure 7.7. All samples were tested with the E6 fluorogenic probe assay.

The experiment shown in figure 7.8 was repeated using 1/2 dilutions of the specimen rather than 1/10 dilutions. Each dilution was repeated 8 times in order to determine what difference in starting copy number could be accurately resolved as different starting copy numbers. The results are shown in figure 7.9.

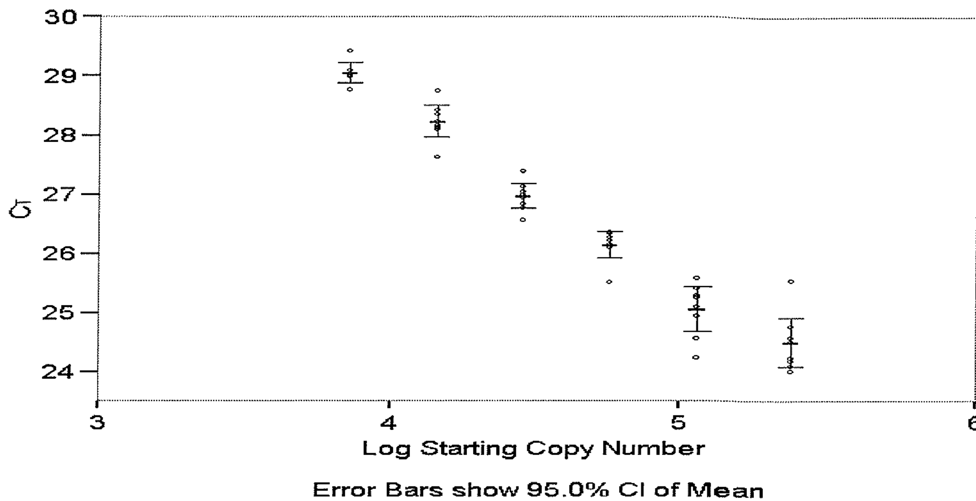


Figure 7.9. Resolution of Fluorogenic Probe Assay. A HPV-16 containing clinical specimen was diluted 1/2 to give a range of genome starting copy number. There were 8 replicates of each sample. The C_T was plotted against the starting copy number. The clinical sample was quantified using the calibration curve shown in figure 7.7. All samples were tested with the E6 fluorogenic probe assay.

Figure 7.9 shows that for the clinical sample resolution was not precise with overlap in the scatter of results for 1/2 dilutions.

7.2.4 Optimisation of cDNA Production

The Trizol RNA extraction method was tested in parallel with 2 spin column RNA extraction methods; S.N.A.P. total RNA isolation kit (Invitrogen) and Oligotex mRNA purification system (Qiagen). The 3 methods were used to extract RNA from the same number of CaSki cells. cDNA was produced using Superscript reverse transcriptase as in section 2.8. The cDNA was quantified using the E6 fluorogenic probe assay. The S.N.A.P. kit produced E6 cDNA at a level 2.5 logs lower than the Trizol method, and the Oligotex gave 1.5 logs lower levels than Trizol. Therefore, the Trizol method was used to extract RNA from all samples.

The DNAse used to eliminate trace DNA contamination of the purified RNA was found to have no activity following inactivation at 65 °C for 10 minutes.

RNA was extracted from SiHa cells, which contain between 1 and 5 copies of HPV-16 integrated into the host genome. cDNA was produced from this RNA pool using three different primers; oligo$(dT)_{15}$ (Promega), $T_{25}VN$, and a pool of the specific reverse primers of all the fluorogenic assays performed in this study. The cDNA

produced was tested with all the PCR reactions in order to determine which cDNA production method detected the widest range of transcripts. Although no significant differences were found between the C_T values in the fluorogenic assays with the different cDNA priming methods, specific primers markedly increased sensitivity of detection of different HPV-16 mRNA splicings (see table 7.2), and were selected for cDNA production.

Table 7.2. Sensitivity of Different cDNA Priming Methods.

Transcript PCR Reaction	Primer Used for cDNA Production		
	Oligo $(dT)_{15}$	$T_{25}VN$	Specific
E6, 880/3357	-	-	+
E6, 880/2708	+	-	++
E6, 880/2582	-	+	+
E6*I, 880/3357	-	-	+
E6*I, 880/2708	++	+	++
E6*I, 880/2582	+	+	++
E6*II, 880/3357	-	-	+
E6*II, 880/2708	+	-	+
E6*II, 880/2582	-	-	+
E6151, 226/3357	+	++	++
E6151, 226/2708	+	++	++

- no PCR product detected, ± weak PCR product detected, + moderate PCR product detected, ++ strong PCR product detected.

Three different priming methods were used to produce cDNA from HPV-16 RNA. Oligo $(dT)_{15}$ consisted of 15 T bases designed to bind to the poly-A tail of mRNA, $T_{25}VN$ consisted of 25 T bases followed by A/G/ or C, then A/T/C/ or G designed to bind to the 5' end of poly-A tail of mRNA, and specific primers. The transcript PCR reaction shows the primers in the PCR reaction used to detect the cDNA produced by the different priming methods.

7.2.5 Quantification of Transcripts in Cell Lines and Cervical Tumours

In order to compare the methods developed, the HPV-16 containing cell lines SiHa (doubling time of 28 hours) and CaSki (which had a doubling time of 36 hours), and two HPV-16 containing cervical tumours; one was a fast growing tumour from a 34 year old patient, the other a slow growing tumour from a women aged 78, were tested for the presence and quantity of the viral transcripts using the E1, E6, E6*I, E6*II, E7, and L1 fluorogenic probe assays. The number of cells in each sample was determined

by PicoGreen DNA assay. In addition the number of copies of each gene (except E6*I and E6*II) were determined, for E6*I and E6*II the number of gene copies of E6 were used for calculations. The nature of the samples tested would determine if the methodologies developed could detect differences in the range and level of transcripts produced.

In order to correct for sample variations, e.g. number of cells or viral copy numbers per cell, viral transcript levels are normalised using a variety of denominators such as number of copies of the particular gene, number of cells in the sample, or number of transcripts from a so-called 'housekeeping gene' of which GAPDH is commonly used. From table 7.3 GAPDH levels per cell varied between cell lines (7 transcripts per CaSki cell and 126 transcripts per SiHa cell) and between tumours (18 GAPDH transcripts per cell in the fast growing tumour and 0.7 per cell in the slow growing tumour). Depending on which denominator is used drastically changes the comparative findings. The affect of using different denominators on the final result are illustrated in table 7.3. For example, in CaSki cells the number of E1 transcripts per viral gene (6×10^{-4}) are similar to those for the slow growing tumour (1×10^{-3}), however when the number of transcripts are calculated per cell there are 100 times more E1 transcripts; 3×10^{-1} in CaSki compared to 3×10^{-3} in the slow growing tumour. In addition when the number of transcripts are standardised per GAPDH transcript there are 10 times as many E1 transcripts in CaSki (4×10^{-2}) compared to the slow growing tumour (5×10^{-3}). Thus, depending on which denominator is used samples can have similar or 100 times different levels of the same transcript. Of the possible denominators, normalising to the number of transcripts per cell consistently correlated with the observed differences in the samples. Therefore, this was used when normalisation was necessary.

Table 7.3. Comparative Analysis of HPV-16 Transcripts Using Different Denominators for Cervical Cancer Cell Lines and Tumour Biopsies.

Transcript	Source											
	CaSki			SiHa			Fast Growing Tumour			Slow Growing Tumour		
	Per Gene	Per Cell	Per GAPDH	Per Gene	Per Cell	Per GAPDH	Per Gene	Per Cell	Per GAPDH	Per Gene	Per Cell	Per GAPDH
E1	$6x10^{-4}$	$3x10^{-1}$	$4x10^{-2}$	1	1	$1x10^{-2}$	5	$7x10^{-1}$	$4x10^{-2}$	$1x10^{-3}$	$3x10^{-3}$	$5x10^{-3}$
E6	$3x10^{-4}$	$2x10^{-1}$	$3x10^{-2}$	$3x10^{-1}$	1	$1x10^{-2}$	3	2	$9x10^{-2}$	$6x10^{-4}$	$8x10^{-3}$	$1x10^{-2}$
E6*I	0	0	0	$3x10^{-1}$	1	$1x10^{-2}$	4	2	$1x10^{-1}$	$1x10^{-4}$	$1x10^{-3}$	$2x10^{-3}$
E6*II	0	0	0	$8x10^{-3}$	$4x10^{-2}$	$3x10^{-4}$	$2x10^{-1}$	$8x10^{-2}$	$5x10^{-3}$	0	0	0
E7	$5x10^{-3}$	6	1	2	9	$7x10^{-2}$	50	42	2	$4x10^{-3}$	$8x10^{-2}$	$1x10^{-1}$
L1	$4x10^{-6}$	$5x10^{-3}$	$8x10^{-4}$	$6x10^{-4}$	$2x10^{-3}$	$2x10^{-5}$	$2x10^{-4}$	$1x10^{-4}$	$6x10^{-6}$	$3x10^{-5}$	$5x10^{-4}$	$7x10^{-4}$
GAPDH	3	7		63	126		9	18		$3x10^{-1}$	$7x10^{-1}$	

DNA and cDNA were extracted from the cervical cancer cell lines containing HPV-16, CaSki and SiHa, and biopsies from 2 patients with a cervical tumour containing HPV-16; a fast growing tumour from a 34 year old women and a slow growing tumour from a women of 78. Gene copy number and transcript quantifications were performed by fluorogenic probe assay. For E6*I and E6*II the number of gene copies of E6 were used for calculations. Cell numbers were determined by PicoGreen assay. The average HPV-16 genome copy number per cell was 815 for CaSki cells, 3 for SiHa cells, $6x10^{-1}$ for the fast growing tumour, and 11 for the slow growing tumour.

7.2.6 Detection of Polycistronic mRNA in Cervical Cell Lines and Cervical Tumour Biopsies

The cDNA, produced using specific primers, from the HPV-16 containing cell lines, CaSki and SiHa, and 2 HPV-16 positive cervical tumour biopsies were also tested for the presence of the polycistronic transcripts shown in figure 7.2. The results are shown in figure 7.10 and table 7.4.

Table 7.4 shows that although some transcripts were detected in all the samples the amount of the transcripts detected varied despite similar numbers of cells from each source being used. Transcripts A (E6, E7, E1^E4, E5), D (E6*III, E5) and H (E6*IV, E2, E5) were detected at high levels from all the samples.

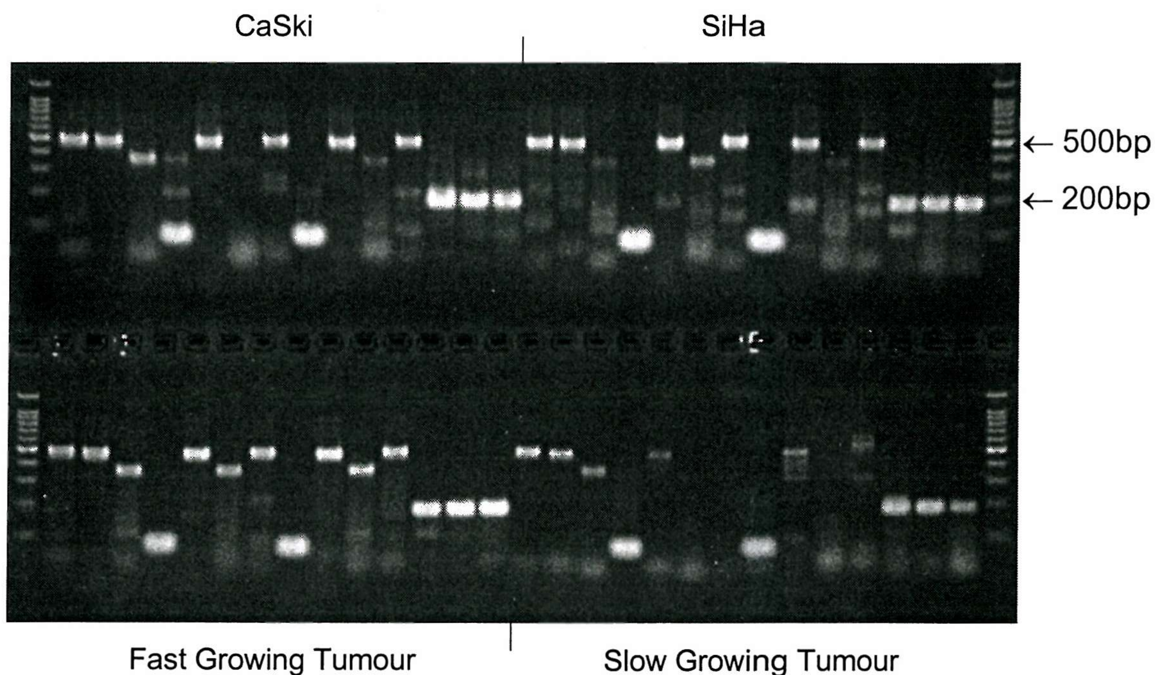


Figure 7.10. Presence of Polycistronic mRNA in Cervical Cell Lines and Cervical Tumours. In both gels lanes 1 and 30 are 100 bp markers, the 500 bp and 200 bp are highlighted. In both portions of the gel lanes 2 to 15 and lanes 16 to 29 represents transcripts A to N as shown in figure 7.2. The top left half of the gel shows transcripts detected in CaSki cells and top right shows transcripts detected in SiHa cells. The bottom left shows transcripts from the fast growing tumour, and bottom right shows transcripts detected in the slow growing tumour biopsy. Transcripts A, B, E, G, I, and K are approximately 500 bp in size, transcripts C, F, and J are around 380 bp, transcripts L, M, and N are 190-191 bp, and transcripts D and H are 84 bp and 85 bp respectively (see table 7.1).

Table 7.4. Presence of HPV-16 Transcripts in Cervical Cancer Cell Lines and Cervical Tumours.

Transcript (see figure 7.2)	Cell Line			
	CaSki	SiHa	Fast Growing Tumour	Slow Growing Tumour
E6, E7, E1^E4, E5	++	++	++	++
E6*I, E7, E1^E4, E5	++	++	++	++
E6*II, E7, E1^E4, E5	++	±	++	+
E6*III, E5	++	++	++	++
E6*I, E7, E2, E5	++	++	++	±
E6*II, E7, E2, E5	-	+	++	-
E6, E7, E2, E5	++	++	++	-
E6*IV, E2, E5	++	++	++	++
E6*I, E7, E1C, E2, E5	++	++	++	±
E6*II, E7, E1C, E2, E5	±	±	++	-
E6, E7, E1C, E2, E5	++	++	++	-

The relative intensities of the products detected are shown: - no PCR product detected, ± weak PCR product detected, + moderate PCR product detected, ++ strong PCR product detected.

PCR reactions were performed in order to detect the transcripts indicated from CaSki or SiHa cervical cancer cell lines or cells from 2 cervical biopsies; a fast growing tumour from a 34 year old women and a slow growing tumour from a women of 78.

7.2.7 Conclusions

In the cervical cancer cell lines and the cervical tumour biopsies, transcripts coding for E7 protein were the most abundant HPV-16 transcripts detected, whereas transcripts coding for L1 protein were rare or absent.

The GAPDH transcript level per gene copy number in the different cell lines were shown to be different. Hence cells in cervical samples may not express similar levels of GAPDH, thus the practice of using GAPDH transcript levels to normalise samples should be performed with caution.

Of all the transcripts detected only transcripts A (E6, E7, E1^E4, E5), B (E6*I, E7, E1^E4, E5), D (E6*III, E5) and H (E6*IV, E2, E5) were consistently detected at high levels from the cell lines and cervical tumour biopsy, however, transcripts D and H produced PCR products of less than 100 bp compared to the other transcripts which were all above 370 bp. Therefore the high level of transcripts D and H may simply be due to higher PCR efficiency with the smaller PCR products.

7.3 Detection and Quantification of HPV-16 Transcripts in Cervical Intraepithelial Neoplasia

7.3.1 Standardisation of Cervical Samples

The results of the comparative analysis of HPV-16 transcripts in two cervical cell lines which differ in growth rate and between a highly malignant and a slow growing cancer clearly demonstrate the variability in transcript production and how this can be compounded by different standardisation protocols. The problem of standardisation will be exaggerated in cervical samples since the proportion of abnormal cells from the lesion relative to the number of normal cervical cells sampled will vary, as will the ratio of inflammatory cells in the specimen.

The total number of cells present in cervical samples from HPV-16 infected women is plotted against the CIN grade in figure 7.11. There were no significant differences between the mean number of cells collected according to CIN grade, but the range within each CIN grade varied 20 to 100 fold. The feasibility of normalisation of HPV-16 transcripts according to host cell GAPDH mRNA production was also assessed (figure 7.12). Again, the mean GAPDH transcript copy number was not significantly different between CIN 0/1 and CIN 2/3 samples, but there were marked variation within each sample group.

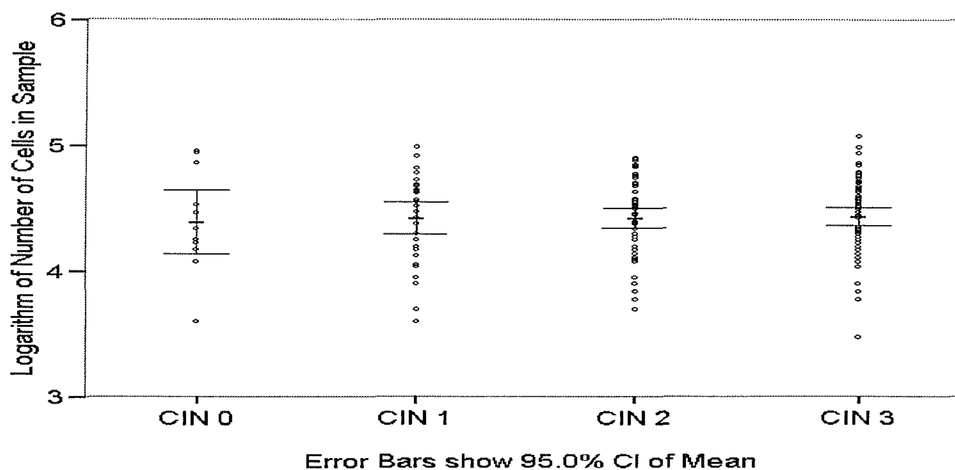


Figure 7.11. Number of Cells in HPV-16 Positive Samples with Different Grades of CIN. The number of cells in the sample was determined using PicoGreen DNA stain as described in section 2.1. The result was plotted against the grade of CIN of the sample determined by histopathology. The mean of the results for each CIN group are shown together with the 95% confidence limits. The number of specimens in CIN 0 was 12, 32 samples in CIN 1, 59 samples in CIN 2, and there were 71 specimens in CIN 3.

