Host-Virus Interactions in Human Papillomavirus Mediated Disease

A Thesis Submitted for the Degree of Doctor of Philosophy

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For Karen

And in Memory of Patient RRP-001

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Abstract

Human Papillomavirus (HPV) is the cause of a wide spectrum of disease ranging from benign cutaneous warts to malignant anogenital tumours.

Two notable features of HPV diseases are that the viruses are highly tissue specific of individual HPV types and the fact that while HPV infections are common only a minority of infected individuals manifest clinical disease, indicating the importance of host virus interactions. In this thesis two HPV mediated diseases have been examined in detail, cervical cancer and Recurrent Respiratory Papillomatosis.

Cervical cancer is a major cause of mortality in women in developing nations. In these countries cervical screening programmes are impractical for logistical reasons and there is much interest in the feasibility of developing preventative vaccines. However, before such agents can be developed it must be established that the same HPV types that cause cervical cancer in industrialised nations are associated with cervical cancer in developing nations and that the local clades of HPV have the same amino acid sequence as putative vaccine strains.

This thesis presents a study of the prevalence of cervical HPV infection, HPV type distribution and viral genetics carried out in a previously unstudied population of 934 women in rural Gambia. A high cervical HPV prevalence is observed, the most common high risk types were found to be HPV-16 & -35, the former being the most common high risk type worldwide and the latter this study show may be underestimated in African populations. Sequencing of the HPV L1 open reading frames provided data which may have implications for vaccine research.

RRP is a rare disease characterised by the presence of papillomata on the larynx and other sites in the upper aerodigestive tract. RRP is usually caused by HPV-6 or -11, two viruses more commonly associated with genital warts. RRP is thought to be contracted from the genital tract. However, genital HPV infection is common yet RRP remains a rare disease, therefore other factors either from the virus or the host must modulate disease pathogenesis.

In order to elucidate if RRP is caused by unique HPV clades that might be particularly well adapted to the larynx, the L1 major capsid gene and the oncogenes E6 & E7 have been sequenced. In order to establish the sequence of "normal" genital HPV-11 and to exclude regional differences between UK and US HPV-6 and HPV-11, genital wart tissue from 37 UK donors has also been analysed and compared to papilloma material from 53 RRP patients.

Significant sequence differences between RRP and Genital Wart E6 & E7 ORF were observed. In order to examine the possible role of host immunogenetic factors in the pathogenesis of RRP, 50 individuals with RRP were then HLA class typed. A significant association between the HLA class II allele DRB*0301 was found along with a negative association with the HLA DQB1*03 allele. Finally, T-cell proliferation studies using Cytokine Bead Array to detect cytokine production revealed that DQB1*03 positive donors produce more IFN-γ in response to HPV peptides than DQB1*03 negative individuals.

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Abbreviations

Units of Measure

°C	degrees Centigrade
cpm	counts per minute
g	gram
G	gravitational units
l	litre
ml	milliliter
mg	milligram
min	minute
mM	millimolar
M	molar
µM	micromolar
µg	microgram
pg	picrogram
rpm	revolutions per minute
s	second

General Abbreviations

95% CI	95% Confidence Interval Using the Approximation of Woolf (Fisher's Exact Test)
AORRP	Adult Onset Recurrent Respiratory Papillomatosis
APC	Antigen Presenting Cells
ASCUS	Atypical Squamous Cells of Undetermined Significance
bp	Base Pairs
BPV	Bovine Papillomavirus
CA	Condyloma Acuminata
CBA	Cytometric Bead Array
CaCx	Cervical Cancer
CIN	Cervical Intraepithelial Neoplasia
CMV	Cytomegalovirus
COPV	Canine Oral Papillomavirus
CRPV	Cottontail Rabbit Papillomavirus
CTL	Cytotoxic T-Lymphocyte
DNA	Deoxyribonucleic Acid
EBV	Epstein-Barr Virus
ELISA	Enzyme-Linked Immunoabsorbent Assay
EV	Epidermodyspiasia Verruciformis
HHV	Human Herpes Virus

HLA	Human Leukocyte Antigen
HPV	Human Papillomavirus
HR	High Oncogenic Risk Human Papillomavirus
HSIL	High Grade Squamous Intraepithelial Lesion
HSV	Herpes Simplex Virus
IL	Interleukin
INF	Interferon
JORRP	Juvenile Onset Recurrent Respiratory Papillomatosis
Kb	Kilobase(s)
LCR	Long Control Region
LR	Low Oncogenic Risk Human Papillomavirus
LSIL	Low Grade Squamous Intraepithelial Lesion
xM (cells)	x Million (cells)
Mb	Megabase(s)
MHC	Major Histocompatibility Complex
MRC	Medical Research Council
NCBI	National Centre for Biotechnology Information
OR	Odds Ratio (Fisher's Exact Test)
ORF	Open Reading Frame
P	Two-Sided P Value (Fisher's Exact Test)
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PBMC	Peripheral Blood Mononuclear Cell
RFLP	Restriction Fragment Length Polymorphism
RMS	Reproductive Morbidity Study
RNA	Ribonucleic Acid
CD45RA+	CD45RA high / CD45RO Low Naïve T-Cell Population
CD45RO+	CD45RA low / CD45RO High Memory T-Cell Population
RRP	Recurrent Respiratory Papillomatosis
SIL	Squamous Intraepithelial Lesion
SSP	Sequence Specific Primers
TAP	Transporter Associated With Antigen Processing
TCR	T-Cell Receptor
TGF-β	Transforming Growth Factor- β
Th	T-Helper
TNF	Tumor Necrosis Factor
VLP	Virus-Like Particles
WHO	World Health Organisation

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1.0 Overview

This thesis examines host virus interactions in Human Papillomaviruses (HPV) mediated diseases, particularly cervical cancer and Recurrent Respiratory Papillomatosis. In order to set the scene for this work the introduction will initially present a comprehensive overview of *Papillomaviridae* including its taxonomy, genetic structure, protein function and life cycle. In the second half of this chapter cervical cancer and RRP are discussed in detail including a review of host immune response to the disease associated HPV types.

1.1 Taxonomy & Classification of the *Papillomaviridae*

1.1.1 The *Papillomaviridae*

The *Papillomaviridae* (PV) are a diverse group of viruses that are members of the papovavirus family which also encompasses picoviruses and polyomaviruses. Papillomaviridae are highly tissue specific and infect only mucosal epithelia or cutaneous cells. They infect only a few mammals and are extremely species specific and so are classified according to the species they infect e.g. bovine (BPV), hamster oral (HaOPV), human (HPV) or cottontail rabbit (CRPV). This latter PV was the very first PV to be isolated in the 1930's and the very first DNA tumour virus to be isolated (Doorbar and Sterling 2001).

1.1.2 The Human Papillomavirus

HPV is classified into types, subtypes and variants by comparative DNA homology based, since the 1995 International HPV Workshop, primarily upon the L1 outer capsid gene and secondarily the E6 and E7 oncogenes (Doorbar and Sterling 2001). These early genes, E6 and E7, are transcribed as nuclear transforming proteins binding p53

and retinoblastoma protein (Rb) respectively. DNA homology of E6 and E7 may be used to define viral clades that may vary in their transforming potential. However to define a new HPV type it must have less than 90% L1 DNA homology to any previously defined types. HPV types can be further characterised into subtypes, 90%-98% L1 DNA homology, and variants, either >98% L1 DNA homology or oncogene variations. To date 118 complete HPV genomes have been characterised (de Villiers, Fauquet et al. 2004), however many more are present on the Genbank data base as subtypes, variants and those types only partially defined (usually in region of L1 amplified by the MY09/11 primer pair discussed extensively in Chapter 3).

1.1.3 HPV and Oncogenic Potential

As well as being taxonomically divided into genetically homologous groups A-H (Figure 1.1) these types and subtypes are usually divided into two supersets defined by oncogenic risk potential. Certain types which behave in a similar fashion clinically, have been found to demonstrate homology (Bauman NM 1996): These are the low oncogenic risk HPV types (LR or low risk) associated usually with benign lesions and the high oncogenic risk HPV types (HR or high risk) which have the potential to disrupt the cell cycle and cause proliferative dysplasia. The mechanism of this cell cycle disruption will be discussed later. Disease outcome in HPV infection is thought to be centred on the expression and function of the HPV early gene proteins E6 and E7 which may potentially be used to define viral clades within the HR and LR types and that may vary in their transforming potential. The range of disease both benign and potentially malignant caused by HPV can be seen in Table 1.1.

Table 1.1: Diseases of HPV and Associated Types (Doorbar and Sterling 2001)

Disease Pathology	Associated HPV Types
Plantar Warts	1, 4 & 65
Palmer Warts	1 & 65
Common Warts	2, 4, 26, 27, 28, 29, 57 & 63
Plane Warts	3, 49 & 65
Epidermodysplasia Verruciformis (EV) - Benign Lesions	3, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25 & 47
EV - Macular Lesions	5, 8 & 9
EV - Flat Warts	5
EV – Squamous Cell Carminoma (SCC)	5 & 8
Genital Warts	6, 11, 26, 31, 40, 43 & 44
Laryngeal Papillomata	6 & 11
Cervical Intraepithelial Neoplasia	6, 11, 16, 18, 31, 33, 34, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 61, 66, 67, 68, 69 & 72
Buschke-Löwenstein Tumours	6, 7, 11 & 54
Butchers' Warts	7 & 28
EV – Plane Warts	8, 9 & 10
Focal Epithelial Hyperplasia	13 & 32
Cutaneous SCC	14 & 47
Vulval Intraepithelial Neoplasia	16, 40, 61, 62 & 64
Anal Intraepithelial Neoplasia	16
Penile Intraepithelial Neoplasia	16, 51, 58 & 66
Cervical SCC	16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 66, 67 & 68
Vulval SCC	16 & 30
Anal SCC	16 & 30
Penile SCC	16 & 30
Laryngeal SCC	30
Oral Warts	32
Anogenital Warts	34, 48, 51, 54, 55, 64, 70 & 73
EV Keratoses	36
Cutaneous Plane Warts	41
Mucosal Warts	57 & 59
Plantar Epidermoid Cyst	60
Multiple Plantar Punctate Keratoses	63
Genital Lesions	74
Skin Lesions	75, 76 & 77

1.2 HPV Protein Structure & Function

1.2.1 Overview

The *Papillomaviridae* are small (diameter ≈ 55nm) non-enveloped viruses with an icosohedral pentameric outer capsid which encloses a circular supercoiled double stranded DNA genome (Fig. 1.1). This genome is around 8kbp and, in most cases, contains eight open reading frames (ORF), located on an single DNA strand (Fuchs *et al.*, 1994, Turek *et al.*, 1994), as well as an untranslated region (UTR) (Fig. 1.2). The UTR is also known as the non-coding region (NCR) or the upstream regulatory region (URR) as it contains numerous sites of gene transcription regulation as well as promoter and enhancer sequences and the replication point of origin. The ORF which encode HPV proteins involved in DNA replication and transcription are situated closer to the replication point of origin and are hence termed the 'early' proteins. These genes are, in order from the origin of replication; E6, E7, E1, E2, E4, & E5 of these, E6 & E7 are also involved in malignant transformation and will be discussed in detail later. The ORF which encode the L1 major and L2 minor capsid proteins are located further from the point of origin of replication and are hence termed the late proteins.

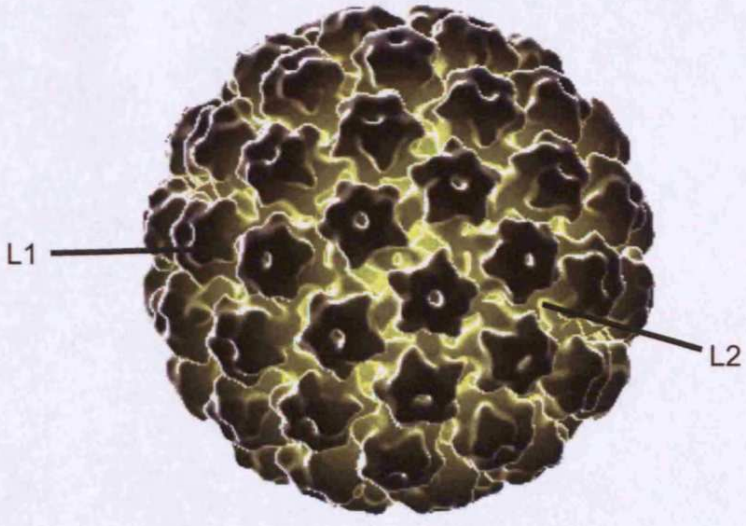

Figure 1.1: HPV Capsid Structure (Baker, Newcomb et al. 1991)

(Used with Kind Permission)

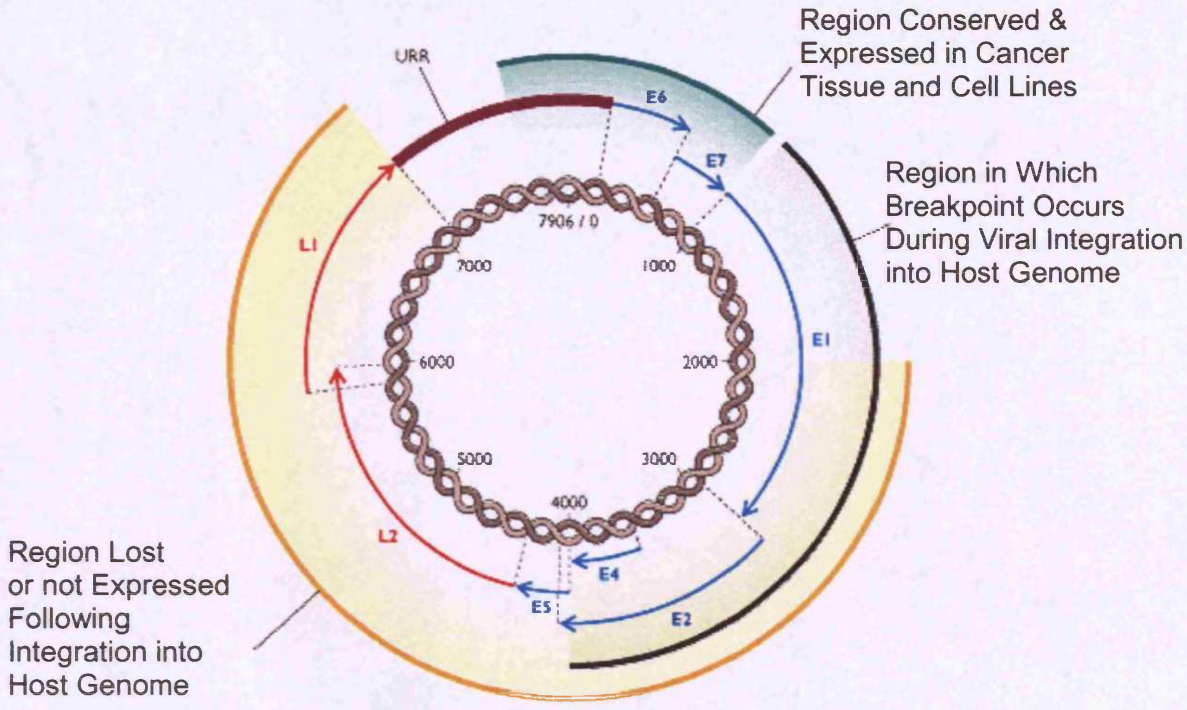

Figure 1.2: HPV Genomic Structure

1.2.2 E6

1.2.2.1 Normal Function of E6

This protein is around 150 amino acids in length and contains two putative zinc finger domains and E6 has been demonstrated to bind zinc (Barbosa, Lowy and Schiller 1989; Grossman and Laimins 1989; Rapp and Chen 1998). It also is involved in the HPV life cycle in high oncogenic risk types, and low oncogenic risk types (Oh, Longworth and Laimins 2004) by facilitating the maintenance of the viral genome as stably replicating episomes within keratinocytes this is discussed in greater detail later. Studies involving substitutions of the cisternines of the zinc finger CxxC motifs with glycine have indicated that the zinc finger nearer the amino terminus (66C), but not that nearer the carboxyl terminus (136C), is essential for *in vitro* zinc binding by HPV-16 E6 (Kanda, Watanabe et al. 1991). Both substitutions reduced the efficiency of E7-induced cellular transformation and the 66C glycine for cysteine substitution, but not 136C, also had the effect of lowering the nuclear E6 levels.

1.2.2.2 The Role of E6 in Cellular Transformation

The HPV E6 protein is, with E7, one of the two HPV oncoproteins. HPV-16 E7 alone is sufficient to induce the immortalisation of mammary epithelial cells, however, both E6 and E7 are required for efficient transformation of human keratinocytes (Tanaka, Noda et al. 1989; Watanabe, Kanda and Yoshiike 1989; Boyer, Wazer and Band 1996). The transformation target of the E6 protein is the tumour suppressor protein p53 to which it binds resulting in degradation of p53 via the ubiquitin proteolysis pathway (Scheffner, Werness et al. 1990; Werness, Levine and Howley 1990). In the absence of E6, p53 is normally targeted by this pathway so E6 may largely have the effect of accelerating p53 turnover or compelling p53 degradation in the presence of cell signals that would ordinarily result in stable p53 turnover (Ciechanover, DiGiuseppe et al. 1991). In native keratinocytes the half life of p53 is around 1-4 hours whilst in HPV immortalised keratinocytes it has been demonstrated to be between 15-30 minutes as a result of E6 mediated degradation (Hubbert, Sedman and Schiller 1992). In the normal course of events, when there is damage to host DNA, levels of p53 rise stimulating G1 cell cycle arrest, permitting DNA repair. In cells where p53 is damaged, cell division continues unchecked resulting in the perpetuation of DNA mutations. The concomitant loss of the G1 cell cycle checkpoint results in genomic instability (Livingstone, White et al. 1992; Yin, Tainsky et al. 1992). At position 72 of p53 is a common polymorphism of either an arginine or a proline the arginine form of which is far more susceptible to E6 mediated degradation (Storey, Thomas et al. 1998).

The E6 protein may also modulate cellular processes by binding the cellular proteins E6-BP (E6 binding protein) and E6-AP (E6 associated protein) (Chen, Reid et al. 1995; Rolfe, Beer-Romero et al. 1995; Elston, Napthine and Doorbar 1998). The E6-E6AP complex can bind *in vitro to* p53 (Huibregtse, Scheffner and Howley 1993) or to E6-BP (Chen et al. 1995). Upon binding p53 the E6-E6AP complex may act as a

ubiquitin ligase, binding and ligating ubiqutin to p53 (Scheffner, Huibregtse et al. 1993). Although the binding of E6 to E6-AP is a prerequisite for p53 binding, the binding of E6 to these cellular proteins can be regarded independently from binding degradation of p53 as E6 may both bind E6-BP in the absence of p53 or form an E6-E6AP-E6BP ternary complex (Chen et al. 1995). Amongst the other cellular proteins which may be bound by E6 are, the transcription factor binding proteins, p300 and CBP (Patel, Huang et al. 1999). As these two proteins bind to cellular transcription factors involved in growth and differentiation, E6 binding results in repression of p53 transcriptional activity (Zimmermann, Degenkolbe et al. 1999). Various other cellular proteins other than p53 are bound and degraded by the HPV E6 protein in the various stages of malignant transformation. These include the degradation, again via the ubiquitin pathway, of the E6 targeted protein 1 (E6-TP1) which is believed to have a regulatory role in meiotic signalling (Gao, Srinivasan et al. 1999; Gao, Singh et al. 2001; Gao, Kumar et al. 2002; Singh, Gao et al. 2003). The discs large protein (DLG) is also targeted by E6 this protein regulates contact between cellular signalling molecules and cytoskeletal proteins and acts to attract cytoskeletal proteins to the site of cell-cell contact (Kiyono, Hiraiwa et al. 1997; Lee, Weiss and Javier 1997). Hence degradation of DLG disrupts cell signalling and adhesion and may therefore play a role in the latter phases of tumour progression. Although the DLG is also degraded via the ubiquitin pathway this degradation has been shown *in vitro* to be independent of E6-AP binding (Grm and Banks 2004). An additional means by which E6 may regulate cellular proliferation is via interaction with human telomerase reverse transcriptase (hTERT). E6 promotes and regulates high levels of hTERT in cervical carcinoma cells via direct stimulation of hTERT promoter and prevention of the inhibitory effects of p53 (Oh, Kyo and Laimins 2001; Veldman, Horikawa et al. 2001; Jeong Seo, Jung Kim et al. 2004). This results in the prevention the normal shortening of the chromosomes with the concomitant induction of cell immortalisation.

1.2.3 E7

1.2.3.1 Normal Function of E7

Like E6, E7 is also involved in viral episome maintenance and has considerable sequence similarity to E6; they may indeed share a common ancestral gene. However E7 is only two thirds of the length of E6 (approximately 100 amino acids) and contains only one putative zinc finger domain. E7 is localised to the nucleus and cytoplasm and is homologous with many other DNA tumour virus transforming proteins. The E7 protein can be divided into three conserved domains (CD 1-3) two of which (CD 1 & 2) are homologous to regions of the SV-40 (another papova virus) large T antigen oncoprotein and the adenovirus 5 E1A oncoprotein (Phelps, Munger et al. 1992).

1.2.3.2 The Role of E7 in Cellular Transformation

The HPV E7 protein binds to the hypophosphorylated form of the retinoblastoma (Rb) tumour suppressor protein and the related proteins p107 and p130 (Dyson, Buchkovich et al. 1989; Munger, Werness et al. 1989; Davies, Hicks et al. 1993). A large number of genes associated with cellular proliferation are activated by the displacement of transcriptional factors which complex with Rb resulting from this binding. This binding affinity of E7 to Rb is ten-fold greater in high risk than in low risk and this disparity in affinity seems to be modulated by a single amino acid residue at position 22 on E7 (Heck, Yee et al. 1992; Sang and Barbosa 1992; Schmitt, Harry et al. 1994). Substitution of the glycine found in HPV-6 at this position with an aspartic acid residue results in an increase in affinity for Rb (Sang and Barbosa 1992). As mentioned above, (HPV)-16 E7 is able, in the absence of E6, to induce the immortalization of mammary epithelial cells (Boyer et al. 1996). However it is when E6 & E7 work synergistically that transformation of human keratinocytes can proceed efficiently (Barbosa and Schlegel 1989).

The cellular proteins Rb, p107 and p130 function as regulators of cellular growth and the main role of Rb is in the G1 and growth arrest phases of the cell cycle where it prevents cell proliferation by segregating the transcription factors necessary for cell cycle progression (DeCaprio, Ludlow et al. 1988; Buchkovich, Duffy and Harlow 1989). As with E6 and p53, the binding of E7 induces the degradation of Rb via the ubiqutin proteolysis pathway (Boyer et al. 1996). At different phases of the cell cycle Rb can be found in it's phosphorylated form (pRb) or it's hypophosphorylated form (Rb) with the latter found during growth arrest and the G1 phase. An important mechanism of the cell cycle modulated by Rb is the binding of the E2F family of transcription factors and the regulation of other genes involved in cell proliferation (Shirodkar, Ewen et al. 1992; Krek, Livingston and Shirodkar 1993). This binding of E2F to Rb results in the restriction of cell proliferation, during the G2/S phase of the cell cycle Rb is phosphorlyated and binding of E2F ends and cell growth may progress (Buchkovich et al. 1989). E2F is one of the proteins mentioned above that is displaced upon the binding of E7 to Rb permitting uncontrolled cell growth as long as the E7 stays bound to Rb. However, the release of E2F alone does not seem to be adequate for efficient transformation thus E7 implicated in other transformation mechanisms (Banks, Edmonds and Vousden 1990; Banks, Moreau et al. 1991). Other such interactions of E7 include interactions with the AP-1 family of transcription factors including c-Fos, c-Jun, JunB and JunD such interactions seem to be key in the progression of tumourigenesis (Antinore, Birrer et al. 1996). As with E6, E7 also binds to p300 forming a complex which may contribute to the regulation of E2 transcriptional activity (Bernat, Avvakumov et al. 2003).

1.2.4 E1

This nuclear phosphoprotein is the longest of all HPV proteins at around 600-650 amino acids in length. It is another protein with regions of homology with the SV-40 large T antigen oncoprotein (Seif 1984). As with large T antigen, E1 is principally associated with the initiation of DNA replication (Clertant and Seif 1984). E1 is an ATP dependant helicase, essential for viral replication, which acts synergistically with E2 to bind to the point of origin of replication and facilitate the unwinding and strand separation of DNA (Frattini and Laimins 1994). Stable binding to the origin of replication and DNA unwinding results in the formation of a hexameric complex (Fouts, Yu et al. 1999). Formation of this complex is followed by a host virus interaction between E1 and host DNA-α polymerase (Conger, Liu et al. 1999) which is recruited to the origin of replication permitting the initiation of DNA replication (Wilson, West et al. 2002).

1.2.5 E2

1.2.5.1 Normal Function of E2

As with E1 this protein is a nuclear phosphoprotein around 350 to 500 amino acids in length. As described above E2 regulates replication and transcription of HPV DNA in conjunction with E1. During this process E1 and E2, which display high affinity for each other, form a multimeric complex which permits stable binding of E1 at the origin of replication (Ham, Steger and Yaniv 1994). The binding of E2 to the origin of replication leads to a host virus interaction between E2 and the host transcription factors TFID and SP1 dislocating these factors from the E6/E7 promoter. This results in repression of E6 & E7 transcription (Tan, Leong et al. 1994). Thus although E2 generally initiates transcription; it has a dual role as not only an activator of transcription but a repressor of early gene expression.

1.2.5.2 The Role of E2 in Cellular Transformation

As described above the main roles of HPV E2 protein are in the regulation of transcription and the inhibition of the E6 & E7 oncoproteins. This regulation is disrupted following integration of the viral genome into the host genome leading to uncontrolled oncoprotein expression of and proliferative malignancy (Schwarz, Freese et al. 1985) Whilst not involved directly in transformation, the HPV E2 protein has been demonstrated to mediate cell proliferation via a number of mechanisms. These include the induction of cell cycle arrest (Dowhanick, McBride and Howley 1995) and apoptosis (Desaintes, Demeret et al. 1997). It has been shown that in cloned HPV-16, deletion of the E2 gene results in failure to immortalise keratinocytes (Storey, Greenfield et al. 1992). However a more recent study has shown that E2 is not so critical for oncogene regulation when the viral DNA is episomal (Bechtold, Beard and Raj 2003).

1.2.5 E4

This is another zinc binding protein (Roberts *et al.*, 1994) around 100 amino acids in length which is the most highly expressed of all HPV proteins. The ORF is totally enclosed within the E2 ORF but is within a different reading frame and thus does not share amino acid sequence with E2 (Howley and Lowy 1996). However it lacks an initiator AUG codon and the E1 reading frame is utilised for initiation of translation and expression of E4 does not occur until the late in the viral life cycle and its expression is limited to the upper spinious epithelial stratum. Consequently the ORF of E4 is translated from spliced transcripts as an E1^E4 fusion protein along with the first five amino acids of the E1 protein (Howley and Lowy 1996). A recent study has found evidence that proteolytic cleavage of this E1^E4 fusion protein modifies its function allowing mediation of G2 arrest in the cell cycle and that alternative forms of HPV-1 E4 cooperate to negatively influence keratinocyte proliferation (Knight, Grainger et al. 2004). The function of this protein is still mainly unidentified, however E4 does form

substantial structured filamentous cytoplasmic complexes which are observed to co-localise with the keratin intermediate filaments. These complexes may potentially impede normal epithelial differentiation and lead to the release of the mature virion (Howley and Lowy 1996).

1.2.6 E5

1.2.6.1 Normal Function of E5

This is a small hydrophobic protein which is generally confined to the Golgi apparatus and endosomal membranes (Conrad, Bubb and Schlegel 1993). The E5 protein is also found, to a lesser extent, in cellular membranes where a recent study has found that expression of HPV-16 E5 has modulatory effects in *in vitro* keratinocytes. E5 is observed to lower the rate of synthesis of phosphatidylglycerol and increases that of phosphatidylcholine and phosphatidylserine and, whilst not changing the total cellular cholesterol, it also increases the availability of easily available free cholesterol from the plasma membrane (Bravo, Crusius and Alonso 2004). In BPV the E5 protein is the principal protein of malignant transformation however there is little homology between E5 in BPV and HPV and they are likely to have different cellular targets (Longworth and Laimins 2004).

The BPV E5 protein has also been demonstrated to downregulate class I major histocompatibility complexes (MHC) by confining them to the golgi apparatus thus preventing them from being exported to the cell surface (Araibi, Marchetti et al. 2004). A more recent study, by the same group, has also demonstrated this phenomenon in HPV-16 (Ashrafi, Haghshenas et al. 2005). It was found that HPV-16 E5 may act to selectively down regulate surface expression of human leukocyte antigen - A (HLA-A) and HLA-B, which present viral peptides to MHC class I-restricted cytotoxic T lymphocytes (CTLs), but not HLA-C and HLA-E the natural killer (NK) cell inhibitory

ligands. This selective down regulation of cell surface HLA class I alleles may well be the underlying process which permits HPV infected cells to avoid immune surveillance by both CTLs and NK cells and establish infection.