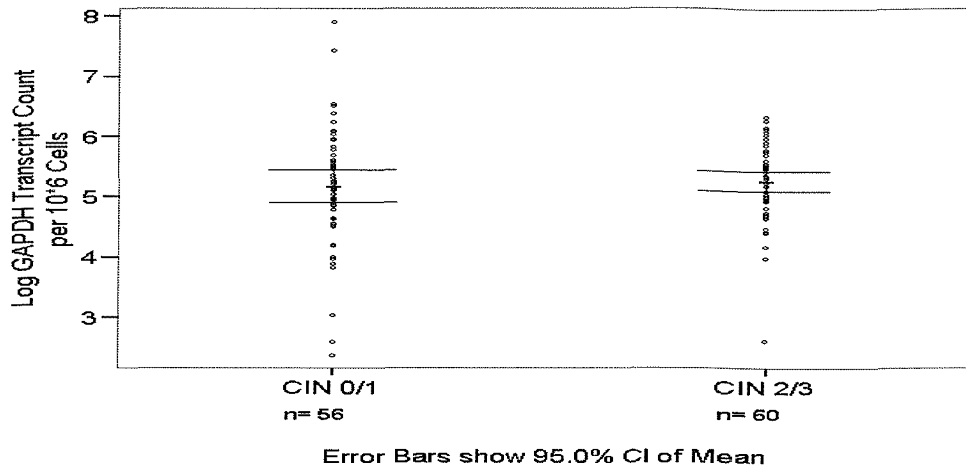


Figure 7.12. GAPDH Transcript Levels in Different Grades of CIN. The number of transcripts in each sample were determined using fluorogenic probe assay. The number of cells in each sample were determined using PicoGreen DNA stain. Transcript levels were normalised to the number of transcripts per 10^6 cells. The number of samples in each group (n) are shown. Error bars show 95% confidence limits of the mean.

The difficulty of normalising HPV-16 transcript copy number according to the HPV genome count is that viruses in clinical samples may be metabolically inactive HPV virions produced by the cells or active viral genes within infected cells. To determine if this is a significant problem 14 HPV-16 positive samples were collected into RPMI + 10% FCS + 0.05% sodium azide. The distribution of virus in these samples was tested by vortexing and then centrifuging at 1,000 x g to pellet the cells. The supernatant was removed to a fresh tube and centrifuged at 60,000 x g to pellet free virus. The DNA from both samples was extracted using ammonium hydroxide and the number of HPV-16 genomes determined with the E6 fluorogenic probe assay. Figure 7.13 shows the proportion of the total HPV genomes detected in the whole sample that occurred in the cell pellet fraction.

From figure 7.13 as only 3 samples were found in total in CIN 0/1 it is difficult to determine the level of cell associated virus in these samples. However, for CIN 2 and 3 the proportion of cell associated HPV genome in the sample ranged from 30% to 100%. The implication is that in high grade lesions the HPV genomes are predominately cell-associated rather than free virus released from the cell. Accordingly, it was decided to pellet the cells from RNAlater solution by centrifugation at 60,000 x g for 5 minutes before analysis of HPV-16 transcripts and gene copy numbers.

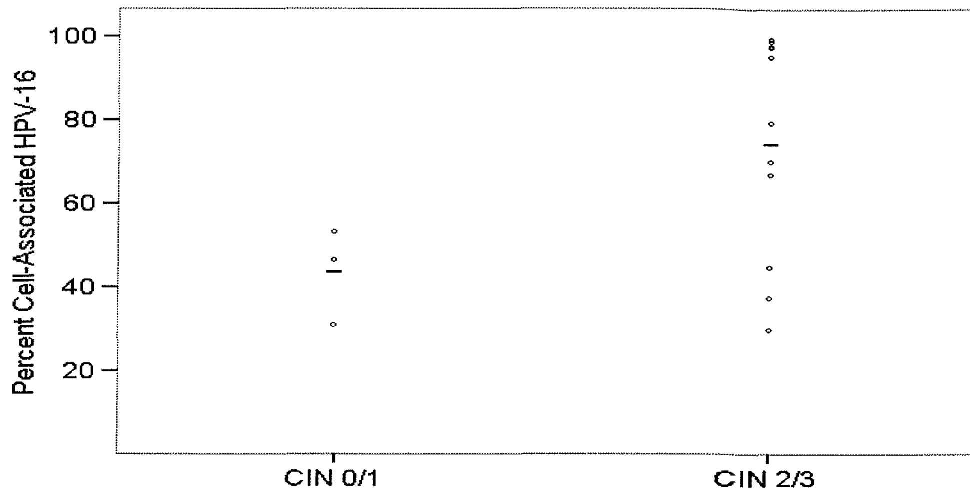


Figure 7.13. Cellular Association of HPV-16 in High Grade CIN Lesions. Fresh samples were centrifuged at 1,000 x g to pellet cells, the supernatant was then centrifuged at 60,000 x g to pellet free virus. Both samples had the DNA extracted with ammonium hydroxide. The number of viral genome copies in the samples was determined using the E6 fluorogenic probe assay. The total number of HPV genomes cell-associated is expressed as a percentage of the total HPV genome count. Horizontal bars represent the mean. The number of samples in CIN 0/1 was 3 and 11 samples in CIN 2/3.

7.3.2 Quantification of Transcripts in Different Grades of CIN Using Fluorogenic Probe Assay

cDNA was produced from 57 samples collected in the colposcopy clinic, all contained HPV-16 DNA as determined by the HPV-16 E7 PCR (see section 2.3). The cDNA was tested using the E1, E6, E6*I, E6*II, E7, and L1 fluorogenic probe assays to quantify transcription of the relevant genes. The GAPDH assay was used to determine mRNA viability from the cells. For analysis, samples were divided into CIN 0/1 and CIN 2/3 to give larger sample sizes.

Figure 7.14 shows the results of 3 assays compared against different grades of CIN. Only samples with detectable levels of the transcripts are shown. No significant differences were found with the expression levels of E6, E7, and L1 transcripts between high and low grade CIN. For CIN 0/1 only 2 specimens expressed E6*I transcripts, 1 specimen expressed E1 transcripts, and no E6*II transcripts were detected.

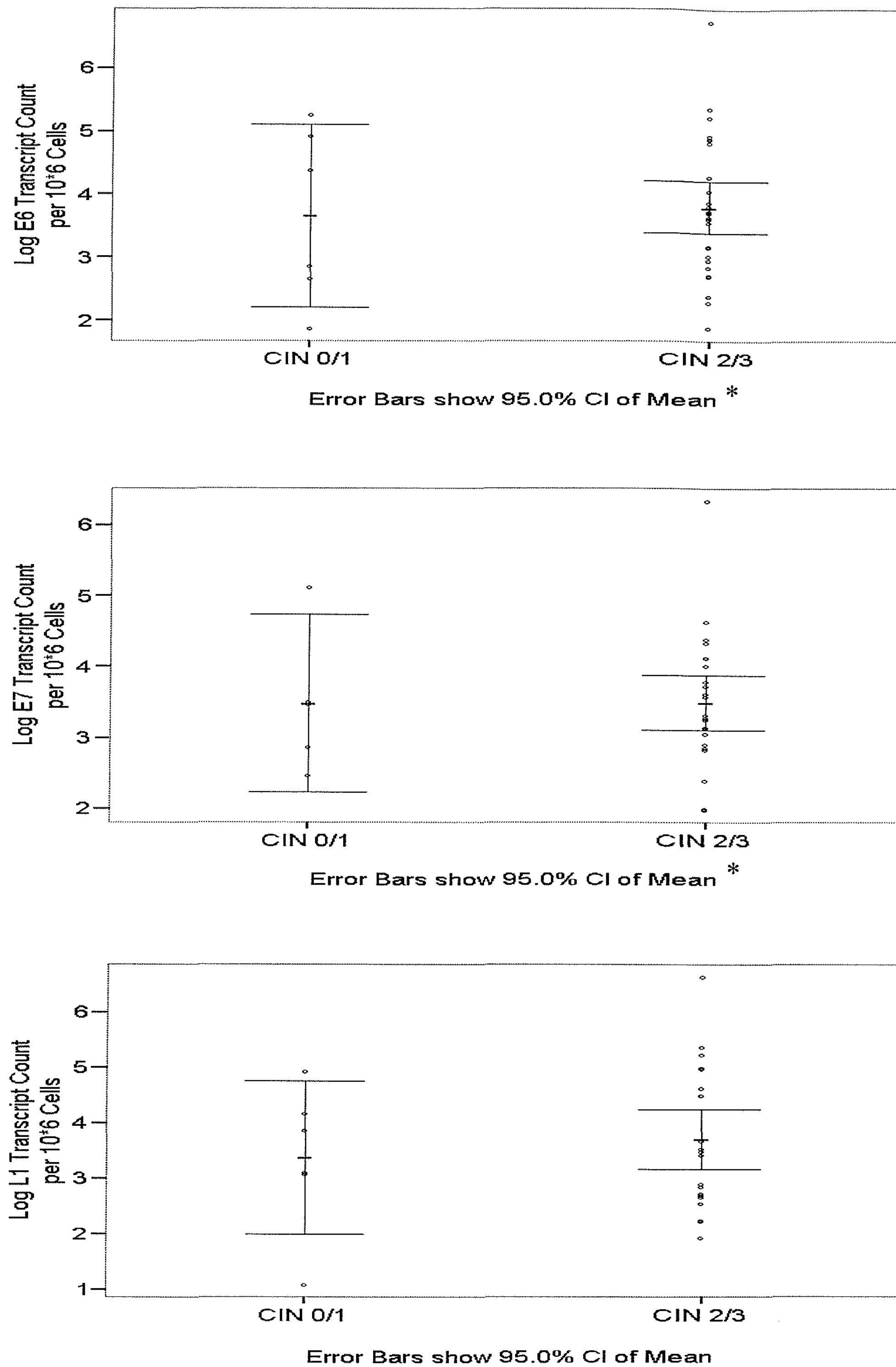


Figure 7.14. Initial Starting Copy Number for HPV-16 E6, E7, and L1 Transcripts in High and Low Grade CIN. Transcript values were normalised to number of transcripts per 10^6 cells. Error bars show 95% confidence limits of the mean. The total number of samples in CIN 0/1 was 17 and 40 samples in CIN 2/3. Only specimens expressing transcripts are shown.

* Findings were confirmed by retesting in triplicate (see table 7.5)

Although the level of expression of the HPV-16 transcripts was not different between high and low grade CIN, there was a difference in the proportion of samples containing detectable transcripts (table 7.5). Transcripts which showed significantly different results between CIN 0/1 and CIN 2/3 after initial testing (E6, E6*I, E6*II, and E7), were repeated in triplicate. Shortage of sample meant E1 and L1 transcripts could not be repeated. All significant results were confirmed after repeating in triplicate.

Table 7.5. Percentage of Specimens Positive for HPV-16 Transcripts.

Transcript	CIN 0/1 (n=17)	CIN 2/3 (n=40)	p-value (χ^2 analysis)
E6	35.3	72.5	**0.007***
E7	29.4	60	**0.043***
E6*I	11.8	42.5	**0.006***
E6*II	0	25	**0.035***
E1	5.9	20	0.18
L1	35.3	57.5	0.13

* After repeating in triplicate.

The statistical significance between the CIN 0/1 and the CIN 2/3 groups was calculated using χ^2 analysis. The corresponding p-values are shown. Values in bold indicate a statistically significant result. The sensitivities of the assays were 10 starting copies for E6, 100 copies for E7, 10 copies for E6*I, 1 copy for E6*II, 1000 copies for E1, and 10 starting copies for L1 assay.

7.3.3 Detection of Polycistronic mRNA in Different Grades of CIN

cDNA was produced from 57 samples taken at the colposcopy clinic. Presence of transcripts A to M shown in figure 7.2 was determined by PCR on the cDNA. The presence of transcripts L, M, and N was determined by production of a PCR product using the appropriate reverse primer together with E7 forward primer, but with the absence of PCR products using the same reverse primer with any of the E6 forward primers, thus indicating the likely transcription of mRNA from the P_{670} site. Figure 7.15 shows examples of the transcript patterns from 2 patients.

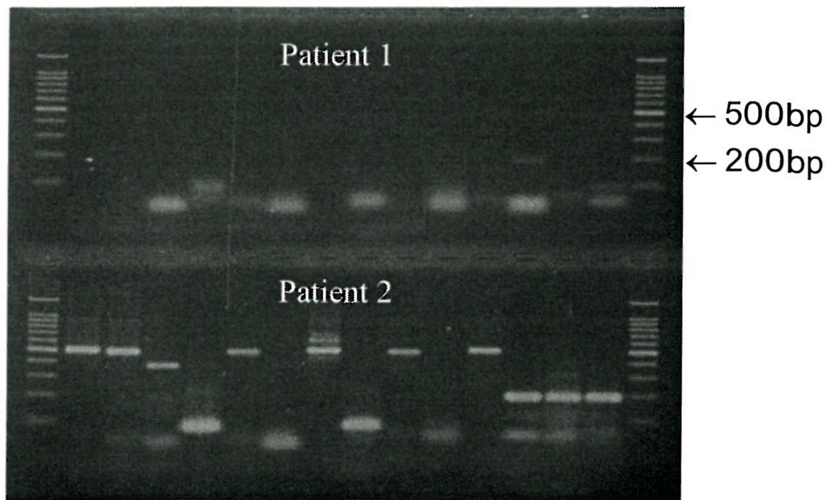


Figure 7.15. HPV-16 Polycistronic Transcripts Expressed by 2 Colposcopy Patients. Transcript patterns of species A to N as shown in figure 7.2 are shown for 2 patients attending the colposcopy clinic. Lanes 1 and 16 on both gels are 100 bp markers, 200 bp and 500 bp markers are highlighted. Transcripts A to N for patients are shown in lanes 2 to 15 respectively. Patient 1 was classified as CIN 2 and patient 2 as CIN 3.

Figure 7.15 illustrates the variability of HPV-16 transcripts in women referred for colposcopy. In patient 2 (CIN 3) all transcripts except F and J are readily detected. By contrast, in patient 1 also diagnosed as a high grade lesion (CIN 2) only transcripts D and L were detected.

The results of screening for the polycistronic transcripts in the 57 samples from patients attending the colposcopy clinic are shown in table 7.6.

Table 7.6. Percentage of Specimens Positive for HPV-16 Polycistronic Transcripts.

Transcript*		CIN 0/1 (n=17)	CIN 2/3 (n=40)	p-value (χ^2 Analysis)
A	E6, E7, E1^E4, E5	11.7	32.5	0.1
B	E6*I, E7, E1^E4, E5	0	32.5	**0.01**
C	E6*II, E7, E1^E4, E5	5.9	15	0.34
D	E6*III, E5	52.9	55	0.87
E	E6*I, E7, E2, E5	11.7	20	0.46
F	E6*II, E7, E2, E5	5.9	2.5	0.53
G	E6, E7, E2, E5	0	17.5	0.07
H	E6*IV, E2, E5	22.2 (n=9)	62.5 (n=24)	**0.04**
I	E6*I, E7, E1C, E2, E5	0	7.5	0.25
J	E6*II, E7, E1C, E2, E5	0	2.5	0.51
K	E6, E7, E1C, E2, E5	0	17.5	0.07
L	E1^E4, E5	31.3 (n=16)	52 (n=25)	0.19
M	E2, E5	18.8 (n=16)	32.3 (n=31)	0.33
N	E1C, E2, E5	11.7	24.2 (n=33)	0.3

* See figure 7.2.

The statistical significance between the CIN 0/1 and the CIN 2/3 groups was calculated using χ^2 analysis. The corresponding p-values are shown. Values in bold indicate a statistically significant result. The number of samples in each group (n) is shown.

Table 7.6 shows that overall, a smaller range of transcripts are produced in CIN 0/1 compared to CIN 2/3. Transcript B, potentially coding for E6*I, E7, E4, and E5 showed a significantly higher incidence of detection in CIN 2/3 than in CIN 0/1. In CIN 0/1 transcript B is not expressed, hence the only transcript coding for E6*I is transcript E, whereas in 32.5% of CIN 2/3 transcript B is the major transcript for E6*I.

Transcripts H also showed a significantly higher incidence in CIN 2/3 (62.5%) compared to CIN 0/1 (22.2%). This transcript potentially codes for E2 and E5 proteins.

Table 7.7 shows the range of transcripts detected in 11 selected patients presenting with different grades of CIN; all patients had similar GAPDH transcript levels. Viral loads are shown normalised to number of viral genomes per cell.

Table 7.7. HPV-16 Transcripts Detected in Selected Patients with Different Grades of CIN.

CIN Grade	Viral Load / per cell	Transcript																			
		E6	E7	E6*I	E6*II	E1	L1	A	B	C	D	E	F	G	H	I	J	K	L	M	N
3	670	3.9	3.3	2.7	2.1	3.5	2.5	+	+	+	+	+	+	+	+	+	-	+			
3	1,900	2.6	2.3	-	-	-	2.2	-	-	-	±	-	-	-		-	-	-	+	-	-
3	260	2	2.3	1.2	-	-	-	+	±	-	+	-	-	-		-	-	-		+	-
2	1,200	3.7	0.8	2.9	1.2	3.6	3.8	+	+	+	+	±	-	+	++	-	-	+			
2	530	2.8	2.1	-	-	-	3.2	-	-	-	+	-	-	-	+	-	-	-	±	±	±
2	810	1.7	2.6	-	-	-	2.4	-	-	-	-	-	-	-		-	-	-	+	-	+
1	3,300	3	3.2	-	-	1.5	3	-	-	-	+	-	-	-		-	-	-	-	-	-
1	10	2.6	2	1.1	-	-	2.4	-	-	-	+	+	-	-	-	-	-	-	-	-	-
1	70	1.4	2	1.1	-	-	1.6	-	-	-	±	-	-	-		-	-	-	+	+	-
0	14,000	-	-	-	-	-	0.3	-	-	-	-	-	-	-	+	-	-	-	-	-	-
0	0.2	-	-	-	-	-	-	-	-	-	+	-	-	-		-	-	-	±	+	+

Results show the presence of transcripts detected by fluorogenic probe assay or PCR detecting transcripts A-N (see figure 7.2). Grade of CIN is that reported at colposcopy. For E6, E7, E6*, E1, and L1 transcripts the value given is the logarithm of the number of transcript detected by fluorogenic probe assay. Presence of transcripts A-N is high (++), moderate (+), low (±), or not detected (-).

The results of the fluorogenic probe assays shown in table 7.7 indicate there are overlapping ranges of expression levels of E6, E7, and L1 transcripts in women referred to colposcopy with cervical smear grades of CIN 1, 2, and 3. The number of viral genome copies per cell also have a similar range in the different grades of CIN.

Table 7.7 also highlights the importance of assay sensitivity when comparing patterns of transcripts detected. For four of the nine samples full-length E6 transcript levels were recorded by fluorogenic probe assay, but no corresponding polycistronic mRNA was detected on agarose gel, i.e. transcripts A, G, or K. Hence the E6 fluorogenic probe assay, sensitive to 10 starting copies, detected more positive patients. In one patient transcripts L, M, and N were detected on agarose gel, but the E7 fluorogenic probe assay, sensitive only to 100 starting copies, did not detect these transcripts.

7.3.4 Conclusions

Although no consistent differences could be found in the expression levels of HPV-16 E1, E6, E7, and L1 transcripts, there were significantly more samples in CIN 2/3 expressing E6, E6*I, E6*II, and E7 transcripts than in the CIN 0/1 group. When related to which polycistronic transcripts could be encoding these transcripts, only transcript B potentially encoding E6*I, E7, E1^E4, E5 and transcript H potentially encoding E6*IV, E2, E5 were present in a significantly higher proportion of CIN 2/3 samples compared to CIN 0/1 samples.

The production of transcripts A to N was highly variable although there was a trend towards high grade CIN samples expressing a wider range of transcripts than low grade lesions. However, high grade lesions expressing very few transcripts as well as low grade lesions expressing many different transcripts were also found. Transcript D potentially encoding E6*III and E5 was the most widespread transcript appearing in over 50% of both high and low grade lesions.

7.4 Analysis of Telomerase and Survivin Production in Cervical Intraepithelial Neoplasia

Although high risk HPV types were found more often in high grade CIN lesions, analysis of the viral load or transcripts produced by the most commonly detected high risk type, HPV-16, did not yield results which accurately predicted the severity of a

cervical lesion. Therefore it was decide to investigate abnormal production of factors which affect epithelial cell growth.

The purpose of this study was to determine the mRNA expression levels of the telomerase RNA component (hTR), telomerase reverse transcriptase (hTERT), and survivin in samples from different grades of CIN and whether this analysis could be used to predict the clinical outcome.

7.4.1 Methods

Calibration curves for the fluorogenic assays were produced for hTR, hTERT, survivin, and GAPDH using cDNA from SiHa cells; PCR reactions were performed with fluorogenic probe assay reagents and cycling conditions, but dTTP in place of dUTP and without the probe. The products were purified using DNA purification kit II (Hybaid) according to manufacturer's instructions and quantified using OD_{260} value. These products were diluted to give known amounts of starting copies. The calibration curve was produced by plotting the logarithm of the starting copy number against the cycle number at which the fluorescent signal reached a threshold set at a level equivalent to 10 standard deviations of the average baseline level of the signal.

To determine any role of hTR, hTERT, and survivin in cervical intraepithelial neoplasia (CIN), the levels of expression of hTR, hTERT, and survivin were performed using fluorogenic probe assay on cDNA from specimens with different grades of CIN collected from patients attending a colposcopy clinic or a health centre.

7.4.2 Preliminary Studies

Purified PCR products for hTR, hTERT, survivin, and GAPDH were used to produce different standards with defined starting copy numbers. Dilution series enabled calibration curves to be made for the assays as shown in figure 7.16. These graphs were used to determine the levels of the corresponding transcripts in specimens.

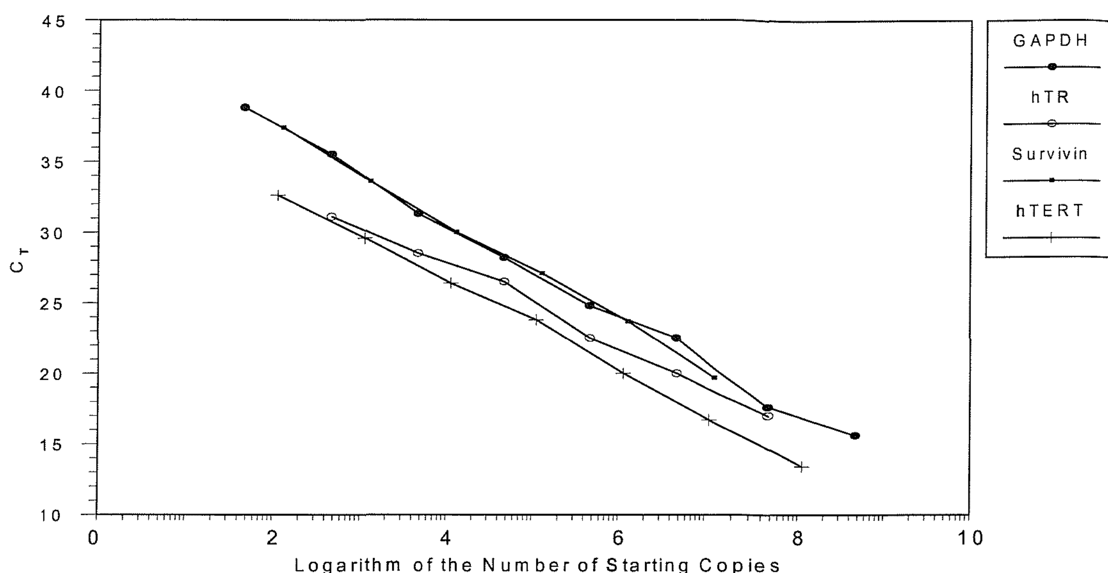


Figure 7.16. Standard Curve for Initial Starting Copy Number for Cellular Factor Fluorogenic Probe Assays. The graphs represent the change in the calculated C_T value with variation in the initial starting copy number for each of the assays. The graphs show linearity down to a level below 1000 starting copies for all assays.

7.4.2.1 Detection of hTR, hTERT, and Survivin in Cervical Cancer Cell Lines and Cervical Tumours

The cervical cell lines SiHa, CaSki, and HeLa and cells from 2 cervical tumours were tested for the presence and quantity of transcripts for hTR, hTERT, survivin, and the housekeeping gene GAPDH. The results are shown in table 7.8.

Table 7.8. Quantification of hTR, hTERT, Survivin, and GAPDH Transcripts in Cervical Cancer Cell Lines and Cervical Tumours.

Cell Line	Number of Transcripts per Cell			
	hTR	hTERT	Survivin	GAPDH
CaSki	1	$6x10^{-5}$	$2x10^{-1}$	7
SiHa	2	$3x10^{-3}$	3	126
HeLa	50	$2x10^{-2}$	20	372
Fast Growing Tumour	4	$8x10^{-4}$	$5x10^{-1}$	18
Slow Growing Tumour	$6x10^{-2}$	-	$3x10^{-3}$	$7x10^{-1}$

The number of cells in the samples was determined by PicoGreen fluorescent dye and the transcripts were quantified using fluorogenic probe assay. The cell lines are derived from cervical cancers. The fast growing tumour was from a 34 year old patient and the slow growing tumour was found in a women of 78. The values shown are the mean of 3 replicate samples. Transcript abbreviations are telomerase RNA component (hTR) and telomerase reverse transcriptase (hTERT).

The number of GAPDH transcripts per cell was highly variable suggesting a relationship to cellular growth rates. We estimated that CaSki cells doubled in number after 36 hours, SiHa cells had a doubling time of 28 hours, whereas HeLa cells had a doubling time of around 10 hours. The inverse relationship between the number of GAPDH transcripts per cell and cell doubling time is shown in figure 7.17. This is paralleled in the cervical cancer samples with the highly malignant tumour having an average of 18 GAPDH transcripts per cell and the slow growing tumour only $7x10^{-1}$ copies.

If the malignancy-associated transcripts detected in the cell lines are normalised as a ratio of the GAPDH transcript level rather than absolute transcript levels per cell then differences in hTR, hTERT, and survivin expression are no longer evident, i.e. all hTERT transcript levels are 10^{-5} per GAPDH transcript and all survivin levels are 10^{-2} per GAPDH transcript for all 3 cell lines. Therefore using GAPDH transcript levels to normalise results can hide important differences between samples.

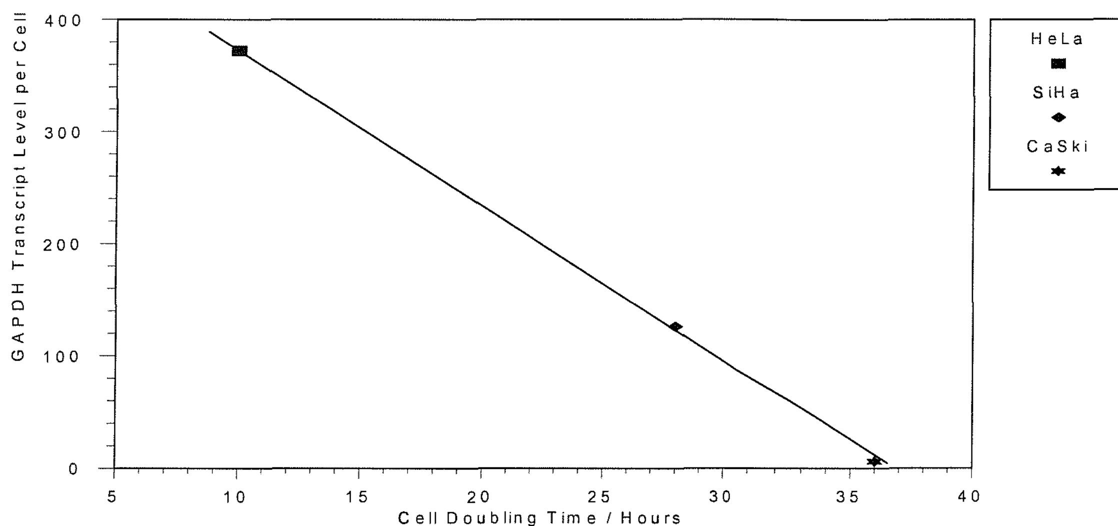


Figure 7.17. Relationship Between GAPDH Transcript Levels in Cervical Cancer Cell Lines and Cell Doubling Time. The doubling time of the slow growing cervical cancer cell lines was determined by counting the cell number in cell culture after 0, 24, 48, and 72 hours incubation. The number of GAPDH transcripts per cell was determined by fluorogenic probe assay. HeLa cells were confluent after 24 hours incubation from a 1 in 4 split, giving an estimated doubling time of around 10 hours.

Expression of high levels of hTR and survivin may play a key role in the rate of tumour growth, hence the rapidly growing HeLa cells have 50 fold more hTR and 100

fold more survivin transcripts per cell than the slow growing CaSki. The validity of this analysis for cervical cancer is suggested by the finding that a rapidly growing tumour from a young woman had 67 fold higher hTR and 167 fold higher survivin transcripts per cell than a slow growing tumour from an elderly patient. These differences disappear when transcripts are expressed as a ratio of GAPDH mRNA.

These findings suggest that presentation of telomerase and survivin transcript production per cell better reflects tumour malignancy than expressing the results as a ratio of GAPDH mRNA.

7.4.2.2 Detection of hTR, hTERT, and Survivin in White Blood Cells

In order to determine if the presence of inflammatory cells would confound the quantification of telomerase and survivin transcripts in cervical smear samples, the level of each of these transcripts was determined for leukocytes isolated from fresh blood of healthy volunteers. Whole blood (1 ml) was centrifuged at 1,000 x g for 5 minutes, the plasma was removed and 1 ml red blood cell lysis solution added (155 mM NH_4Cl, 10 mM $KHCO_3$, 100 mM Na_2EDTA, pH 7.2) and incubated at room temperature for 10 minutes. The cells were centrifuged once more, the supernatant removed and 0.5 ml Trizol added. mRNA was extracted and cDNA produced as for clinical samples (see section 2.8). Lymphocytes were also isolated from the blood samples using Lymphoprep (Nycomed); 1 ml whole blood was mixed with 1 ml PBS and carefully layered onto 3 ml Lymphoprep. The sample was then centrifuged at 800 x g for 20 minutes. Red blood cells and neutrophils pass through the solution and are pelleted at the bottom of the tube, whereas the lymphocytes collect at the interface of the Lymphoprep and the blood. The lymphocytes were collected, washed with PBS, and resuspended in 0.5 ml Trizol. mRNA was extracted and cDNA produced as for clinical samples (see section 2.8).

All 9 leukocyte and lymphocyte samples tested contained hTR transcripts as shown in figure 7.18. Mean level of hTR transcripts per 10^6 GAPDH transcripts in leukocytes and lymphocytes was 115,000. Survivin and hTERT transcripts were not detected in any of the samples.

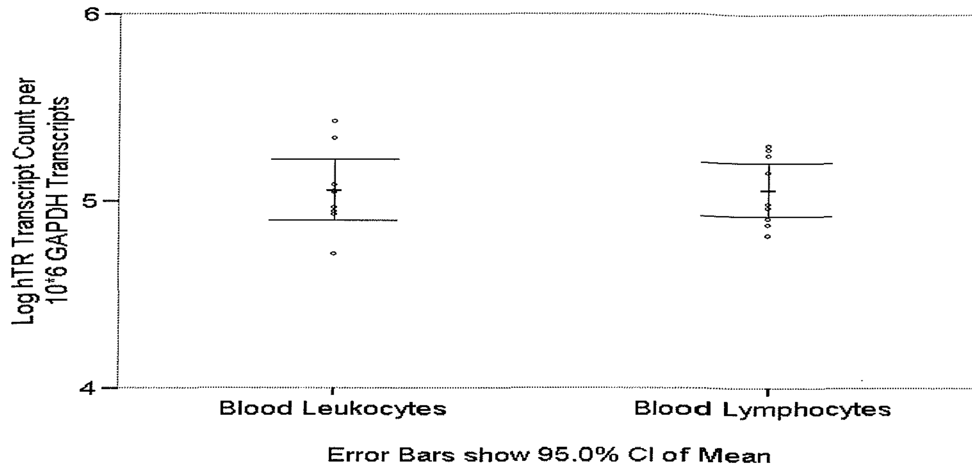


Figure 7.18. Transcription Level of hTR in Blood. Leukocytes and lymphocytes from 9 fresh blood samples were analysed for the number of transcripts of hTR. The results show the logarithm of the hTR transcript count normalised per 10^6 GAPDH transcripts. All 9 samples are shown, together with the mean and 95% confidence limits.

Figure 7.18 shows that lymphocytes and other leukocytes have similar levels of hTR when compared to GAPDH transcript levels. If inflammatory cells or whole blood contaminates the cervical specimens the hTR and GAPDH levels would be affected, but hTERT or survivin transcripts detected most likely originate from the cervical cells and not from contamination. For lymphocytes the mean transcript count per cell was 0.4 for GAPDH and 0.05 for hTR.

7.4.2.3 Conclusions

Contamination of cervical samples with inflammatory cells or menstrual blood could affect GAPDH or hTR levels detected in the cervical samples, but was unlikely to alter the levels of hTERT or survivin transcripts.

In the cervical cell lines CaSki, HeLa, and SiHa transcript levels of hTR and survivin were expressed at relatively high rates. The production of GAPDH transcripts also varied and appeared to be related to the growth rate of the cell. Hence standardisation of telomerase and survivin transcript levels to the GAPDH mRNA level did not show differences between cell lines or cervical cancers, which were evident when results were normalised to cell number.

7.4.3 Detection of hTR, hTERT, and Survivin in Patient Samples

Cervical scrapes from a total of 141 women were tested for the presence and quantity of transcripts coding for GAPDH, hTR, hTERT, and survivin. The samples included 25 Normal smears, 56 with CIN 0/1, and 60 with CIN 2/3. The frequency of occurrence of the transcripts are shown in table 7.9.

Table 7.9. Percentage Frequency of Cellular Transcripts.

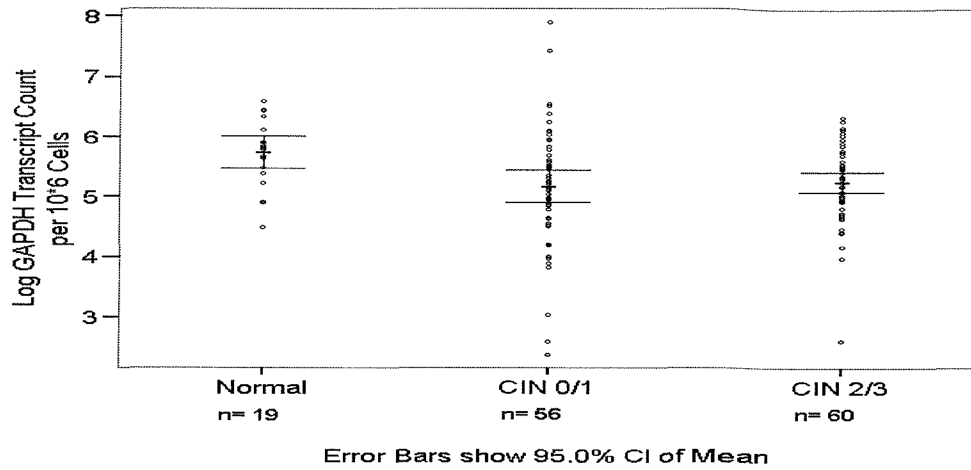
Histology	Transcript			
	GAPDH	hTR	hTERT	Survivin
Normal (n=25)	100	100	4	0
CIN 0/1 (n=56)	100	83.9^{+}	4	28.6*
CIN 2/3 (n=60)	100	96.7	0	33.3*

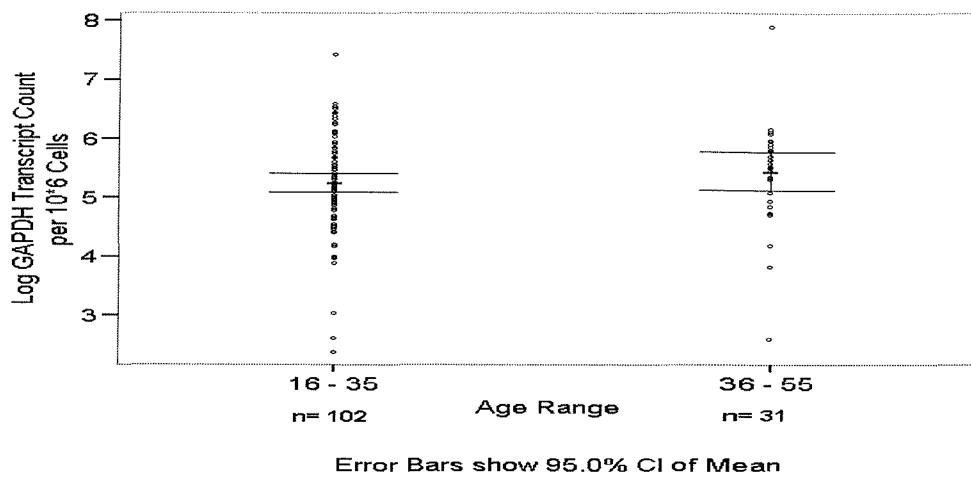
* p-value<0.003, $^{+}$ p-value 0.03 compared to Normals using χ^2 analysis. Transcript abbreviations are telomerase RNA component (hTR) and telomerase reverse transcriptase (hTERT).

Figure 7.19 shows the transcript counts for GAPDH against grade of CIN, age of the patient, and presence of high risk HPV. Results were normalised to the number of transcripts per 10^6 cells. There were higher mean levels of GAPDH in Normal samples compared to CIN samples (p-value 0.0004 using Student t-test analysis). However, there was considerable overlap in the range of values recorded.

Figure 7.20 shows the transcript counts for telomerase RNA component (hTR) against grade of CIN, age of the patient, and presence of high risk HPV type. Results were normalised to the number of transcripts per 10^6 cells. Using Student t-test analysis, there were significantly higher levels of hTR in Normal samples compared to CIN (p-value 0.008), in patients aged 36 to 55 (p-value 0.03), and in HPV negative samples (p-value 0.02).

Survivin was not found in any Normal samples, but occurred in 29% of samples with CIN 0/1 and 33% of samples with CIN 2/3. Figure 7.21 shows the number of survivin transcripts per 10^6 cells for different grades of CIN. No significant differences were found between high and low grade CIN using Student t-test analysis.