1.2.6.2 The Role of E5 in Cellular Transformation

In BPV the E5 protein is the major transforming protein (Yang, Spalholz et al. 1985) whilst in HPV it displays only weak transforming potential (Leechanachai, Banks et al. 1992; Pim, Collins and Banks 1992). The E5 ORF is usually deleted following integration of the viral DNA into the host genome in cervical cancer (Howley and Lowy 1996). However, it is found that when a premature stop codon is present in the HPV-16 E5 gene, immortalisation efficiency is reduced by 90% (Stoppler, Straight et al. 1996). Hence although E5 does not play an essential role in transformation, like E2, it does increase the efficiency of transformation.

1.2.7 L2

The HPV L2 protein is the largely internalised, minor capsid protein around 500 amnio acids in length which associates with L1 pentamers to spontaneously form icosahedral capsids (Finnen, Erickson et al. 2003). The L2 protein is synthesised late in the viral life cycle and is expressed in the cytoplasm of the differentiated cells of the infected epithelium and is then transported, along with L1, to the nucleus where assembly of the replicated viral DNA into virions occurs.

1.2.8 L1

The L1 protein is the major outer capsid protein and is, like L2, around 500 amino acids in length but is approximately thirty times more abundant than the L2 protein (Kirnbauer, Taub et al. 1993). Whilst L2 is largely internalised the majority of the L1 protein projects outwards forming the 72 capsomeres of the icosahedral capsid. (Fig.

1.1) (Baker et al. 1991; Hagensee, Olson et al. 1994). When expressed in eukaryotic, bacterial or yeast systems L1 alone or L1 in conjunction with L2 spontaneously forms the highly immunogenic virus like particle (VLP). Although synergistic expression of L1 with L2 increases the production of VLP *in vitro* there is no concurrent increase in immunogenicity. Like the viral capsid, the VLP is icosahedral in construction. Electron microscopy shows the VLP to be superficially identical to the natural virion, though lacking the viral genome.

1.3 The HPV Life Cycle

1.3.1 Virus Entry

1.3.1.1 Mechanisms of HPV Entry

HPV infection is thought to be facilitated by rupture in the infected epithelium whereupon it infects the mitotically active basal epithelial cells (Schneider and Koutsky 1992). A number of cell surface molecules have been implicated in the mediation of HPV entry into the cell. These molecules include; α_6 integrin (Evander, Frazer et al. 1997; Yoon, Kim et al. 2001), CD16 (Da Silva, Velders et al. 2001) and glycosaminoglycans such as heparin and heparan sulphate (Joyce, Tung et al. 1999; Selinka, Giroglou and Sapp 2002; Drobni, Mistry et al. 2003). Another study found that lactoferrin may inhibit HPV entry most likely by preventing binding to heparan sulphate and it has also been proposed that HPV entry into the cell is occurs via clathrin-dependent receptor-mediated endocytosis (Day, Lowy and Schiller 2003; Drobni, Naslund and Evander 2004).

1.3.1.2 Potential Explanation for HPV Tropism

This multitude of molecular mediators may imply that different HPV types use different cellular receptors and may help explain the specific tissue tropism observed

between HPV types. For example heparin was found to bind to HPV-11 L1 whilst the HPV-16 L1 region found to bind to α_6 integrin was not homologous to the same region of HPV-11 (Joyce et al. 1999; Yoon et al. 2001). Thus host-virus interactions at the cell surface may play a crucial role in high degree of tissue specificity observed in HPV and as result disease, so it is possible that mutations may effect these interactions. Therefore there is the potential for the emergence of specific viral clades that may vary in their interactions from the majority of the same HPV type that may lead to a specific disease such as RRP, this possibility will be addressed in Chapter 4.

1.3.2 Incubation and Latency of Infection

In high risk HPV types there is a lag phase of around 14-21 days before the formation of any tumours (Kreider and Bartlett 1981). HPV may remain in the cell in a quiescent state of latency, indeed the vast majority of HPV infection is transient in nature and is normally cleared by the immune system without progressing to disease (Hildesheim, Schiffman et al. 1994).

1.3.3 Early Events in HPV Replication

After entering the cell HPV loses its coat and the viral genes may be expressed, the HPV genome is conserved as episomes within the cell nucleus where they are maintained at a copy number of 50 to 100 genomes per cell (Doorbar and Sterling 2001). Due to the simple nature of *Papillomaviridae*, they do not encode their own polymerase are is thus dependant upon host mechanisms to replicate. During division of a HPV infected cell, one of the daughter cells will migrate from the basal layer and start to differentiate (Howley and Lowy 1996). The remaining daughter cell remains in the basal layer and performs the role of a viral DNA pool for subsequent cell divisions (Howley and Lowy 1996). Primary HPV infection occurs in the poorly differentiated basal epithelium; however viral replication takes place in the terminally differentiating

epithelial strata which are approaching growth arrest. These cells therefore contain scarce or none of the enzymes such as polymerase required for DNA replication. In order to counter this, the HPV early genes E6, E7 & E5 contribute to prevent the termination of the cell cycle driving the infected differentiated supra-basal cells into the last S-phase of the cell cycle. This subversion of the cell cycle thus facilities high level replication of HPV (Doorbar and Sterling 2001).

1.3.3 Late Events in HPV Replication

As the HPV infected cells advance up through granular layer HPV DNA is amplified and the L1 and L2 late gene products are expressed. The viral particles are assembled within the outermost epithelial layer (the stratum corneum) where they are released in course of natural exfoliation (Howley and Lowy 1996).

1.4 Disease Mediated by Low Risk HPV Types

1.4.1 Overview

Low risk HPV types typically cause benign cutaneous hyperproliferative lesions known as warts which usually regress spontaneously, with no need for treatment (Tagami, Takigawa et al. 1977). HPV types commonly causing cutaneous warts are HPV-1, -2, -3- & -4 and the range of lesions caused by these low risk HPV types are shown in Table 1.1. As discussed above, individual HPV types preferentially infect certain tissue types and another classification division of HPV is between the types which infect cutaneous tissues and those which infect mucosal epithelia. The HPV which preferentially infect mucosa can themselves be split into types the so called "low risk" types which are classically associated with benign warts and the "high risk types" which are associated with cancer. Examples of low risk HPV types are HPV-6 & -11. These two types commonly infect the anogenital mucosa causing anogenital warts and

are the most commonly found genital low risk types in Europe and the USA (Muñoz, Bosch et al. 2003) HPV-6 & -11 may also infect the laryngeal mucosa, and it is thought that laryngeal infection with these HPV types is derived from genital infection (discussed in more detail below). In certain individuals, laryngeal infection with HPV-6 or -11 leads to the development of Recurrent Respiratory Papillomatosis (RRP). This is a disease where warts form within the larynx and can spread to the trachea, lung, pharynx, nose and oesophagus. While cutaneous warts are usually innocuous warts or papillomata on the larynx and other sites in the airway have the potential to cause fatal airway obstruction.

1.4.2 The Role of E6 & E7 in Low Risk HPV Type Mediated Disease

As described above, in high oncogenic risk HPV types the E6 protein complexes with the E6 associated protein (E6-AP) leading to the degradation of the tumour suppressor protein p53 via the ubiqutin proteolysis pathway (Scheffner et al. 1990; Howley, Munger et al. 1991; Munger, Scheffner et al. 1992). The E7 protein in high oncogenic risk types binds to, and inhibits the function of, the cell cycle regulatory protein; retinoblastoma protein (Rb) (Scheffner et al. 1990; Howley et al. 1991). Binding of E6 to p53 and E7 to Rb has also been observed in low-risk types, such as HPV-6 & -11, but with greatly reduced affinity (Li and Coffino 1996). In high-risk types the E6 and E7 proteins have been shown to have a role in the HPV life cycle by helping to maintain the viral genome as stably replicating episomes. In addition a recent study has found evidence suggesting that E6 and E7 in low-risk types such as HPV-11 may have this same function (Oh et al. 2004).

1.4.3 Genital Warts

1.4.3.1 The Pathogenesis of Genital Warts

The genital wart (condyloma acuminatum) is a benign growth predominantly associated with HPV-6 and -11, DNA of one or both of these HPV types is found in greater than 90% of genital warts (Shah 1996). HPV–6 and -11 infection is common in the ano-genital region (Koutsky 1997) with one study reporting a 54% genital HPV-6 or -11 infection rate amongst women with normal cervical cytology. Genital warts are, also common; with prevalence rates of approximately 1.5% reported in the US general population and 4-13% in US Genitourinary Medicine (GUM) clinic attendees (Koutsky 1997).

1.4.3.2 Transformation in Genital Warts

Only in extremely rare cases do genital warts undergo malignant transformation. into Buschke Löwenstein tumours. Unlike other HPV mediated malignancies such as cervical cancer (discussed later) the Buscheke Lowenstein tumours are only locally destructive tumours which do not metastasise (Jongen, Reh et al. 2001), and surgical excision is curative (Dr Humphrey Birley, Genitourinary Consultant, Genitourinary Medicine, Cardiff Royal Infirmary - Personal Communication).

1.4.4 Recurrent Respiratory Papillomatosis

1.4.4.1 Overview

RRP is characterised by the presence of papillomatous masses in the larynx and upper aerodigestive tract (including the trachea, bronchi, lung, pharynx, nasal air passages, and oesophagus). Although first documented in the 17th Century (*from*: (Webb 1956)) the communicable nature of RRP only established in the 1920s (Ullman 1923). The viral origin of RRP was verified in the 1970s by electron microscopy (Boyle, Riggs et al. 1973) and HPV DNA detected by Southern blotting in laryngeal

papillomata in the 1980s (Quick, Watts et al. 1980). Despite being a relatively rare disease RRP is the most common benign tumour of the larynx (Jones, Myers and Barnes 1984; Derkay 2001).

Although most papillomata in RRP are non-malignant, they have the potential to cause fatal airway obstruction if left untreated. There is no universally effective medical treatment for RRP, and thus many patients require repeated surgery to maintain their airway. Indeed it is not uncommon for children to require in excess of 200 surgical procedures during the course of their disease (Kashima, Leventhal et al. 1988; Kimberlin 2004; Silverberg, Thorsen et al. 2004).

1.4.4.2 HPV Type Association with RRP

The first HPV type to be associated with RRP was HPV-6 (Mounts, Shah and Kashima 1982), where the HPV type most commonly associated with RRP (>50%) was a subtype they designated HPV-6c. This HPV-6c subtype was latterly identified as HPV-11 (Metcalfe, Chen and Mounts 1989). HPV-11 had, in the same year as the Mounts study, been isolated from laryngeal papillomata by another group (Gissmann, Diehl et al. 1982) and by the same group the following year in genital warts (Gissmann, Wolnik et al. 1983). Although there are currently 118 completely characterised HPV genomes (de Villiers et al. 2004) it is still HPV-6 and -11 that are almost exclusively responsible for respiratory papillomata (Wiatrak, Wiatrak et al. 2004). However, a recent study in New Mexico (Penaloza-Plascencia, Montoya-Fuentes et al. 2000) found the presence of HPV-16, -31, -33, -35, and -39 in laryngeal papillomata but in much smaller numbers than reported for HPV-6 and -11.

Previous studies have suggested that HPV-11 is more likely to cause more aggressive disease in RRP, an earlier age of onset and with spread of disease into the trachea and lungs. (Rabah, Lancaster et al. 2001; Obchinnikov Iu, Kiselev et al. 2004;

Wiatrak et al. 2004). Thus HPV type appears to be a crucial determinant of disease pathology; however HPV-6 & –11 share a high degree of homology. Therefore RRP may be a viral clade specific disease mediated by polymorphisms within the HPV genome.

1.4.4.3 Disease Pathogenesis

RRP is thought to be contracted through oral exposure to genital HPV-6 and -11 (Shah, Stern et al. 1998; Silverberg, Thorsen et al. 2003). In infants and young children this exposure to HPV is thought to occur in the birth canal (Lindeberg and Elbrond 1989; Shah et al. 1998; Silverberg et al. 2003), and in older individuals the exposure is thought to have occured during oro-genital intercourse. However as genital HPV infection, genital warts and oral HPV infection appear to be common other factors must be implicated in the pathogenesis of RRP other than just HPV infection. In this thesis, host and virus mechanisms that may be such factors are investigated.

1.4.4.4 The Epidemiology of RRP

RRP displays a bimodal age distribution and has been arbitrarily divided into juvenile onset RRP (JORRP) when the onset of disease occurs before the age of 15 years, and Adult Onset RRP if the disease presents after this age. JORRP has an incidence of about 4 per 100,000, and usually presents before the age of 5 years)(Derkay 1995; Silverberg et al. 2003) Adult onset RRP (AORRP) has an incidence oft about 1.8 per 100,000 population and a peak incidence between the ages of 20 and 30 years (Derkay 1995). AORRP is associated with middle-class, married, heterosexuals (Derkay 1995).

In JORRP it has been postulated that HPV is transmitted from mother to through the birth canal (Shah et al. 1998) and this is supported by a rate of birth by caesarean

section in JORRP patients that is 4.6 times less than average (Rabah et al. 2001). JORRP has been statistically associated with firstborn, vaginal delivery and the lower social class (Shah et al. 1998). In addition JORRP patients with mothers with a history of genital warts have been shown to have an earlier age of RRP onset (Silverberg et al. 2004). In AORRP HPV infection is believed to be contracted through oro-genital intercourse (Kashima, Shah et al. 1992). Previous studies have found HPV-11 to be more prevalent than HPV-6 in JORRP (Gissmann, deVilliers and zur Hausen 1982; Gissmann et al. 1983; Mounts and Shah 1984; Abramson, Steinberg and Winkler 1987; Gabbott, Cossart et al. 1997; Wiatrak et al. 2004) whilst in AORRP the reverse is found (Corbitt, Zarod et al. 1988; Pou, Rimell et al. 1995).

1.4.4.5 The Clinical Features of RRP

Papillomata of the larynx are the most common manifestation of RRP and are seen in greater than 95% of patients. Spread of papillomata to other parts of the aerodigestive tract is observed in around 30% of JORRP patients and 16% of AORRP patients (Kashima et al. 1992; Kashima, Mounts et al. 1993; Derkay, Rimell and Thompson 1998). With papillomata of the trachea occurring in 11%, papillomata in the bronchi in 5% (Kashima et al. 1993), and papillomata of the lung parenchyma in 1% (Doyle, Henderson et al. 1994; Armstrong, Derkay and Reeves 1999). Lung parenchymal papillomata often undergo malignant transformation and are the resultant carcinomas are usually fatal (Doyle et al. 1994). Such malignant transformation in a disease mediated by low oncogenic risk HPV types has been estimated to occur in around 1% of cases (Reidy, Dedo et al. 2004).

1.4.4.6 Herpes Viruses as Potential Co-Factors in the Development of RRP

It has been observed that in many virally mediated diseases certain viruses of the herpes virus family act as co-factors which facilitate disease progression. Examples

include human herpes virus (HHV), herpes simplex virus (HSV), Epstein-Barr Virus (EBV) and cytomegalovirus (CMV) acting as co-factor viruses to HIV in the development of AIDS (Lau 1990; Fiala, Mosca et al. 1991; Polstra, Van Den Burg et al. 2003). HHV-6 has also been shown to be an important co-factor to HPV in the development of cervical carcinoma. A previous study has found that 2/9 RRP patients with aggressive disease were also co-infected with a herpes viruses as a group (Pou et al. 1995).

1.5 Disease Mediated by High Risk HPV Types

1.5.1 Overview

High risk HPV types are associated with anogenital cancers such as vaginal intraepithelial neoplasia (VIN), penile intraepithelial neoplasia (PIN), and anal neoplasia intraepithelial (AIN) (Rueda-Leverone, Di Paola et al. 1987; Planner and Hobbs 1988; Syrjanen, von Krogh and Syrjanen 1989; Gomousa-Michael, Gialama et al. 2000; Zbar, Fenger et al. 2002). However it is cervical cancer and its precursor cervical intraepithelial neoplasia (CIN) that is the most common disease mediated by high risk HPV and that with the highest morbidity rates (Munoz and Bosch 1997). Worldwide cervical cancer is a major cause of female mortality and in terms of cancer death is second only to second only to breast cancer with around 500,000 new cases annually, (Franco, Schlecht and Saslow 2003).

1.5.2 Cervical Cancer - A HPV Mediated Disease

The sexual transmission of high oncogenic risk HPV types has been demonstrated on a cellular and epidemiological basis to be the essential prerequisite for the progression of cervical carcinogenesis (zur Hausen 1977; IARC_Monographs 1995; Muñoz and Bosch 1996; Walboomers, Jacobs et al. 1999). Indeed, studies have shown as many as 99.7% of cervical cancers contain HPV DNA (Walboomers et al. 1999). Some studies have found slightly lower figures (zur Hausen 1991; Bosch 1995; Bosch, Manos et al. 1995). This may be because of the HPV detection methods used as some are less sensitive at detecting certain HPV types (Qu, Jiang et al. 1997), or could, in some cases, be due to integration of HPV into the host genome. To date 118 complete HPV genomes have been characterised (de Villiers et al. 2004), however only 15 are definitely, and a further three most likely, associated with cervical cancer (Table. 1.2) (Muñoz et al. 2003). Of these cervical types the most prevalent in cervical

lesions on a worldwide basis are (in order of prevalence) HPV-16, -18, -45, -31, -33, -52, -58, & -35 and in normal cervixes the order is found to be: HPV-16, -18, -45, -31, -6, -58, -35, & -33 (Muñoz et al. 2003).

Table 1.2: Relative Risk Association of HPV types and Cervical Cancer Worldwide

CaCx Risk	Associated HPV Types (Muñoz et al. 2003)
HR	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82
Probably HR	26, 53, 66
LR	6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, CP6108

1.5.3 Cervical Cancer and the Developing World

As many as 80% of new cases of cervical cancer occur in the developing world (Lazcano-Ponce, Moss et al. 1999) and indeed in West Africa it is the most common female malignancy (Koulibaly, Kabba et al. 1997; Bah, Parkin et al. 2001). In the Western world cervical screening programmes have reduced the rate of cervical cancer (Parkin, Nguyen-Dinh and Day 1985; Laara, Day and Hakama 1987; Sparen, Gustafsson et al. 1995; Bailie, Selvey et al. 1996; Gustafsson, Ponten et al. 1997; Ng, Wilkins et al. 2004; Wyatt, Huang et al. 2004). However, conventional cervical screening in developing rural populations is logistically impractical, therefore the World Health Organisation (WHO) is keen to explore other methods of cervical cancer prevention in such regions.

1.5.3 Prophylactic HPV Vaccines

Recently there has been a high degree of interest in prophylactic vaccination for the prevention of cervical cancer in developing countries (Dupont 1984; Munoz and Bosch 1997; Sherman, Schiffman et al. 1998; Goldie 2003; Lowy and Frazer 2003; Jansen 2004; Mandic and Vujkov 2004). However, current HPV vaccine research is based on high risk HPV types prevalent in the industrialised Western world. In order to rationally

design vaccines for the developing world, the circulating HPV types must be determined and high risk and low risk type associations resolved for the population. In addition, vaccine efficacy may be reduced or nullified if classified HPV types diverge from those variants upon which a vaccine is based. This is especially true if those variations fall within epitopes, it is therefore crucial that specific HPV type genetic variants (<98% L1 DNA homology) are defined by sequencing.

1.5.4 HIV Infection: A Confounding Factor in the Study of HPV

HIV (Human Immunodeficiency Virus) can be a co-factor in the development of cervical cancer. It is thought that HPV mediated cervical abnormalities are exacerbated by HIV induced immunosuppression and may thus distort the linkage between HPV and cervical disease development (Feingold, Vermund et al. 1990; Lefevre, Hankins et al. 2004, Baay, 2004; Moscicki, Ellenberg et al. 2004; Moscicki, Ellenberg et al. 2004). Sub-Saharan Africa has highest rates of HIV/AIDS (Acquired Immunodeficiency Syndrome) in the world, with an estimated 25 million infected individuals (UNAIDS 2004),

1.6 HPV Interactions with the Host Immune System

1.6.1 MHC Associations with HPV

1.6.1.1 HLA Associations with RRP

Only a handful of studies have investigated the role of HLA in the mediation of host immune response to HPV in RRP. The first of these examined only 16 individuals with RRP and found significant associations with the HLA class II alleles DRB1*11 and DQB1*03, although the DRB1*11 association was thought to be through DQB1*03 linkage (Bonagura, Siegal et al. 1994). A follow up study by another group examined only the HLA class II DQA1 and DQB1 alleles of 45 Finnish AORRP patients with no significant associations found (Aaltonen, Partanen et al. 1999).

More recently our group has found novel significant associations between RRP and the HLA class II alleles DRB1*0301 and the ancestral haplotype 8.1 (AH 8.1) as well as a negative association with DQB1*03 (Gelder, Williams et al. 2003). The AH 8.1 is a highly conserved, evolutionarily stable, cassette of genes which are inherited together and encompasses the HLA Class I alleles; A*0101, Cw*0701 and B*0801 and HLA Class II alleles DRB*0301, DQB1*02 as well as the. Of theses alleles of the AH 8.1 however only DRB1*0301 was still found to be significantly associated with RRP outside of the AH 8.1 association. The DRB1*0301 allele was also found to be associated with more aggressive disease with the patients with the most severe disease being homozygous for this allele. In this study an array of T-cell proliferative responses to the HPV-11 VLP were observed in DRB1*0301-positive JORRP patients, the magnitude of which was inversely associated with disease severity. This association between RRP and DRB1*0301 has recently been confirmed in a secondary US population by another study by Bonagura *et al* (Bonagura, Vambutas et al. 2004). Although this study did not find the AH 8.1 association they did find another HLA class

II allele, DRB1*0602, to be negatively associated with aggressive RRP in a caucasoid disease subset.

1.6.1.2 HLA Associations with Cervical Cancer

Extensive study has been made of associations between HLA alleles and cervical cancer. Such studies have often reported an association between susceptibility to cervical cancer and HLA class II allele DQB1*03. Whilst the initial study to report this association was in a US population (Wank and Thomssen 1991) it has also been observed in a variety of populations and ethnicities in studies in the UK (David, Taylor et al. 1992; Mehal, Lo et al. 1994; Odunsi, Terry et al. 1996; Cuzick, Terry et al. 2000), Norway (Helland, Borresen et al. 1992; Helland, Borresen et al. 1994), Spain (Montoya, Saiz et al. 1998), Sweden (Sanjeevi, Hjelmstrom et al. 1996), Japan (Nawa, Nishiyama et al. 1995), Honduras (Ferrera, Olivo et al. 1999). Additional studies of US ethnic groups have also found an association between this allele and cervical cancer susceptibility in African-American (Gregoire, Lawrence et al. 1994) and Hispanic (Apple, Erlich et al. 1994) populations. In the studies listed above, where HLA class II DRB1*1301-5 was investigated it was found to have an opposite effect to that of DQB1*03 and was associated with a protective effect against cervical cancer. Other investigations have reported a significant association between progression to cervical cancer and the DQB1*06 allele in Scandinavian (Sanjeevi et al. 1996; Helland, Olsen et al. 1998) and Tanzanian (Wank, Meulen et al. 1993) populations. However, some studies have failed to confirm the DQB1*03 association with cervical cancer noted above in British (Glew, Duggan-Keen et al. 1993; Mehal et al. 1994), Dutch (Krul, Schipper et al. 1999), French (Sastre-Garau, Loste et al. 1996), Swedish (Allen, Kalantari et al. 1996) and Sengalese (Lin, Koutsky et al. 2001) populations. Of these studies two (Sastre-Garau et al. 1996; Krul et al. 1999) have also found an association between DRB1*03 and progression to cervical cancer. Of the HLA Class I alleles only

B*07 has been associated to an in susceptibility to cervical cancer and it appears to act in synergy with DRB1*0302 as women positive for both of these alleles have been shown to display the highest susceptibility to cervical cancer (Duggan-Keen, Keating et al. 1996; Hildesheim, Schiffman et al. 1998).

1.6.2 Cytokine Expression in HPV Infection

1.6.2.1 Overview

The differentiation of $CD4^+$ T cells into Th1, generating IL-2, TNF-α & IFN-γ, and Th2 producing; IL-4, IL-5, IL-6, IL-10 & IL-13 was first proposed by Mosmann and co-workers (Mosmann, Cherwinski et al. 1986; Cher and Mosmann 1987; Mosmann and Coffman 1989). In mice Th1 cytokines induce a cellular response while the Th2 cytokines elicit a humoral response. This is also observed in humans, but is less clear cut as cytokines do not have identical effects in all mammals (Del Prete, De Carli et al. 1993). Most viruses invoke a Th1 response, although a few such as HIV elicit a Th2 response (Clerici and Shearer 1993; Romagnani, Del Prete et al. 1994) more commonly associated with parasitic infection. Accordingly, HPV infection, like most viruses has been found to induce predominantly a Th1 like pattern of cytokine expression with the production of IFN-γ and reduced expression of IL-4 preceding viral clearance (Labeit, Labeit et al. 1995; Jackson, McKenzie et al. 1996; Scott, Stites and Moscicki 1999; Goncalves and Donadi 2004)

1.6.2.2 IFN-γ

The Interferons (IFN) play a key role in the antiviral, anti-parasitic, anti-tumour and immunoregulatory functions of the innate immune response especially in their antiviral capacity. Interferon gamma (IFN-γ) is a 20 or 25kDa glycoprotein produced by both $CD4^+$ & $CD8^+$ T lymphocytes and NK cells, specific IFN-γ receptors are observed on the

majority of immune cells which react by upregulating the expression of MHC class I molecules (Gray and Goeddel 1982; Farrar and Schreiber 1993) also IgG2A synthesis. IFN-γ also modulates MHC class II gene expression and is the only interferon able to perform this function (Borish and Steinke 2003). IFN-γ inhibits the proliferation of a number of normal and transformed cells and has antiviral and antiparasitic activities (Farrar and Schreiber 1993). Whilst the main host defence function of IFN-α & IFN-γ is in an antiviral capacity, IFN-γ appears to have a largely immunomodulatory role (Farrar and Schreiber 1993). IFN-γ acts (to a greater degree than IFN-α & IFN-γ) in inhibiting cellular proliferation of various cell types in conjunction with TNF-α & TNF-γ, of particular interest is its effect of inhibiting Th2 cell growth. IFN-γ also functions in combination with IL-1 and IL-2 (inhibition of the IL-2 receptor also inhibits IFN-γ synthesis) and has been found to be necessary for the expression of IL2 receptors on the T-lymphocyte cell surface. IFN-γ is therefore involved in the modulation of T-cell growth and cell mediated mechanisms of cytotoxicity (Borish and Steinke 2003).

Viruses associated with malignant transformation, such as HPV, have evolved means of evading the host immune response such as hiding within keratinocytes minimising exposure to the host immune system (Tindle 2002). This strategy is not effective against IFN-γ mediated intracellular defence, however E7 has the effect of inhibiting the interferon-stimulated gene factor 3 transcription complex (ISGF3) resulting in a weakening of IFN-α & IFN β function (Barnard and McMillan 1999). IFN-α has been used as a therapy for both genital warts and RRP and it has been seen that patients with genital warts who respond to IFN-α treatment have lower levels of E7 gene expression prior to treatment than non-responders (Arany, Goel and Tyring 1995).

An investigation into the effects of autologous papillomata upon T cell cytokine

mRNA levels *in vitro* showed significant IFN-γ mRNA levels in patients with mild disease and low levels in patients with severe disease (Bonagura, Hatam et al. 1999). However, our group has also previously demonstrated by ELISpot that IFN-γ is the primary cytokine response in RRP patients with the HLA class II allele DRB1*0301, which we have shown to be associated with severe RRP (Gelder et al. 2003). Another study has found that cervical $CD4^+$ and $CD8^+$ T cells *in vitro* from patients positive for HPV-16 express IFN-γ in response to HPV-16 L1 stimulation (Passmore, Burch et al. 2002).

1.6.2.3 TNF-α

Tumour necrosis factor alpha (TNF-α) is a pro-inflamatory cytokine secreted by numerous cells including T cells but is chiefly expressed by keratinocytes. It is cytotoxic to a diverse range of tumours in which it may induce cytolysis or cytostasis (Herbein and O'Brien 2000). It is capable of stimulating apoptosis directly or indirectly largely by binding to the TNF receptor-1 (TNFR-1) or TNFR-2. TNF-α also induces the expression of HLA class I and II molecules and differentiation antigens in cellular precursors of lymphocytes and leukocytes (Herbein and O'Brien 2000). TNF-α augments T-cell proliferation in the absence of IL2 and various T-cell subpopulations only react to IL2 in the presence of TNF-α.