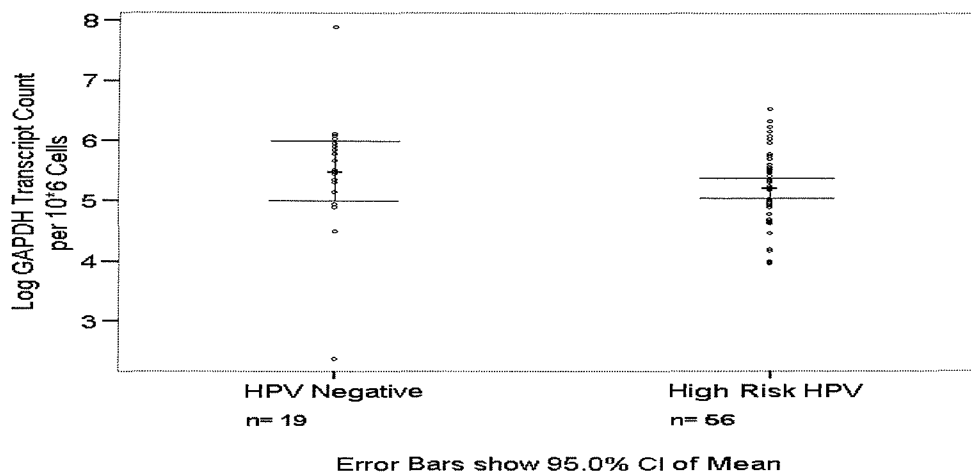
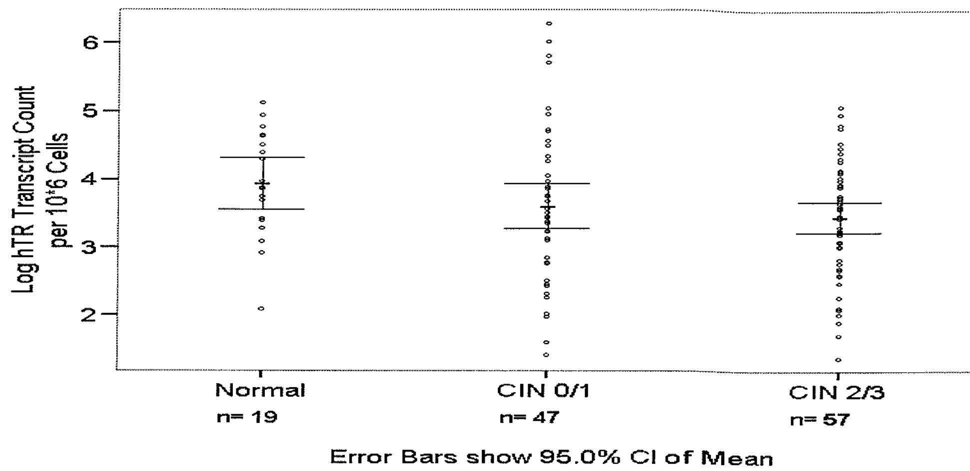
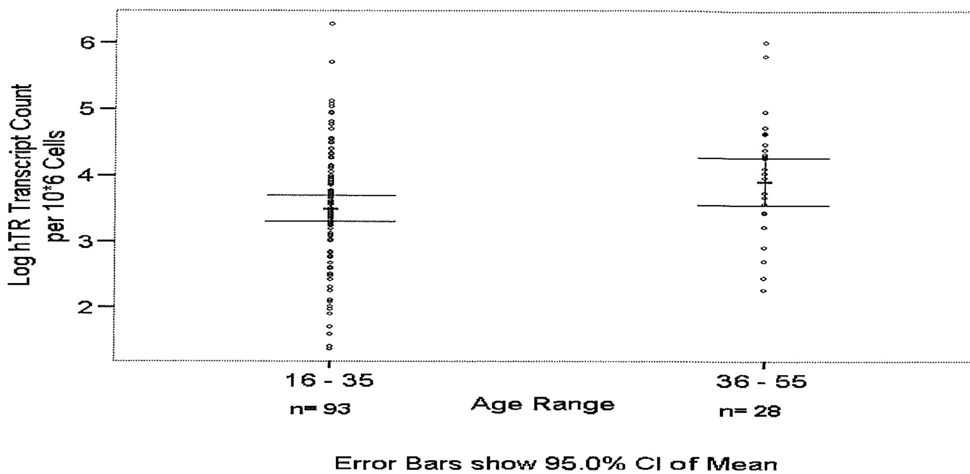


Figure 7.19. Expression Levels of GAPDH in Patient Samples. Graphs show the scatter of GAPDH transcript counts per 10^6 cells in different grades of CIN (top), age of the patient (middle), and presence or absence of HPV (bottom). The mean and 95% confidence limits are also shown. The number in each group (n) is indicated.





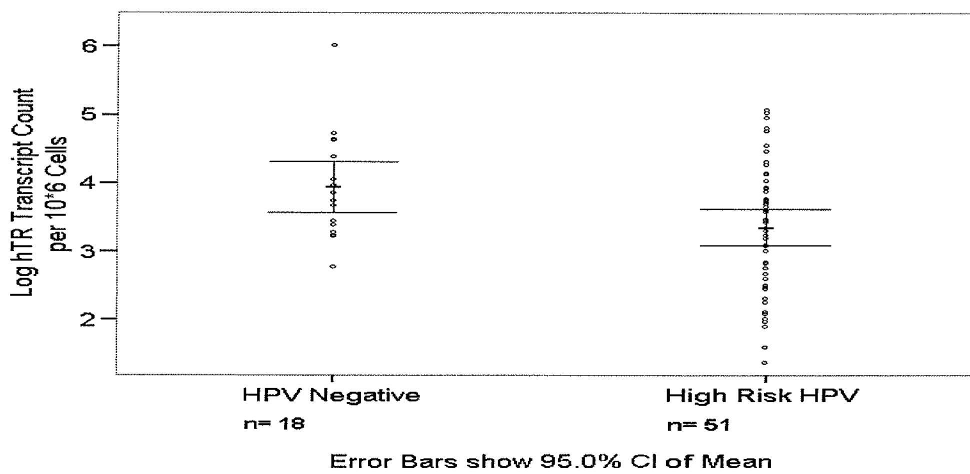


Figure 7.20. Expression Levels of hTR in Patient Samples. Graphs show the scatter of hTR transcript counts per 10^6 cells in different grades of CIN (top), age of the patient (middle), and presence or absence of HPV (bottom). The mean and 95% confidence limits are also shown. The number in each group (n) is indicated.

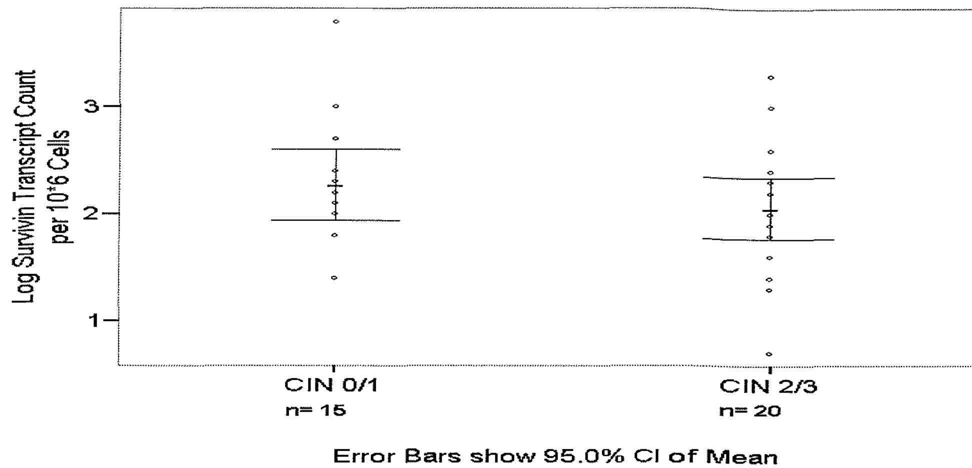


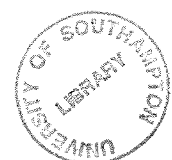
Figure 7.21. Expression Levels of Survivin in Different Grades of CIN. The scatter of the survivin transcript levels in different grades of CIN are shown with the mean and the 95% confidence limits. The number in each group (n) is also indicated.

7.4.4 Conclusions

GAPDH transcript levels were significantly higher in Normal patients compared to CIN patients. This is unlikely to be due to transportation conditions, since, all samples were transported in RNAlater which preserves mRNA even at ambient temperatures in the time delay of 4hrs involved in sample processing. Further, the CIN samples which showed lower values had shorter transportation times. No differences were found in the detection rates, and quantity, of transcripts for hTERT between Normal patients and patients with CIN 0/1 or CIN 2/3. However, transcripts coding for survivin were only found in 31% of patients with CIN and not in Normal controls, but there was no difference in the mean level of expression of survivin between CIN 0/1 and CIN 2/3. Levels of telomerase RNA component (hTR) apparently increased in older patients and HPV negative patients.

7.5 Overview

Tables 7.10 and 7.11 show the detection of transcripts potentially coding for survivin and the HPV-16 E6, E7, E1, E2, E4, E5, L1, and L2 proteins, according to the polycistronic transcripts detected. The findings are presented in relation to viral load per cell. Data are divided into low grade (CIN 0/1, table 7.10) and high grade (CIN 2/3, table 7.11) lesions. To establish if the detection of survivin mRNA correlated with the presence of any particular HPV-16 transcripts the tables are divided into survivin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mRNA positive and negative sections. Only specimens with viral loads of 10 per cell or above are shown as these were considered to have sufficient starting material for reliable results.

Unfortunately, insufficient number of samples were obtained for CIN 0/1 specimens in order to determine any significant differences with CIN 2/3 specimens or survivin positive or negative samples.

Some specimens had repeatedly different HPV-16 gene counts which may indicate integration of the genome into the host chromosome. However, most of these samples with different gene counts had detectable E2, E4, or E5 transcripts which would not be expected if the integration site was in the E1 or E2 genes. In addition, all the samples have very low viral load values and hence failure to detect similar gene copy number may be due to insufficient starting material.

No differences were found in the proportion of specimens expressing E5 or L2 transcripts with grade of CIN or survivin expression. E4 transcripts were significantly less common in CIN 0/1 samples than in CIN 2/3 (p-value 0.006 using χ^2 analysis). Similar levels of survivin transcripts are found in both high and low viral loads as well as between high and low grade CIN. In HPV-16 positive patients the proportion of samples expressing survivin was 47.1% in CIN 0/1 and 40.5% in CIN 2/3; these results were not significantly different to those obtained from all CIN 0/1 and CIN 2/3 specimens. Interestingly, the mean expression level of E6 transcripts was seven times lower in CIN 2/3 samples co-expressing survivin transcripts (p-value 0.04 using Student t-test analysis), whereas the mean expression levels of E7 (p-value 0.92) and L1 (p-value 0.11) were not different in these samples. Expression of survivin protein allows the cell to replicate, as does the binding of HPV-16 E6 protein to the cellular p53 protein. Co-expression of survivin may enable the virus to induce cellular replication with lower levels of E6 protein.

For all grades of CIN there was an increase in the proportion of samples expressing E2 transcripts in the absence of survivin (p-value 0.005 using χ^2 analysis) and in CIN 2/3 patients detection of transcripts for E1 protein was mostly in survivin negative samples although not at significant levels (p-value 0.08 using χ^2 analysis). HPV-16 E1 protein initiates viral DNA replication and E2 protein controls viral DNA replication and transcription. Presence of both E1 and E2 transcripts may indicate viral

DNA replication is possible and hence this is only likely to occur in survivin negative samples.

No obvious patterns are present which could be used to predict the outcome of HPV infection, although only one CIN 2/3 sample did not express viral or cellular transcripts (specimen 27 in table 7.11). Therefore the potential cause of CIN cannot be accounted for in this sample.

Table 7.10. HPV-16 Positive Samples from Colposcopy Patients with CIN 0/1.

Specimen	Viral	Transcripts Potentially Coding for:								
	Load	Survivin	E6	E7	E1	E2	E4	E5	L1	L2
1	3,300	0.8	3	3.2	1.5	-	-	+	3	+
2	260	2.2	-	-	-	-	+	+	-	+
3	100	1.8	-	-	-	-	-	+	-	-
4	70	2	1.4	2	-	+	+	+	1.6	-
5	10	1.8	2.6	2	-	+	-	+	2.4	+
6	14,000	-	-	-	-	+	-	+	0.3	-
7	3,600	-	1.3	-	-	-	+	+	2.5	+
8	560	-	2.9	-	-	+	+	+	-	+
9	50	-	-	-	-	+	-	+	-	-
10	40	-	-	-	-	+	-	+	1.8	+

Viral load is per cell. Transcripts are shown as absent (-), present (+), or the logarithm of the number of starting copies per sample. Specimens with viral load below 10 per cell are not shown.

Table 7.11. HPV-16 Positive Samples from Colposcopy Patients with CIN 2/3.

Specimen	Viral Load	Transcripts Potentially Coding for:								
		Survivin	E6	E7	E1	E2	E4	E5	L1	L2
1	2,600	1.4	1.5	1.2	-	+	+	+	-	-
2	1,900	2.2	2.6	2.3	-	-	+	+	2.2	+
3	330	1.4	-	-	-	-	+	+	-	+
4	260	2.6	2	2.3	-	+	+	+	-	+
5	190	2.4	3.5	3	2.2	+	+	+	3.1	+
6	100	2.2	2.4	2.3	-	+	+	+	2.1	+
7	70	3.3	1.3	1.7	-	-	+	+	1.3	+
8	60	2.4	1	-	-	+	+	+	-	+
9	20	1.6	-	-	-	+	+	+	-	-
10	10	2.4	1.9	1.9	-	-	+	+	1	-
11	10	1.9	1.1	-	-	-	-	-	1.6	-
12	10	1.6	1.6	1.4	-	-	+	+	0.5	-
13	27,500	-	4.5	4.1	3.3	+	+	+	4.4	+
14	1,200	-	3.7	0.8	3.6	+	+	+	3.8	+
15	810	-	1.7	2.6	-	+	+	+	2.4	+
16	680	-	3.9	3.3	3.5	+	+	+	2.5	+
17	590	-	3.5	1.7	1.9	+	+	+	3.5	+
18	520	-	2.8	2.1	-	+	+	+	3.2	+
19	500	-	-	0.9	-	+	+	+	-	-
20	150	-	3.5	2.4	2.4	+	+	+	3.6	+
21	100	-	3.8	3.2	3.9	+	+	+	3.2	+
22	50	-	1.9	1.5	-	+	-	+	1.7	+
23	50	-	2.3	1.6	-	+	+	+	1.4	+
24	40	-	-	2	-	+	+	+	1.1	-
25	30	-	2.7	-	3	+	+	+	1.6	+
26	30	-	0.6	1.1	-	-	-	-	-	-
27	10	-	-	-	-	-	-	-	-	-
28	10	-	1.6	1.7	-	+	+	+	-	-
29	10	-	2.5	1.9	-	+	-	+	1	+
30	10	-	2	-	-	+	+	+	1.5	+
31	10	-	-	-	-	+	+	+	-	-

Viral load is per cell. Transcripts are shown as absent (-), present (+), or the logarithm of the number of starting copies per sample. Specimens with viral load below 10 per cell are not shown.

7.6 Final Conclusions

In patients with high grade cervical lesions (CIN 2/3) there was a larger proportion of samples expressing transcripts for HPV-16 E6, E6*I, E6*II, and E7 compared to patients with low grade lesions (CIN 0/1). This may be an indication of loss of control of viral transcription.

There was a trend for high grade lesions to express a larger range of polycistronic mRNA than low grade lesions, however, there was overlap where patients with CIN 1 expressed a wide range of transcripts and patients with CIN 2/3 expressed very few transcripts.

Survivin transcript expression was not found in samples from Normal control patients. Although CIN specimens expressed survivin this only accounted for 30% of both CIN 0/1 and CIN 2/3 samples. The presence of survivin also reduced the proportion of samples expressing HPV-16 E1 and E2 transcripts. However, in CIN 2/3 samples where survivin expression was found there was a reduction in the expression level of HPV-16 E6 transcripts, but the proportion of samples expressing E6 transcripts was the same and transcript levels of HPV-16 E7 and L1 were similar in samples expressing or not expressing survivin. It is possible lower levels of HPV-16 E6 protein are required in survivin positive samples in order for the virus to induce cellular replication.

Chapter 8

Susceptibility to Cervical Intraepithelial Neoplasia of *p53* Polymorphisms and Haplotypes

8.1 Introduction

The p53 gene is comprised of 11 exons (see figure 8.1) coding for a protein of 393 amino acids which primarily functions as a transcriptional transactivator with sequence specific DNA binding activity. The N-terminal domain, residues 1-42, functions as the transcriptional transactivator binding to members of the general transcription initiation complex. Next, a proline rich region (residues 64-91) which has been implicated in non-transcriptional control of apoptosis. Residues 102-292 comprise a central region with specific DNA binding activity; most tumour derived mutations occur in this area. A region required for oligomerisation of p53 extends from residues 324-355. The C-terminal tail (residues 368-393) binds single-stranded DNA and regulates specific DNA binding by the core region (Levine, 1997). The p53 protein plays an essential role in normal cellular growth; at least 50% of human cancers possess p53 mutations; this being the most commonly found genetic change in malignant cells (Hollstein *et al.*, 1994).

Polymorphisms are naturally occurring variations in the nucleotide sequence of DNA arising through non-lethal mutations during evolution. At least 10 polymorphisms have been found in the wild-type p53 gene; five in the coding region and five in the non-coding region. These include a 16 bp insertion in intron 3, a 5 base difference in sequence over an 8 bp span revealed by a G→A substitution in intron 6, and a G→C substitution resulting in replacement of arginine by proline at residue 72 of the p53 protein.

There is a higher incidence of the 16 bp insertion in intron 3 of Caucasian women with breast cancer (Weston *et al.*, 1997) and the intron 6 A form is reported to be more prevalent in patients with ovarian cancer (Mavridou *et al.*, 1998). At present, no specific genetic traits have been identified which account for the familial clustering of cervical tumours (Magnusson, Sparén, and Gyllensten, 1999). The significance of polymorphisms in *p53* introns is worthy of examination. Polymorphisms in exons which alter the amino acid sequence of this protein may make individuals more susceptible to

malignancies. Interestingly, the arginine polymorphism at codon 72 increases susceptibility of p53 to oncogenic HPV E6 protein degradation relative to the proline form (Storey *et al.*, 1998). The E6 proteins of all high risk HPVs degrade the tumour suppressor protein, p53, and may play a key role in oncogenesis since, unlike the many tumours where p53 is mutated, in early cervical cancer p53 is usually wild-type (Crook *et al.*, 1992).

No studies so far have evaluated the significance of *p53* intron and exon polymorphisms in cervical intraepithelial neoplasia, although Storey's group suggested arginine at the exon 4 codon 72 position may predispose women to cervical cancer.

8.2 Methods

This study was undertaken in collaboration with Dr. Robert Gornall, Department of Obstetrics and Gynaecology, Princess Anne Hospital, Southampton. The control population DNA was extracted from blood leukocytes of 254 randomly chosen women attending gynaecology clinics for non-malignant disorders. Control women were resident within the same area as the patients attending the colposcopy clinic. The DNA was extracted from 10 ml blood by lysis of the cellular fraction with an equal volume of ice cold 320 mM Sucrose, 5 mM $MgCl_2$, 1% Triton X100, pH 7.5 then centrifugation at 10,000 x g for 10 minutes at 4 °C to pellet the nuclei. The nuclei were lysed with 5 ml 75 nM NaCl, 25 mM EDTA, 1% SDS, pH 8.3, 50 µl 40 mg/ml proteinase K added then incubated overnight at 55 °C. Purification of the DNA was achieved with the addition of 1/3 volume of saturated NaCl and an equal volume of chloroform. After mixing the sample was centrifuged at 10,000 x g for 10 minutes at 4 °C, the aqueous layer removed to a new tube and the DNA precipitated with the addition of an equal volume of isopropanol. The DNA was pelleted by centrifugation at 15,000 x g for 20 minutes, followed by a wash with 5 ml 75% ethanol. Any remaining ethanol was removed after centrifugation by vacuum drying, and the DNA resuspended in 700 µl 10 mM Tris, 1 mM EDTA, pH 8.0. For all PCR reactions, 50-100 ng DNA was used.

In addition, the DNA was extracted from archival paraffin histology specimens from women with cervical cancer using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. For all PCR reactions, 50-100 ng DNA was used.

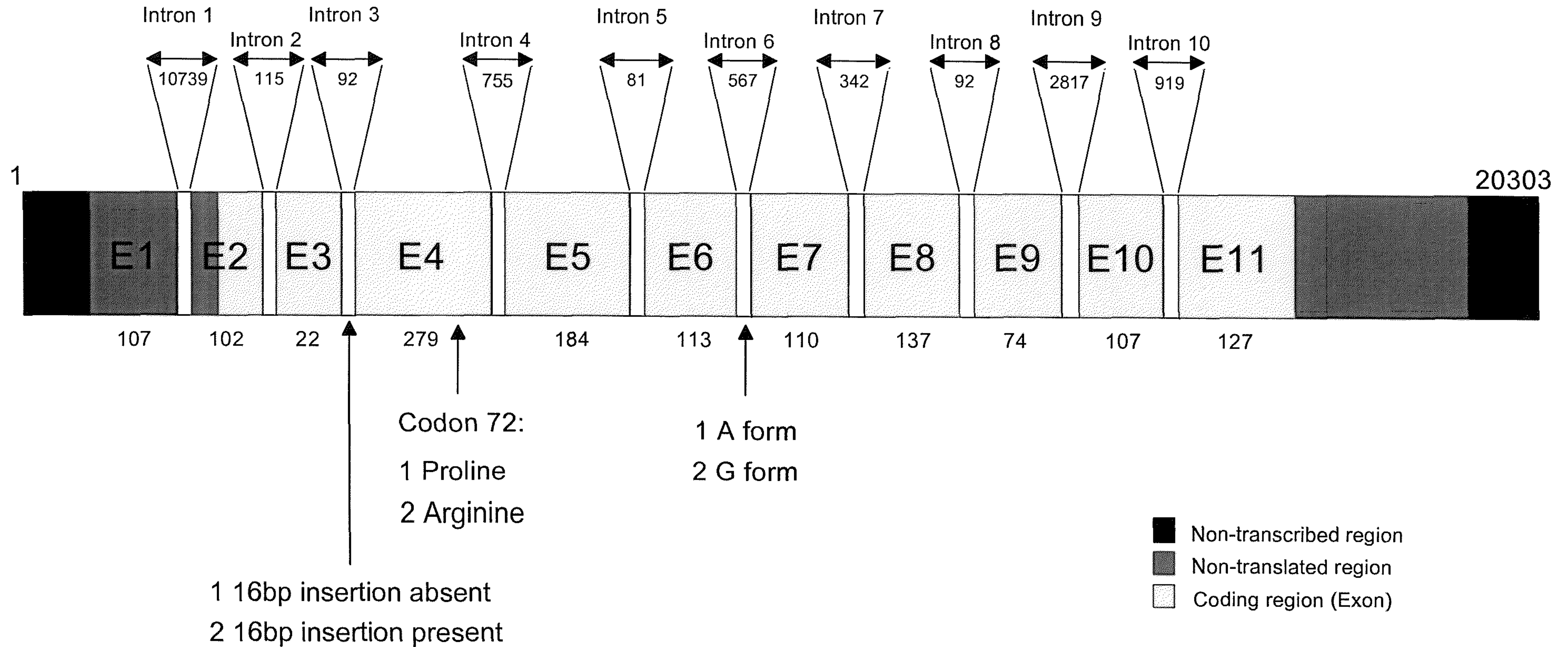

Figure 8.1. p53 Gene Showing Position of Polymorphisms Investigated. The size in base-pairs of introns and exons are indicated, as well as the allele designation for the 3 polymorphisms as used by Weston *et al.* (1997) for haplotype description.

For PCR analysis of all polymorphisms the reaction mix contained PCR buffer (Promega), 1.5 mM $MgCl_2$, deoxynucleotide triphosphates (Promega), each at 300 μM, appropriate primers at 1 mM, and 1.5 units of *Taq* polymerase (Promega). The PCR was performed on a MJ Research PTC-225 machine using an initial denaturing step of 94 °C for 5 minutes, followed by 40 cycles of denaturing at 94 °C for 30 seconds, annealing at 50 °C for 50 seconds, followed by extension at 72 °C for 20 seconds. The primer sets used were:

Exon 4 polymorphism (Buller *et al.*, 1997):

Forward primer: $^{nt\ 12111}$ 5' GCTCCCAGAATGCCAGAGGCT 3' $^{nt\ 12131}$, Reverse primer: $^{nt\ 12344}$ 5' CAGGCATTGAAGTCTCATGG 3' $^{nt\ 12325}$. Following the PCR, 10 μl of the PCR product was digested with 2 units *Bst* U1 (New England Biolabs) for 2 hours at 60 °C. The 234 bp product corresponded to proline at codon 72, and the 205 bp product indicated arginine at position 72 of the protein.

Intron 3 16 bp duplication polymorphism:

Forward primer: $^{nt\ 11875}$ 5' ATGGGACTGACTTTCTGCTCTT 3' $^{nt\ 11896}$, Reverse primer: $^{nt\ 12126}$ 5' CTGGCATTCTGGGAGCTTCA 3' $^{nt\ 12107}$. The duplication increased the PCR product size from 252 bp to 268 bp.

Intron 6 polymorphism:

Forward primer: $^{nt\ 13451}$ 5' GTCTCTGGGAGGAGGGGTTA 3' $^{nt\ 13470}$, Reverse primer: $^{nt\ 13549}$ 5' TGGGGTTATAGGGAGGTCAA 3' $^{nt\ 13530}$. Following the PCR, 10 μl of the PCR product was digested with 5 units *Msp* 1 (Promega) for 2 hours at 37 °C. The undigested product of 99 bp indicated no polymorphism, digestion products of around 60 bp and 40 bp showed an A→G substitution indicating the presence of the polymorphism.

All numbering is based on Genebank accession number U94788. All reaction products were detected on 3% SeaKem agarose gel (Flowgen) stained with ethidium bromide.

8.3 Results

The frequency of *p53* polymorphisms in the control population were tested against the population of women with cervical abnormalities graded according to the histopathology result of the sample. Although the frequency of each allele is reported to change with latitude, the control population had similar allele frequencies to other

studies based on Caucasians in New York (Weston *et al.*, 1997), Finns (Själander *et al.*, 1995), and Swedes (Birgander *et al.*, 1995), see table 8.1. The findings from the Southampton patients are shown in table 8.2.

Table 8.1. Percentage Frequency of *p53* Polymorphisms in Control Populations

Polymorphism		This Study	New York Caucasians*	Finns†	Swedes‡
Exon 4	Proline	6	2.6	5.2	9.6
	Arginine	56.8	61.5	56.6	52.8
	Arg/Pro	37.2	35.9	38.2	37.6
Intron 3	Absent	75	79.5	79.2	73.8
	Present Homo	1.6	0.9	1.1	2.8
	Present Heter	23.4	19.7	19.6	23.4
Intron 6	A Form	1.6	0	1.2	3.2
	G Form	81.9	81.2	80.3	74.5
	A/G	16.5	18.8	18.5	22.3

References for study population are: *Weston *et al.*, 1997, †Själander *et al.*, 1995, and ‡Birgander *et al.*, 1995.

The results shown in table 8.2 were tested using χ^2 test. Only the intron 6 polymorphism showed a significant result; presence of the G form was less prevalent in women with cervical abnormalities compared to Normal controls (p-value 0.008), and specimens with the A form of the polymorphism occurred more in samples with abnormalities than controls (p-value 0.002). The proportion of specimens with heterozygous presence of the polymorphism was similar in women with cervical abnormalities and the control group. Thus, homozygous presence of the G form of the intron 6 polymorphism may confer some resistance to cervical abnormalities. In the patients tested there was no evidence of the codon 72 polymorphisms alone influencing the development of cervical intraepithelial neoplasia. Similar findings for these polymorphisms have recently been published by many groups including Rosenthal *et al.* (1998) on a larger number of samples including those initially tested by Storey *et al.* (1998), and on patients with CIN by Hayes *et al.* (1998).

Table 8.2. Variation of *p53* Polymorphisms with Grade of Histopathology.

Polymorphism		Controls		CIN 0		CIN 1		CIN 2		CIN 3		Cancer		All CIN/Cancer	
Exon 4	Proline	15	(6.0)	4	(7.3)	10	(10.8)	6	(8.0)	3	(3.1)	3	(7.9)	26	(7.3)
	Arginine	142	(56.8)	24	(43.6)	44	(47.3)	39	(52.0)	48	(49.5)	20	(52.6)	175	(48.9)
	Arg/Pro	93	(37.2)	27	(49.1)	39	(41.9)	30	(40.0)	46	(47.4)	15	(39.5)	157	(43.9)
Intron 3	Absent	189	(75.0)	38	(77.6)	68	(76.4)	53	(75.7)	66	(68.0)	28	(80.0)	253	(74.4)
	Present Homo	4	(1.6)	1	(2.0)	3	(3.4)	1	(1.4)	3	(3.1)	0		8	(2.4)
	Present Heter	59	(23.4)	10	(20.4)	18	(20.2)	16	(22.9)	28	(28.9)	7	(20.0)	79	(23.2)
Intron 6	A Form	4	(1.6)	3	(6.0)	8	(8.9)*	6	(8.2)*	5	(5.1)	2	(5.9)	24	(6.9)*
	G Form	208	(81.9)	38	(76.0)	66	(73.3)*	50	(68.5)*	69	(69.7)*	28	(82.4)	251	(72.5)*
	A/G	42	(16.5)	9	(18.0)	16	(17.8)	17	(23.3)	25	(25.3)	4	(11.8)	71	(20.5)

The number of specimens is shown together with the percentage value in brackets. Controls refers to random females, taken from the same geographic region as those patients attending colposcopy, but with no history of cervical abnormalities.

* p-value < 0.014

The polymorphisms were compared with the HPV risk type detected in the sample (table 8.3). No correlation was found between *p53* polymorphisms and HPV infection of any risk type. Only 7.3% of women referred for colposcopy were HPV negative, and of these only one patient had biopsy confirmed CIN 2. A much larger population would be required to establish if *p53* polymorphisms are implicated in HPV negative cervical cancer.

Table 8.3. Variation of *p53* Polymorphisms with HPV Risk-Type Present.

Polymorphism		HPV Negative		Low Risk		Moderate		High Risk		HPV Positive	
Exon 4	Proline	3	(12.0)	0		10	(13.9)	9	(4.7)	19	(6.4)
	Arginine	13	(52.0)	18	(60.0)	33	(45.8)	93	(48.2)	144	(48.8)
	Arg/Pro	9	(36.0)	12	(40.0)	29	(40.3)	91	(47.2)	132	(44.7)
Intron 3	Absent	21	(91.3)	20	(76.9)	47	(70.1)	140	(72.5)	207	(72.4)*
	Present Homo	1	(4.3)	0		3	(4.5)	3	(1.6)	6	(2.1)
	Present Heter	1	(4.3)	6	(23.1)	17	(25.4)	50	(25.9)	73	(25.5)*
Intron 6	A Form	1	(4.2)	3	(11.5)	11	(15.9)	7	(3.6)	21	(7.3)
	G Form	21	(87.5)	20	(76.9)	45	(65.2)	139	(71.6)	204	(70.6)
	A/G	2	(8.3)	3	(11.5)	13	(18.8)	48	(24.7)	64	(22.1)

The figure in brackets is the percentage of specimens.

* p-value < 0.05 compared to Normal controls (see table 8.2).

Pair-wise haplotype frequencies were determined using the methods of Hill (1974) by Dr. Andrew Collins, Wessex Clinical Genetics Service, Princess Anne Hospital, Southampton, the results of which are shown in table 8.4. For codon 72 proline and A form of the intron 6 polymorphism there was a significant increase in the number of patients with all grades of CIN compared to the control group. Also, there was a significant decrease in the number of patients with CIN 1 and CIN 2 compared with the control group for homozygous presence of the intron 3 insertion and G form of

the intron 6 polymorphism. Although the cancer specimens did not fit this pattern, this may be due to the low number of specimens tested.

Table 8.4. Estimated *p53* Pair-Wise Haplotype Frequencies.

	1-1	1-2	2-1	2-2
Codon 72 - Intron 3				
Controls	0.125	0.121	0.731	0.024
CIN 1	0.197*	0.118	0.668	0.017
CIN 2	0.157	0.121	0.714	0.007
CIN 3	0.109	0.157	0.71	0.024
Cancer	0.207	0.069	0.724	0
Codon 72 - Intron 6				
Controls	0.089	0.157	0.021	0.733
CIN 1	0.145*	0.169	0.023	0.663
CIN 2	0.157*	0.121	0.014	0.707
CIN 3	0.154*	0.122	0.016	0.718
Cancer	0.017	0.259	0.069*	0.657
Intron 3 - Intron 6				
Controls	0.03	0.826	0.08	0.065
CIN 1	0.057	0.808	0.112	0.023*
CIN 2	0.05	0.821	0.121	0.007*
CIN 3	0.033	0.786	0.137*	0.044
Cancer	0.069	0.861	0.017	0.052

For each locus the alleles are designated 1 for proline and 2 for arginine at codon 72, 1 for deletion and 2 for insertion in intron 3, 1 for A form in intron 6 and 2 for G form.

*p-value<0.04 compared with controls.

The results of the estimated haplotype frequencies for significant combinations of all 3 polymorphisms are shown in table 8.5.

For the haplotypes containing all three polymorphisms, only the intron 6 polymorphism in combination with codon 72 proline and presence of the intron 3

polymorphism showed consistent results; the A form of the intron 6 polymorphism had a significantly higher incidence in patients with CIN, whereas G form of the polymorphism was significantly less common in patients with CIN, compared to controls.

Table 8.5. Estimated *p53* Haplotype Frequencies.

Study Group	1-1-2	1-2-1	1-2-2	2-1-1
Controls	0.109	0.073	0.048	0.014
CIN 1	0.164	0.112	0.006*	0.023
CIN 2	0.122	0.121	0*	0.015
CIN 3	0.093	0.137*	0.02	0.017
All CIN	0.127	0.124*	0.009*	0.019
Cancer	0.204*	0.017	0.052	0.067*

For each locus the alleles are designated 1 for proline and 2 for arginine at codon 72, 1 for deletion and 2 for insertion in intron 3, 1 for A form in intron 6 and 2 for G form. Order of alleles is codon 72, intron 3, intron 6. For clarity, only haplotypes containing significant results are shown.

*p-value<0.04 compared with controls.

Although the A form of the intron 6 polymorphism appears to offer some degree of susceptibility to CIN, it does not account for the majority of cases of CIN; only around 5% of cases of CIN or cervical cancer in the Southampton population .

Chapter 9

Discussion

World-wide, cervical cancer is the second most common cancer in women and accounts for 200,000 deaths per year. In the UK, annual deaths from cervical cancer fell from 2000 in the 1980s to 1369 in 1994 mainly due to the cervical cancer screening programme. However, it is estimated that between 5 and 30% of women with abnormalities are missed using the Pap smear test (Fricker, 1997); samples may not be suitable for examination, inadequate smears maybe reported as normal, or misinterpretation of positive smears due to human error. Women are only screened every 3 to 5 years in the UK, so any women with undetected cervical abnormalities could develop cancer in the interval between tests. In order to prevent women 'slipping through the screening net' there is considerable political pressure to improve smear testing. HPV detection is becoming more likely as an additional screen to the cervical smear to prevent cervical cancer.

Dyskaryotic cells from the cervix show varying degrees of differentiation. In general, the more differentiated the cell the milder the form of dyskaryosis. The World Health Organisation (Riotton and Christopherson, 1973) classifies mild dyskaryosis as superficial or intermediate cells, rarely parabasal in origin, with a uniform chromatin pattern. Severe dyskaryosis shows cells of mainly parabasal or small intermediate types with dense, uniform chromatin staining and no nucleoli. Moderate dyskaryosis shows cellular patterns between those of mild and severe.

This study used specimens collected at a colposcopy clinic from women referred with an abnormal smear test. In disparent cases the referral smear was confirmed by a senior cytologist and the colposcopy findings confirmed by biopsy taken at the time of the HPV specimen. The smear result correlated well with the colposcopy biopsy result. For patients with a referral smear of borderline nuclear abnormalities (BNA) 73.7% of samples subsequently had low grade lesions (CIN 0/1), similarly 70.8% of patients with a referral smear result of mild dyskaryosis had CIN 0/1. In contrast, 60.2% of patients with moderate dyskaryosis at referral had high grade lesions (CIN 2/3) at colposcopy and for patients with referral smears with severe dyskaryosis 90.7% of samples

subsequently had CIN 2/3. These results indicate the high predictive value of determining dyskaryosis using the current cervical screening methodology.

HPV Detection by PCR. Consensus PCR primer pairs are used for HPV detection in population surveys and studies on cervical cancer. The MY or GP consensus primers against the L1 gene used in the majority of studies were compared by Karlsen's group (1996) using biopsy specimens from cervical carcinomas; they found similar detection rates with both consensus primer pairs. However the 1997 study by Qu and colleagues found overall the MY primer set detected HPV in more samples than the GP primer set and more mixed infections than the GP primers. When individual HPV types were compared in clinical samples consisting of cervicovaginal lavages, the GP primer set detected HPV type 35 better than the MY primers, which in turn detected types -52, -53, -58, and -61 more often than the GP primer set. In dilution series using cloned DNA, the only differences in sensitivity of the primer sets were found with types -35 (GP primers were 3-logs more sensitive), -53, and -61 (both detected to 3-log greater dilution with MY primers). Therefore neither consensus primer set detects adequately all HPV types.

The aim of the work reported in this thesis was to combine consensus primer sets with specific primers for HPV-16 and 18 in order to define true HPV infection rates in a Southampton clinic population.