TNF-α has been demonstrated to suppress expression of HPV-16 E6 and E7 at the transcriptional level in a HPV-16 immortalised keratinocyte cell line (Kyo, Inoue et al. 1994). TNF-α has also been observed to have an anti-proliferative effect on epithelial cells infected with HPV-16, although not on HPV-18 immortalised cells (Malejczyk, Malejczyk et al. 1992; Villa, Vieira et al. 1992) Further investigation has shown this that this anti-proliferative effect is caused an accumulation of hypophosphorylated Rb in HPV-16 infected cells resulting growth arrest during the G_0-G_1 cell cycle phase whilst

no such effect on Rb phosphorylation was observed for HPV-18 immortalised cells (Vieira, Goldstein and Villa 1996). A more recent study has investigated this apparent resistance of HPV-18 immortalised cell lines to the anti-proliferative effect of TNF-α in primary human keratinocytes transfected with HPV-18 whole genome or infected with recombinant retroviruses containing only E6/E7 or E7 with results which indicated that E7 alone confers partial resistance to TNF-α (Boccardo, Noya et al. 2004).

1.6.2.4 IL-2

IL-2 is produced by T-cells in response to antigenic or mitogenic stimulation, and is an influential regulator of immunity. IL-2/IL-2R signaling is required for T-cell proliferation and other important functions of the immune response and IL-2 is a growth factor for all subpopulations of T-lymphocytes (Taniguchi and Minami 1993). It is an antigen-unspecific proliferation factor for T-cells that induces cell cycle progression in resting cells and thus allows clonal expansion of activated T-lymphocytes (Smith 1988). IL-2 in the presence of IL-4 and other factors helps stimulate the proliferation of activated B-cells. Because of these roles with T-cells and B-cells IL-2 is a vital modulator of host immune responses and is involved in tumour surveillance, as well as being used extensively as an anticancer agent, especially in renal cancer (Smith 1988; Atkins, Regan and McDermott 2004; Baaten, Voogd and Wagstaff 2004; Schrama, Xiang et al. 2004). IL-2 also induces IFN-γ production in peripheral leukocytes and stimulates the secretion of TNF-α and TNF-β and IL1.

1.6.2.5 IL-4

IL-4 regulates a range of T-cell and B-cell responses including gene expression and cellular proliferation and the differentiation of naïve $CD4^+$ T-cells into Th2 cells (Jansen, Fibbe et al. 1990; Paul 1991). It is produced largely by a subpopulation of activated T-cells as well as by by mast cells, basophils and eosinophils and induces the expression

of class II MHC antigens(Paul 1991) and production of IgG1 and IgE. Like IL-2, IL-4 has also been used in cancer therapy especially in the treatment of breast and colon cancer (Dullens and De Wit 1991; Majhail, Hussein et al. 2004). Also, as many tumours express IL-4 receptor on the cell surface it has also been used for targeting tumours with other anticancer agents (Husain, Kreitman et al. 1999; Zhang, Hu et al. 2004).

1.6.2.6 IL-5

IL-5 is produced by T-cells, mast cells, and eosinophils and stimulates the activation, differentiation and proliferation of eosinophils and promotes the generation of cytotoxic T lymphocytes (CTL) from thymocytes (Yamaguchi, Hayashi et al. 1988; Takatsu and Tominaga 1991; Takatsu 1992). In humans it is produced by Th2 cells and it is also a vital regulator of immune response to parasites and some tumours (Takatsu 1992; Kataoka, Konishi et al. 2004).

1.6.2.7 IL-10

IL-10 is produced by a variety of cells including T-cells, B-cells, keratinocytes, macrophages and monocytes (Howard and O'Garra 1992; Howard, O'Garra et al. 1992; Spits and de Waal Malefyt 1992). It has a largely immunosuppressive role and enhances humoral responses whilst diminishing the cellular immune response. IL-10 also augments MHC class II expression on B-cells whilst it inhibition it on monocytes (Spits and de Waal Malefyt 1992). A recent study has shown that in response to HPV-11 E6 PBMC from RRP patients express greater levels of IL-10 than of IFN-γ (DeVoti, Steinberg et al. 2004). IL-10 causes inhibition of IFN-γ, IL-2 and TNF-β production from Th1 cells

1.6.3 The $CD4^+$ T-Cell Response to HPV Infection

1.6.3.1 Overview

In the normal course of the cellular immune response, MHC class II molecules are expressed only by professional antigen presenting cells (APC) such as B-cells, dendritic cells (DC) or macrophages. Such APC utilise endocytosis or phagosomes to procure exogenous antigen which is subsequently degraded into short peptide fragments within endosomes. These endosomes subsequently fuse with vesicles containing the MHC class II molecules which associate with the peptides whereupon this complex is exported to the cell surface where they are presented to $CD4^+$ T cells. $CD4^+$ T-cells typically identify the exogenous antigens in the context of MHC class II molecules priming them for their relevant function.

1.6.3.2 Evidence for a Role of $CD4^+$ T-Cells in HPV Infection

$CD4^+$ T-cells are observed in abundance in regressing genital warts, the bulk of which are found to be from the $CD45RO^+$ memory subset, with a significant increase in the $CD4^+$:$CD8^+$ T-cell ratio between regressing and non-regressing warts (Stanley, Coleman and Chambers 1994). There is also a significant induction of the accessory molecules intercellular adhesion molecule 1 (ICAM-1) and HLA DR within such regressing warts and this overall immune response is synonymous with a delayed-type hypersensitivity (DTH) response to foreign antigen (Coleman, Birley et al. 1994; Stanley et al. 1994). The antigenic stimului of the wart against which this response is initiated are not known but in an experimental murine model and humans imply that viral proteins are immune targets. Keratinocytes expressing E6 & E7 grafted onto immunocompetent mice when inoculated with a recombinant vaccinia virus expressing HPV-16 E6 or E7 display a $CD4^+$ T-cell dependent DTH response (McLean, Sterling et al. 1993; Chambers, Wei et al. 1994). If low levels of viral antigen are used to prime in

this murine model this leads to non-responsiveness and the loss of the DTH response associated with a Th1-Th2 cytokine expression switch in $CD8^+$ T-cells.

1.6.3.3 In Vitro $CD4^+$ T-Cell Proliferative Responses to HPV Proteins

HPV-specific $CD4^+$ T-cell proliferative responses using PBMC or short-term T-cell cultures are usually used in the study of potential HPV antigens, and a number of HPV-specific helper T-cell epitopes have been identified in this manner (Luxton, Rowe et al. 1996; Kadish, Ho et al. 1997; Luxton, Rose et al. 1997; de Gruijl, Bontkes et al. 1998; Gill, Bible et al. 1998; Hopfl, Heim et al. 2000; van der Burg, Ressing et al. 2001; de Jong, van der Burg et al. 2002; Williams, Hart et al. 2002; Welters, de Jong et al. 2003). Most such studies have concentrated upon HPV-16 as the most prevalent high risk HPV type overall worldwide among the oncogenic and the type most associated with cervical cancer risk. Such proliferative T-cell responses have been identified against HPV 16 L1 peptide in patients with various grades of cervical cancer / CIN and healthy control populations (Shepherd, Rowe et al. 1996; Luxton et al. 1997). Such experiments have also been employed by our group in a study where proliferation from T-cell lines generated from non-related healthy donors has been observed in response to 15mer peptides spanning the length of HPV-11 L1 (Williams et al. 2002). It was determined by cell sorting that the bulk of response was in the $CD45RO^+$ memory subset of $CD4^+$ T-cells, these responses were cross-reactive and the degree of cross-reactivity was inversely proportional to the degree of L1 sequence diversity between the HPV types. Proliferative responses were also observed to stimulation by HPV-11 L1 peptides in $CD45RO^+$ PBMC confirming that recognition of HPV-11 is a specific memory response in this first study of $CD4^+$ T-cell responses to HPV-11 (Williams et al. 2002). In addition to L1 such studies have investigated stimulation by peptides based upon the HPV early proteins also. Peptides based upon HPV-16 E2, E5, E6 & E7 have been shown to stimulate responses variously in healthy controls, individuals with HPV-

16 positive cervical lesions and CIN patients (Luxton et al. 1996; de Gruijl et al. 1998; Gill et al. 1998; Hopfl et al. 2000; van der Burg et al. 2001; de Jong et al. 2002; Welters et al. 2003). These studies demonstrated that such proliferative responses to stimulation with peptides based upon HPV-16 proteins were associated with persistence of HPV infection and progression to CIN III. Conversely, the results of another, similar, study found HPV-16 E6 and E7 specific proliferative responses to be associated with viral clearance (Kadish et al. 1997). Our group has also recently found proliferative responses in both RRP patients and healthy donors to HPV-11 peptides derived from E4, E6 and E7 proteins (Hillyer, Wall & Gelder – Unpublished observations).

Thus a number of HPV proteins are able to stimulate T-cell responses from individuals in both a disease and non-disease state these latter responses are likely to be a result of previous exposure to HPV. Such responses are potentially cross reactive responses, as discussed above, where the immune system has been primed by previous interaction with a different HPV type to that which it produces a proliferative response, mainly to HPV-16 in these studies.

Chapter Two:

Materials & Methods

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2.2.5 HLA Typing by Sequence-Specific Primer PCR

2.2.5.1 Overview

2.2.5.2 Molecular HLA Class I and II Typing Using Polymerase Chain Reaction with Sequence-Specific Primers (PCR-SSP)

2.3 Tissue Culture

2.1 Collection Of Samples

2.1.1 Cervical Cytobrush Samples

Cervical cytobrushes (Digene Corporation, Gaithersburg, USA) were used to swab the cervical canal, and shipped to Cardiff on dry ice. They were then stored at -70°C until the DNA was extracted.

2.1.2 Laryngeal papillomata

Laryngeal papillomata were obtained during routine debulking surgery under general anaesthetic. Then, using aseptic technique, were placed in labelled 1ml cryovials (Greiner BioOne, Stonehouse, Glos. UK). The samples were transported on ice and the biopsies snap frozen and stored in liquid nitrogen at -180°C until DNA extraction Samples sent by courier from sites other than UHW were transported frozen on dry ice at –78.5°C.

2.1.3 Genital Warts

Genital warts were obtained during routine removal, and transferred to cyrovials they were then transported to UWCM, snap frozen on liquid nitrogen and stored as above.

2.2 Molecular Biology

2.2.1 DNA Extraction

2.2.1.1 Extraction of DNA From Fresh EDTA Anticoagulated Blood

The ethylenediaminetetraacetic (EDTA) vacutainer (Becton Dickinson, Franklin Lakes, NJ, USA) whole blood sample was centrifuged at 700 x g for ten minutes followed by the the careful transfer, using a Pasteur pipette, of the PBMC 'buffy coat' at the interface between the red cells and plasma into clean 15 ml polypropylene

tubes (Greiner BioOne). To the tube was added of 10ml red cell lysis buffer (RCLB) followed by incubation at room temperature for 10 minutes then centrifuged for 10 minutes at 700 x g. This last step was repeated until no red cells were visble in the pellet followed by resuspension in 3 ml of nuclear lysis buffer with sodium dodecyl sulphate (NLB/SDS) followed by vigorous mixing by hand and the addition of 1ml 6M NaCl, then 3ml of chloroform. This was followed by another vigorous mix by hand until the sample was a uniform milky white, followed by centrifugation for 10 minutes at 700 x g. Using a Pasteur pipette, the aqueous top phase containing the DNA was carefully transferred into a clean polypropylene tube. The DNA was then precipitated by adding of 2 volumes of 95% ethanol and gently rocked back and forth until the thread like DNA precipitate was visible. This ethanol was carefully removed and the DNA washed twice in 70% ethanol after which residual ethanol was removed in a rotary evaporator for 1 hour on medium with no heat (DNA Speed Vac, Savant Instruments Inc., Farmingdale, NY). The DNA was resuspended in between 100-300µl of dH_2O dependant upon the size of the DNA pellet.

2.2.1.2 DNA Extraction from Frozen Blood

Four volumes of Swelling Buffer A (at 4°C) were added to 5ml of frozen blood that had been thawed at 37°C in a carefully labelled polypropylene tube. The sample was then vortex mixed, incubated on ice for 5 minutes then centrifuged at 1100 x g for 10 minutes. The supernatant was discarded and the pellet resuspended in 20ml of Swelling Buffer B and incubated on ice for 5 minutes followed by centrifugation at 1100 x g for 10 minutes. The pellet was resuspended in 20 ml RCLB and incubated at room temperature for 10 minutes after which it was centrifuged at 1100 x g for 10 minutes. The pellet was then resuspended in 3ml of NLB/SDS and 120µl 10mg/ml proteinase K (Sigma, Poole, UK) and incubated in a covered water bath at 42°C for 16 hours. To the sample was then added 1 ml of 6M NaCl followed by vigorous agitation for 30 seconds before 2 ml of chloroform was added. The sample was then agitated for 30 seconds until a uniform milky white colour was observed and

centrifuged at 1200 x g for 30 minutes. The upper aqueous phase containing the DNA was carefully transferred into a carefully labelled fresh polypropylene tube with a Pasteur pipette. This chloroform extraction step was then repeated. The DNA was then precipitated by the addition of 2 volumes of 95% ethanol and gently rocked back and forth until the thread like DNA precipitate was visible. The ethanol was then carefully removed leaving the DNA, which was washed twice in 70% ethanol after which residual ethanol was removed in a rotary evaporator for 1 hour on medium with no heat (DNA Speed Vac, Savant Instruments). The DNA was resuspended in between 100-300µl of dH_2O dependant upon the size of the DNA pellet.

2.2.2 Viral DNA Extraction

2.2.2.1 Viral DNA Extraction From Cervical Cytobrush Samples

After thawing samples were vortex mixed and the cytobrush removed with forceps the cellular matter and fluid were then transferred to a carefully labelled 1.5ml tube (Greiner BioOne) using a Pasteur pipette. The samples were then centrifuged at 700 x g for 10 minutes and the supernatants discarded. If the resultant pellet was blood stained then it was resuspended in 1ml RCLB, mixed then centrifuged at 700 x g for 5 minutes and the supernatant discarded. The pellet was then thoroughly resuspended in 1ml 10mMol TRIS HCl then 100µl was then aliquoted into a carefully labelled 0.5ml tube (Greiner BioOne) and the remainder aliquoted into a carefully labelled 1.5ml tube (Greiner BioOne) both were then frozen at -70°C and left overnight. The 100µl samples were then defrosted and boiled in a water bath for 10 minutes followed by immediate cooling on ice. These were then centrifuged for 10 minutes at 700 x g then transferred into a fresh carefully labelled 100µl 0.5ml tube (Greiner BioOne) then frozen at -70°C until required for further processing.

2.2.2.2 Viral DNA Extraction From Laryngeal & Genital Biopsies

Biopsies were allowed to thaw and then macerated in a petrie dish (Greiner BioOne) using a pair of scalpels and transferred to a carefully labelled 2ml tube (Greiner BioOne). DNA extraction was carried out by incubation with regular agitation in 1ml 10mM Tris-HCl (pH 7.4) containing 500µg/ml proteinase K (Sigma, Poole, UK, Poole, UK) at Pharmaceuticals 56°C for 1hour. Samples were then transferred to a boiling water bath for 10 minutes then placed immediately on ice for 5 minutes followed by centrifugation at 13krpm for 3 minutes to pellet cellular detritus then aliquoted into carefully labelled fresh 0.5ml tubes (Greiner BioOne) at 100µl per tube then frozen and stored at -70 °C.

2.2.3 Detection & Typing of HPV by PCR-ELISA

All PCR were carried out in 200µl domed thin walled PCR tubes (Greiner BioOne) negating the need for mineral oil. The primers used for this are shown in table 2.1 (Eurogentec, Southhampton, UK).

Table 2.1: HPV Detection and Control Primers

Name	Role	5' Primer Sequence 3'
GP5+	HPV Consensus Sense	TTTGTTACTGTGGTAGATACTAC
bioGP6+	HPV Consensus Antisense (Biotinylated)	GGAAAAATAAACTGTAAATCATATTC
PCO3	β-Globin Sense	ACACAACTGTGTTCACTAGC
PCO5	β-Globin Antisense	GAAACCCAAGAGTCTTCTCT

2.2.3.1 Amplification of HPV DNA

HPV DNA was detected using GP5+ / biotinylated GP6+ (bioGP6+) primer pair (Jacobs, Snijders et al. 1997). A PCR was setup in a total volume of 50µl containing the the PCR mixture listed below to which was added 5µl DNA.

PCR Setup for HPV Detection

10X dNTPs (Bioline): 5µl

10X PCR Buffer II (Perkin Elmer): 5µl

$MgCl_2$ (Perkin Elmer): 7µl

Amplitaq (Perkin Elmer): 0.2µl

GP5+ / bioGP6+: 5µl Each

dH_2O: 17.8µl

PCR Program for HPV Detection

4 min @ 94°C

60 secs @ 94°C
90 secs @ 40°C
120 secs @ 72°C
— X 40

4 min @ 72°C

2.2.3.2 Amplification of β-Globin Gene

For each sample a separate control sample was setup using the PCO3 & PCO5 primer pair amplifing a ≈200bp region of the human housekeeping gene β-globin. Amplification of the β-Globin segment was carried out to ensure that the absence of a product in the GP5+/GP6+ reaction did not just indicate a failed PCR reaction for example due to the presence of haemoglobin. The PCR setup to which was added 5µl DNA is listed below.

PCR Setup for β-Globin Detection

10X dNTPs (Bioline): 5µl

10X PCR Buffer II (Perkin Elmer): 5µl

$MgCl_2$ (Perkin Elmer): 5µl

Amplitaq (Perkin Elmer): 0.2µl

PCO3/ PCO5: 5µl Each

dH_2O: 14.8µl

PCR for β-Globin Detection

4 min @ 94°C

60 secs @ 94°C
90 secs @ 55°C
120 secs @ 72°C
— X 40

4 min @ 72°C

For each set of samples one positive and two negative control tubes were included which were the identical to the test samples. For the positive control, 5µl DNA from the HPV-16 positive cell line CaSki was added, DNA was extracted from CaSki cells (A kind gift from Dr K. Thomas & Dr S. Man) using the technique outlined in 2.2.2.1. In the two negative control tubes 5µl dH_20 was substituted for the DNA. One of the negative control tubes was closed before any DNA samples were added, and acted as an internal control to ensure that the stock reagents had not become contaminated with HPV DNA. The second negative control tube remained open whilst the DNA was added to the test PCR tubes, and served as a control for potential contamination during the PCR set-up process. Amplification was carried out on a Helena Bioscience Phoenix Thermocycler. Visual determination of a positive sample was determined by running the PCR products on a 1.5% (w/v) agarose gel containing 0.5µg/ml ethidium bromide at 15V/cm in 1x TBE buffer for 60 minutes with Orange G loading buffer and visualised with UV transillumination and photographed with a Polaroid camera. The HPV PCR gives a product of 142bp and the β-globin PCR gives a product of 186bp.

2.2.3.3 HPV Typing by DNA ELISA

After amplification and a positive agarose gel HPV type was determined by enzyme linked immunosorbent assay (ELISA) using streptavidin coated microtitre plates to capture PCR product. HPV type detection was performed using two cocktails of digoxigenin labelled probes, one for common low risk types and one for common high risk types, listed below. (Probe sequences shown in Appendix 2B)

Low Risk Cocktail

HPV-6, -11, -40, -42, -43 & -44

High Risk Cocktail

HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66 & -68

2.2.3.4 ELISA Protocol

To a streptavidin-coated well of a 96 well microtitre plate (Roche, Welwyn Garden City, UK) 5µl of the biotinylated PCR product was added into each followed by 50µl 1xSSC/ 0.5% tween. The plates were then sealed with an adhesive plate sealing strip, and incubated at 37°C for 1 hour then washed 3 times with 1x Sodium chloride – Sodium Citric acid (SSC) / tween solution on an automated plate washer. The DNA was then denatured by the addition of 100µl 0.2N NaOH and incubated at room temperature for 15mins. This was followed by another 3 washes with 1x SSC / tween the digoxigenin labelled probes were then hybridised by the addition 50µl of the appropriate probe mixture to each well and incubated for 1 hour at 37°C. The wells were washed a further 3 times, and 50µl of diluted conjugate (Anti-digioxigenin-alkaline phosphatase F_{AB} fragments, diluted 1:5000 in 1x SSC/0.5% Tween 20) (Roche) was added to each well and incubation at 37°C for 1 hour. After a final 5 washes, 100µl substrate solution (1 tablet of pNPP with 1 tablet of Tris (Sigma) in 5ml dH_20) was added to each well. Plates were then incubated at 37°C, and optical densities (OD) measured with a UV/Vis spectrophotometric plate reader with the absorbance measured at the dual wavelength, 415 & 595nm at 1, 3, and 24 hours. For the distinction between positive and negative samples, the OD values obtained at 24 hours after substrate incubation were taken into account, and an OD greater than 3 times background was taken as a positive reading.

After high- or low-risk (or both) HPV type had been determined, the specific HPV-type(s) present were identified using the individual oligonucleotide probes. The ELISA was performed as above for determining high- and low-risk types, but used individual probes at a 0.5pmol/ well, the sequences for these probes are shown in table 2.3 and 2.4.

2.2.4 Sequencing

2.2.4.1 Initial Amplification to Provide DNA for the Sequencing Reaction

The initial reaction used 5µl DNA, 5µl of each of the two primers (both at 5µM); 30µl Master Mix {Amplitaq (Perkin Elmer) 200µM dNTP's (Roche), 10X PCR Buffer II (Perkin Elmer), 2.9mM $MgCl_2$ (Perkin Elmer) & dH_2O pre-mixed) and 5µl of additional dH_2O. PCR program: (94°C 4mins) x1, (94°C 1min 30s, 60°C 1min 30s , 72°C 2mins) x10, (94°C 1min 30s, 55°C 1min 30s, 72°C 2min 30s) x20, (94°C 2mins, 50°C 1min 30s, 72°C 2mins) x10, (72°C 5mins) x1

2.2.4.2 Sequencing using the Beckman Coulter CEQ 2000

Sample Preparation

DNA from the amplification PCR was purified using the 'Spin Prep' purification kit (Novagen, Nottingham, UK). Reaction mix: 100fmol DNA (0.5-10.0µl depending on concentration), 8µl DTCS 'Quick Start' mastermix (Beckman Coulter) 2µl primer at 1.6µM (Oswel), dH_2O to total volume of 20µl. These were then run on the following PCR program: 30 cycles of 96°C for 20s, 50°C for 20s, 60s for 4mins. These samples were then transferred to 5µl microfuge tubes along with 4µl of stop solution (1.5M NaOAc (Sigma) & 50mM EDTA (Sigma)), 1µl of 20mg.ml^{-1} glycogen (Beckman Coulter), 1µl of 'pellet paint' (Novagen), and 60µl 95% (v/v) EtOH (kept at -20°C). This mixture was then vortexed briefly and centrifuged at 14K rpm at 4°C for 15mins after which the supernatant was then removed. The pellet was then washed in 200µl 70% (v/v) EtOH (kept at -20°C) twice, after each rinse the samples were centrifuged at 14krpm at 4°C for 2mins followed by removal of the supernatant. This was followed by drying in the rotor evaporator (DNA Speed Vac, Savant Instruments) for 40mins with no heat after which they were resuspended in 'sample loading solution' (Beckman Coulter) and transferred to a 96 well plate (Beckman Coulter) and overlaid with 1 drop of mineral oil (Beckman Coulter)

Sample Loading

The sample plate is loaded on the sequencer along with the wetting tray (filled with dH_2O), the buffer plate (with the wells corresponding to the sample wells filled with loading buffer (Beckman Coulter)). The capillary array was then put in place as well as the sequencing gel (Beckman Coulter) and the instrument set for a 'long fast run'.

Sequencing Primers

The sequences of the MY09/11 primer pair used in the sequencing of L1 in Chapter 3 are shown in Table 2.2. Sequencing primers used in Chapter 4 for the Sequencing of HPV-6 and HPV-11 are shown in full in Appendix 2C

Table 2.2: Consensus HPV Primers Used for sequencing

Name	5' Primer Sequence 3'
MY09	GATCAGTWTCCYYTKGGACG
MY11	GCMCAGGGWCATAAYAATGG

M=A or C

R=A or G

W=A or T

Y=C or T

2.2.5 HLA Typing by Sequence-Specific Primer PCR

2.2.5.1 Overview

Typing of HLA class I and II alleles was performed using PCR with sequence specific primers (PCR-SSP) based phototyping system developed by Dr M. Bunce and Dr K. Welsh (Bunce, O'Neill et al. 1995).

2.2.5.2 Molecular HLA Class I and II Typing Using Polymerase Chain Reaction with Sequence-Specific Primers (PCR-SSP)

PCR Setup

A panel of sequence specific primers (Eurogentec) representing individual HLA alleles were used in individual reactions on a 200µl 96 well PCR plate (ABgene, Epsom, Surrey) In a total reaction volume of 13µl were 5µl of diluted primers and 8µl of master mix (250U Amplitaq (Perkin Elmer) & 10ml of TDMH (see Solutions)) with10µl of mineral oil (Sigma). A total of 144 reactions were used to perform a complete Class I and II typing.

PCR Program for HLA Class I & II Typing

DNA amplification were performed using Helena Bioscience Phoenix Thermocyclers with the following cycling paramaters: 1 minute at 96°C; 5 cycles of 25s at 96°C, 45s at 70°C, 45s at 72°C followed by 21 cycles of 25s at 96°c, 50s at 65°C, 45s at 73°C, and finally 4 cycles of 25s at 96°c, 60s at 55°c and 120s at 72°c. Following PCR 10 µl of loading buffer consisting of 0.25% Orange G, 30% v/v glycerol and 0.5% TBE (89mM Tris base, 89mM boric acid, 2mM EDTA, pH 8.0) was added to each reaction mixture. PCR products were then size fractionated on a 1.5% (w/v) agarose gel containing 0.5µg/ml ethidium bromide at 15V/cm in 1x TBE buffer for 30 minutes and visualised with UV transillumination and photographed with a Polaroid camera.

2.3 Tissue Culture

2.3.1 Preparation of AB Serum

After a batch test selection a single batch of pooled human AB serum was obtained from the Welsh blood Transfusion Service, (Pontyclun, Rhondda-Cynon-Taf, UK) and stored at -70°C until prepared. The serum was thawed and centrifuged at 30K rpm under vacuum for 1 hour at 4°C to remove any lipid contamination. The serum was carefully transferred into sterile Schott bottles and heat-inactivated at 56°C for 30 minutes with frequent mixing. The serum was then sterile filtered through a 0.2μ filter, aliquoted and stored at -40°C until required.

2.3.2 Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells PBMC were isolated from fresh heparinised peripheral venous blood, using centrifugation on a discontinuous Histopaque 1077 gradient (Sigma). Fresh whole blood was then added to an equal volume of heparinised medium and gently mixed. This mixture was then carefully layered on top of an equal volume of chilled Histopaque in polypropylene (Greiner BioOne, UK) tubes. Tubes were then centrifuged at 700 x g for 30 minutes in a centrifuge pre-cooled at 4°C and allowed to stop slowly with no brake. PBMC were removed from the plasma / density gradient interface, and transferred to a fresh polypropylene tube to which at least an equal volume of heparinised medium was added. Samples were centrifuged at 500 x g for 20 minutes at room temperature. The pellet was resuspended and washed in 50ml of heparinised medium and centrifuged at 400xg for 15 minutes. After a further 2 washes the PBMC were resuspended in a total volume of 25 ml heparinised medium. The number of cells was determined using white cell counting fluid (2% glacial acetic acid with gentian violet). Yields varied from donor to donor but were normally about $2.0x10^6$/ml (range 0.8–3.0 $x10^6$ cells/ ml of venous blood).

2.3.3 Freezing of PBMC

Freshly isolated PBMC were surplus to requirements were frozen in dimethyl sulphoxide (DMSO) (Sigma) at a concentration of 15 to $20x10^6$ cells/ml. Unused cells were centrifuged at 400xg for 15 minutes and resuspended in 0.5 ml of ice-cold neat heat-inactivated human AB serum. The cells were then incubated on ice for 20 minutes and a solution of 20% v/v DMSO in heat-inactivated serum was made up, and 0.5ml added to labelled cryovials (Greiner BioOne). The cold cells in serum were gently resuspended and 0.5ml added to each cryovial, transferred into Nalgene Cryo 1°C freezing chambers, and frozen overnight at -70°C. If cells were not to be used within 1 to 2 days the cryovials were stored at -180°C in liquid nitrogen.

2.3.4 Thawing of Frozen PBMC

PBMC, stored at -180°C in liquid nitrogen, were thawed rapidly in a 37°C water bath and transferred to a sterile 20ml universal container. Drop-wise with continuous mixing, 5ml of thawing medium, at 37°C, was gradually added over 5 minutes followed by the rapid addition of a further 5ml. The cells were then centrifuged at 400 x g for 10 minutes. The supernatant was discarded and the pellet resuspended in 10ml of warmed thawing medium. After a further centrifugation the cells were resuspended in complete medium and viability was assessed using 0.4% Trypan Blue (Sigma) under a light microscope.

2.3.5 Tritiated Thymidine Incorporation (Proliferation) Assay

2.3.5.1 MoFlo Cell Sorting

Following isolation and resuspension in 5 ml complete media (see above), PBMC were stained by the addition of 200µl FITC conjugated anti-CD45RA (Becton Dickinson biosciences) and 200µl PE conjugated anti-CD45RO (BD biosciences) for 30 minutes at 4°C in the dark. Stained PBMCs were washed once using fresh media

and resuspended at approx. $1x10^7$ cells /ml in complete medium before sorting on a MoFlo cell sorter (Cytomation, Freiburg, Germany) into CD45RA high / CD45RO low (RA+) and CD45RA low / CD45RO high (RO+). All cell sorts were checked by FACS analysis and were >98% pure in all cases.

2.3.5.3 Proliferation Assay Protocol

Peptides (detailed in Chapter 6) were added to individual wells in 48 well flat bottomed plates (Greiner BioOne) at 500µg/ml (10µl/well). In separate wells were added 5µl HPV-11 L1 VLP @ 1µg/ ml, 1µl influenza haemagglutinin @ 0.1µg/ml, 5µl tetanus toxoid @ 15µl/ml, 2µl tuberculin purified protein derivative @ 2µl/ml, each acting as a positive control with an empty well acting as a negative control. To all of these wells were added PBMC irradiated with 3000rads at $3x10^5$ cells/ml (1ml per well) followed by incubation for 3hrs at 37°C, in air supplemented with 5.0% CO_2. The plates were then centrifuged for 5 minutes at 700xg and the media discarded followed by immediate washing with the addition of complete media (1ml per well) and the previous step repeated. Autologous PBMC were then added to each well @ 4×10^6 (1ml per well) and incubated for 8 days at 37°C, in air supplemented with 5.0% CO_2

On days 7 & 8 150µl aliquots from each well were transferred in triplicate to 96 well plates (Greiner BioOne) and pulsed with 0.5µCi/ well of tritiated thymidine (Amersham Pharmacia Biotech). These plates were incubated for 16 hours at 37°C, in air supplemented with 5.0% CO_2 followed by harvesting using a cell harvester (Tomtec, Hamden, USA) and quantification of proliferation by liquid scintillation spectroscopy. Stimulation indices (SI) (cpm of proliferative response of PBMC to antigen/ cpm of proliferative response of PBMC to media control) were calculated and a response three times the geometric mean of the controls was taken to be significant.

On days 2 & 7 two separate aliquots of 60µl were taken from each well, transferred to separate cryovials (Greiner BioOne), snap frozen in liquid nitrogen and stored at -90°C ready for the CBA assay. On day 2 200µl of complete media was added after removal of the aliquots.

2.3.6 Cytometric Bead Array for the Detection of Cytokines

Quantitative analysis of the cytokines IFN-γ, TNF-α, IL-10, IL-5, IL-4 and IL-2 produced in the proliferation assay was performed supernatant at day 2 and day 7 (see Chapter 6) using a human Th1/Th2 CBA kit (Becton Dickinson biosciences) following the manufacturers guidelines. This involved incubating 50µl of supernatant with a mixture of cytokine specific antibody coated beads of different fluorescence intensities. The amount of cytokine bound to each bead was detected by PE conjugated antibodies specific to each of the six cytokines. The samples were then washed, and analysed using a FACScalibur (Becton Dickinson biosciences) and concentrations found by measuring against known concentrations of cytokine standards. Results were analysed using Cellquest (Becton Dickinson), Cricket Graph (Computer Associates International) and the CBA analysis software (Becton Dickinson biosciences). The specific application of this protocol in this thesis is outlined in detail in Chapter 6.

Appendix 2A: Reagents

DNA preparation

Red cell lysis buffer (RCLB)

0.144M ammonium chloride (NH4Cl)1mM sodium bicarbonate (NaHCO3)

Dissolve 15.4g of NH4Cl and 1.68g of NaHCO3 in 2L dH_20

Nuclear lysis buffer (NLB)

10mM Tris-HCl pH8.2

0.4M Sodium chloride (NaCl)

2mM di-sodium EDTA pH 8.0 (Na2EDTA)

23.37g of NaCl in 900ml of dH_20

10ml 1M Tris-HCl pH8.2

10ml Na2EDTA pH8.0 made up to 1L dH_20

10% w/v sodium dodecyl sulphate (SDS)

100g of sodium dodecyl sulphate in 1L dH_20

Stored at room temperature to prevent precipitate forming

NLB+SDS buffer

300ml NLB

20ml 10% w/v SDS

Stored at room temperature to prevent precipitate forming

Red Cell Lysis Buffer (RCLB)

0.144 M NH_4Cl

1.0mM $NaHCO_3$

dH_2O to 1L

Swelling Buffer A

10mM Tris-HCL, pH 8.2,

20mM NaCl

dH_2O to 1L

Swelling Buffer B

10mM Tris-HCl, pH8.2,

20mM NaCl, 1% v/v NP40

dH_2O to 1L

PCR

TDMH

33.3ml x10 base buffer

25.1ml 25mM fresh MgCl

140.4ml dH_20

1.215ml dNTP mix (i.e. all 4 mixed together)

10x Base buffer

670mM Tris-base pH8.9

166mM Ammonium sulphate

1%v/v Tween 20 (Polyoxyethylenesorbitan monolaurate)

Dissolve 40.568g Tris base in 400ml dH_2O and adjust to pH 8.9 with conc. HCl. Dissolve 10.96g of ammonium sulphate in the Tris solution. Filtered through a 0.22 micron filter into an autoclaved bottle. Add 5ml of Tween and make up to 500ml with dH_20. Store at -70°C.

Electrophoresis

Orange G loading buffer

Combine 300ml of glycerol

250ml of 2xTBE

550ml dwater

0.25g orange G.

TBE Electrophoresis Buffer

2x TBE (Stock)

216g of Tris-base

110g of boric acid

9L dH_2O

8ml of 0.5M EDTA pH 8.3 and make up to 10L with dH_20.

0.5x TBE

750ml of dwater with 250ml of 2xTBE.

5.3. 1% agarose (one litre).

10g Electrophoresis grade agarose.

One litre 0.5x TBE

10µl 10mg/ml ethidium bromide.

Appendix 2B: ELISA Probes

Table 2.3: Low Risk HPV ELISA Probes

HPV Type	5' (Dig.) Probe Sequence 3'
6	atccgtaactacatcttccacatacaccaa
11	atctgtgtctaaatctgctacatacactaa
40	gctgccacacagtcccccacaccaacccca
42	ctgcaacatctggtgatacatatacagctg
43	tctactgaccctactgtgcccagtacatat
44	gccactacacagtcccctccgtctacatat

Dig: Digoxigenin Label

Table 2.4: High Risk HPV ELISA Probes

HPV Type	5' (Dig.) Probe Sequence 3'
16	gtcattatgtgctgccatatctacttcaga
18	tgcttctacacagtctcctgtacctgggca
31	tgtttgtgctgcaattgcaaacagtgatac
33	tttatgcacacaagtaactagtgacagtac
35	gtctgtgtgttctgctgtgtcttctagtga
39	tctacctctatagagtcttccataccttct
45	acacaaaatcctgtgccaagtacatatgac
51	agcactgccactgctgcggtttccccaaca
52	tgctgaggttaaaaaggaaagcacatataa
56	gtactgctacagaacagttaagtaaatatg
58	attatgcactgaagtaactaaggaaggtac
59	tctactactgcttctattcctaatgtatac
66	tattaatgcagctaaaagcacattaactaa
68	tctactactactgaatcagctgtaccaaat

Appendix 2C: PCR Primers

Table 2.5: HPV-11 L1 Primers

Primer	Range		Orientation	5'	Sequence	3'
L1	5771	7276				
P1	5701	5721	Sense	g a c t t c t a t t t g c a t c c t a c a		
P3	6251	6271	Sense	c c g t t a g g t g a a c a t t g g g g t		
P4	6751	6771	Sense	t g t t a c t g t g g t a g a t a c c a c		
P5	6271	6291	Antisense	t t t g a a c a t t g t g t a c c c t t a		
P6	6771	6791	Antisense	g t g t c a t a t t t g t a c t g c g t g		
P8	7326	7346	Antisense	a t a c a c a a t a c a a g a a a c a t a		
P9	6945	6945	Sense	c t t c t g t t t t g g a g g a c t g g		

Table 2.6: HPV-11 E6 Primers

Primer	Range		Orientation	5'	Sequence	3'
E6	102	554				
P1	32	52	Sense	g g g a c c g a a a a c g g t t c a a c		
P2	604	624	Antisense	t g t c t t c t a a t t g c t c a t a g		

Table 2.7: HPV-11 E7 Primers

Primer	Range		Orientation	5'	Sequence	3'
E7	530	826				
P1	460	480	Sense	a t t g g g a a a g g c a c g c t t c a		
P2	876	896	Antisense	c t t c t a c c a t a a a c c a t c c t		

Table 2.8: HPV-11 L1 Primers

Primer	Range		Orientation	5'	Sequence	3'
L1	5790	7292				
P1	5720	5740	Sense	g g a t t t a t t t g c a t c c t g c a		
P3	5770	5790	Sense	t t g g g c g a g c a t t g g g g t a a a		
P4	6270	6290	Sense	t a c t g t g g t a g a t a c c a c a c a c g		
P5	6770	6790	Sense	g t a t t a g t a c a c t g t t t a c c t		
P6	6290	6310	Antisense	a t a a t g t c a t g t t g g t a c t g c		
P8	6790	6810	Antisense	t a a c a g t a c a t a a a c a c a t a t		
P9	7292	7312	Antisense	c a t t a c c t g t c a a a a g c c c a c		

Table 2.9: HPV-11 E6 Primers

Primer	Range		Orientation	5'	Sequence	3'
E6	103	555				
P1	33	53	Sense	g g g a c c g a a a a a c g g t t g g g a		
P2	605	625	Antisense	t g t c t a c t a a t t g c t c a t a g		

Table 2.10: HPV-11 E7 Primers

Primer	Range		Orientation	5'	Sequence	3'
E7	531	827				
1	461	481	Sense	t a c t a a c c a a g g c a c g g t t c		
2	877	897	Antisense	c t t c t a c c a t a a a c c a t c c t		

Appendix 2D: Tissue Culture Media

Heparinised Medium

Minimum Essential Medium Eagle (MEM) (Invitrogen)

10 units/ml preservative free sodium heparin (CP Pharmaceuticals, Wrexham, UK)

1% v/v foetal calf serum (Invitrogen).

Wash Medium

MEM (Invitrogen)

2mM L-glutamine (Invitrogen)

100IU/ml penicillin/streptomycin (P/S) (Invitrogen)

Complete Medium

Dulbecco's Modified Eagles Media (DMEM) (Sigma) *

2mM L-glutamine (Invitrogen)

100IU/ml (P/S) (Invitrogen)

7.5% v/v of heat-inactivated pooled human AB serum

*Single batch of DMEM was selected by batch testing & used in all T-cell experiments

Sigma Lot Number: 62K2389

Thawing Medium

Minimum Essential Medium Eagle (Invitrogen)

2mM L-glutamine (Invitrogen)

100IU/ml (P/S) (Invitrogen)

20% heat-inactivated foetal calf serum (Invitrogen).

Appendix 2E: Control Antigens Used in Proliferation Assay

HPV-11 L1 Virus-Like Particles (VLP)

Purified recombinant HPV-11 L1 capsid protein was a kind gift from Dr James Cook, Merck Research Labs, West Point, PA, USA. The preparation was produced using a *Saccharomyces cerevisiae* expression system of a full length HPV-6/-11 hybrid gene which self-assembled into virus-like particles (VLPs) (as described in Chapter 1). The purity of the protein was >98% by Coomassie-stained SDS/PAGE and was suspended in a solution containing 1.25M NaCl and 50mM MOPS buffer, pH 7.0. The VLP was aliquoted into 1ml volumes and stored at -70°C until needed, but appeared stable for a short period when stored at 4°C.

Adsorbed Tetanus Vaccine

Adsorbed Tetanus vaccine was obtained from Evans Medical Limited, Regent Park, Leatherhead, England, and contained not less than 80IU/ml (27 lf/ml (limitation of flocculation) of tetanus toxoid adsorbed onto aluminium hydroxide. It was used at 15μl/ml (equivalent to 1.2IU or 0.405lf).

Haemagglutinin

Haemagglutinin from Influenza A/Beijing/32/92 (H3N2) was a generous gift of Dr R. Brands Solvay Duphar BV, Weesp, The Netherlands, and was used at a working concentration of 0.1μg/ml.

Tuberculin Purified Protein Derivative (PPD)

Tuberculin PPD was (100,000 units/ ml) was obtained from Evans Medical Limited, Regent Park, Leatherhead, England, and was used at 2μl/ml (200 units/ ml).

Chapter Three:

Host-Virus Interactions in a High Risk HPV Mediated Disease in a Rural, Unselected Non-Caucasoid Cohort

Contents

3.4 Discussion

3.5 Concluding Remarks

3.1 Introduction

3.1.1 Aims and Scope of Chapter 3

As described in Chapter 1 HPV is the mediator in both benign and malignant disease and that this is frequently determined by HPV type (low or high risk) but that variable host factors also play a major role in disease progression. Any comprehensive study of such host-virus interactions in HPV mediated disease and how they influence disease pathology should therefore include both malignant and non-malignant disease. As discussed in 1.4, cervical cancer is a disease mediated by HPV and is the most common malignant disease caused by HPV responsible for 450,000 deaths worldwide each year worldwide (Koulibaly, Kabba et al. 1997; Bah, Parkin et al. 2001; Crum and Rivera 2003). The greatest burden of this disease is carried by the developing world where cervical screening programs are either not affordable or impractical whilst in industrialised nations, such as the USA, the cancer rate is low (13,500 cases per year) (Crum and Rivera 2003). Prophylactic vaccines are proposed for such populations to decrease this disease burden. It is essential in such developing populations to find determine if the same HPV associations found in industrialised predominantly caucasoid nations apply to a rural non-caucasoid population with dissimilar host immune genetics and how it affects disease pathology. It is also critical in terms of the host versus virus relationship to investigate the viral genetics and how this compares to the same HPV types in industrialised populations.

3.1.2 Worldwide HPV Prevalence Variation

Cervical cancer is the second most frequent cause of cancer death in women world-wide (Franco, Schlecht et al. 2003). Regardless of the detection method used, the relative prevalence of HPV types varies throughout the world. This may be due to a variety of factors; sociological factors e.g. religious practices or sexual behaviour, demographic e.g. age or age range of sexual activity or host factors e.g. HIV status or

HLA type (Burk, Kelly et al. 1996; Koutsky 1997; Svare, Kjaer et al. 1998). Therefore HPV type prevalence studies in one part of the world cannot automatically be applied to another. This means that although, worldwide, HPV-16 is most frequently found type in cervical cancers (Muñoz, Bosch et al. 2003) the spectrum of HPV prevalence from country to country and from urban to rural populations can be quite diverse (Koutsky 1997; Nindl, Lotz et al. 1999).

3.1.3 Cervical Cancer and HPV Prevalence in The Gambia

No previous unselected studies currently exist from a rural West African population, so the distribution of HPV types here is unknown. The population of the West African state of The Gambia, due to its largely dispersed, rural nature is difficult to screen conventionally for cervical cancer. The Gambia also has a relatively stable population and, unlike most of sub-Saharan Africa has relatively low rates of HIV/AIDS which, as described in Chapter 1 is a confounding factor in the study of HPV prevalence (Ramsay 1993, Da Costa, 1994; Schim van der Loeff, Sarge-Njie et al. 2003). This study aims to assess the feasibility of a prophilactic strategy in The Gambia and potentially other rural populations in developing nations. It is the largest HPV point prevalence investigation into type, genetic variation and relationship to cervical cancer in an unselected rural sub-Saharan African population ever conducted (Tables 3.19-3.21).

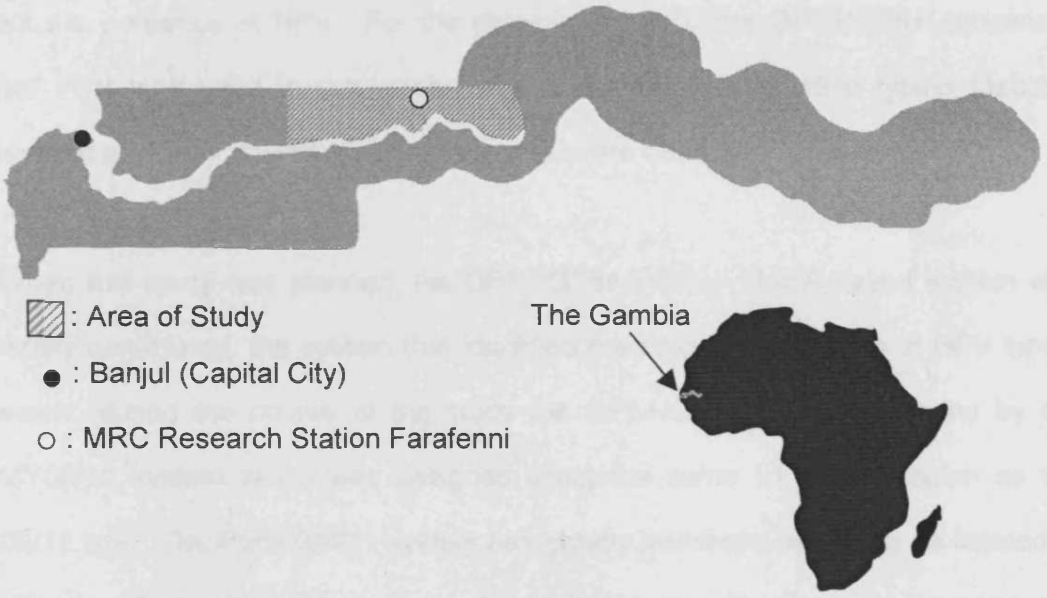
3.2 Methodology

3.2.1 Epidemiology

The samples for this study were obtained between January and July 1999 during the course of a major reproductive morbidity study (RMS) carried out under the auspices of the MRC (Walraven, Scherf et al. 2001). Women of reproductive age (15-

54), in twenty villages surrounding the MRC research station in Farafenni in rural Gambia were contacted and invited to take part. Of those approached 1,348 (72% of those suitable within the population) agreed to participate.The collection of samples was based at the MRC research station in Farafenni, Gambia, the area included in the study is shown in Figure 3.1.

Figure 3.1: Map of Gambia Showing Study Area



3.2.2 Ethics

Informed consent was obtained from all participants and this study was approved by the ethics committee of The Gambian Government / MRC Laboratories. All work was conducted in accordance with the Helsinki Declaration of 1975 as revised in 1983.

3.2.3 Sampling

Cervical cytobrushes (Digene Corporation, Gaithersburg, USA) were used to swab the cervical canal, and shipped to Cardiff on dry ice. Cervical swabs were collected by Dr Gjis Waldraven (MRC, Gambia), Dr Caroline Scherf, Prof. Alison Fiander, and Dr Mererid Evans (UWCM). Cervical cytology was carried out by the Department of Cytology Llandough Hospital.

3.2.4 HPV Detection and Identification

Substantial evolution in HPV detection methods in recent years has led to enhanced detection sensitivity and type specificity. Despite this there is still no 'gold standard' method for HPV detection therefore comparisons between studies are problematic. The main divergence in methodology is in the method used to actually type the HPV as most studies are based on initial consensus primer PCR amplification of HPV DNA to detect the presence of HPV. For the detection of HPV the GP5+/GP6+ consensus primer PCR was used in conjunction with a DNA ELISA for HPV typing (Jacobs, Snijders et al. 1997). Detailed methods for these are described in Chapter 2.

When this study was planned, the GP5+/GP6+ PCR – ELISA based system was generally considered, the system that identified the broadest spectrum of HPV types. However, during the course of the study the GP5+/GP6+ was superseded by the PGMY09/11 system which was designed using the same L1 primer region as the MY09/11 pair. The PGMY09/11 system has greatly increased sensitivity as instead of the degenerate nucleotides used by the MY09/11 pair to allow for heterogeneity between types a battery of five sense (PGMY11) and thirteen antisense (PGMY09) primers are employed (Gravitt, Peyton et al. 2000). All samples in this study were HPV typed by the Author. Some initial HPV typing was carried out by Dr CS Scherf and Dr KW Hart; all samples were rechecked at least once by the Author.

3.2.5 Sample Controls

In order to guarantee the existence of an adequate DNA in the cervical samples and to ensure the absence of PCR inhibitors, a PCR for the human housekeeping β-globin gene was carried out in tandem with each HPV PCR. This was performed using the primers and PCR protocol for β-globin described in Chapter 2.

3.2.6 Sequencing

Sequencing was carried out on a Beckman Coulter CEQ2000 Automated Sequencer (Beckman Coulter, Fullerton, CA, USA) by the protocol described in Chapter 2 to a >98% accuracy. An NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) search was carried out for each of the consensus strands produced by sequencing at least twice in each direction to generate a consensus sequence. Alignment, comparative DNA analysis, and phylogenetic tree construction was carried out using the Lasergene suit of software (DNA* Inc. Madison, Wisconsin, USA).

Early attempts at sequencing samples utilised the GP5+/GP6+ primer pair however this approach proved problematic as this primer pair amplifies a region of the HPV L1 gene 142 bp in length. This is unsuitable for identifying HPV types or isolating variations by sequencing. Subsequently the MY09/MY11 primer pair was selected which amplifies a 455 bp region of the HPV L1 gene that encompasses that amplified by the GP5+/GP6+ pair. These degenerate primers have been used extensively to classify HPV-types from this region alone (Chan, Delius et al. 1995) as the region they amplify contains highly conserved amino acids, which simplifies sequence alignment.

3.3 Results

3.3.1 Sample Suitability for HPV Analysis

Of the 934 cervical cytobrush samples taken, β-Globin was not successfully amplified in 224 samples despite repeated testing, in none of these samples was HPV detected. These samples may have been incorrectly taken or damaged during transfer from the Gambia to the UK. Prevalence figures are therefore based upon the cohort of 710 samples positive for β-Globin.

3.3.2 HPV Typing

3.3.2.0 HPV Typing Overview

HPV DNA was detected in 93 of 710 cytobrush samples giving a HPV prevalence of 13.09%. HPV-16 (19%) and HPV-35 (10%) were most common HPV types and other prevalent types were HPV-18, -33 and -58 (each 8%), HPV-31 (7%) and HPV-42 (5%). An overall summary is shown in Table 3.2, HPV type distribution is shown in Table 3.3 and Figure 3.5. Dual HPV infection was found in 16/93 samples (17%) and no instances of multiple infection greater than two were found. The distribution of dual infection combinations is shown in Table 3.4 and Fig. 3.6. Samples found to be positive by PCR but negative by ELISA were were typed by sequencing. These were potentially novel, previously unidentified types, however all samples successfully sequenced were found to be types previously characterised.

Table 3.2: Summary of Overall HPV Typing Results

Total of HPV Infections	**109**
Total of Identified Infections	105
Total Infected Individuals	93
LR : HR	11 : 89
%HPV +ve Individuals	13.09%

Table 3.3: Distribution of All HPV Types (inc. Multiple Infections & Sequence Typing)

HPV Type	6	11	16	18	31	33	35	39	40	42	43	44	45	51	52	56	58	59	66	67	68	70	82	X
n	1	1	21	9	8	9	11	2	1	6	1	2	5	4	6	3	9	0	1	1	2	1	1	4
% Total*	1	1	20	9	8	9	10	2	1	6	1	2	5	4	6	3	9	-	1	1	2	1	1	-

* Total Known HPV Types

Table 3.4 Totals of All Multiple HPV Infection Combinations

HPV Type	& 16	11 & 44	16 & 35	16 & 45	16 & 58	18 & 45	31 & 33	31 & 35	33 & 58
n	1	1	1	2	1	1	2	5	2
% Total*	6	6	6	13	6	6	13	31	13

*Total of Multiple infections

HPV types in white are low oncogenic risk and black are high oncogenic risk

3.3.2.1 HPV Typing by PCR-ELISA

HPV typing by PCR-ELISA was successful in 93 samples (Tables 3.3 & 3.4); six of the HPV types found in these have been classified in studies worldwide as low oncogenic risk and thirteen as high oncogenic risk types. Of these 93 samples, 16 had dual infection and no instances of multiple infection greater than two types were found.

3.3.2.2 HPV Typing by Sequencing

Of the 104 cervical samples where HPV DNA was successfully amplified by PCR, 11 could not be typed by ELISA. Five had possibly degraded in storage, two contained multiple templates and, therefore, with consensus primers produced unreadable sequences. Four samples; 2006D, 2712F, 2781F and 2919F, were successfully sequenced and identified using the MY 09/11 primer pair. All discussion of sequencing pertains to the 415bp region of the MY09/11 amplified region excludes the primer regions, as the degenerate nature of the primers makes the sequence unreliable along their length.

Sample 2006D

The amplified region of sample 2006D possesses a 90.8% DNA homology with the reference strain of HPV-82 (Table 3.5 & Fig.3.2). HPV-82 is a high oncogenic risk HPV type for both CIN & vaginal intraepithelial neoplasia (VIN), first isolated in 2000, it is closely related to HPV-26, -51, and -69. Moreover, there is a published HPV-82 subtype identified by the MY09/MY11 primer pair identical in sequence to sample

2006D. The HPV-82 Subtype HPV-AE2 (Genbank accession No. U01532) is sourced from a study in New York, USA (Prof. R.D. Burk RD Burk, Albert Einstein College of Medicine, New York, USA – Personal Communication) and is fully homologous to sample 2006D. An additional HPV-82 subtype from the same study (Genbank accession No. AF293961) has two silent single point mutations to sample 2006D whilst a sample originating from Argentina, HPV-IS039 (Genbank accession No.U12481), has a single silent single point mutation to 2006D.

Table 3.5: Comparison of Most Closely Homologous Sequences to Sample 2006D

Genbank Accession No.	Isolate ID (Isolate Type)	Origin	Homology to Gambian Sample		
			% DNA Homology	Silent Mutations	Polymorphisms
U01532	AE2 (MY9/11)	USA	100	0	0
U12481	IS039 (MY9/11)	Argentina	99.8	1	0
AF293961	AE2/IS039 (Full)	New York, USA	99.5	2	0
AB027021	HPV-82 (Ref.)	Japan	89.0	38	8

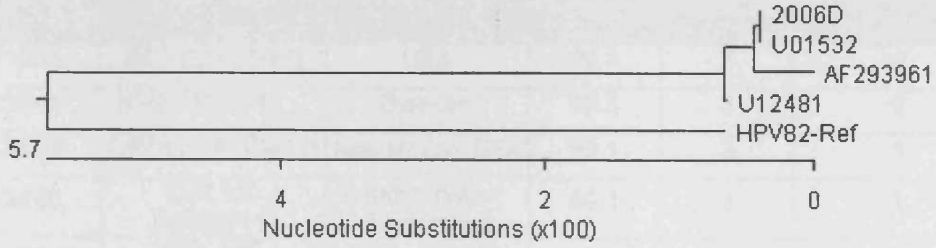


Figure 3.2: Phylogenetic Tree of BLAST Results for Sample 2006D

Sample 2712F

Sample 2712F displays 99% DNA homology to the reference strain of HPV-67, with 3 missense and 1 silent mutation (Table 3.6). It therefore appears to be a variant of this rare high oncogenic risk type, not included in the high risk HPV probe cocktail (described in Chapter 2), first isolated from a VIN but most frequently found in CIN and squamous cell carcinoma.

Table 3.6: Comparison of Most Closely Homologous Sequences to Sample 2712C

Genbank Accession No.	Isolate ID (Isolate Type)	Origin	Homology to Gambian Sample		
			% DNA Homology	Silent Mutations	Polymorphisms
D21208	HPV-67 (Ref.)	NK	98.3	4	3
U12492	(MY09/11)	NK	97.8	6	3

Sample 2781F

Sample 2781F shows 99.3% DNA homology (3 silent mutations) from the reference strain of HPV-70 (Table 3.7 & Figure.3.3) and only two silent single point mutations compared to the AE1 sub-type of HPV-70 (Genbank accession No. U01535). HPV-70 is a rare high-risk type, first isolated from a cervical wart and found in other anogenital warts. It was not included in the high risk probe cocktail. Sample 2781F is, therefore, infected with the AE2 subtype of HPV-70.