Using only the GP consensus primer set 18% of 62 control samples from women attending a GP cervical screening clinic were HPV positive, whereas using the panel of primer sets 42% of the same specimens contained HPV. In CIN 0 the HPV positivity rate rose from 61% using GP alone to 79% with the panel of primers. For CIN 1 the figures were increased from 74% to 90%, and for CIN 2 and CIN 3 using GP primers detected HPV in 92% of samples, but the panel of primer sets detected HPV in over 99% of samples (100% in CIN 3). In total, using the GP primers alone would have missed 57 samples of the 416 tested (13.7%) accounting for 16% of HPV positive samples detected. The addition of CP primers targeting the E1 gene detected an additional 6.5% of positive samples and the MY primers detected a further 5.6%, but these consensus primers still missed a further 4% of samples with HPV-16 (10 samples) and 18 (4 samples). Using the GP primer set Cromme's group (1993) detected HPV in 68% of CIN 1 samples, 91% of CIN 2, and 100% of samples with CIN 3, whereas Kalantari's group (1997) detected 69% HPV positive samples in CIN 0, 71% in CIN 1,

81% in CIN 2, and 84% in CIN 3 using MY primers. However, Kleter and colleagues (1998) used the SPF primer set containing 4 modified MY11 forward primers and 2 modified GP5+ reverse primers and detected 23% HPV positive samples in the control patients, 97% in CIN 1, and 99% in CIN 3. Hence, each primer set alone will detect the majority of infections; the SPF set appearing to be more sensitive than the GP or MY sets. However, all 3 primer sets detect the same L1 portion of the genome which may not be present in all cervical cancers, therefore using other consensus or specific primers targeting other parts of the HPV genome are likely to give maximum detection of HPV types.

The HPV types detected in the population studied here differed depending on the grade of CIN. A higher proportion of low risk HPV types were detected in Normal/CIN 0/1 (p-value 0.017), and most high risk types were detected in CIN 2/3 (p-value $3x10^{-17}$). These data fit with the designation of high and low risk HPV types. Interestingly, moderate risk types were detected in similar amounts from all grades of CIN (p-value 0.14 between high and low grade lesions), occurring more often than low risk HPV types and less often than high risk types. Again, this fits with the cancer risk designation of moderate. Infection with high risk HPV types was found to correlate with the severity and course of disease; 80% of patients with high risk HPV infection had high grade CIN or the lesion severity increased in the 3 to 4 month interval between the referral smear being taken and the patient being seen at the colposcopy clinic. By contrast, 60% of samples with moderate risk HPV types, and only 40% of patients with low risk HPV types having high grade CIN or progressing disease. One samples was found to have a high grade lesion (CIN 2) and was HPV negative. This sample failed to react with the GP, MY and CP consensus primer sets, the newly described SPF primers (Kleter *et al.*, 1998), and specific primers for HPV-16 and HPV-18. This may represents a non-HPV induced high grade lesion. If so this is only 0.5% of the high grade lesions tested. Alternatively this sample may contain an unknown HPV type which can only be detected with specific primers.

Prevalence of HPV. With increasing patient age there was a reduction in the incidence of high risk HPV infection. This could be due to the development of anti-HPV immunity over a number of years against high risk types that produce pathogenic lesions, but less effective against moderate and low risk types that do not cause such severe lesions. Across the age groups there were some differences in the CIN grades

detected with a greater proportion of CIN 3 cases in 26-35 age range and control cases in 46-55 age group. HPV type 16 was the most prevalent HPV type detected in specimens from all age groups studied here; 40% in age range 16 to 25, 44% in range 26 to 35, 26% in ages 36 to 45, and 21% in patients aged 46 to 55. This is in agreement with many studies undertaken throughout the world.

The incidence of mixed HPV infections was found to decrease with increasing age of the patient (p-value 0.0001) and increased with increasing grade of CIN (p-value 0.002). The differences with age may reflect the younger age groups being more likely to encounter different HPV types, and/or having less acquired immunity to HPVs; having not yet been exposed to many different types. Certainly, mixed HPV infections have been reported as associated with an increased risk of CIN (Becker *et al.*, 1994), and more prevalent in high grade squamous intraepithelial lesions (Syrjänen *et al.*, 1992). However, Kalantari's group (1997) reported similar amounts of mixed HPV infections with all grades of CIN and Chang and co-workers (1997) found a higher proportion of mixed HPV infections in low grade lesions. In Southampton, the finding of raised mixed HPV incidence with increasing severity of histopathology implies multiple types cause more severe lesions either due to synergy between the two types producing more severe infections, or interference leading to abnormal transcription and loss of regulation and hence abnormal infection.

A commercially available HPV hybrid capture detection kit divides HPV types into 2 groups; low risk (HPV types 6, 11, 42, 43, and 44) and intermediate/high risk (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). Examination of women referred for colposcopy in Southampton using a combination of intermediate/high and low risk HPV tests would have missed HPV infection in 4.3% in CIN 2/3 and 15.4% in CIN 0/1. One problem is that 18 of the 35 genital HPV types detected in the Southampton group are not included in the commercial test panels. Of particular interest in the Southampton population was the han831 isolate which was detected in 3 CIN 2/3 patients. In addition, only HPV types in different screening panels would be identified as mixed infections. Presence of mixed HPV infections in the Southampton group was found to be more prevalent in CIN 2/3 patients. The data presented in my study of colposcopy clinic patients with CIN 0/1 and CIN 2/3 clearly showed an association between the presence of high risk HPV types and CIN 2/3 (p-value 3×10^{-17}), whereas moderate risk types were not significantly linked with CIN

2/3 (p-value 0.14). In addition, for the Southampton population studied detection of a high risk type was 67.5% predictive of CIN 2/3, whereas detection of a moderate risk type was only 42% predictive of CIN 2/3. Combining both high and moderate risk together was only 59.7% predictive of high grade CIN. Therefore detection of a moderate risk type is less predictive of a pre-cancerous lesion than detection of a high risk type. Linking these two risk types in the same pool in a HPV detection kit may give misleading results.

The four most common HPV types detected were HPV-16 (30%), 18 (5%), 31 (7%), and 33 (7%). Of these HPV-16, 18, and 31 are classified as high risk types whereas HPV-33 as a moderate risk type. HPV-18 did not show an increased incidence with CIN in the Southampton population studied; 7% incidence in CIN 0/1 and 4% in CIN 2/3 (p-value 0.16). However, HPV-18 frequently occurs in adenocarcinoma of the cervix (Tenti *et al.*, 1996) which occurs higher up the cervix than the squamous epithelium readily sampled using a spatula. Thus, the inclusion of HPV testing in the UK screening programme may prevent women with early adenocarcinoma being missed. Further, HPV-18 is more prevalent in developing countries (Bosch *et al.*, 1995). In addition, HPV-18 appears to be frequently disrupted in the L1 region (detected by the MY and GP primer sets) and this may also lead to underestimation of prevalence (Walboomers *et al.*, 1999).

In conclusion, the HPV risk types detected in women referred to the colposcopy clinic with abnormal cervical cytology correlated with the severity of the cervical lesion. Although, high risk HPV types were detected in around 40% of low grade lesions, detection of a high risk HPV type was 67.5% predictive of CIN 2/3. Conversely, detecting a low risk HPV type was 67.7% predictive of CIN 0/1, but absence of any HPV type was 96.2% predictive of CIN 0/1 indicating the importance of a confirmed negative HPV result. Persistence of infection appears necessary for progression to high grade lesions (Nobbenhuis *et al.*, 1999); the host factors which lead to persistence of infection are unknown. Impaired host immunity, for example women with HIV (Cappiello *et al.*, 1997) undoubtedly increases the risk of cervical cancer. One possibility is that uncontrolled replication of high risk HPV increases the chance of an oncogenic event.

Analysis of HPV Load. In this context, some researchers have found higher numbers of HPV-16 genomes in more severe lesions, and high viral load predicts

persistence of an infection (Villa, 1997). Indeed, Cuzick and colleagues (1994) found in a semi-quantitative study detection of HPV-16 at high viral numbers was 93% predictive for CIN 2/3 based on PCR product band intensity on an ethidium bromide stained agarose gel. In the colposcopy patients studied in Southampton the mean HPV viral load was measured using Genescan; significantly higher mean viral loads were found in CIN 1/2/3 compared to CIN 0 samples for all HPV types together. However, no significant difference was found between HPV-16 viral load with high and low grade CIN (p-value 0.11); a result which was confirmed using the fluorogenic probe assay (p-value 0.44). Further, the scatter of the results from both Genescan and fluorogenic probe assay demonstrate viral load cannot be used as a predictor of CIN or disease status. Swan and co-workers (1999) used a fluorogenic probe assay to determine viral loads of HPV types 16, 18, 31, and 45 in cervical cytobrush specimens. HPV counts were normalised to cellular DNA content. They found HPV-16 DNA increased with grade of CIN, but the other HPV types did not when using median HPV load values. Swan however, as in my study, found the range of viral load meant a cut-off value predictive of high grade lesion was not possible.

After infection HPV genomes are stably maintained within basal cells at a copy number of about 50-200 copies per cell. In productive infections this increases to 1,000-10,000 copies per cell during epithelial cell differentiation (Bedell *et al.*, 1991). These viral loads were determined in a cell line derived from a low grade lesion grown in raft culture, however high grade lesions may contain a mixture of differentiated and undifferentiated cells in the uppermost layers and may not be producing progeny virus, so the actual viral loads per cell may be lower in high grade CIN than low grade CIN. Determining the number of viruses per cell may or may not be of value in determining the outcome of a HPV infection. There are three commonly used cervical cancer cell lines containing high risk HPVs; CaSki cells contain 60-600 copies of HPV-16 per cell, HeLa cells each have 10-50 copies of HPV-18, and SiHa cells have 1-2 copies of HPV-16 (Meissner, 1999). Thus, all the cell lines are derived from cervical cancer and all contain varying numbers of virus genome per cell. Of course, all these cell lines contain HPV DNA integrated into the cellular genome and not episomal virus, whereas pre-cancerous lesions tend to have episomal virus only (Cullen *et al.*, 1991).

In my study cervical samples were taken with a combination of spatula and a cytobrush resulting in the efficient sampling of the outermost layers of the epithelium.

However, for lower grades of CIN these outermost layers may not necessarily contain abnormal cells, whereas for CIN 3 the whole thickness of the epithelium is abnormal (see figure 1.1). Therefore, if cells become dysplastic due to the presence of abnormally replicating HPV, then infected cells will not differentiate to produce progeny virus, thus no HPV virions would be released from the cell. The implication is that the persistently infected epithelial cells are more likely to occur at the outermost layers of the epithelium in severe lesions (CIN 2/3) than in low grade lesions (CIN 0/1). This can explain reports of higher viral loads in severe lesions compared to low grade lesions. The confounding factor is productive HPV infection when large numbers of HPV genomes occur in the surface epithelium. Such women with no detectable cervical abnormalities may have very high viral loads. In my study cervical samples were centrifuged at 60,000 x g for 30 minutes to pellet free HPV virions and increase sensitivity of HPV detection.

The use of quantitative PCR cannot determine how many cells in the sample are infected, and if all abnormal cells are infected with equal numbers of virus. Thus, cellular viral load values can be misleading. If we accept that the proportion of abnormal cells increases with the grade of CIN, then an increase in HPV load in women with CIN 3 does not necessarily mean a higher viral copy number per cell. For example, if the mean number of HPV copies per cell was calculated as 100, 200, 300, and 400 for CIN 0, CIN 1, CIN 2, and CIN 3, but the actual proportion of infected cells was 10% for CIN 0, 20% for CIN 1, 50% for CIN 2, and 80% for CIN 3, then the true picture would be that the number of viruses per infected cell decreases with increasing CIN.

The number of viruses per cell calculated in my study was 800 for CaSki and 3 for SiHa using fluorogenic probe assays. Therefore, these fluorogenic assays give comparable results to the 'official' viral copy numbers in these cell lines. Using the Genescan methodology the values calculated were 1100 HPV-16 genomes per cell in CaSki and 13 HPV-18 genomes in HeLa cells.

Hence, determination of viral load has no obvious correlation with lesion severity. However, differences may be hidden if the samples contain varying amounts of HPV infected cells and if the virus is either cell associated or free virions.

Prevalence of other Genital Infections. A history of inflammatory genital infection is more common in women with cervical cancer suggesting these agents also increase the risk of developing this cancer. However, these additional infections may

simply be markers of sexual activity and hence correlate with the risk of acquisition of HPV infection, but have no additional etiological significance.

A very low incidence of previous sexually transmitted diseases was reported by the patients studied in Southampton, so the influence of past STDs, in relation to CIN and HPV, is minor in this group of patients. Only current gonorrhoea and chlamydia infections could be screened for in the samples. No cases of gonorrhoea were detected. Although chlamydia was detected there was no correlation with infection and grade of CIN, however chlamydia infection occurred more often in patients less than 26 years old (7%) compared to older women (1%) attending the colposcopy clinic. Ideally then, women attending a colposcopy clinic, especially if less than 30 years old, should be routinely screened for chlamydia infection, but seeking patient permission to screen for STDs could significantly compromise the cervical smear programme.

The herpesviruses and adeno-associated virus (AAV) cause latent infections hence detection of DNA genome from these viruses in cervical cells establishes persistent infection at this site or a carrier state. The most common infections detected in my study were HHV-7 (45%), HHV-6 (15%), and AAV (12%). The remaining herpesviruses were detected at a rate of 3% or less.

In humans the replication cycle of CMV results in lysis of infected cells, hence for cellular transformation to occur requires non-productive infection. This could happen with a spontaneously produced mutant virus, or more likely the wild-type virus may only express certain genes in a particular cell type, e.g. cervical epithelial cells. The induction of cervical neoplasia by human CMV in mice (Heggie *et al.*, 1986) may be due to an abortive infection from few viral proteins being produced. The immediate-early proteins of human CMV, IE1 and IE2, are mutagenic in primary rat cells (Shen, Zhu, and Shenk, 1997), but are only transiently required and viral DNA is frequently absent in the transformed cells. Therefore, the immediate-early proteins of human CMV could induce transformation of infected cells in human cervical epithelial cells. If these cells were not permissive for the full replication cycle of the virus the cells could become malignant. However, the continued presence of the viral proteins could become detrimental to the cell and thus cellular growth would favour cells which have lost the viral genome. This may suggest a 'hit-and-run' role for CMV in cervical neoplasia, but which is difficult to establish in the patients studied in Southampton as only 8 patients had evidence of cervical CMV infection.

Epstein-Barr virus was found in specimens with CIN 1 (3%) and CIN 3 (5%). These results are comparable with those published by Landers *et al.* (1993) who found 8% of CIN 2 and CIN 3 samples tested positive for EBV DNA. Again, the presence of EBV DNA within the cervix raises the possibility this virus may be sexually transmitted. Interestingly, Landers' group found of 18 cases of cervical cancer studied, 8 contained EBV DNA (44%). *In situ* hybridisation showed the EBV DNA to be located in the nucleus of malignant cells, suggesting the EBV genome had integrated into the host cell chromosome. Whether this integration event was a cause of malignancy or a consequence is unknown, but not all cases showed integration of EBV DNA so it may have been a chance event.

Evidence for HSV having a causal role in CIN is uncertain (Boyle and Smith, 1999), possibly due to some studies screening for HSV antibodies and others detecting HSV DNA. However Koffa *et al.* (1995) found that detection of HSV DNA correlated with cervical cancer in specimens that did not contain HPV DNA, suggesting HSV may have a role in HPV negative cervical cancer. However, the single case of HPV negative CIN 2 found in the Southampton study did not contain detectable HSV DNA.

In Southampton, AAV was found more often in patients with CIN than in the control population (p-value 0.006). This is not in agreement with the hypothesis that AAV can prevent the replication of HPVs and therefore protect infected patients from CIN (Hermonat, 1994). However, Hermonat *et al.* (1998) analysed the effect of AAV on BPV infection. They discovered that the time between infection by the two viruses critically affected the outcome of infection; if infection of both viruses occurred within 10 hours, there was significant inhibition of BPV, provided there was infection with between 1 and 10 AAVs per cell. If AAV infection occurred more than 10 hours before or after BPV infection, there was no affect on BPV replication. Most importantly, if the cells were latently infected with AAV, they were more susceptible to BPV transformation than AAV-uninfected cells. If HPVs infecting the endocervix behave similarly to BPV with regard to AAV infection, and women acquired AAV infection before HPV infection, this could explain my finding of an increased incidence of AAV in patients with CIN. It is known that primary infection with AAV usually occurs in childhood, but an unstable AAV antibody response may allow lifelong reinfection or reactivation of persisting virus (Erles, Sebökovà, and Schlehofer, 1999). In the Southampton population AAV occurred in around 12% of all age groups studied (16-25,

26-35, 36-45, and 46-55) indicating re-activation of latent infection rather than reinfection would be the most likely source of AAV. This suggests many women will have latent AAV infection from early age and hence possible susceptibility to HPV transformation if cells in the endocervix are infected.

Han and co-workers (1996) found AAV to be present in 50% of cervical brushing from undiseased patients and Tobiasch's group (1994) detected AAV in 63% of Normal cervical mucosa samples tested; the same figure as that published by Walz *et al.* (1998) from high grade CIN biopsies. The patients studies were from Northern Europe or North America. However in a larger study by Strickler *et al.* (1999) on cervical samples from women in Jamaica and university students in the United States found no evidence of AAV infection in either CIN samples or Normal cervical samples. The authors offer no explanations for non-detection of AAV in either group. All the groups screened for AAV using the same primers as in the Southampton study. The difference in incidence of AAV in the Southampton colposcopy patients (14.1% infected) and the control population (1.6% infected) may be because the control population were attendants from one GP cervical screening clinic and may represent a subset of women in the Southampton area. Regardless of the differences in detection of AAV in CIN by specific groups no evidence was found to suggest AAV plays a role in reducing the incidence of pre-malignant cervical lesions and subsequent cervical cancer.

Two groups have studied the prevalence of HHV-6 and HHV-7 in the female genital tract. Leach *et al.* (1994) found HHV-6 to be present in 10% of vaginal swabs of women attending a STD clinic, whereas Okuno's group (1995) found HHV-6 (variant B only) in 19.4% of cervical swabs from 72 pregnant women and 6% of 34 nonpregnant controls, and HHV-7 in 2.7% of pregnant women and none in the nonpregnant controls. In my study, HHV-6 was detected overall in 15% of specimens and of 14 samples typed, 12 were HHV-6 variant B and 2 were variant A. HHV-7 was detected in 45% of cases with CIN, but only 18% of Normal samples. Therefore, HHV-7 was more prevalent in patients with cervical abnormalities (p-value 0.007). In addition, HHV-7 was detected more often in samples containing high risk HPV types (p-value 0.008). The high incidence of HHV-6 and HHV-7 in cervical samples may indicate these viruses are sexually transmitted.

Unlike HHV-6, HHV-7 can be reactivated from latently infected peripheral blood mononuclear cells by T cell activation (Katsafanas *et al.*, 1996) and the

ongoing/active HHV-7 replication can, in turn, reactivate latent HHV-6 infections. Detection of DNA from HHV-6 and HHV-7 in my study does not indicate if the viruses are latent or re-activated. Thus, it may be possible for a latent HHV-7 infection in the cervix to be reactivated by T cell activation due to the immune response to HPV infection, which could in turn re-activate HHV-6, and therefore both viruses could be shed and sexually transmitted.

However, in the submandibular salivary gland HHV-6 DNA has been detected in 88% and HHV-7 DNA in 100% of healthy individuals (Sada *et al.*, 1996) suggesting saliva is the important route for transmission of the virus, especially as most people are seropositive for both viruses by the age of 2 years. The presence of HHV-6 and HHV-7 in the cervical samples could be due to macrophages or lymphocytes from the tissue or blood being collected with the cervical cells or latently infected epithelial cells. Detection in the cervix may therefore represent dissemination of the patient's systemic infection of the immune system rather than sexually acquired infection.