Table 3.7: Comparison of Most Closely Homologous Sequences to Sample 2781F

Genbank Accession No.	Isolate ID (Isolate Type)	Origin	Homology to Gambian Sample		
			% DNA Homology	Silent Mutations	Polymorphisms
U01535	AE1 (MY09/11)	USA	99.5	2	0
U21941	HPV-70 (Ref.)	Sweden	99.3	3	0
U12476	CP141 (MY09/11)	New Mexico, USA	99.1	3	1
U12486	LVX160 (MY09/11)	Indigenous Amazonian	99.1	3	1
AF538717	SDL105 (MY09/11)	Minnesota, USA	99.0	4	0

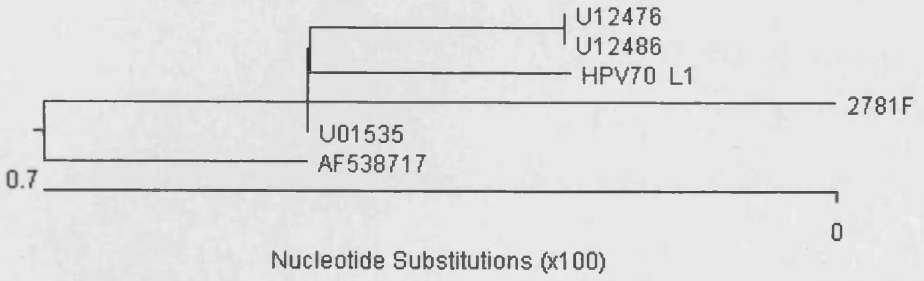


Figure 3.3: Phylogenetic Tree for Sample 2781F

Sample 2919F

The BLAST search on sample 2919F gave only one result, a 99.8% DNA match and exact amino acid homology to a HPV-68 sub-type originally isolated from a cervical

sample from a black Senegalese woman with a normal Pap smear (Astori, Beltrame et al. 1999) (Table 3.8 & Fig. 3.4).

Table 3.8: Comparison of Most Closely Homologous Sequences to Sample 2919

Genbank Accession No.	Isolate ID (Isolate Type)	Origin	Homology to Gambian Sample		
			% DNA Homology	Silent mutations	Polymorphisms
Y17206	GA115 (MY09/11)	Senegal	99.8	1	0
M73258	ME180 (Cell Line)	NK	98.3	5	2
U45934	IS362 (MY09/11)	Germany	97.8	7	2
X67161	HPV-68 (Ref.)	NK	93.0	22	7

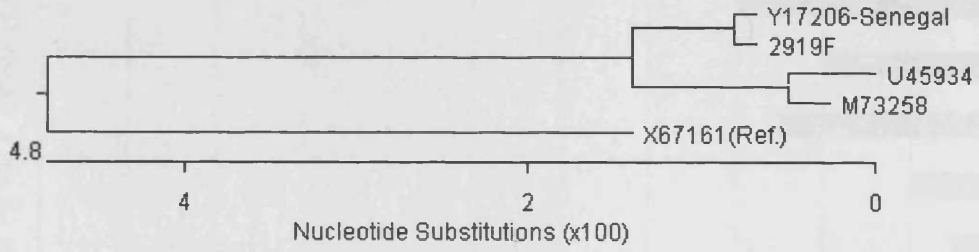


Figure 3.4: Phylogenetic Tree for Sample 2919F

Figure 3.5: Totals of All HPV Types (inc. Multiple Infections)

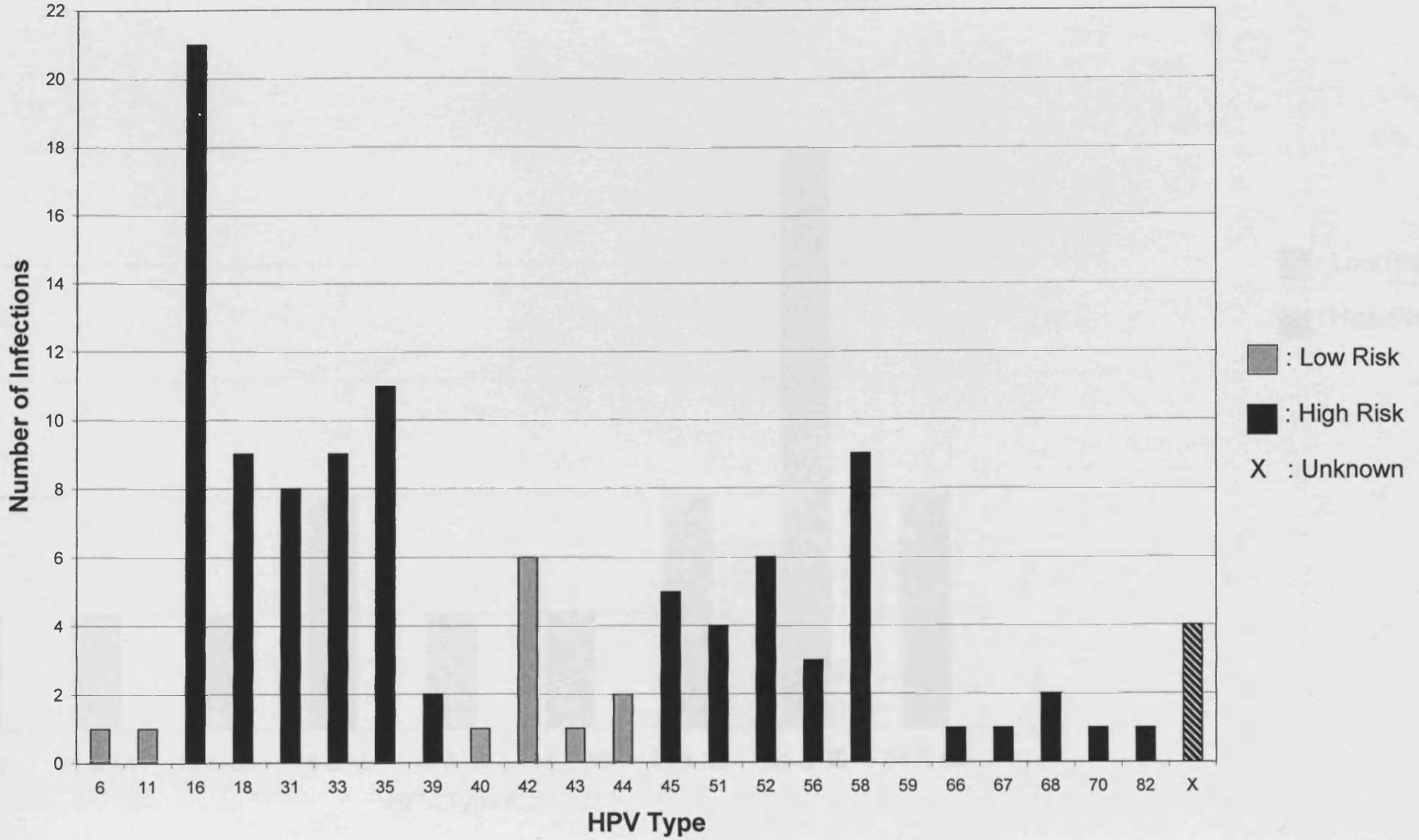
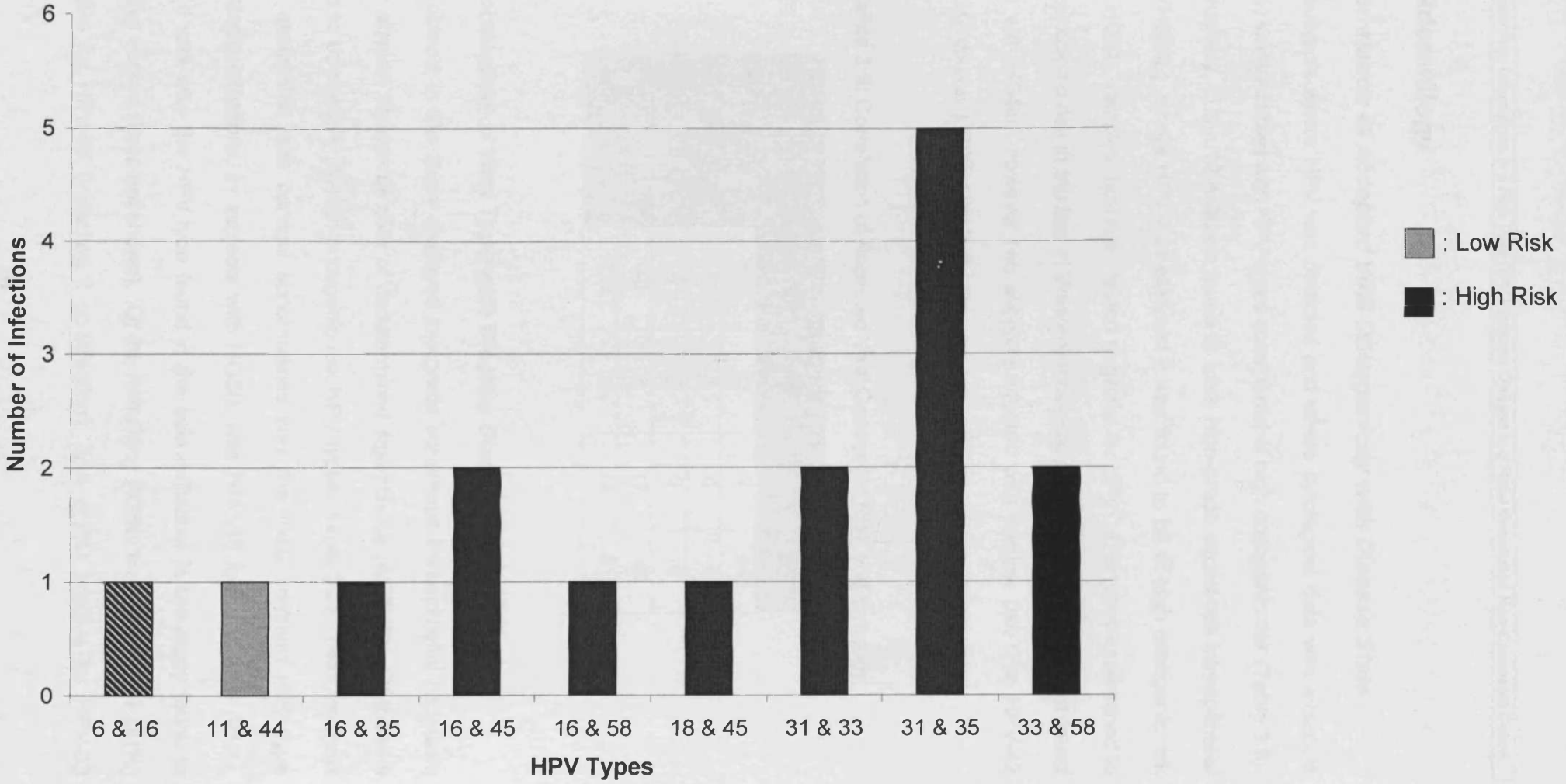


Figure 3.6: Totals of All Multiple HPV Infections



3.3.3 Epidemiology

3.3.3.1 Correlation of Accepted Viral Oncogenicity with Disease State

Of the subjects where HPV was detected and where cytological data was available 59/67 (88%) were infected with HPV types considered of high oncogenic risk (Table 3.9). More conclusively, in the 12 subjects found to have high-grade squamous intraepithelial neoplasia (HGSIL), where HPV was detected it was found to be of high oncogenic risk type. Two HGSIL samples, however, tested negative for HPV. The types established to be of low oncogenic risk in studies in Western industrialised populations were not found in subjects with HGSIL, however two subjects infected with the low risk type HPV-42 were found to display LGSIL (Table 3.14).

Table 3.9: Correlation of Accepted Viral Oncogenic Risk and Cytology

Cytology	Normal	LGSIL or ASCUS	HGSIL	Total
HPV Negative	525	13	2	540
Unknown HPV	3	1	0	4
LR HPV	6	2	0	8
HR HPV	40	8	12	60
Total	574	24	14	612

3.3.3.2 Correlation of Viral Type with Disease State

Thirty subjects in this study displayed low-grade squamous intraepithelial neoplasia (LGSIL) or atypical squamous cells of undetermined significance (ASCUS). Of these 8 were found to be positive for high oncogenic risk HPV types (Table 3.9). The type most commonly associated with cervical abnormalities was the most common HPV type found, in single infections, in subjects with HGSIL was HPV-16 found in 3/14 (21%). Moreover, it was also the HPV type found in the sole individual in the study found to have invasive cancer (Data not shown). Of the remaining HGSIL individuals 3/14 (21%) were positive for HPV-58 (including 1 co-infection), 3/14 (21%) positive for HPV-33

(including 2 co-infections). The high risk types; HPV-18, -35, -39 and -45 (7% each) were each found in a separate single HGSIL subject and HPV-31 was found in 1/14 (7%) as a co-infection (Tables 3.10 & 3.11). Of the subjects displaying normal cytology 66/565 (12%) were positive for HPV infection. Correlation of cytology with HPV type for individual samples is shown in Tables 3.12 & 3.13).

Table 3.10: HPV Types found in Subjects with HGSIL (Inc. Multiple Infections)

HPV Type(s)	16	18	31	33	35	39	45	58	Total
n HGSIL	3	1	1	3	1	1	1	3	14

Table 3.11: Multiple HPV Infections found in Subjects with HGSIL

HPV Type(s)	31 & 33	33 & 58	Total
n HGSIL	1	1	2

Table 3.12: All Multiple HPV Infections Correlated With Histology

Sample	Types	Histology	Sample	Types	Histology
0058H	31, 33	HGSIL	**2774G**	*6*, 16	Normal
0184C	33, 58	LGSIL	**2791H**	31, 33	Normal
2452B	31, 35	Normal	**2811E**	16, 58	Normal
2463E	31, 35	Normal	**2885C**	16, 35	Normal
2523J	31, 35	Normal	**2996I**	18, 45	Normal
2561F	31, 35	Normal	**3016J**	16, 45	NK
2595F	*11, 44*	Normal	**3142E**	31, 35	Normal
2772E	33, 58	HGSIL	**3216F**	16, 45	LGSIL

NK= Cytology not known due to inadequate smear

HPV types in italics are low oncogenic risk all others are high oncogenic risk

Table 3.13: All Single HPV Infections Correlated With Histology

Sample	HPV Type	Cytology	Sample	HPV Type	Cytology	Sample	HPV Type	Cytology
0013E	18	Normal	**2402B**	X	LGSIL	**2784I**	39	HGSIL
0019A	16	Normal	**2405E**	52	Normal	**2795B**	33	Normal
0028B	X	Normal	**2416H**	16	NK	**2802D**	18	Normal
0056F	39	Normal	**2436B**	16	Normal	**2809A**	16	HGSIL
0064F	*42*	Normal	**2453C**	16	Normal	**2810D**	16	Normal
0069A	18	Normal	**2462D**	*43*	Normal	**2835C**	16	Normal
0083I	35	Normal	**2466H**	52	Normal	**2843C**	52	Normal
0089E	X	Normal	**2470D**	16	NK	**2848H**	16	Normal
0093A	16	HGSIL	**2474H**	*44*	NK	**2849I**	33	Normal
0096D	18	Normal	**2484J**	16	Normal	**2854F**	51	Normal
0098F	45	Normal	**2493A**	66	LGSIL	**2867A**	35	Normal
0104G	58	HGSIL	**2501D**	35	Normal	**2898H**	56	ASCUS
0126C	56	Normal	**2503F**	*42*	Normal	**2919F**	68	NK
0145F	18	Normal	**2524A**	*40*	NK	**2927F**	*42*	Normal
0155H	16	Normal	**2565J**	33	Normal	**2935F**	58	Normal
0185D	18	Normal	**2631C**	51	Normal	**2958C**	X	Normal
0206B	*42*	LGSIL	**2696J**	52	Normal	**2981B**	33	HGSIL
0220J	31	LGSIL	**2712C**	67	Normal	**2983D**	58	Normal
0263A	18	HGSIL	**2718I**	35	HGSIL	**2987H**	56	Normal
2006D	82	LGSIL	**2734I**	16	HGSIL	**3067A**	51	Normal
2030D	51	Normal	**2739D**	*42*	LGSIL	**3086D**	33	Normal
2062B	58	LGSIL	**2741H**	33	Normal	**3153H**	52	LGSIL
2331D	45	HGSIL	**2747D**	16	Normal	**3189J**	*42*	Normal
2362A	16	Inflam.	**2754C**	68	Normal	**3227I**	16	NK
2381D	58	HGSIL	**2758G**	35	Normal	**3229A**	18	NK
2400J	58	Polyp	**2781F**	70(AE 2)	Normal			

NK= Cytology not known due to inadequate smear

HPV types in italics are low oncogenic risk all others are high oncogenic risk

3.3.4 Sequencing of L1 in HPV Types Associated with HGSIL

3.3.4.0 Overview

The HPV protein which has been the primary focus of vaccine research is the L1 outer capsid protein (Lowy and Frazer 2003; Mandic and Vujkov 2004). Thus, in order to assess the viability of a vaccine it is necessary to sequence the L1 gene of the HPV types found to be associated with HGSIL to identify the presence of HPV subtypes and variants. To this end, sequencing using the MY09/MY11 primer pair was carried out on samples positive for the types associated with HGSIL in this study. The MY09/11 primer pair have been used extensively to classify HPV-types from this region alone (Chan, Delius et al. 1995) as the region they amplify contains highly conserved amino acids, which simplifies sequence alignment. The only HPV type in which HGSIL was observed that was not sequenced successfully by this method was HPV-35 as these primers are ineffective in amplifying HPV-35, this is discussed in detail later.

3.3.4.1 HPV-16

Three HPV-16 positive samples were successfully sequenced in the MY09/MY11 amplified region of the L1 gene. These were samples; 0093A, 2809A and 2885A, the latter was positive as a dual infection with HPV-35 by ELISA based typing. These three samples proved to be identical to each other within the MY09/MY11 amplified region and have four silent point mutations when compared to the HPV-16 reference strain, a composite sequence designated HPV-16R (Table 3.14 & Fig 3.7). However the three Gambian HPV-16 samples sequenced are completely homologous with a variant of HPV-16 African Type 1 (Genbank accession No.AF536180) and a sample from the USA which also appears to be the same HPV-16 Af1 variant (Genbank accession No. U34188). Protein and DNA sequence alignments with the BLAST results are shown in Figs. 3.8 & 3.9 with nucleotide / amino acid divergence from reference HPV type shown boxed.

Table 3.14: Comparison of Most Closely Homologous Sequences to Gambian HPV-16

Genbank Accession No.	Isolate ID (Type)	Origin	Homology to Gambian Samples		
			% DNA Homology	Silent Mutations	Polymorphisms
AF536180	Af1 Variant (Full)	Africa	100	0	0
U34188	OR7587 (L1)	USA	100	0	0
AF472508	Af1 Variant (Full)	Africa	99.8	1	0
U34189	OR7632 (L1)	USA	99.8	1	0
U34183	OR6106 (L1)	USA	99.5	1	1
AF472509	Af2 (Full)	Africa	99.3	2	1
AF134178	GU2 (L1)	NK	99.3	2	1
U37217	(L1 + L2)	Zaire	99.3	2	1
U34186	OR7145 (L1)	USA	99.3	2	1
-	HPV16R (Ref.)	Composite	99.0	4	0

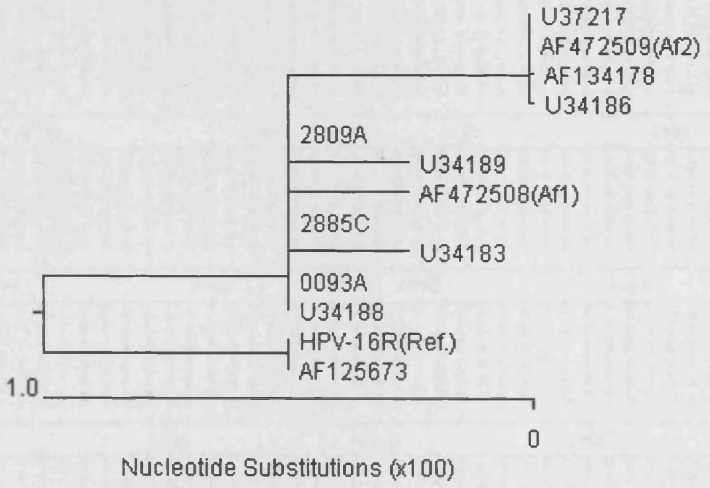


Figure 3.7: Phylogenetic Tree for Gambian HPV-16 Samples

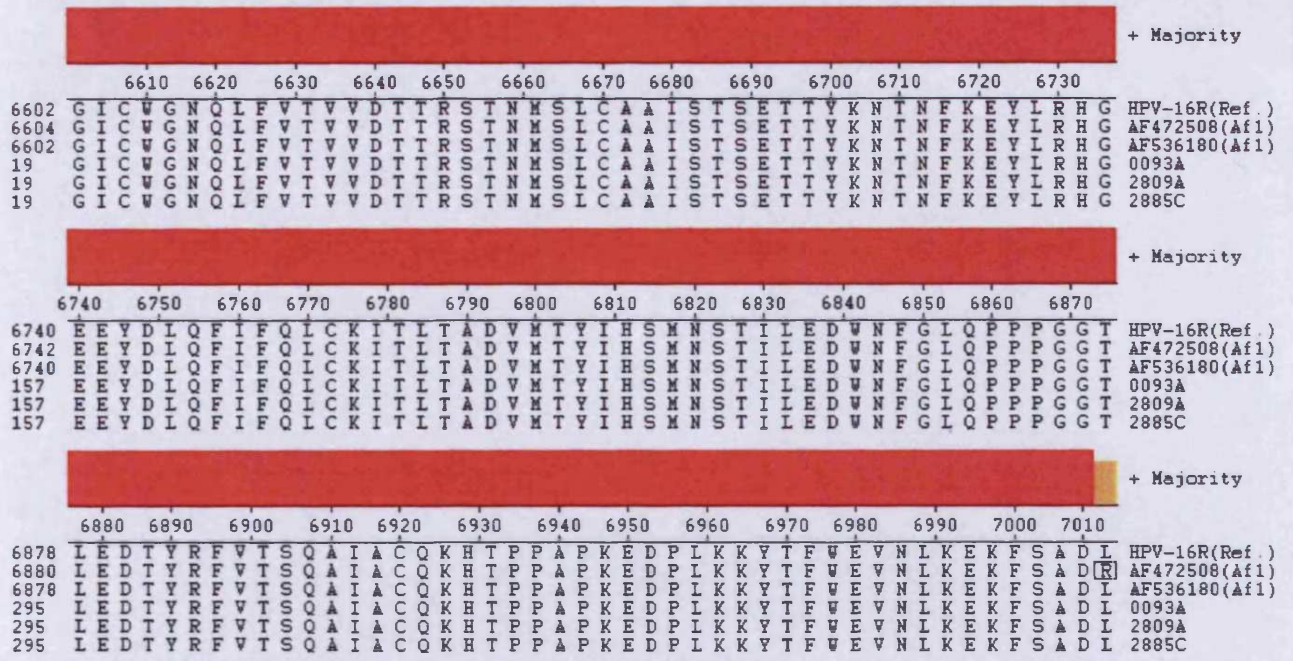


Figure 3.8: Protein Alignment of Gambian HPV-16 Samples with Af1 Variant and Ref. Type

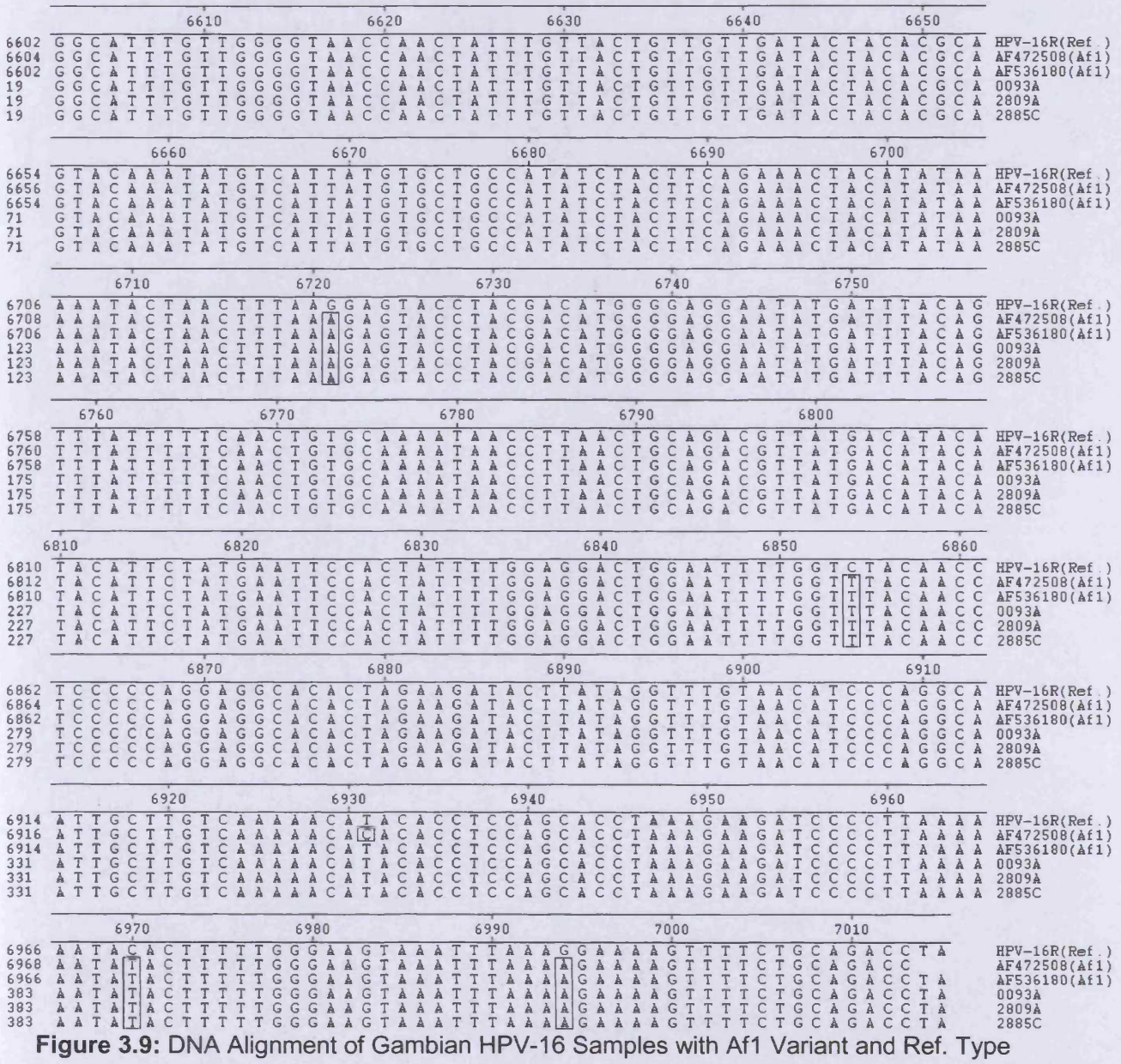


Figure 3.9: DNA Alignment of Gambian HPV-16 Samples with Af1 Variant and Ref. Type

3.3.4.2 HPV-18

The two successfully sequenced HPV-18 Gambia samples, 0069A & 0096D have a number of missense and silent mutations (Table 3.15 & Fig 3.10) when compared the reference HPV-18 strain (Genbank accession No. X0505). They also have two silent DNA differences to each other at bases 1197* and 1416* (*relative to the start of the ATG of the reference strain L1). Sample 0069A has a cytosine and 0096D a thymine at base 1197* and 0069A a cytosine, 0096D an adenine at 1416* where the reference HPV-18 also has an adenine (*relative to the start of the ATG of the reference strain L1).

The BLAST search for these sequences showed 0096D to be homologous on the DNA level to a sequence sourced from Benin in West Africa (Genbank accession No. U45894). Another from Benin (Genbank accession No. U45892) and one from Uganda (Genbank accession No. U45893) both have the same silent mutations (Dartmann, Schwarz et al. 1986) to 0096D and the Ugandan sample also has an additional polymorphism. Protein and DNA sequence alignments with the BLAST results are shown in Figs. 3.11 & 3.12 with nucleotide / amino acid divergence from reference HPV type shown boxed.

Table 3.15: Comparison of Most Closely Homologous Sequences to Gambian HPV-18

Genbank Accession No.	Isolate ID (Type)	Origin	Homology to Gambian Samples		
			% DNA Homology	Silent Mutations	Polymorphisms
U45894	IS172 (MY09/11)	Benin	100	0	0
U45892	IS168 (MY09/11)	Benin	99.8	1	0
U45893	IS768 (MY09/11)	Uganda	99.3	2	1
	0069A*(MY09/11)	Gambia	99.5	2	0
X05015	HPV-18R (Ref.)	Brazil	97.6	8	2

* Dissimilar HPV-18 Sample from this Study

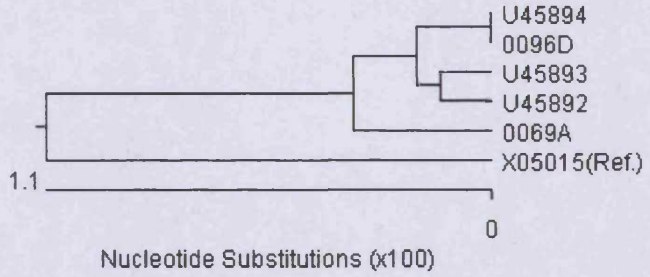


Figure 3.10: Phylogenetic Tree for Gambian HPV-18 Samples

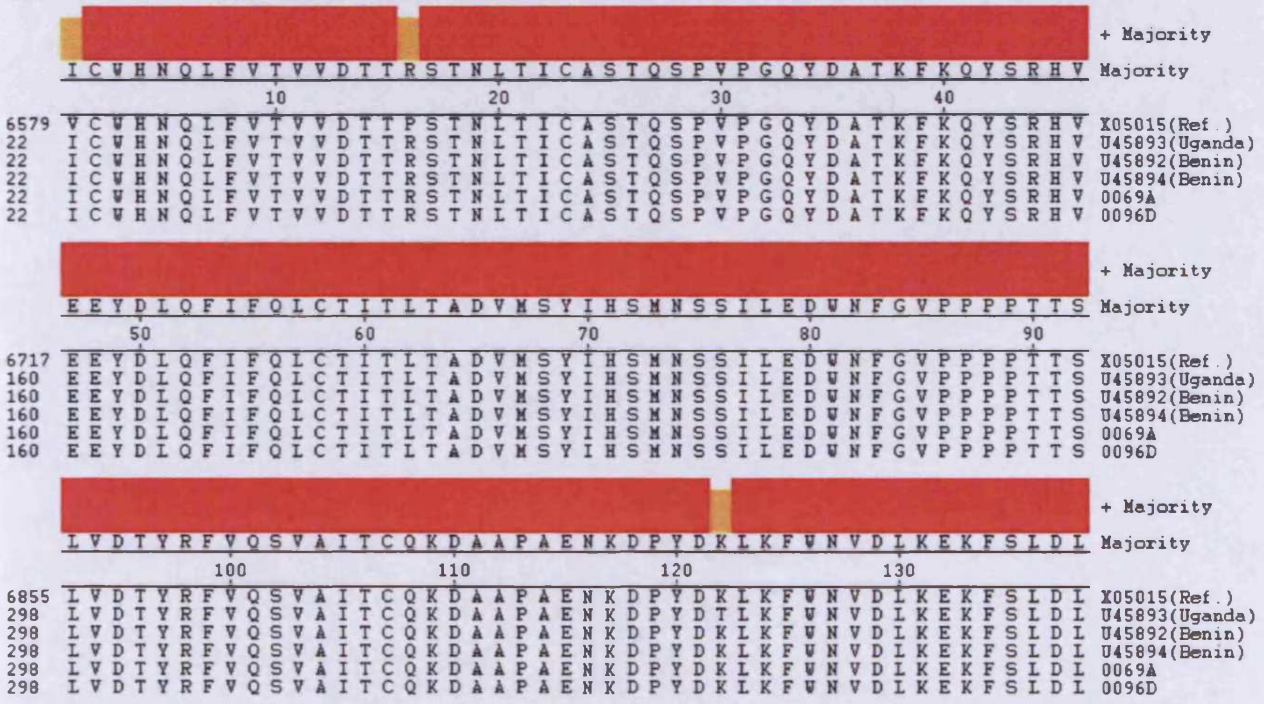


Figure 3.11: Protein Alignment of Gambian HPV-18 Samples with BLAST Hits and Ref. Type

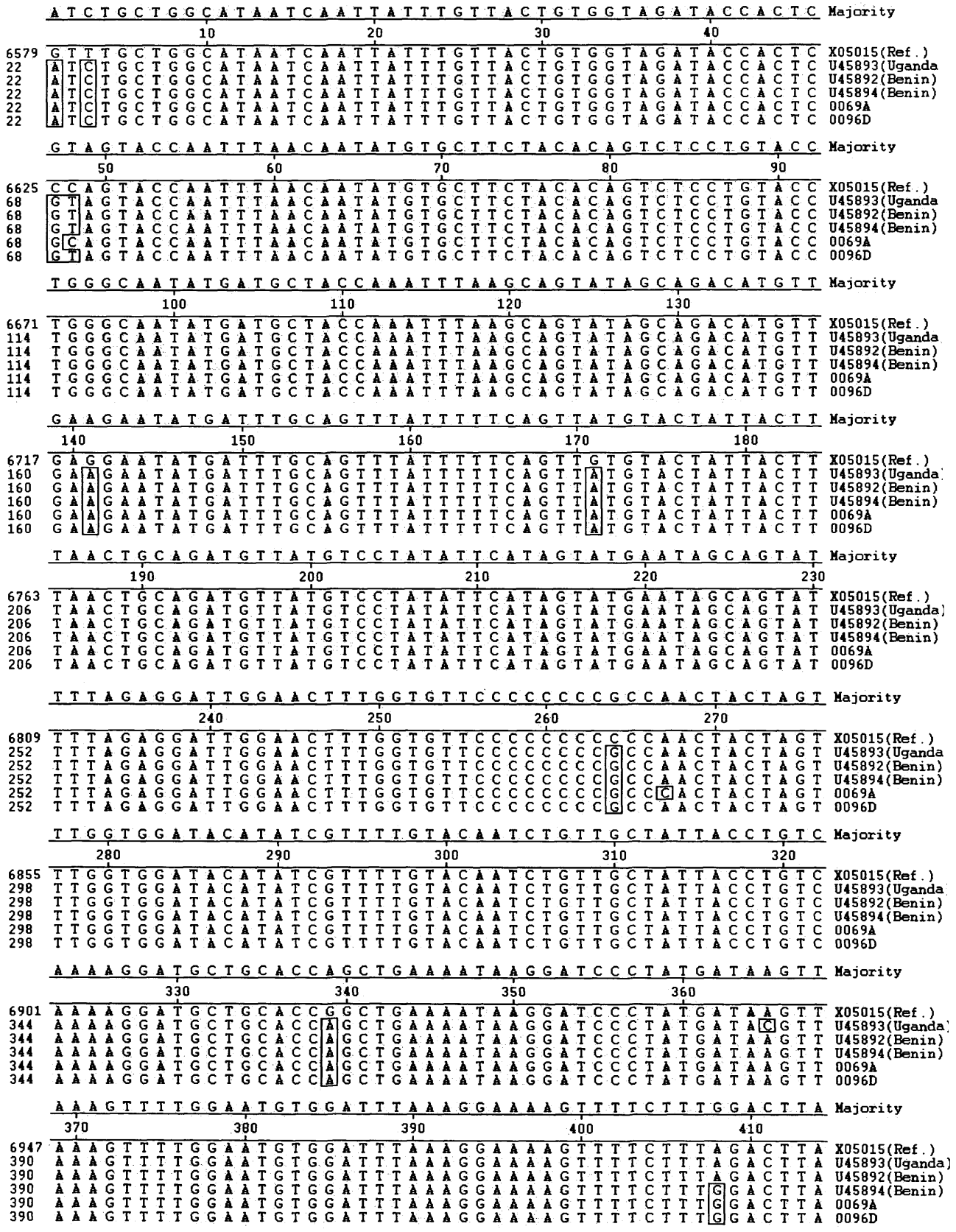


Figure 3.12: DNA Alignment of Gambian HPV-18 Samples with BLAST Hits and Ref. Type

3.3.4.3 HPV-31

The one unambiguously sequenced HPV-31 sample, 2561F, gave only two close hits when a BLAST search was carried out (Table 3.16) neither of which were extremely close. One of these was the HPV-31 reference strain (Genbank accession No. J04353) with 10 silent point mutations and 2 polymorphisms compared to the Gambian sample. The other (Genbank accession number U37410) possessed 5 silent mutation and 2 polymorphisms compared to the Gambian sample. Protein and DNA sequence alignments with the BLAST results are shown in Figs. 3.8 & 3.9 with nucleotide / amino acid divergence from reference HPV type shown boxed. Protein and DNA sequence alignments with the BLAST results are shown in Figs. 3.13 & 3.14 with nucleotide / amino acid divergence from reference HPV type shown boxed.

Table 3.16: Comparison of BLAST results for Gambian HPV-31

Genbank Accession No.	Isolate ID (Type)	Origin	Homology to Gambian Samples		
			% DNA Homology	Silent Mutations	Polymorphisms
U37410	(L1 & L2)	NK	98.3	5	2
J04353	HPV-31 (Ref.)	NK	97.1	10	2

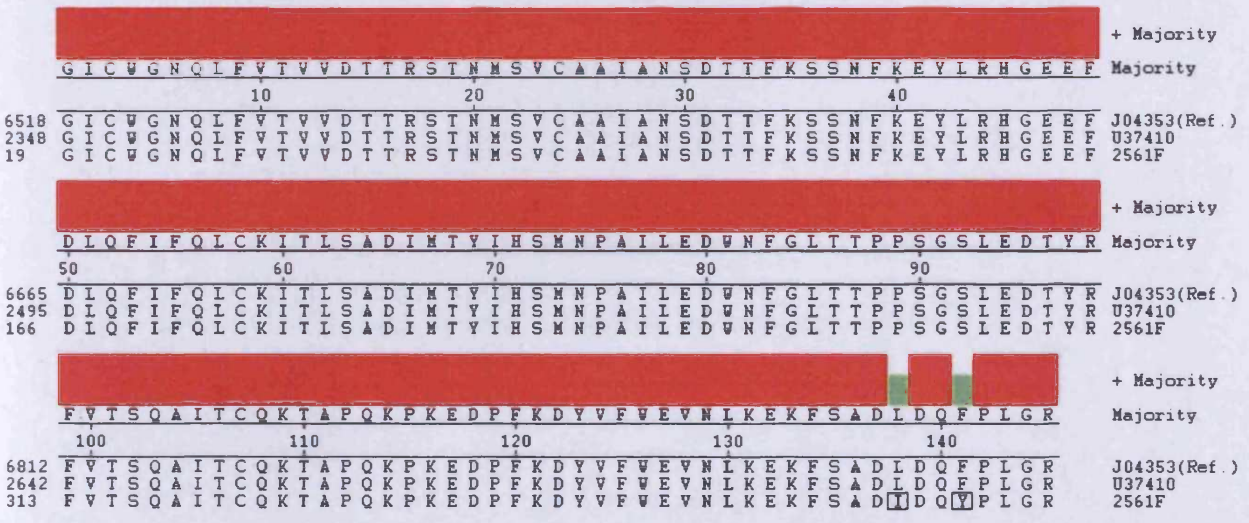


Figure 3.13: Protein Alignment of Gambian HPV-31 Samples with BLAST Hits and Ref. Type

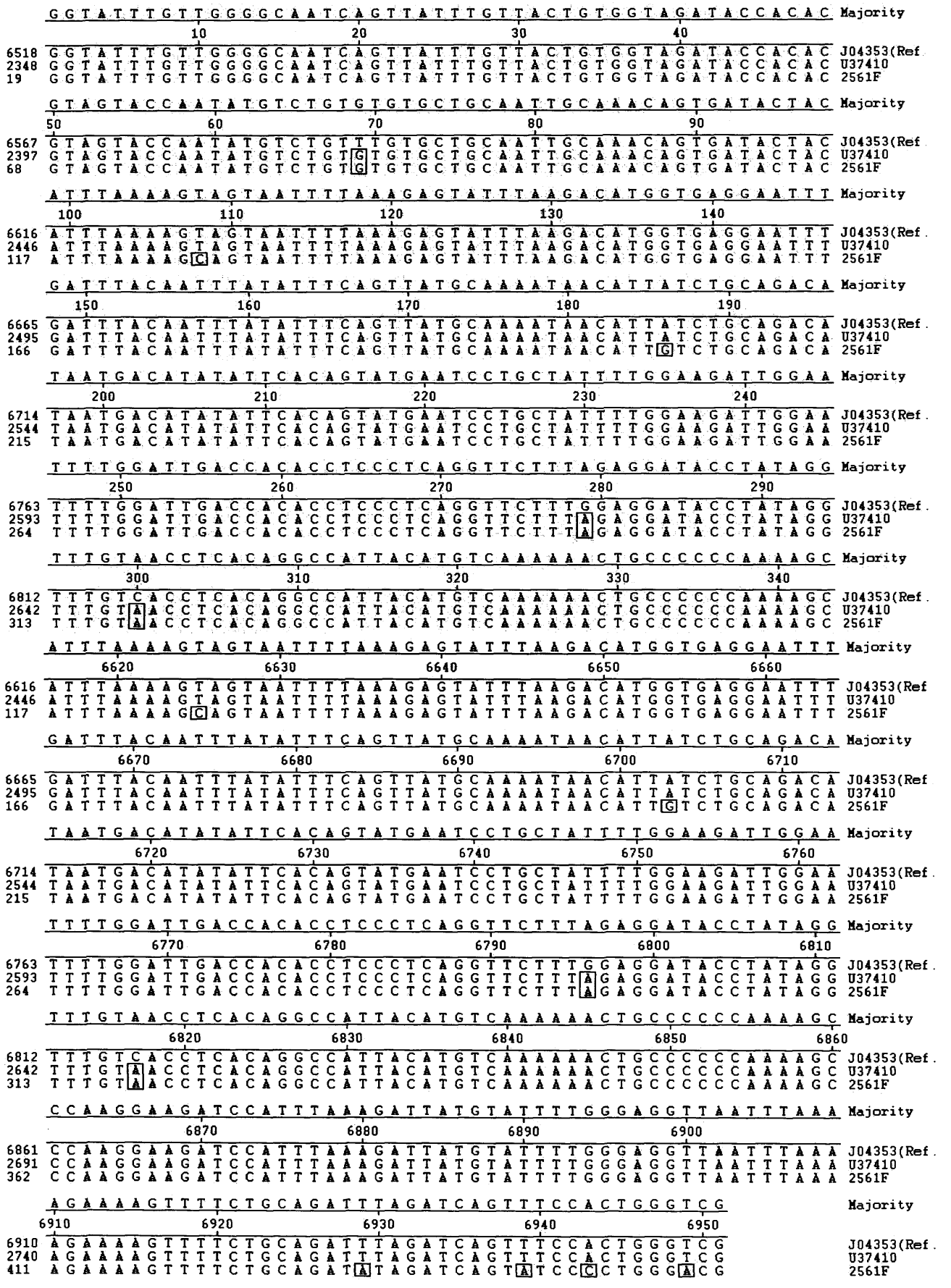


Figure 3.14: DNA Alignment of Gambian HPV-31 Samples with BLAST Hits and Ref. Type

3.3.4.4 HPV-33

The three HPV-33 samples sequenced were 0184C, 2565J & 2981F, the latter two are identical whilst 0184C has a missense mutation at position 1284 (relative to the start of ATG at the L1 of the reference strain). This mutation is an adenine to a cytosine which results in a polymorphism of a histadine for a glycine in 0184C. The two identical samples display one silent mutation compared to the reference HPV-33 strain (Genbank accession No. M12732) and another silent point mutation compared to a sample originating in the West African State of Guinea (Table 3.17 2 & Fig. 3.15). Protein and DNA sequence alignments with the BLAST results are shown in Figs. 3.16 & 3.17 with nucleotide / amino acid divergence from reference HPV type shown boxed.

Table 3.17: Comparison of BLAST results for Gambian HPV-33

Genbank Accession No.	Isolate ID (Type)	Origin	Homology to Gambian Samples		
			% DNA Homology	Silent Mutations	Polymorphisms
U45897	IS827 (MY09/11)	Guinea	99.8	1	0
U45896	IS549 (MY09/11)	Paraguay	99.5	2	0
M12732	HPV-33 (Ref.)	NK	99.8	1	0

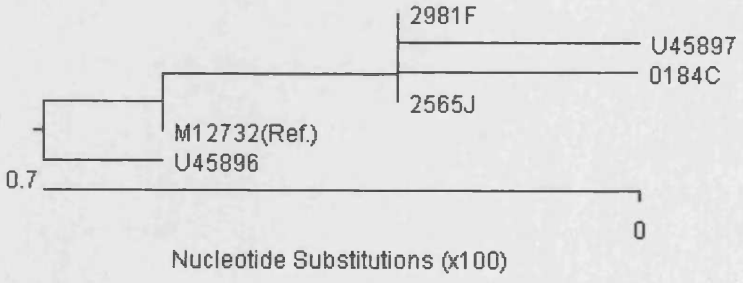


Figure 3.15: Phylogenetic Tree for HPV-33 Samples

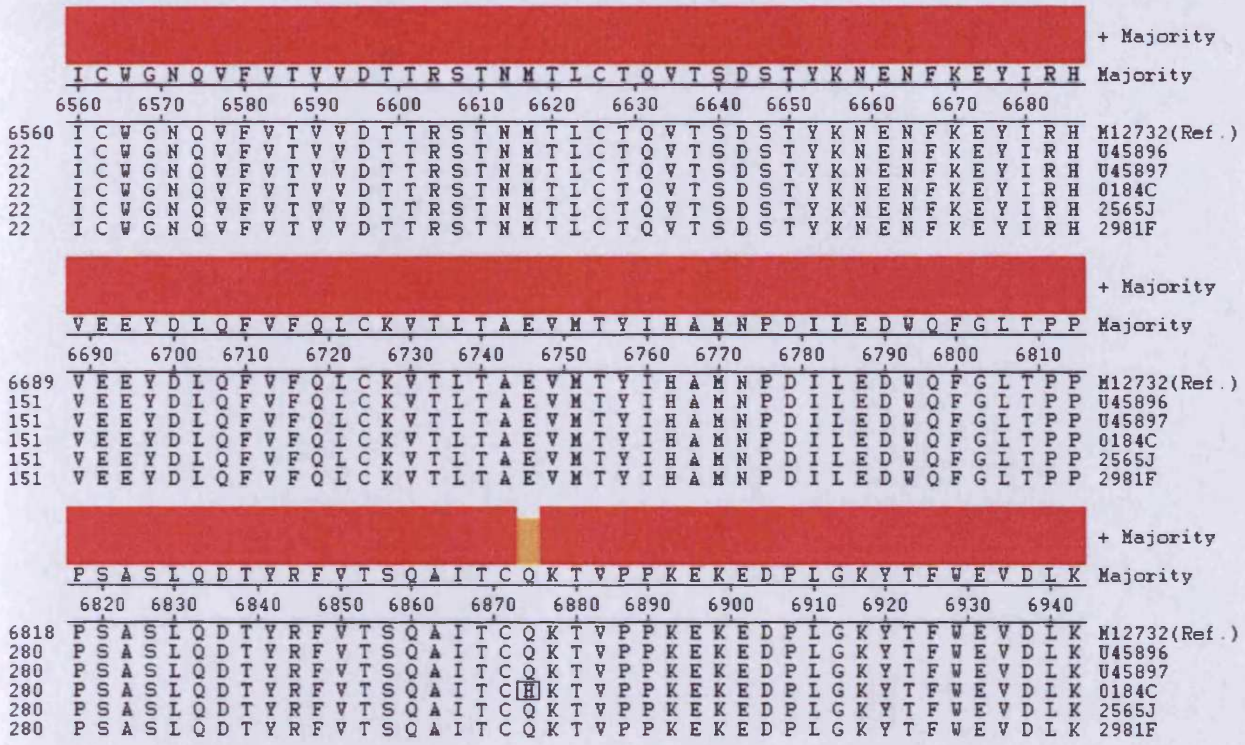


Figure 3.16: Protein Alignment of Gambian HPV-31 Samples with BLAST Hits and Ref. Type

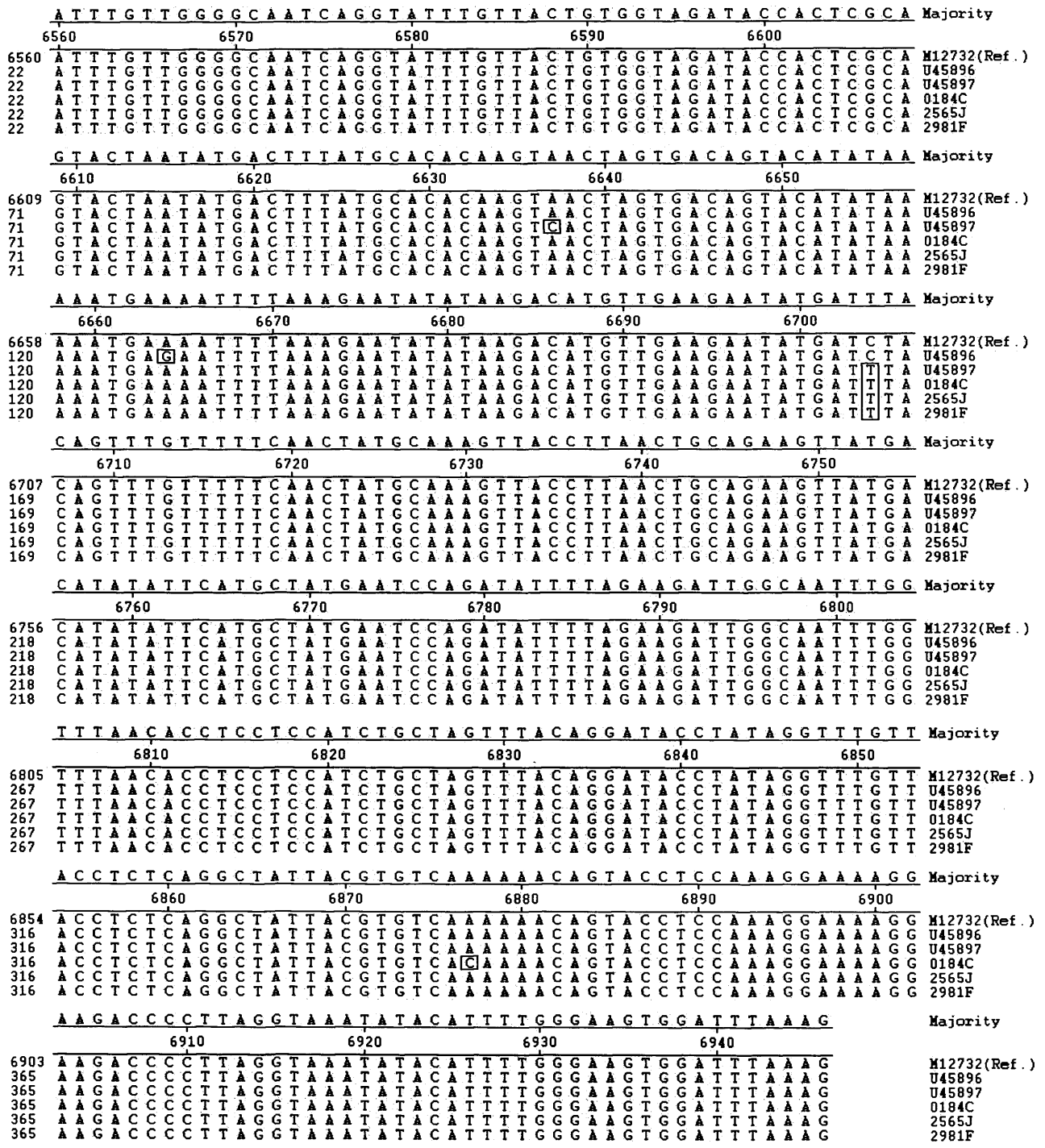


Figure 3.17: DNA Alignment of Gambian HPV-33 Samples with BLAST Hits and Ref. Type

3.3.4.5 HPV-58

The one unambiguously sequenced HPV-58 sample, 0104FG, gave three near matches when a BLAST search was carried out (Table 3.18). Two of these were from the West African nation of Mali, one of which was an exact match to the Gambian HPV-58 sample. The other Malian sample displays one silent point mutation and one polymorphism compared to the Gambian sample. The other near match was a sample from Brazil which showed one polymorphism compared to the Gambian sample. The HPV-58 reference strain (Genbank accession No. D90400) possessed 1 silent point mutations and 3 polymorphisms and another (Genbank accession number U37410) compared to the Gambian sample. Protein and DNA sequence alignments with the BLAST results are shown in Figs. 3.19 & 3.20 with nucleotide / amino acid divergence from reference HPV type shown boxed.

Table 3.18: Comparison of BLAST results for Gambian HPV-58

Genbank Accession No.	Isolate ID (Type)	Origin	Homology to Gambian Samples		
			% DNA Homology	Silent Mutations	Polymorphisms
U45928	IS417 (MY09/11)	Mali	100	0	0
AY101598	Bsb-2 (MY09/11)	Brazil	99.7	0	1
U45929	IS404 (MY09/11)	Mali	99.2	1	2
D90400	HPV-58 (Ref.)	Japan	98.9	1	3

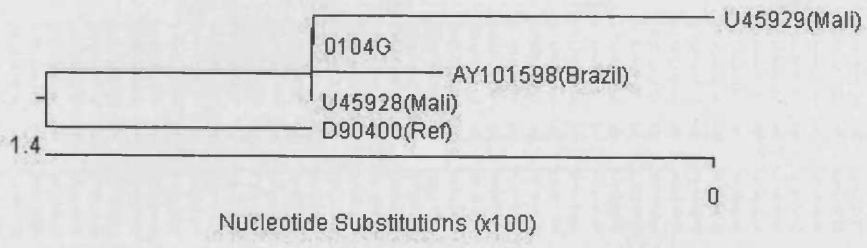


Figure 3.18: Phylogenetic Tree for HPV-58 Samples

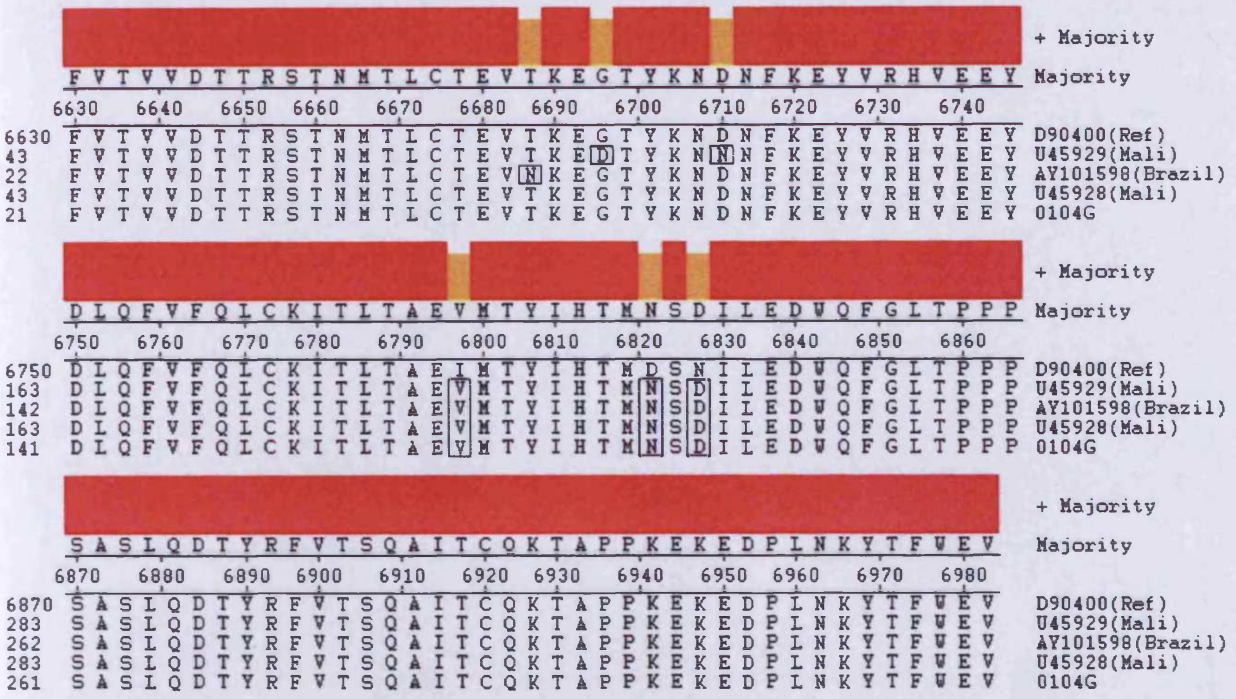


Figure 3.19: Protein Alignment of Gambian HPV-31 Samples with BLAST Hits and Ref. Type

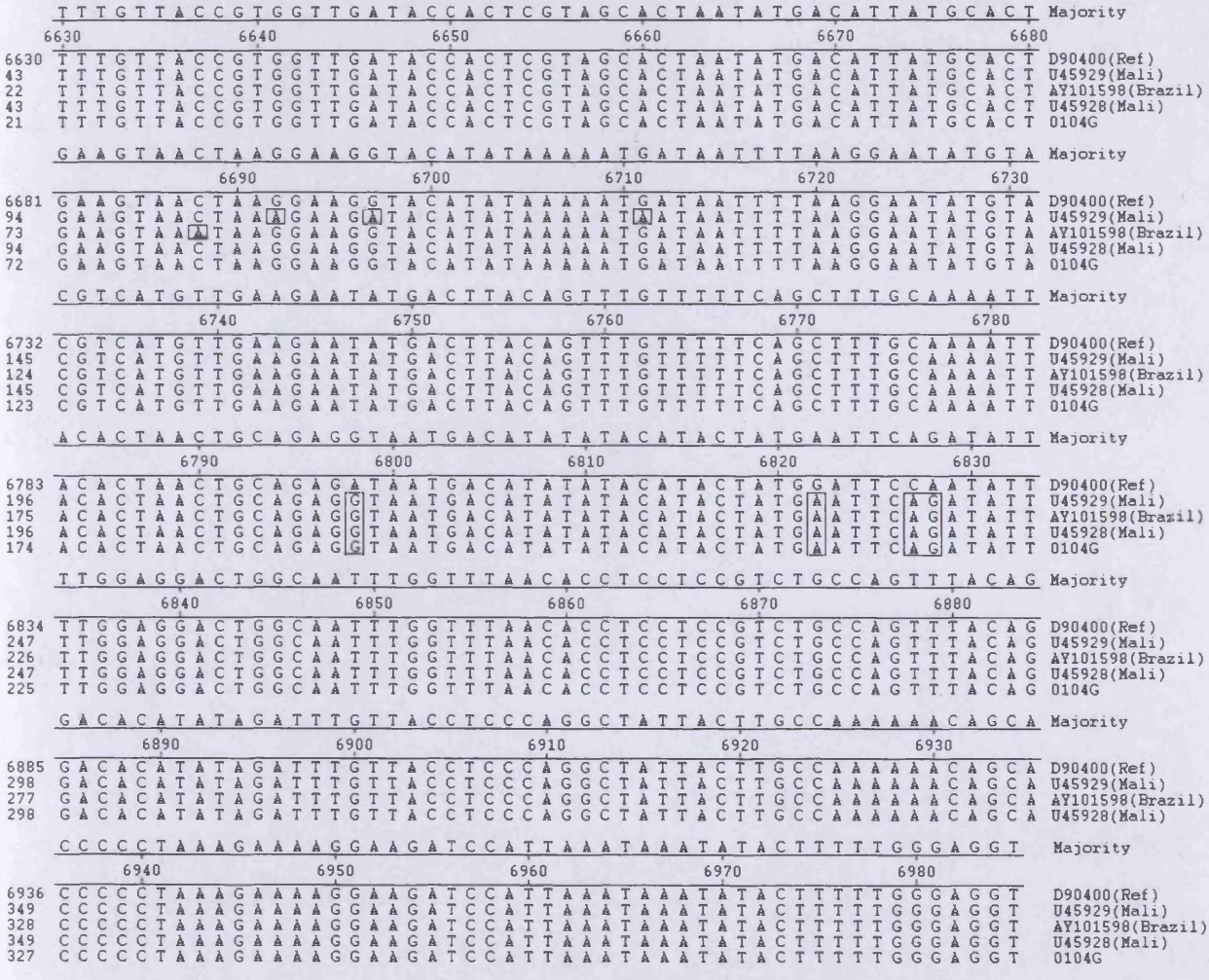


Figure 3.20: DNA Alignment of Gambian HPV-58 Samples with BLAST Hits and Ref. Type

3.4 Discussion

3.4.0 Summary of Results

- Overall HPV prevalence =13.0%
- Most Common HR Types: HPV-16 (19%), HPV-35 (10%), HPV-18, -33 & -58 (All 8%) and HPV-31 (7%)
- Most common LR Type: HPV-42 5% of all types, 50% of low risk types
- Low risk types uncommon
- No multiple HPV infections greater than 2
- None of the HPV types identified by sequencing were novel
- Accepted HPV oncogenic risk matched patient cytology

3.4.1 Comparison With Other African HPV Studies

3.4.1.1 Overall HPV Prevalence

Overall HPV prevalence was found to be 13.0% in this first, unselected large-scale, study of HPV and cervical neoplasia in a rural African population (Tables 3.19-3.22). Comparable overall prevalence figures of 13.6% and 13.9% have been observed in cytologically normal populations in two studies from Senegal, the only nation with which The Gambia shares a border (Astori, Beltrame et al. 1999; Xi, Toure et al. 2003). However, another recent West African study from Nigeria has found a HPV prevalence figure of 26.3% (Thomas, Herrero et al. 2004). The Nigerian study is an unselected study of similar size which used the same detection and typing techniques as this study. However, the primary difference between these two studies is that the Nigerian study is in the urban setting of Ibadan (pop >1M) whilst this study is based in a rural population (Table 3.19 & Fig. 3.9). This higher Nigerian prevalence, however, may be distorted by a much higher proportion of low risk types than are found in this study and possible reasons for this are discussed later.