In the colposcopy patients tested in Southampton 161 samples were infected with HHV-6 or HHV-7, but only 15 samples were positive for HSV, CMV, EBV, or HHV-8; 10 of which were also infected with HHV-6 or HHV-7. Thus, in my study, the presence of any herpesvirus essentially means presence of HHV-6 or HHV-7. When Southampton colposcopy patients were grouped into those containing or not containing any herpesvirus in the cervical sample significantly more of the herpesvirus containing samples had CIN 2/3 or lesions which had become more severe in the time between the smear sample and the biopsy being taken at the colposcopy clinic (54.4% versus 38.9%, p-value 0.017). Hence the presence of herpesviruses may induce the production of paracrine growth factors which cause HPV lesions to become more severe. Both HHV-6 and HHV-7 produce a G protein-coupled receptor homologue (Davis-Poynter *et al.*, 1997) which in HHV-6B acts as a β-chemokine receptor with affinity for RANTES, MIP-1β, and MCP-1 (Isegawa *et al.*, 1998). The receptors may bind chemokines and hence reduce the local inflammatory response or in latent infections the receptors may be activated by chemokines and reactivate the virus. The former possibility may affect HPV by reducing the local cell-mediated immune response.

In this model the presence of any herpesvirus DNA in cervical samples would be a co-factor in disease progression of HPV lesions. Of course, it remains to be

established whether herpesvirus infection causes HPV infected cells to become neoplastic or if herpesviruses simply allow an existing lesion to progress faster.

Cytokine Analysis. It is possible HHV-6 and/or HHV-7 cause, rather than are re-activated by, inflammation and hence the production of cytokines which may affect the growth of HPV infected cells and influence the development of CIN. In the Southampton patients, although the presence of human herpesvirus type 7 (HHV-7) appeared to increase the levels of IL-8 in CIN 0 specimens (1200 pg/ml in HHV-7 infected samples versus 500 pg/ml in uninfected samples, p-value 0.03), this is probably coincidental as the IL-8 levels are independent of HHV-7 infection in other grades of CIN.

Woodworth and Simpson (1993) measured cytokine levels in cultured cervical cells. For IL-1β Normal ectocervical cells produced around 47 pg/10^6 cells/24 hrs and endocervical cells produced 74 pg/10^6 cells/24 hrs. SiHa cells only produced 3 pg/10^6 cells/24 hrs of IL-1β. IL-6 levels were 14 pg/10^6 cells/24 hrs in ectocervical cells, 55 pg/10^6 cells/24 hrs in endocervical cells, and 125 pg/10^6 cells/24 hrs in SiHa cells. IL-8 levels were 1216 pg/10^6 cells/24 hrs in ectocervical cells, 60000 pg/10^6 cells/24 hrs in endocervical cells, and 141 pg/10^6 cells/24 hrs in SiHa cells. Levels of IL-1β, IL-6, IL-8, and TNF-α were reduced in expression in HPV-16 or 18 immortalised cervical cells. The authors concluded that cervical keratinocytes produced a large number of cytokines which were important in initiating inflammation or specific immunity in the cervical mucosa. The down regulation of these cytokines provided a favourable environment for HPV infected cells. However, these results are contradictory to the increased levels of IL-6 and IL-8 found in cervical washings from CIN and cervical cancer patients by Tjiong *et al.* (1999). Bauknecht *et al.* (1999) found IL-6 did not induce activity of the LCR of HPV-18 probably due to low level expression of the IL-6 receptor in these cells. In my study IL-8 was detected at a concentration of 300 pg/ml after 3 days and 120 pg/ml after 4 days culture of SiHa cells and IL-6 was detected at 5 ng/ml after 4 days, but IL-1β was not detected in the cells. The high levels of IL-6 I detected in the SiHa cells may indicate these cells are no longer affected by the autocrine action of IL-6, probably due to down-regulation of the IL-6 receptor. If this occurred *in vivo* then normal cells infiltrating and surrounding the tumour would be affected by IL-6, but not the tumour itself. This could produce a local T_H2 antibody

immune response and increase blood supply to the tumour by angiogenesis which would aid tumour growth.

The mean level of IL-6 in the Southampton colposcopy samples tested was 100 pg/ml, for IL-1β it was 20 pg/ml in CIN 0 and 60 pg/ml in higher grades of CIN, and for IL-8 there was 870 pg/ml in CIN 0 and 1240 pg/ml in CIN 1/2/3. TNF-α was not detected in the specimens. These levels are in agreement with the results found by Tjiong *et al.* (1999), who detected 2 to 73 pg/ml IL-6 in specimens with CIN and 248 to 1158 pg/ml IL-8 in the same specimens. The authors found significantly higher levels of both cytokines in cervical cancer specimens and significantly lower mean levels of the cytokines in patients without cervical abnormalities. However, no breakdown of the CIN group was reported. The samples tested by Tjiong's group (1999) were ectocervical/vaginal washings using 20 ml PBS, whereas the samples tested in my study were 2.5 ml medium containing a spatula sample from the ectocervix and a cytobrush sample from the endocervix. These two methods, therefore, appear to give comparable results.

Fichorova and Anderson (1999) showed endocervical and ectocervical cells produced a different range, and quantity, of cytokines. For instance endocervical cells produced high concentrations of IL-6 and IL-8 whereas ectocervical cells produced low levels of IL-8 and IL-6 was undetectable. Low levels of IL-1β and TNF-α were detected in both types of cells when lysed, but not in cell supernatants suggesting storage of these cytokines in the cells. Cervical infections with HPV normally occurs at the transformation zone where the endocervix and ectocervix meet. Hence cytokine production by cells in the transformation zone may be different from endocervical or ectocervical cells which may affect HPV infection. The cervical scrapes taken in the Southampton study would sample cytokines produced by both endocervical and ectocervical cells.

Of the two forms of IL-1, IL-1α is produced by keratinocytes whereas IL-1β is found in macrophages, although keratinocytes contain IL-1β, but in the inactive form (Mizutani, Black, and Kupper, 1991). The ELISA used only detected the active form of IL-1β and therefore detection of IL-1β indicated an inflammatory response by activated macrophages. The detection of IL-6 and IL-8 also indicated a generalised immune reaction as IL-6 causes T and B cell proliferation and IL-8 attracts and activates neutrophils. IL-1β causes naïve T helper cells (T_H0) to secrete IL-2. If these T_H0 cells

are activated in the presence of IFN-γ they differentiate into T_H1 cells producing cytokines which cause a cell mediated immune response and in the presence of IL-4 they differentiate into T_H2 cells producing cytokines which cause an antibody mediated response. Scott, Stites, and Moscicki (1999) found the cervical cells from women who subsequently cleared HPV infection had a T_H1 cytokine profile (expression of IFN-γ and absence of IL-4) according to mRNA analysis. In addition, IL-1 stimulates synthesis of IL-6, but IL-6 inhibits IL-1 (Ulich *et al.*, 1991) and IL-8 (Barker *et al.*, 1991). Many studies have shown a reduced incidence of IFN-γ in CIN and cancer samples compared to Normal controls (Pao *et al.*, 1995, Clerici *et al.*, 1997, de Gruijl *et al.*, 1999), suggesting a reduction in the T_H1 immune response and favouring a T_H2 antibody mediated response instead which does not inhibit growth of HPV transformed cervical cells. However, in my study it was not possible to collect specimens from a normal control population for cytokine assays, so it is difficult to determine if the levels of the cytokines found in the CIN samples are reduced or increased compared to those found in the normal cervix. Therefore, for a complete understanding of the role of cytokines in both the immune response to HPV infection and in enhancing/inhibiting transformed cervical cells requires time-course studies on a group of women with early HPV infection.

Transcript Detection. In order to preserve unstable mRNA cervical scrape samples were collected into RNAlater solution and total RNA extracted from the pool of cells. However, only a minority of cervical cells in a single sample will show HPV induced abnormalities and the abnormal cells are not clonal in origin. This problem of a spectrum of HPV induced abnormalities occurring in the cervix of individual women was confirmed by the colposcopy biopsy findings. The typical histopathological report on women recorded as CIN 1 was "CIN 1 with adjacent florid papillomavirus effect." Colposcopy biopsies are taken from both the anterior and posterior aspects of the cervix and these can differ in the grade of CIN reported. Thus in about 10% of the situations where one biopsy showed CIN 2 the second showed CIN 3. Biopsies showing different focal areas of CIN 1 and CIN 3 were also recorded. Thus, my study was designed to answer the pragmatic question "Can we detect transcripts of HPV oncogenes in all women with high grade intraepithelial neoplasia" anticipating that HPV transcripts associated with normal viral replication may well be present in the same sample.

Similarly, analysis of the cervical cell regulatory protein transcripts represent the mixture of cells present in the specimen.

Normalisation of Samples. In order to allow for differences in samples, e.g. number of cells or viral copy numbers per cell, viral transcript levels are normalised using a variety of denominators such as number of copies of the particular gene, number of cells in the sample, or as a ratio of the number of transcripts from a so-called 'housekeeping gene' of which GAPDH is commonly used. Depending on which is used can drastically change the overall appearance of the results. For example, in CaSki cells the number of E1 transcripts per gene ($6x10^{-4}$) was found to be similar to those from the slow growing tumour ($1x10^{-3}$), however when the number of transcripts are calculated per cell CaSki had 100 times more E1 transcripts, 0.3, compared to $3x10^{-3}$ in the slow growing tumour. In addition when the number of transcripts was determined per GAPDH transcript there were 10 times as many E1 transcripts in CaSki cells ($4x10^{-2}$) compared to the slow growing tumour ($5x10^{-3}$). Thus, depending on which denominator was used samples produced similar or 100 times different levels of the same transcript. Possibly the number of viral transcript per cell is more important than the number of transcripts per viral gene, as it is the number of transcripts within a cell which will affect cellular behaviour rather than the number of viral genes producing transcripts. Although this is practical for cell lines where all cells are the same, in clinical samples there are likely to be many different cell types present (epithelial, fibroblast, blood cells, etc.) many of which may not contain virus. Hence using the total cell number as a denominator will 'dilute' the apparent number of viral transcripts in infected cells. Similarly using GAPDH levels; as all cells will be producing transcripts. In addition, we showed that the level of GAPDH transcripts in different cell lines varied depending on how quickly the cells were growing. Cancer cells, and possibly pre-malignant CIN cells, may be growing faster than the variable number of shed normal cells in the sample hence normalisation of HPV transcripts to host cell GAPDH mRNA levels will affect results. Using viral gene counts will give an idea of viral gene expression levels, but these will not take account of cells containing multiple viral genome copies where some of these viral genomes may not produce transcripts.

HPV-16 Transcript Analysis. Using HPV-16 containing cell lines the fluorogenic probe assays were found to be sensitive and reproducible, and the polycistronic transcript PCRs gave products of the expected sizes from cDNA. In the

cervical cancer cell lines and the cervical tumour biopsies, transcripts coding for E6 and E7 proteins were the most abundant HPV-16 transcripts detected, whereas transcripts coding for L1 protein were rare or absent. These results are consistent with viral E6 and E7 proteins causing cell immortalisation and the virus having an abnormal life cycle with no capsid production.

In the Southampton patients with high grade cervical lesions (CIN 2/3) there was a larger proportion of samples expressing transcripts for HPV-16 E6, E6*I, E6*II, and E7 compared to patients with low grade lesions (CIN 0/1), although in positive samples no differences were found in the level of transcription. Only a longitudinal study could establish if the CIN 0/1 samples expressing these transcripts indicates those lesions likely to progress to high grade CIN. In addition, there was a trend for high grade lesions to express a larger range of polycistronic mRNA than low grade lesions, although some patients with CIN 1 expressed a wide range of transcripts and almost half of the patients with CIN 2/3 expressed very few transcripts.

The role of the different splice products from polycistronic mRNA in the life-cycle of HPV-16 is not clear, although the presence of many different splicing events gives multiple opportunities for post-transcriptional regulation of HPV gene expression, plus splicing allows for differential gene expression from a single pre-mRNA. Certainly, splicing of E6 to give the E6* species reduces the coding potential for E6 protein and in addition the different E6* species may have different functions.

Although all the E6 splice variants (E6I-E6IV, see figure 7.2) have been shown to be translated, only the protein from full length E6 degraded p53 (Shally *et al.*, 1996). This is in agreement with the view that the E6* transcripts are to increase the likelihood of the E7 transcripts being translated after the E6 region as ribosomes appeared to 'fall off' the transcript after the full length E6 coding region (Alloul and Sherman, 1999). However, Braunstein *et al.* (1999) have reported a potential promoter at nt 542 in HPV-16 which may control synthesis of the E7 protein from a monocistronic mRNA.

No other HPV types studied produce an equivalent of the HPV-16 E6*II transcript, including the closely related HPV-31 (Baker and Calef, 1996). As this transcript is rare in the samples studied here, production of this transcript may be 'accidental' and not provide any function in the viral life-cycle. Interestingly, HPV-33 produces an E6*II transcript using the equivalent to the 226 donor site in HPV-16, but

the acceptor site is within the E7 gene (Snijders *et al.*, 1992). The equivalent transcript has not been found in HPV-16. However, HPV-31, 33, and BPV produce transcripts equivalent to E6*I produced by HPV-16. The production of the E6*I transcript in different papillomavirus types argues for it having a role in the papillomavirus life-cycle.

As E6, E6*I, and E6*II transcripts can potentially code for E7 protein, E7 transcripts should occur at higher levels than the E6 transcripts. Indeed, the E7 transcript level should be equal to the sum of the E6, E6*I, and E6*II transcripts. For the SiHa cell line and the cervical cancer biopsies E7 transcript levels were within 1 log of the sum of the E6 and E6* transcript levels. For CaSki cells the E7 transcript level was 1.5 logs higher than the E6 transcript levels. Of 18 specimens with E6 and E7 transcript counts above 100 starting copies, all E7 transcript levels were within 1.1 logs of the total E6, E6*I, and E6*II transcript levels. Therefore E7 transcript level are the same as the total E6, E6*I, and E6*II transcript levels. If the E7 transcript level exceeded the total E6 transcript levels this could be due to the detection of a transcript from the P_{670} promoter. This shows the validity of the fluorogenic probe assays and the low level of transcription from the P_{670} promoter.

The E6*III and E6*IV transcripts (species D and H respectively) were detected at high levels from all the Southampton samples. These transcripts produced PCR products of less than 100 bp compared to the other transcripts which were all above 370 bp. Thus, the high level of transcripts D and H may simply be due to higher PCR efficiency with the smaller PCR products. In future, the small size of these PCR products means the transcripts could be quantified using a fluorogenic probe assay.

Although polycistronic transcripts are produced, the full range of potential proteins may not be produced. For instance, according to Alloul and Sherman (1999) translation of E2 protein from transcript H (see figure 7.2) was more efficient than from transcripts E and I, as translation must re-initiate after termination of the E7 upstream ORF in these transcripts. Therefore transcripts E and I coded for E7 protein rather than E2. Similar selection may occur with other transcripts, e.g. transcripts A, G, and K may only code efficiently for E6 protein, whereas transcripts B, C, E, F, I, and J may only code for E7 protein, although transcripts containing full length *E6* and *E7* have been shown to produce both proteins (Tan *et al.*, 1994). Transcript D may only code for E5 protein, transcript L for E4 protein, and transcripts M and N may only code for E2

protein. To enable production of these proteins under control of two differentiation dependent promoters (P_{97} and P_{670}) requires splicing of transcript using 2 donor sites (226 and 880) and acceptor sites (409, 2708, and 3357). Combinations of these donor/acceptor sites and the promoters would account for transcripts A, B, D, E, G, H, L, and M. However, for unknown reasons 2 additional acceptor sites are present in HPV-16; 526 and 2582. These sites may be 'accidental' and serve no function to the virus and no equivalent sites have been found in other HPVs (Baker and Calef, 1996), also transcripts using these sites are rare in the Southampton samples. In addition, these sites may have different translational properties than the other donor and acceptor sites. These additional sites account for the remaining transcripts. Hence the different transcripts can be accounted for by the necessary presence of splicing donor/acceptor sites to code for individual proteins using only 2 promoters, one in undifferentiated cells and the other in differentiated cells. Once the transcripts are produced there is no reason why different grades of CIN should have different ratios of each transcript, unless a further level of post-transcriptional regulation is present in HPV-16.

In the normal viral life cycle of HPV-16, the E2 protein represses transcription from the viral early promoter, P_{97}, upstream of the E6/E7 ORFs, therefore enabling transcription from the differentiation specific promoter, P_{670}, to occur (Grassmann *et al.*, 1996), and thus favouring production of transcripts L, M, and N. For CIN 0/1 HPV-16 infected specimens, 53.3% only expressed transcripts from the P_{97} promoter compared to 21.4% of CIN 2/3 (p-value 0.033 using χ^2 analysis). In contrast, 26.7% of CIN 0/1 expressed transcripts from both P_{97} and P_{670}, whereas 71.4% of CIN 2/3 produced transcripts from both promoters (p-value 0.005 using χ^2 analysis). This apparent switch to additional use of the P_{670} promoter may simply indicate a higher level of cellular differentiation in high grade CIN. However, epithelial cells in high grade CIN are undifferentiated unlike epithelial cells in low grade CIN which closely resemble normal epithelium (Laimins, 1993). This apparent discrepancy can be explained by the cervical scrape samples containing a range of CIN grades as indicated on the histopathological reports.

The transcript map in figure 7.2 only shows transcripts detected in high grade CIN and cervical cancer specimens; no E1 or L2 protein mRNAs have been reported. However, these mRNAs have been detected with the closely-related HPV-31 grown in monolayer cultures and in organotypic raft cultures. In HPV-31 the E1 protein was

upregulated during viral genome amplification, and may regulate DNA packaging and virion production (Ozbun and Meyers, 1998). In addition, differentiation induced E1 message in HPV-31 (Klumpp and Laimins, 1999). In the HPV-16 positive samples analysed here E1 transcripts were detected in 5.9% of CIN 0/1 and 20% of CIN 2/3. In addition, similar proportions of L2 transcripts were detected in CIN 0/1 (52.9%) and CIN 2/3 (57.5%) and the proportion of L1 transcripts was not significantly different between the two groups, 35.3% of CIN 0/1 and 57.5% of CIN 2/3. Although there were relatively high proportions of samples expressing L1 and L2 transcripts in the samples the mRNA may not be translated into protein due to specific tRNAs not being available in the cells (Zhou *et al.*, 1999). In future, the levels of protein expression would need to be tested in order to determine if the E1, L1, and L2 proteins are being produced at different levels in different grades of CIN. In addition, Stubenrauch *et al.* (2000) found the transcript in HPV-31 equivalent to transcript O in figure 7.2 produced a protein which strongly repressed the major early promoter and was a negative regulator of viral replication. Also, mutations in the gene coding for this protein prevented episomal maintenance of viral genomes. Analysis of this transcript in patient samples containing HPV-16 is necessary in future studies.

The biopsy of a highly malignant tumour from a young patient showed similar HPV-16 transcript levels as the cell lines. Interestingly, the biopsy sample from an elderly patient with a slow growing cervical tumour expressed lower levels of transcripts than the other biopsy or cell lines. The level of HPV-16 transcripts detected per cell in the slow growing tumour were 100 to 1000 fold less than in the fast growing tumour. Certainly, for the cervical cancers the level of HPV-16 transcripts per cell fitted the clinical picture of the disease; the highly malignant specimen expressing high levels of transcripts and the slow growing sample expressing very low levels.