Prevalence figures of more than double the prevalence in this study have been observed in both urban and rural, unselected studies from Eastern and Southern African (Tables 3.20 & 3.21). These range from 34% in a rural unselected study in Zimbabwe (Baay, Kjetland et al. 2004), to 44% in urban clinic attendees in Kenya. Such high prevalence figures are most likely to be because much higher rates of HIV infection are found in Eastern and Southern Africa than in West Africa (UNAIDS 2004). HPV can act as an opportunist infection taking advantage of immunosuppression induced by HIV/AIDS and HIV infection is an acknowledged contributory factor in the progression of cervical cancer (Feingold, Vermund et al. 1990; Moscicki, Ellenberg et al. 2004; Moscicki, Ellenberg et al. 2004). Thus, although most HPV infections are cleared by the immune system (Hildesheim, 1994; Bosch, 1995), if HIV is present as a co-infection HPV is more likely to persist The Gambia in particular, has one of the lowest HIV infection rates in Africa (Ramsay 1993, Da Costa, 1994; Schim van der Loeff, Sarge-Njie et al. 2003).

3.4.1.2 Prevalence of Multiple HPV Infection

Multiple cervical HPV infection is frequently reported in African studies (Chabaud, Le Cann et al. 1996; Castellsague, Menendez et al. 2001; Gravitt, Kamath et al. 2002; Stanczuk, Kay et al. 2003; Xi, Toure et al. 2003; Baay, Kjetland et al. 2004) yet this study showed no multiple HPV infections greater than two. This may, again, be linked to the low HIV rates in The Gambia (Da Costa 1994; Schim van der Loeff, Sarge-Njie et al. 2003) compared to the rest of sub-Saharan Africa (UNAIDS 2004) as studies have shown multiple HPV infection to be linked to HIV immunosuppression of (Levi, Fink et al. 2002; Levi, Kleter et al. 2002; Moscicki, Ellenberg et al. 2004). Indeed this linkage between HIV and multiple HPV infection has significantly shown (χ^2 P= <.001) in a Zimbabwean population where HIV infected individuals have been shown to exhibit up to quintuple HPV infection (Baay, Kjetland et al. 2004).

3.4.1.3 High Oncogenic Risk HPV Type Prevalence

The HPV types observed displayed comparative diversity of type and a relatively even distribution among subjects (Table 3.1 & Fig. 3.7). HPV-type 16 the most common cervical high risk type worldwide (Muñoz, Bosch et al. 2003; Bosch, 1995) and is also the most frequently detected type in this study. HPV-16 is part of the triumvirate of types, along with HPV-18 and -45. It has also been shown to preferentially progress to cervical cancer and is widely regarded to have the highest oncogenic potential of any HPV type (Clifford, Smith et al. 2003; Clifford, Smith et al. 2003). In this study HPV-35 was the second most common type (10%) which is unexpected as a meta-analysis of worldwide HPV prevalence studies has found the overall prevalence of HPV-35 to be no higher than around 2% even in Africa (Clifford, Smith et al. 2003). HPV-35, is a high oncogenic risk type belonging to the same Group as HPV-16 (Group A). It was first found in a cervical adenocarcinoma sample (Lorincz, Quinn et al. 1987) and preferentially affects the cervix and the lower anogenital tract.

With regard to HPV-35 our study differs distinctly from two studies, both from selective urban populations in neighbouring Senegal. The more recent of these studies, (Xi, Toure et al. 2003), found a HPV-35 prevalence of only 1.4% of the total HPV positive samples, the other two found no HPV-35 positive samples at all (Astori, Beltrame et al. 1999) (Chabaud, Le Cann et al. 1996) (Table 3.16). This inconsistency between studies in two nations with overlapping ethnic groups, religious practices and trade routes initially appears incongruous. This discrepancy however, can be explained by technical differences between the studies in question. is most likely the consequence of the differing sensitivities of the PCR primers used. The GP5+/6+ primers used in the Gambian Study are 5000 times more sensitive in the detection of HPV-35 than the MY09/MY11 primer pair used in the Xi and Astori studies (Qu, Jiang et al. 1997). This is likely to have resulted in an underestimation of the true frequency of HPV-35 infection in Senegal. This hypothesis is supported, by other studies from West Africa (Table 3.19)

and other parts of the continent (Table 3.19-3.22 & Fig. 3.22) where high rates of HPV-35 are observed in African studies in which the MY09/11 primer pair is not exclusively used in the detection of HPV. This clearly demonstrates the importance of careful consideration of all experimental shortcomings in the design of viral epidemiological studies. In all but one of the African studies where the MY09/11 primers were not used, or were supplemented with more sensitive primers, HPV-35 is widespread. This is seen in studies from; Nigeria (Thomas, Herrero et al. 2004), Kenya (De Vuyst, Steyaert et al. 2003), Zimbabwe (Baay, Kjetland et al. 2004) and two from Mozambique (one urban (Naucler, Da Costa et al. 2004), one rural (Castellsague, Menendez et al. 2001)) where HPV-35 is seen at least in the top four of the most prevalent types. In the rural Zimbabwean study (Baay, Kjetland et al. 2004), where the same HPV detection and typing method as this study were used HPV-35 was the most prevalent type within an unusually even distribution of HPV types (Table 3.19). Due to this even distribution of types although HPV-35 is the most prevalent type in the Zimbabwean study it actually makes up a lower proportion (6%) of the total HPV types as this study (10%) with HPV-33 & -58 joint second (5.5% each) and HPV-18 & 45 joint third prevalent (5% each). An even distribution of HPV types with HPV-35 most prevalent was also seen in the rural study in neighbouring Mozambique (Castellsague, Menendez et al. 2001) where the PGMY09/11 set of eighteen primers was used for HPV amplification. Although HPV-35 is most prevalent HPV-35 makes up the same proportion of the total HPV types as in this study (10%) with HPV-16 (8%) and HPV-18 (7%) second and third most prevalent respectively.

The scarcity of HPV-35 in studies using the MY09/11 primer set is also seen in East Africa where two similarly sized studies from neighbouring countries, in urban selected populations, both using reverse line blot for typing show highly contrasting rates of HPV-35 prevalence. In a study in Tanzania where the MY09/11 primers were used no HPV-35 was found whilst a study in neighbouring Kenya (De Vuyst, Steyaert et al. 2003),

which used the SPF-10 primer set, found HPV-35 to be the third most common high risk type (12%) after HPV-52 (18%) and -16 (15%). Furthermore, in this selected urban Kenyan study HPV-35 and -16 displayed the joint highest risk for the development of cervical cancer.

The Nigerian study (Thomas, Herrero et al. 2004) has reported HPV-16 and -35 as jointly the most prevalent types (Table 3.19 Fig. 3.9). The Nigerian study has a very similar distribution of HPV types to this study excepting that the HPV-35 rate is equal to HPV-16. It therefore appears that HPV-35 is highly prevalent throughout sub-Saharan Africa rivalling HPV-16 in most cases, however it seems that the extent of this high prevalence is underestimated by the use of the MY09/11 primer pair.

3.4.1.4 Low Oncogenic Risk HPV Type Prevalence

Although not causative of cervical cancer, low oncogenic risk HPV types can be indicative of sexual behaviour which could lead to infection with high oncogenic risk types. Overall 13% (12/93) of the total HPV types were classified as being of low oncogenic risk types. Of these, HPV-42 was by far the most common forming 50% of the total low risk types and 6% of the total identified HPV types. This is a shift from the USA and Europe where HPV-6 and HPV-11 are dominant low risk types (Muñoz, Bosch et al. 2003) and confirms findings of other African studies (Castellsague, Menendez et al. 2001; De Vuyst, Steyaert et al. 2003; Xi, Toure et al. 2003), where very few HPV-6 and -11 infections are detected and HPV-53 and -54 are the dominant low risk types. However another study where HPV-42 is by far the most common low risk type is the recent Nigerian study discussed in 3.4.1.1 (Thomas, Herrero et al. 2004). Indeed HPV-42 is the most common HPV type in the entire of the Nigerian study. Interestingly it is the low risk types which predominantly increase the overall HPV prevalence in the Nigerian study. Here the LR:HR HPV ratio is 35:65 whilst in rural Gambia this ratio is 11:89 (Table 3.5). HPV-42 has been linked to the development of genital warts (Burd

2003), and this may be due to a higher prevalence of genital warts in the urban setting of the Nigerian study. Indeed an STI (Sexually Transmitted Infection) studies in Ibabdan Nigeria, the same city where the Thomas *et al* HPV study was carried out have found genital warts in 7% of sex workers studied and 8% attendees at a GUM clinic.

3.4.1.5 Samples Identified by Sequencing

None of the types that could only be identified by sequencing were novel types, merely types that not been included in the ELISA probe cocktail. However some of these were unusual sub-types only recently characterised. Any future studies of HPV prevalence in West Africa should include probes for these types

One of the samples identified by sequencing, 2919F, was a 99.8% DNA match to a HPV-68 sub-type originally isolated from a cervical sample from a study in Senegal (Astori, Beltrame et al. 1999). This study used PCR-RFLP to detect human papilloma virus infection in a group of Senegalese women attending a STD clinic. This sub-type along with 2919F has a polymorphism at nucleotide 1060 (relative to ATG at the start codon of the L1 gene) which falls within the region of the DNA ELISA probe used to detect HPV-68. However, another HPV-68 positive sample in this study, which was identified by ELISA this was sequenced using the MY09/11 primer pair (data not shown) is also of this particular sub-type and includes the same polymorphism, so why this sample failed to bind to the HPV-68 probe upon repeated testing is unclear. However it is encouraging that a rare sub-type found previously only in Senegal, the only country with which the Gambia shares a land border has appeared in this study.

3.4.2 Comparison With HPV Studies From the Rest of the World

The overall prevalence is difficult to compare to industrialised western countries as most cervical studies tend to be selected from colposcopy clinics rather than unselected studies. However two unselected studies from Northern Europe (Sweden and Finland)

(Syrjanen, Nurmi et al. 1996; Forslund, Antonsson et al. 2002) both show overall cervical HPV prevalence rates of around 7%. Thus the overall HPV prevalence of around 13% found in this study is around twice the rate of these studies from these affluent European nations. A large unselected rural study from the Central American nation of Costa Rica (Herrero, Hildesheim et al. 2000) found a comparable overall prevalence rate to this study of around 16%.

The high prevalence of HPV-35 seen in this study contrasts greatly with studies from the rest of the world where HPV-35 is uncommon and HPV 16 dominates. This can be seen in Table 3.23 and Figure 3.11 which show studies from all other continents (except Australasia) although four of these studies did use the MY09/11 primer pair. It therefore appears that high levels of HPV-35 are a significant feature of HPV infection on the continent of African as a whole.

Figure 3.21: Comparison of HPV types in Rural Gambia and Urban

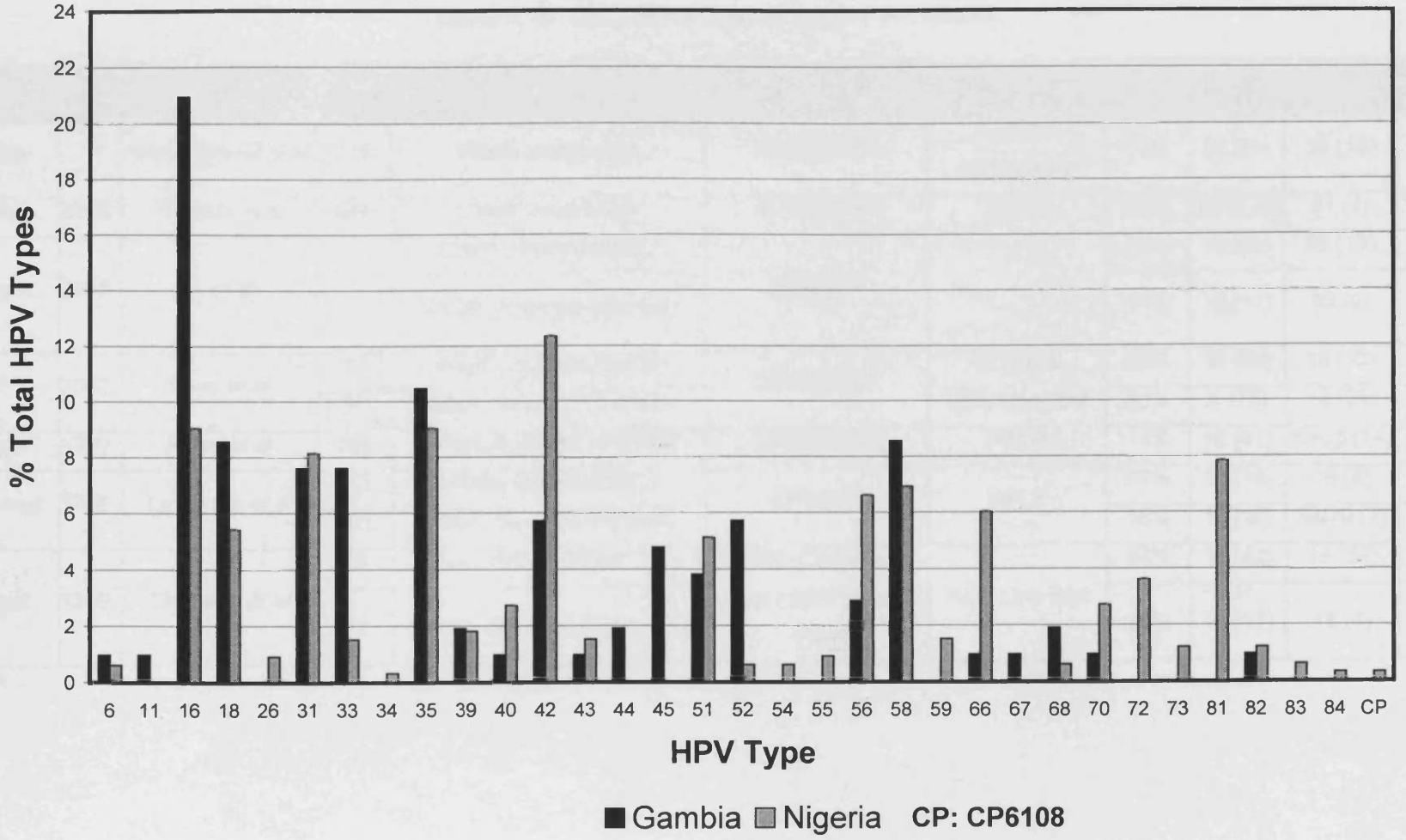


Table 3.19: West Africa Studies of HPV Prevalence

Map	Country	Year Pub.	Ref.	n	Study Population	Amplification Primers	Typing Method	HPV +ve	Top 3 HR (% Total HPV)			Top LR
									1st	2nd	3rd	
1	Gambia	*	Wall & Scherf *et al*	710	Rural, Unselected	GP5+/GP6+	ELISA / Sequencing	13%	16 (21)	35 (10)	18/58 (9)	42
2	Nigeria	2004	Thomas *et al*	844	Urban, Unselected	GP5+/GP6+	ELISA	26%	16/35 (8)	31 (7)	45/56 (6)	42
3	Senegal	2003	Xi *et al*		Urban, Selected (SIL)	MY09/11	-	61%	16 (21)	58 (13)	33 (8)	6
					Urban, Selected (Normal)			14%	16 (11)	83 (9)	33/53/58 (8)	54**
4	Mali	2002	Bayo *et al*	65	Mixed, Selected (CaCx)	GP5+/GP6+	ELISA & Rev. Line Blot	97%	16 (50)	18 (13)	45 (11)	NT
				12	Mixed, Selected (Normal)			33%	X (75)	18 (25)	-	NT
5	Senegal	1999	Astori *et al*	158	Urban, Selected (Normal)	MY09/MY11	RFLP	14%	16 (41)	53/58 (14)	58 (14)	NT
6	Ivory Coast	1998	La Ruche *et al*	211	Urban, Selected (SIL)	GP5/GP6	RFLP	87%	16 (18)	18 (8)	33 (7)	6
				391	Urban, Selected (Normal)			16%	16 (10)	58/70 (7)	31/68 (5)	6
7	Senegal	1996	Chabaud *et al*	65	Urban, Selected (Ab. Cyt.)	X1/X2 Consensus 1°ers, / HPV Specific 1°ers	Rev. Line Blot	94%	16 (42)	18 (39)	45 (10)	6
				72	Urban, Selected (Pregnant)			23%	16 (17)	18 (7)	-	6

*Submitted

Table 3.20: Southern African Studies of HPV Prevalence

Map	Country	Year Pub.	Ref.	n	Study Population	Amplification Primers	Typing Method	HPV +ve	Top 3 HR (% Total HPV)			Top LR
									1st	2nd	3rd	
8	Zimbabwe	2004	Baay *et al*	236	Rural, Unselected	GP5+/GP6+	ELISA	34%	35 (6)	33/58 (6)	18/45 (5)	54†
9	Mozambique	2004	Naucler *et al*	72	Urban, Selected (CaCx)	GP5+/GP6+ & MY09/MY11	Rev. Line Blot	92%	16 (42)	18 (20)	45 (18)**	NT
10	South Africa	2003	Kay *et al*	179	Selected (CIN/CaCx)	L1 Consensus Nested	RFLP	90%	16 (67)	33 (14)	31 (9)	11
11	Zimbabwe	2003	Stanczuk *et al*	98	Urban, Selected (CaCx)	L1 Consensus Nested	RFLP	97%	16 (61)	33 (39)	18 (18)	NT
12	Zimbabwe	2002	Gravitt *et al*	210	Urban, Selected (HGSIL)	MY09/MY11	Rev. Line Blot	77%	16 (12)	18 (8)	31 (5)	6
				213	Urban, Selected (Normal)			31%	52 (11)	51 (9)	16/18 (8)	53**
13	Mozambique	2001	Castellsague *et al*	262	Rural, Selected (Clinic)	PGMY09/MY11	Rev. Line Blot	36%	35 (10)	16 (8)	18 (7)	53**

† 42+43 joint second low risk.

** HPV35 = 4th most prevalent (15%).

Table 3.21: East African Studies of HPV Prevalence

Map	Country	Year Pub.	Ref.	n	Study Population	Amplification Primers	Typing Method	HPV +ve	Top 3 HR (% Total HPV)			Top LR
									1st	2nd	3rd	
14	Kenya	2003	de Vuyst *et al*	429	Urban, Selected (Clinic)	SPF10	Rev. Line Blot	44%	52 (18)	16 (15)	35 (12)	44
15	Tanzania	2003	Mayaud *et al*	190	Urban, Selected (Clinic)	MY09/MY11	Rev. Line Blot	34%	16 (18)	58 (12)	33 (11)	6
16	Tanzania	2001	Mayaud *et al*	612	Urban, Selected	MY09/MY11	Rev. Line Blot	34%	16 (29)	58 (18)	MM7 (17)	NT

Table 3.22: North African Studies of HPV Prevalence

Map	Country	Year Pub.	Ref.	n	Study Population	Amplification Primers	Typing Method	HPV +ve	Top 3 HR (% Total HPV)			Top LR
									1st	2nd	3rd	
17	Tunisia	2003	Hassen *et al*	106	Selected (Clinic)	MY09/MY11	Type Specific 1°ers	14%	-	-	-	6*
				51	Selected (Prostitutes)			39%	16 (37)	-	-	-
18	Morocco	2003	Amrani *et al*	147	Urban, Selected (CaCx)	MY09/MY11	Rev. Line Blot	62%	16	18	-	NT
19	Morocco	2003	Lalaoui *et al*	129	Urban, Selected (Clinic)	MY09/MY11	Rev. Line Blot	71%	16 (49)	18 (22)	NT	NT

Table 3.23: Studies of HPV Prevalence from the Rest of the World

Map	Country	Year Pub.	Ref.	n	Study Population		Amplification Primers	Typing Method	HPV +ve	Top 3 HR (% Total HPV)			Top LR
										1st	2nd	3rd	
20	Iran	2003	Farjadian *et al*	101	Urban, Selected (CaCx)		GP5/GP6	Type Specific 1°ers (HPV-16, -18)	85%	16 (27)	-	-	NT
21	Sweden	2002	Forslund *et al*	6123	Urban, Unselected		GP5+/GP6+	ELISA	7%	16	31		NT
22	Canada	2001	Feoli-Fonseca *et al*	531	Urban, Selected (CaCx)		GP5+/GP6+ & MY09/MY11	Direct Sequencing	70%	16 (20)	31 (7)	56 (3)	6
23	Costa-Rica	2000	*Herrero *et a*	9175	Rural, Unselected		MY09/MY11	Rev. Line Blot	16%	16	18	58	6
24	Brazil	1999	**Franco *et al*	1425	Urban, Selected (Clinic)	Entry	MY09/MY11	RFLP	14%	16 (21)	53 (11)	58 (9)	6/11
						Cumulative			25%	16 (18)	53 (14)	58 (10)	6/11
25	Hong Kong	1999	Chan *et al*	332	Urban, Selected (Ab. Cyt.)		MY09/MY11	RFLP	44%	16 (33)	58 (24)	18 /31(9)	11
26	USA	1999	Liaw *et al*	277	Urban, Selected (SIL)		MY09/MY11	Rev. Line Blot	78%	16 (13)	51 (10)	56 (7)	53
				961	Urban, Selected (Normal)				15%	16 (20)	51 (15)	31/45/58 (7)	53

*HPV prevalence estimated by selectively testing women with SIL.
** Cumulative total of testing at entry and after 18 months.
† Breakdown of HPV types included non-cervical samples

Figure 3.22: Map of African HPV Prevalence Studies in Tables 3.23-3.27

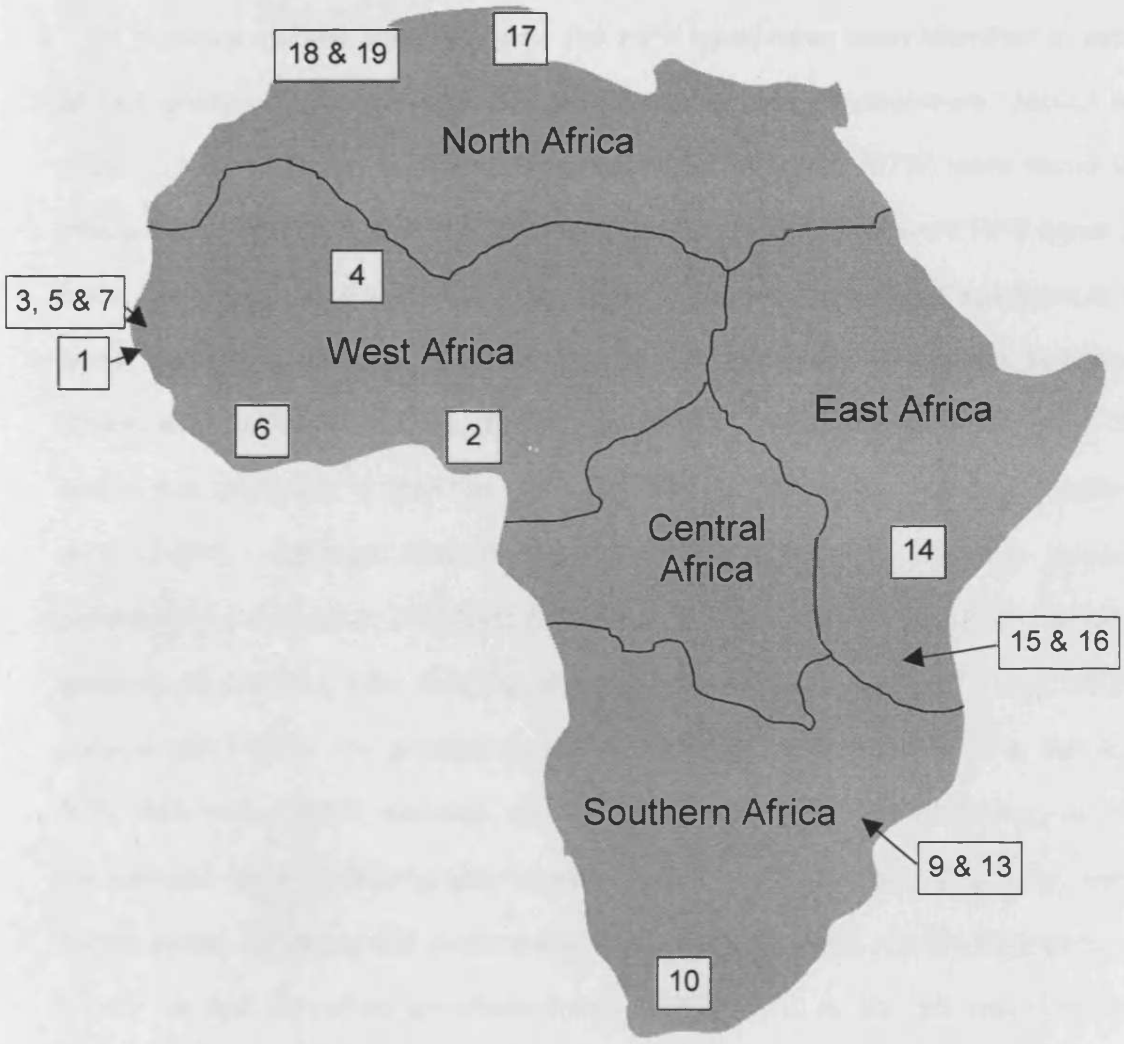
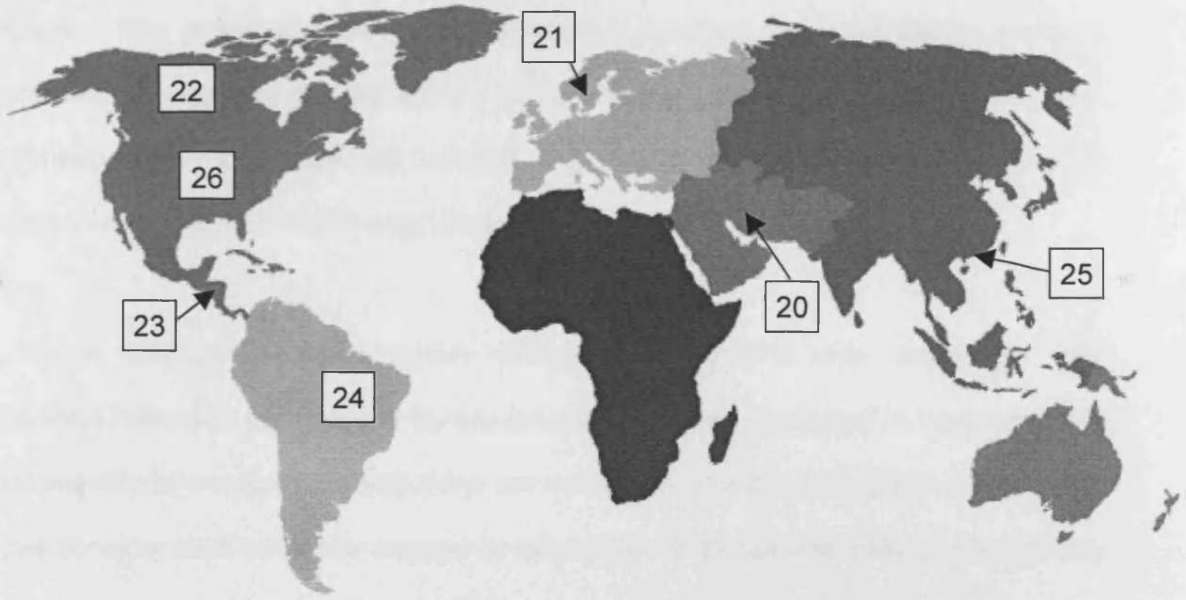