Analysis of Cellular Transcripts. Two cellular proteins which have been shown to be absent in normal tissue, but active in different types of cancer are telomerase and survivin. These proteins appear to be specifically expressed in cancers and therefore may have a role in the development of cancer. Survivin expression has been found in many cancer cell lines and importantly in primary cancers from breast, lung, prostate, colon, pancreas, stomach, neuroblastoma, and lymphoma (Jäättelä, 1999). However, no studies have been published to date which analyse survivin expression in CIN samples.

Survivin transcripts were detected in three cervical tumour cell lines and in two primary cervical cancers analysed. Telomerase assays showed the rapidly growing cell lines HeLa having $5x10^7$ hTR and $2x10^5$ hTERT transcripts per 10^6 cells and SiHa $2x10^6$ hTR and $3x10^3$ hTERT transcripts per 10^6 cells, while the slow growing CaSki cell line had 10^6 hTR and $6x10^1$ hTERT transcripts per 10^6 cells. These findings were mirrored in analysis of primary cervical cancers with a fast growing tumour having $4x10^6$ hTR and $8x10^2$ hTERT transcripts per 10^6 cells while hTERT transcripts were not detected in a very slow growing tumour and hTR transcripts were detected at a level of $6x10^4$ per 10^6 cells.

It seemed likely the cervical cells in the colposcopy samples would be contaminated with mucosal-derived immune cells and/or menstrual blood. Therefore it was necessary to test if immune cells expressed significant levels of the survivin and telomerase transcripts under investigation and hence confound the results from the colposcopy samples. No telomerase reverse transcriptase (hTERT) or survivin transcripts were detected although blood mononuclear cells were found to have ten times higher levels of telomerase RNA component (hTR) compared to equivalent numbers of cells in cervical smear samples. This may explain why hTR was present in all Normal cervical samples, 84% of CIN 0/1 samples, and 97% of CIN 2/3 samples. hTR was present at significantly higher levels in samples from patients 36-55 years of age compared to patients aged 16-35 (p-value 0.03), and in HPV negative samples compared to those containing HPV-16 (p-value 0.02). This would be expected as the older age group contained the majority of HPV negative samples. However, it is not clear why older, HPV negative patients should express higher levels of hTR. In contrast, hTERT was only detected in 3 of the 141 samples tested, interestingly, one sample was a from a Normal patient and the other two were from CIN 0 and CIN 1. If expression of the reverse transcriptase component was indicative of potentially cancerous lesions then constant expression would be expected in more severe lesions.

Takakura and colleagues (1998) analysed the expression of different components of telomerase in cervical cancer specimens and scrapes from normal cervixes. They too, detected hTR RNA in all Normal and cancer specimens tested, in contrast hTERT mRNA was found only in cervical cancer tissue. In addition, the group performed TRAP assay to determine telomerase protein activity in the samples. They concluded hTR although necessary, was not sufficient for telomerase activity. Thus detection of

hTERT mRNA is more indicative of malignant disease. The same group had previously reported the results of TRAP assay on CIN samples (Kyo *et al.*, 1997) and found low level telomerase activity in all grades of CIN and Normal cervical tissue, only high grade lesions and cervical cancer had high/moderate levels of telomerase activity. The group suggest a combination of TRAP and hTERT mRNA expression to screen cervical smears to determine potentially malignant tissues as neither TRAP nor hTERT PCR detected all positive samples. However, to maximise mRNA stability the Southampton samples were collected into RNAlater solution containing agents which destroy all enzymatic activity in the specimen.

Survivin transcripts were detected in all three cervical cell lines and the two cervical cancer samples although the quantity of transcripts varied; HeLa cells expressed $2x10^7$ transcripts per 10^6 cells, SiHa possessed $3x10^6$ survivin transcripts per 10^6 cells, and CaSki had $2x10^5$ transcripts per 10^6 cells. The two cervical cancer specimens also showed different expression levels with $5x10^5$ survivin transcripts per 10^6 cells from the fast growing tumour and $3x10^3$ transcripts per 10^6 cells in the slow growing tumour. Survivin transcript levels detected in these malignant cells were higher than the hTERT transcript levels suggesting that survivin may represent a more easily detected indicator of potential malignancy in colposcopy clinic patients. Indeed, in the Southampton patients survivin mRNA was detected in 29% of CIN 0/1 samples and 33% of CIN 2/3 samples, but was not detected in Normal cervical samples. Individually, the survivin mRNA was detected in 14.8% of CIN 0, 41.4% CIN 1, 28.9% CIN 2, and 40.1% CIN 3. There were significantly fewer samples in CIN 0 samples expressing survivin transcripts than in CIN 1/2/3 (p-value 0.04 using χ^2 analysis). This suggests expression of survivin commonly occurs early in CIN and increases in prevalence with persistent CIN. Survivin is expressed in G_2/M phase of the cell cycle and rapidly downregulates following cell cycle arrest (Li *et al.*, 1998). At the beginning of mitosis, the survivin associates with the mitotic spindle and disruption of this interaction causes apoptosis. Thus, survivin counteracts a default induction of apoptosis in the G_2/M phase. Detection of survivin mRNA in CIN, but not Normal cervical samples indicates that cervical cells in CIN samples are replicating whereas Normal tissue is not. This would be expected if the cells are immortalised by HPV. The low level of expression of survivin in the CIN samples may be due to only a minority of cells being HPV infected and only infected cells expressing survivin. The direct collection of cervical samples into RNAlater

solution would have prevented the decay of survivin mRNA during sample processing. Interestingly, HPV-16 E6 transcripts were found to be expressed at a significantly lower level in samples also expressing survivin compared to those without detectable survivin although the proportion of samples expressing E6 transcripts was the same in both groups. One interesting possibility is that enhanced cellular proliferation in cells expressing survivin may require lower levels of E6 protein.

In addition to a reduced level of HPV-16 E6 transcript expression in the presence of survivin transcripts, the proportion of samples expressing HPV-16 E1 and E2 transcripts was also reduced in the presence of survivin. HPV-16 E1 protein initiates viral DNA replication and E2 protein controls viral DNA replication and transcription. Presence of both E1 and E2 transcripts may indicate viral genome replication is possible and this is more likely to occur in survivin negative samples where a productive viral life cycle is possible.

In summary, survivin is expressed in 30 to 40% of patients with CIN and in samples expressing survivin transcripts the expression levels of HPV-16 E6, E1, and E2 transcripts were reduced. While it is possible that frank cervical carcinoma would be more likely to develop in untreated women where the CIN expresses survivin than in survivin negative women, a longitudinal study to confirm this is unethical. At a practical level, monitoring survivin transcripts cannot be used to differentiate between high and low grade CIN.

Analysis of p53 Polymorphisms. Finally, during the course of this study, a polymorphism in the p53 gene was reported as being a potential risk factor for CIN. The suggestion was that an arginine polymorphism was more susceptible to HPV E7 degradation than a proline form (Storey *et al.*, 1998). As well as investigating this codon 72 polymorphism two other polymorphisms, both in introns of the p53 gene, were also investigated as these had been implicated as risk factors in other cancers.

None of the p53 polymorphisms detected could be implicated in the majority of cases of CIN or cancer. However, the A form of a polymorphism in intron 6 revealed by a G→A substitution may indicate a small group of women at risk from CIN. If this were the case, then less than 5% of women would be at additional risk of CIN as the frequency of homozygous A form is less than 5% in populations studied (see table 10.1) with the majority of women having homozygous G form. Women with the proline form at codon 72 showed increased susceptibility to high grade CIN when associated with

intron 6 A form, however, the number of patients with intron 6 A form and proline at codon 72 represent a small proportion of the population (8.9% according to the figures calculated in table 10.4). Of course, the fact that the expected frequency of this haplotype is not 1 in the CIN or cancer groups shows this is not the cause of CIN simply an increase in risk, especially as there was no correlation with HPV type.

Loss of heterozygosity (LOH) in the cervical samples favouring the A form of the intron 6 polymorphism would produce similar results to those found. Unfortunately, we did not have ethical permission to obtain blood samples from the colposcopy patients which would have enabled any LOH to have been determined. However, as the codon 72 and intron 3 allelic frequencies showed no differences with the control population, determined from blood samples, any LOH within colposcopy patients would be specific to this portion of the p53 gene. Peller's group (1995) found the intron 6 A form was preferentially retained in gastrointestinal tumours, but preferentially lost in breast cancer. Investigation of the 17p13.1 chromosomal region containing the p53 gene has found LOH occurs in around 20% of cervical carcinomas (Kersemaekers *et al.*, 1998), however no data are available for LOH of this region in CIN samples. As the intron 6 allelic frequency was significantly different to the control population, even in CIN 1 patients, then if this difference was due to LOH then this must be a very early event in cervical dysplasia. Although, why an intron should be susceptible to LOH is unknown, especially as the codon 72 and intron 3 polymorphism frequencies were unchanged from controls.

The increased incidence of CIN with intron 6 A form and proline at codon 72 disagrees with the findings of Storey *et al.* (1998) who found the arginine form at codon 72 was more susceptible to high risk HPV E6 degradation, but other workers have also failed to confirm Storey's finding including Hayes *et al.* (1998), Helland *et al.* (1998), and Hildesheim *et al.* (1998). However, when related to HPV risk type, there was no consistent differences in the incidence of any of the polymorphism compared to samples with no detectable HPV; absence of the 16 bp duplication in intron 3 occurred more frequently in HPV negative samples compared to HPV positive samples, although no difference in presence of the duplication was found. It would appear unusual for the heterozygous form to show differences with HPV type and the differences are more likely due to the low number of HPV negative samples tested rather than any biological difference, especially when no differences are found between individual HPV risk types

only HPV positive samples grouped together. Similarly for cervical cancer specimens; lack of significant results may be due to low number of samples tested. Given I was comparing women with cancer and a local control population it was difficult to increase the number of cervical samples tested as very few cases of cervical cancer are seen within the Southampton area; those tested represented approximately 5 years of sample collection.

There are a number of possibilities as to how a polymorphism in an intron may affect the properties of the protein. Firstly, the polymorphism may be at a splice site, in which case the polymorphism will alter the size and translation of the mRNA. The intronic polymorphism studied here appear in the middle of the intron so do not disrupt splice sites, in addition the polymorphisms have the wrong sequence to produce new splice sites (Mount, 1996). Another possibilities is altered stability of the mRNA; Shen, Basilion, and Stanton (1999) found polymorphism altered the folding of mRNA, which in turn may influence mRNA processing, translational control, and regulation. Finally, polymorphisms may function as a marker linked to another alteration in the p53 gene or a gene linked with the p53 gene.

Study Findings. This study was undertaken in order to determine if HPV type, load, and oncogenic transcript production, evidence of other cervical infections and/or inflammation, plus host genetic factors might have a high predictive value for pre-malignant disease. Firstly, the HPV type was found to correlate with severity of the cervical lesion. High risk HPV types were detected in 80% of CIN 3 specimens. In contrast, low risk HPV types were most prevalent in low grade lesions (11%) and totally absent in high grade lesions. Moderate risk HPV types were present in similar proportions in all CIN grades; 34% in CIN 1 and 19% in CIN 3. In addition, mixed HPV infections were more common in high grade lesions. However, viral load showed no positive predictive value for lesion severity. The transcriptional activity of the most commonly detected high risk type, HPV-16, was also investigated. Expression of E6 and E7 transcripts was associated with the presence of high grade CIN. Other sexually transmitted diseases have been implicated as co-factors. In the population studied here the herpesviruses were found more often in women with progressing disease. Cytokines, too, have been implicated as affecting growth of HPV infected cells and IL-1β, IL-6, and IL-8 were found at higher levels in high grade CIN. However, it was not possible to determine if the levels detected were an increase or decrease compared to those found in

the Normal cervix. Host cellular factors were analysed; the expression of survivin occurred in 30% of CIN specimens and a polymorphisms in the p53 gene found in a small group of patients may provide a protective affect against CIN. However, as this is not a longitudinal study the proportion of women with CIN 0/1 who would self-cure and the proportion of women with CIN 2/3 who, if untreated, would develop cervical cancer cannot be determined.

The HPV type detected showed the best correlation to disease severity. However, in the Southampton patients detection of a high risk HPV type was only 67.5% predictive of CIN 2/3. Conversely, detecting a low risk HPV type was 67.7% predictive of CIN 0/1, but absence of any HPV type was 96.2% predictive of CIN 0/1 indicating the importance of a confirmed negative HPV result. Whereas a referral smear result of mild/borderline abnormalities was found to be around 70% predictive for low grade lesions (CIN 0/1) and for patients referred with severe dyskaryosis 90.7% of samples subsequently had CIN 2/3. These results indicate the high predictive value of determining lesion severity using the current cervical screening methodology and that HPV detection, if used, should be as an adjunct to the screening programme and not a replacement.

Appendix 1

HPV-16 Transcripts in Patient Samples

The following table shows the results of the fluorogenic probe assays and the polycistronic mRNA detected in patient samples from the colposcopy clinic. The fluorogenic probe result indicate the logarithm of the number of starting copies for each transcript. The polycistronic mRNA results show the presence of the transcripts shown in figure 7.2. Presence of transcripts A-N is high (++), moderate (+), low (±), or not detected (-).

CIN	Viral Load	Transcript																			
Grade	/ per cell	E6	E7	E6*I	E6*II	E1	L1	A	B	C	D	E	F	G	H	I	J	K	L	M	N
3	680	3.9	3.3	2.7	2.1	3.5	2.5	+	+	+	+	+	+	+	+	+	-	+			
3	1,900	2.6	2.3	-	-	-	2.2	-	-	-	±	-	-	-		-	-	-	+	-	-
3	260	2	2.3	1.2	-	-	-	+	±	-	+	-	-	-		-	-	-		+	-
3	10	2.5	1.9	0.5	-	-	1	-	-	-	+	+	-	±		-	-	-	-	-	-
3	190	3.5	3	1.8	1.2	2.2	3.1	+	++	+	+	+	-	-		-	-	±			
3	1	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-
3	100	2.4	2.3	1.2	-	-	2.1	-	-	-	+	-	-	+		-	-	-	±		+
3	100	3.8	3.2	2.5	1.3	3.9	3.2	+	++	+	++	±	-	+	++	+	-	+			
3	40	-	2	-	-	-	1.1	+	±	+	+	-	-	-	+	-	-	-		-	-
3	10	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	±	-	-
3	460	2.6	-	1	-	-	-	-	±	-	+	-	-	-	++	-	±	-		+	-
3	150	3.5	2.4	2.4	0.9	2.4	3.6	+	+	-	+	±	-	+	++	±	-	-			
3	590	3.5	1.7	1.1	-	1.9	3.5	-	-	-	+	-	-	-	-	-	-	-	±	+	+
3	10^{-4}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	30	0.6	1.1	-	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	280	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	1,200	3.7	0.8	2.9	1.2	3.6	3.8	+	+	+	+	±	-	+	++	-	-	+			

CIN	Viral Load	Transcript																			
Grade	/ per cell	E6	E7	E6*I	E6*II	E1	L1	A	B	C	D	E	F	G	H	I	J	K	L	M	N
2	520	2.8	2.1	-	-	-	3.2	-	-	-	+	-	-	-	+	-	-	-	±	±	±
2	810	1.7	2.6	-	-	-	2.4	-	-	-	-	-	-	-		-	-	-	+	-	+
2	50	2.3	1.6	1.3	0.8	-	1.4	±	±	-	++	+	-	-		-	-	-			+
2	10	1.1	-	-	-	-	1.6	-	-	-	-	-	-	-		-	-	-	-	-	-
2	2,600	1.5	1.2	-	-	-	-	-	-	-	-	-	-	-		-	-	-	±	+	+
2	70	1.3	1.7	-	-	-	1.3	-	-	-	-	-	-	-		-	-	-	+	-	-
2	27,500	4.5	4.1	1.4	-	3.3	4.4	-	±	-	+	-	-	-		-	-	-		++	++
2	10	1.9	1.9	-	-	-	1	-	-	-	-	-	-	-		-	-	-	+	-	-
2	330	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	+	-	-
2	10	1.7	-	-	-	-	-	-	-	-	±	-	-	-		-	-	-	-	+	-
2	10	1.6	1.4	-	-	-	0.5	-	-	-	-	-	-	-		-	-	-	+	-	-
2	10	2	-	1.1	-	-	1.5	±	+	-	+	-	-	-	±	-	-	-		±	-
2	1	1.9	-	-	0.3	-	2.4	-	-	-	+	-	-	-	-	-	-	-	-	-	-
2	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-	-
2	20	-	-	-	-	-	-	±	-	-	-	-	-	±	+	-	-	-	-	-	-
2	1	2.6	1.6	1.9	-	-	3.3	+	-	-	+	-	-	-	++	-	-	+		-	-
2	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

CIN Grade	Viral Load / per cell	Transcript																			
		E6	E7	E6*I	E6*II	E1	L1	A	B	C	D	E	F	G	H	I	J	K	L	M	N
2	30	2.7	-	1.8	0.5	3	1.6	+	+	-	+	+	-	-	+	-	-	-			±
2	10^{-3}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	60	1	-	0.8	-	-	-	+	±	-	-	-	-	-	+	-	-	-		+	-
2	500	-	0.9	-	0.4	-	-	-	-	-	-	-	-	-	+	-	-	-	±	-	-
2	50	1.9	1.5	-	-	-	1.7	-	-	-	-	-	-	-	+	-	-	+	-	-	-
2	10	1.6	1.7	0.7	-	-	-	-	-	+	+	-	-	-	-	-	-	+		±	-
1	260	-	-	-	-	-	-	-	-	-	±	-	-	-		-	-	-	±	-	-
1	-	-	1.8	-	-	-	-	-	-	-	-	-	-	-		-	-	-	±	-	-
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	±	-	-
1	100	-	-	-	-	-	-	-	-	-	±	-	-	-		-	-	-	-	-	-
1	3,600	1.3	-	-	-	-	2.5	±	-	-	+	-	-	-	-	-	-	-	-	-	-
1	40	-	-	-	-	-	1.8	-	-	-	±	±	-	-	-	-	-	-	-	--	-
1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1	10^{-4}	-	-	-	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-
1	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-
1	50	-	-	-	-	-	-	-	-	-	-	-	±	-	-	-	-	-	-		+
1	3,300	3	3.2	-	-	1.5	3	-	-	-	+	-	-	-		-	-	-	-	-	-

CIN Grade	Viral Load / per cell	Transcript																			
		E6	E7	E6*I	E6*II	E1	L1	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	70	1.4	2	1.1	-	-	1.6	-	-	-	±	-	-	-		-	-	-	+	+	-
1	10	2.6	2	1.1	-	-	2.4	-	-	-	+	+	-	-	-	-	-	-	-	-	-
0	14,000	-	-	-	-	-	0.3	-	-	-	-	-	-	-	+	-	-	-	-	-	-
0	7	-	-	-	-	-	-	-	-	-	+	-	-	-		-	-	-	±	+	+
0	0.1	1	1.6	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-
0	560	2.9	-	-	-	-	-	-	-	±	+	-	-	-	+	-	-	-		-	-

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