Figure 3.23: Map of HPV Prevalence Studies From the Rest of the World in Table 3.28



3.4.4 Host-Virus Interactions

In previous studies high oncogenic risk HPV types have been identified in virtually all high-grade pre-invasive and invasive cervical lesions (Walboomers, Jacobs et al. 1999). Here, of fifteen samples displaying HGSIL, thirteen (87%) were found to be infected with high oncogenic risk HPV types. A total of eight different HPV types were found to be associated with HGSIL (Table 3.10), reflecting the broad spectrum of HPV types found in this study. Worldwide HPV-16 is generally considered to have the closest association with HGSIL (Clifford, Smith et al. 2003; Clifford, Smith et al. 2003) and in this study this is also the case with 3/14 (21%) HGSIL samples positive for HPV-16 DNA. Although HPV-58 and -33 are also present in the same number of patients these include co-infections (Table 3.11). Thus crucially the HPV type with the greatest association with cervical abnormalities in rural Gambia is that which is considered to be of the greatest oncogenic potential. It is possible that, the lack of HPV DNA in two HGSIL samples and 13 samples with LGSIL / ASCUS may be due to the samples either contained poor quality DNA, or a PCR confounding factor such as haemoglobin. However this is extremely unlikely as both were positive for β-globin. So it may be that they may be infected with HPV types that are not amplified by the GP5+/GP6+ pair. Alternatively the viral DNA may have integrated into the host genomic DNA with associated deletion of the target site for the consensus PCR primers. This is a likely event, as discussed in Chapter 1, as integration leads to increased HPV genetic stability and a selective cellular growth advantage (Jeon, Allen-Hoffmann et al. 1995; Jeon and Lambert 1995) and has been shown to be the most common explanation for HPV negative cervical cancers (Walboomers, 1999).

Of the subjects displaying normal cytology 49/574 (8.5%) were positive for HPV infection (Table 3.9) but this is to be expected because, as discussed in Chapter 1, the vast majority of cervical HPV infections are transient in nature (Hildesheim, 1994) The types considered to be of low oncogenic were found in all but one case in cytologically

normal individuals. The one case where HPV-42 was found in LGSIL may be a rare but possible case of a low risk type leading to malignancy, albeit low grade, or there maybe an undetected high oncogenic risk co-infection.

3.4.5 Sequencing of L1 in HPV Types Associated with HGSIL

Globally the most common HPV type and that most associated with HGSIL and thus the target of nearly all HPV vaccine research is HPV-16 (Clifford, Smith et al. 2003; Muñoz, Bosch et al. 2003, Bosch, 1995, Clifford, 2003). Thus the sub-typing, and variant analysis by sequencing of HPV-16 is crucial to vaccine planning as differences within epitope regions may impair or even completely inhibit vaccine efficacy. All HPV-16 samples sequenced in this study are homologous with a variant of HPV-16 African Type 1 (Table 3.14, Figs. 3.7-3.9). This is consistent with other studies that have found this type to be the most prevalent of HPV-16 sub-type in Africa as a whole (Yamada, Manos et al. 1997). Within the MY09/11 region of L1 sequenced, the Gambian HPV-16 samples and the Af1 variant which matches them are homologous on the protein level to the reference strain. However the other HPV-16 Af1 variant which was found in the BLAST search is seen to possess a polymorphism compared to the reference sequence (Fig. 3.8). This illustrates the importance of sequencing to screen for sub-types and variants. This is especially true of the most prevalent types and those associated with malignancy as these are the types upon which vaccine strategy will be developed and polymorphisms may have ramifications for vaccine efficacy. One of the HPV-18 samples sequenced is homologous to a sample from Benin in West Africa. Whilst the differences the other HPV-18 sample sequenced displayed are seen in another sample from Benin a sample from Uganda in East Africa (Table 3.21, Figs. 3.10-3.12). None of these samples however differ on the protein level to the reference HPV-18 (Fig. 3.11). The Gambian HPV-31 sample has 2 polymorphisms compared with the reference sequence and no exact matches to this Gambian type were found

on the BLAST search. The HPV-33 samples sequenced possessed a single silent point mutation compared to the reference type and a separate silent point mutation compared to a sample originating in the West African State of Guinea (Table 3.19 & Figs. 3.15-3.17). The HPV-58 sample sequenced showed complete homology to a sample from another West African nation, this time Mali, with both showing 3 polymorphisms compared to the reference sequence. Thus a with the non HPV-16 samples associated in this study with HGSIL a number of polymorphisms are observed compared to the reference types. However most of these types are identical or similar to types found in other African studies. These local variations may have to be considered when a vaccine strategy is planned.

3.5 Concluding Remarks

Overall prevalence of Cervical HPV infection in rural Gambia was found to be around 13% this is concurrent with other African rural studies and unselected Western prevalence studies. There was a divergence from the common low oncogenic risk types seen in Western industrialised countries, HPV-06 and -11, to HPV-42 which constituted 50% of all low risk types. It is possible that this may be due to host immunogenetic differences or just localised differences. However, significantly the type most commonly associated with HPV worldwide, HPV-16, was found to be the most prevalent high risk type. Thus as far as the most oncogenic risk type is concerned a non-caucasoid and potentially a differing set of host responses is seemingly unimportant. However, HPV-35 is the second most prevalent type, this type prevalence where HPV-16 and -35 are the two most prevalent type is largely in accordance with other African studies where the MY09/11 primer pair is not used, and the use of this primer pair may have lead to the underestimation of HPV-35 levels throughout Africa. Thus as HPV-35 is rarely seen in caucasoid HPV studies but is highly prevalent in Africa, host immunogenetics appears play a significant part with

some HPV types. An immunogenetic study of the population examined here would help to determine this host virus relationship and is discussed in detail in Chapter 7. Sequencing revealed HPV types to be closely related to other African types, principally HPV-16 found to be a variant of African type 1, the most common HPV 16 variant in Africa. HPV types universally considered of high or low oncogenic risk were found to relate to histological data from this study. Of these high oncogenic risk types 70% were observed to be members of HPV group A, the same group as HPV-16. Overall, these findings may have a vital impact upon vaccine research and this is discussed at length in Chapter 7. This study is unique as a non-caucasoid population has been examined the ethnic composition of which has never previously been examined in the manner used here. We have seen here that previous studies in the only nation which shares similar tribal groups to the Gambia, Senegal, used methods which will have grossly underestimated the second most common high risk HPV type in this study. Other studies in African populations which used the same methods to those used here show differing LR/HR ratios and disease associations implying that there is more to cervical cancer than just HPV infection. In the next chapter such viral genetics and type ratios shall be examined in a disease mediated by low risk HPV which is nonetheless rare but lethal.

Chapter Four:

HPV Polymorphisms & Viral Co-Factors in Recurrent Respiratory Papillomatosis

Contents

4.1 Introduction

4.1.1 Aims and Scope of Chapter 4

In the previous chapter cervical cancer, a malignant disease caused by high oncogenic risk HPV was examined. This chapter presents an examination of viral factors in the pathogenesis of RRP; a rare but potentially fatal infection caused by the "low risk" HPV types -6 and -11. As discussed in Chapter 1, RRP is characterised by the presence of recurrent papilloma on the larynx and other sites in the aerodigestive tract (trachea, lung, oesophagus, nose and pharynx). Untreated these lesions increase in size and fatal airway obstruction may occur. In addition in around 1% of patients malignant transformation occurs and fatal laryngeal and lung carcinomas develop in children and young adults (Reidy, Dedo et al. 2004).

4.1.2 RRP Aetiology

As described in Chapter 1 HPV types -6 and -11 also cause genital warts, and RRP is believed to be contracted oral-genital contact (Kashima, Shah et al. 1992; Shah, Stern et al. 1998; Silverberg, Thorsen et al. 2003; Silverberg, Thorsen et al. 2004). Genital warts are present in up 1% of the sexually active US population with sub-clinical genital HPV infection present in around 15% of such a population (Koutsky 1997; Maw 2004). Oral HPV infection with HPV types-6 and -11 have been reported in 10% of the UK population (Miller and Johnstone 2001). So there are similar infection rates of HPV-6 & -11 in the oral and genital regions yet RRP is a rare disease occurring in only about 1 per 100,000 individuals. This implies that other factors either from the virus or the host, or both must play an important role in disease pathogenesis. This chapter presents an investigation into possible viral factors in RRP, Chapters 5 and 6 present investigations into host factors in RRP, and Chapter 7 will discuss host virus interactions in detail in this disease.

4.1.3 Potential Mechanisms for Evasion of Host Immune Response by HPV in RRP

As with many other viruses such as; HIV, HBV and EBV, HPV has a number of mechanisms for evasion of immune surveillance (Gallo, 2002; Tindle, 2002). Such mechanisms in HPV infection include; subversion of IFN responses, poor, unstimulated, immune response to E6 and E7, and preferred codon usage in the L1 and L2 outer capsid protein (Zhou, 1999).

As discussed in detail in Chapter 1, our group has previously found the HLA class II allele DRB*0301 and the ancestral haplotype 8.1 (AH 8.1) to be significantly associated with RRP (Gelder, Williams et al. 2003) and this relationship is extended in Chapter 5. We have also demonstrated that the magnitude of proliferative T-cell response to HPV-11 L1 in RRP patients is inversely proportional to their disease status (Gelder et al. 2003). Thus patients in remission or with minimal disease displayed strong proliferative responses whereas those with advanced disease made minimal or no proliferative responses to L1.

We have also characterised an epitope in the L1 outer capsid gene of HPV-11; p139 (amino acids 139 to 153 of HPV-11 L1) which appears to be immunodominant in the HLA DRB01*301 restricted CD4+ T-cell response to L1 of both healthy controls and patients with RRP. This HPV-11 epitope is homologous, on the amino acid level, with the same region in HPV-6a, -6b and 6vc (Table 4.1). Moreover, it contains an appropriate DRB01*0301 restricted motif (Fig. 4.1) and has a strong binding affinity to DRB1*0301 in peptide binding competition assays (Gelder et al. 2003). This raised the interesting possibility that mutations within this region might have accounted for the weak or absent T-cell responses observed in DRB1*0301 positive individuals with severe RRP. Also that other mutations within L1 might have modulated the immune

responses of other DRB1*0301 negative individuals. In order to address this possibility HPV-11 has been sequenced from papilloma tissue collected from 53 individuals with RRP. One complicating factor in this investigation was that the Genebank HPV-11 reference sequences are from individuals with RRP. Thus in order to establish the "normal sequence" of HPV-11 from genital warts and to exclude geographical variation between North American and UK HPV-11 had initially to be obtained from genital warts in the UK, and then HPV typed and sequenced.

Table 4.1: Comparison of HPV-11 and -6 in L1 Peptide 139 region

HPV Type	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153
HPV-11	D	N	R	V	N	V	G	M	D	Y	K	Q	T	Q	L
HPV-6a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HPV-6b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HPV-6vc	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

D N R V N V G M D Y K Q L

Figure 4.1: Peptide 139 (DRB01*0301 Motif in Grey & Anchoring Residues in White)

HPV late genes utilise codons and consequent tRNA that are used only rarely in mammals. This inherent redundancy of the genetic code results in minimal expression of L1 and L2. This minimal expression is localised to differentiated epithelial cells by translation and transcription control mechanisms that inhibit HPV late gene expression in epithelial undifferentiated proximal strata (Schwartz, 2000). The consequence of these factors is the reduced exposure of L1 and L2 to the host immune system and subsequently little chance of priming the immune system to a response against HPV. It is possible therefore that the lack of antibody response to the HPV oncogenes in, for instance, 20-40% of HPV-16 positive patients with cervical cancer (Nindl, 2000), is due

to the same subversion of the genetic code redundancy observed in the HPV late genes. For this reason any even, consistent, silent coding mutations observed in the HPV oncogenes of RRP patients may be the consequence of such an escape mechanism.

4.1.4 RRP May Potentially be Caused by Unique Viral Clade or Subtype

It is possible that RRP is caused by a unique HPV viral sub-type or clade which may as described above, able to avoid immune surveillance or may just be better suited to the infection of laryngeal tissue. The L1 major capsid gene is an important gene to sequence for reasons outlined above. As discussed in Chapter 1 malignant transformation in genital warts does not occur, whilst in RRP there is a 1% frequency of malignancy (Reidy et al. 2004). Therefore, changes adapting the virus to the larynx may potentially be present in the E6 & E7 oncogenes.

Also we have seen in Chapter 1 that a number of different mechanisms are implicated in HPV entry into the cell mediated by L1 and that such mechanisms appear to vary between HPV types and may explain HPV tissue specificity between types. Therefore any repeated polymorphisms within L1 of RRP patient HPV may be a potential mechanism which results in adapting HPV-11 & -6 to laryngeal tissue in RRP.

It is thus essential to sequence these genes in HPV from both RRP papillomata and genital wart biopsies. As three main subtypes of HPV-6 are well defined it is possible that RRP mediated by HPV-6 is caused by a single subtype which is better adapted for laryngeal infection. Therefore HPV-6 subtype distribution is also examined in this Chapter.

4.1.5 Potential Co-Factor Viruses Interactions in RRP

As discussed in Chapter 3 viral co-factors are relevant to the progression of other HPV mediated diseases, such as cervical carcinoma where HIV acts to exacerbate cervical cancer. In this chapter associations with putative herpes virus co-factors are examined by nested PCR and compared with disease aggression.

4.2 Methodology

4.2.1 Clinical Samples

Individuals with RRP were recruited for this study by contacting all Ear, Nose and Throat surgeons throughout Wales and those in England known to be interested in RRP research. A diagnosis of RRP was established by either distinctive recurrent papillomata or the necessity of at least surgical debulking procedures, or both. Laryngeal papillomata biopsies were obtained, with informed consent, from patients receiving treatment for RRP.

These originated from the University Hospital of Wales (UHW), Cardiff, as well as samples from: Sheffield Children's Hospital, Birmingham Children's Hospital, St Michaels Hospital, Bristol, Great Ormond St Hospital (GOS), London and the Royal Sussex Hospital, Brighton. Genital wart biopsies were obtained anonymously from consenting patients undergoing treatment at the Genitourinary Medicine clinic at the Cardiff Royal Infirmary (CRI).

4.2.2 Ethics

Written informed consent was obtained from patients, and ethical approval was obtained from the Multi Centre Research Ethics Committee for Wales, the Bro Taf Local Research Ethics Committee, and other appropriate Local Research Ethics Committees. All work was conducted in accordance with the Helsinki Declaration of 1975 as revised in 1983.

4.2.2 DNA Purification

Viral DNA was extracted using the 'freeze thaw' technique described in Chapter 2.

4.2.3 HPV Detection and Typing

HPV DNA was detected by the amplification of a 140bp region of the L1 outer capsid gene with the $GP5^{+}$/biotinylated $GP6^{+}$ primer pair described in Chapter 2. HPV-6 subtyping was performed by sequencing using specifically designed primers described below.

4.2.4 Sequencing

Sequencing was carried out on a Beckman Coulter CEQ2000 Automated Sequencer (Beckman Coulter, Fullerton, CA, USA) by the protocol described in Chapter 2 to a >98% accuracy. The full length of the E6, E7 and L1 ORF were sequenced using specifically designed primers listed in Chapter which flanked each ORF by 50bp at each end. An NCBI BLAST search was carried out for each of the consensus strands produced by sequencing at least twice in each direction. DNA alignment and analysis was carried out using the Lasergene suite of software (DNA* Inc. Madison, Wisconsin, USA).

4.2.5 Detection of Herpes Viruses

Detection of the presence of, CMV, EBV, HHV-6,-7 and -8, HSV-1 and 2 and VZV was carried out using a nested PCR protocol after (Wakefield, Fox et al. 1992).

4.3 Results

4.3.1 A Comparative Study of HPV Type Prevalence in Genital warts & RRP by PCR-ELISA

HPV DNA was found in 95% (37/39) of the genital warts studied with the majority being single HPV-6 or -11 infections (Table 4.3). Multiple infections accounted for only 14% (5/37) of the genital warts where HPV was observed. In all instances of multiple infection, the additional HPV types were high oncogenic risk types. HPV-16 was present in all of these multiple infections, and in three samples a third HPV type was also observed; two HPV-33 and one HPV-58 (Table 4.4, Fig. 4.3). However amongst the laryngeal warts no dual infections were observed and the only HPV types found were HPV-6 and -11 (Table 4.5, Fig. 4.2). All genital and laryngeal warts that were positive for HPV DNA by PCR-ELISA were found to possess either HPV-06 or HPV-11. Of the genital warts 26% (10/38) were positive for HPV-11 and 74% (28/38) for HPV-6 (including one HPV-6/11 co-infection). The laryngeal warts however, exhibited 40% (19/47) HPV-11 positive and 60% (28/47) HPV-6 positive (Table 4.1 and Figs. 4.2 and 4.3).

Table 4.2: Comparison of HPV-6 & -11 in Genital and Laryngeal Warts

HPV Type	Laryngeal Papillomata		Condyloma Acuminata*	
11	21	40%	10	26%
06	32	60%	28	74%

* Includes 1 HPV-06 / 11 Co-infection

Fishers Exact Test 2 Sided P Value: 0.2623, Odds Ratio: 1.838,

95% Confidence Interval: 0.7410 to 4.556

Table 4.3: HPV Typing Details of all Genital Warts

Sample	Site	HPV Type		Sub-Type	Sample	Site	HPV Type		Sub-Type
		LR	HR				LR	HR	
GW01	Vulval	6	16	6a	GW21	Vulval	6	16 / 58	6vc
GW02	Perineal	6		6a	GW22	Scrotal	6		6a
GW03	NK	6		6a	GW23	NK	6		6a
GW04	Penile	6		6a	GW24	NK	11		-
GW05	Perineal	6		6a	GW25	NK	6		6vc
GW06	NK	11		-	GW26	Penile	6	16 / 33	6a
GW07	NK	6		6vc	GW27	Penile	6		6a
GW08	NK	6		6a	GW28	NK	6		6a
GW09	Penile	11		-	GW29	NK	11	16 / 33	-
GW10	NK	6		6a	GW30	NK	11	16	-
GW11	Penile	6		6a	GW31	NK	6		6a
GW12	NK	11		-	GW32	NK	6		6vc
GW13	NK	11		-	GW33	NK	6		6a
GW14	NK	11		-	GW34	NK	6		6vc
GW15	Penile	6		6vc	GW35	NK	6		6a
GW16	Introitus	6 / 11		6a	GW36	NK	6		6vc
GW17	Fourchette	6		6vc	GW37	NK	6		-
GW19	Penile	6		6a	GW38	NK	6		6b
GW20	perineal	11		-					

NK: Not Known

HPV Type	6	11	16	18	31	33	35	39	40	42	43	44	45	51	52	56	58	59	66	68
n	28	10	5	0	0	2	0	0	0	0	0	0	0	0	0	0	1	0	0	0
% Total*	61	22	11	-	-	4	-	-	-	-	-	-	-	-	-	-	2	-	-	-

Table 4.4: Distribution of all Genital Wart HPV Infections

Types listed in white are Low Risk types, types in black are High Risk

Table 4.5: HPV Typing Details of all Laryngeal Warts

Sample	Onset*	HPV Type	Sub-Type	Sample	Onset*	HPV Type	Sub-Type
RRP-001	J	11	-	RRP-050	J	11	-
RRP-002	J	11	-	RRP-053	J	6	-
RRP-003	J	11	-	RRP-054	A	6	-
RRP-004	J	11	-	RRP-055	J	6	-
RRP-005	J	11	-	RRP-056	J	6	6a
RRP-007	J	11	-	RRP-057	A	6	6vc
RRP-008	J	6	6b	RRP-058	A	6	6vc
RRP-011	A	6	6b	RRP-060	A	6	6vc
RRP-012	A	6	6a	RRP-061	A	11	-
RRP-020	J	6	-	RRP-064	A	11	-
RRP-033	J	11	-	RRP-067		6	-
RRP-034	J	11	-	RRP-081	J	11	-
RRP-035	J	6	-	RRP-083	J	11	-
RRP-036	J	6	-	RRP-084	J	11	-
RRP-037	J	6	-	RRP-085	J	6	6a
RRP-038	J	6	6a	RRP-086	J	11	-
RRP-039	J	11	-	RRP-087	J	6	6vc
RRP-040	J	6	6a	RRP-088	J	6	6a
RRP-041	J	11	-	RRP-090	J	6	6vc
RRP-042	J	6	-	RRP-091	J	6	6vc
RRP-043	J	6	6vc	RRP-108	J	11	-
RRP-044	J	6	6vc	RRP-109	J	6	6a
RRP-045	J	6	6a	RRP-110	J	6	6a
RRP-046	J	11	-	RRP-111	J	6	6a
RRP-047	A	6	-	RRP-112	J	11	-
RRP-048	J	6	-	RRP-115	J	6	6vc
RRP-049	J	11	-				

*J:JORRP, A:AORRP

Patient RRP-1: Progressed to lung involvement which leading to fatal malignancy

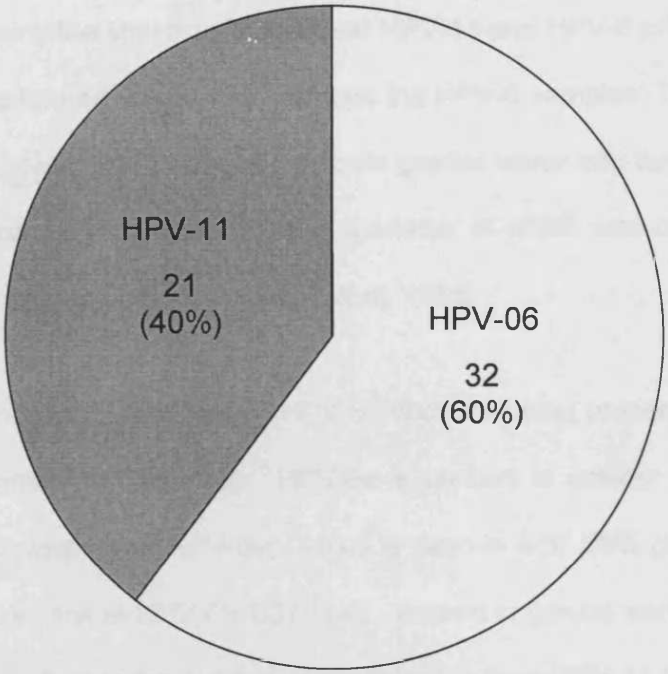


Figure 4.2: Laryngeal Wart HPV Types

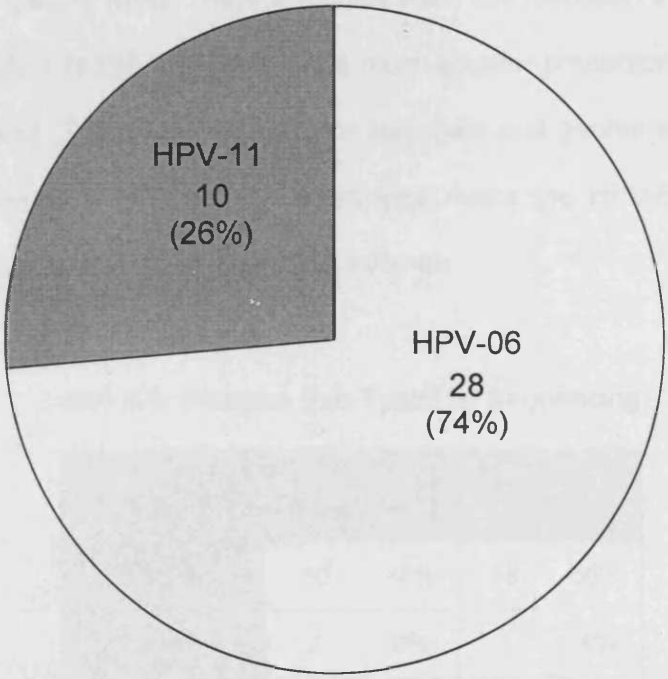


Figure 4.3: Low Risk Genital Wart HPV Types

4.3.2 HPV-6 Sub-Typing by Sequencing

Sequencing using the specifically designed HPV-11 and HPV-6 primers detailed in Chapter 2 was performed, in order to sub-type the HPV-6 samples. This revealed the presence of the three HPV-6 subtypes in both genital warts and laryngeal biopsies. These are HPV-6a, HPV-6b and HPV-6_{VC}, the latter of which was originally isolated from a vulval carcinoma (Rando, Sedlacek et al. 1986).

HPV-6a dominates the genital wart HPV-6 infections being present in 18/37 (66%) genital warts examined (Table 4.6). HPV-6a is present in greater than double the number of genital warts than HPV-6vc, which is seen in 8/37 30% genital warts, and eighteen times the level of HPV-6b 1/37 (4%). Indeed in genital warts HPV-6a alone is present in nearly double the number of genital warts than HPV-11 (Table 4.2).

The laryngeal warts displayed a very different breakdown of HPV-6 subtypes compared to the genital warts. Here a roughly even split between the HPV-6a 10/21 (48%) and -6vc 9/21 (43%) subtypes and a much smaller proportion of HPV-6b 2/21 (9%) was observed (Table 4.6). In both the laryngeal and genital warts the HPV-6a and -6vc far outnumber HPV-6b in the laryngeal warts the HPV-6a and 6vc were present approximately five times more than HPV-6b.

Table 4.6: Samples Sub-Typed by Sequencing

HPV Sub-Type	Laryngeal Papillomata		Condyloma Acuminata	
HPV-06a	10	48%	18	66%
HPV-06b	2	9%	1	4%
HPV-06_{VC}	9	43%	8	30%

4.3.3 L1 Outer Capsid Protein Genetic Variations

Our group has previously found the strength of the immune response of RRP patients to the L1 outer capsid protein to be in inverse proportion to disease severity. Therefore it is possible that this lessened response is caused by a mutation in L1 so the L1 gene of HPV from both RRP patients and genital warts were sequenced and compared. Genbank reference Sequences of HPV-11 are derived from laryngeal papillomata so it is crucial to examine the sequence of genital warts to establish 'normal' HPV-11 sequence.

Polymorphisms and silent point mutations were seen in the L1 major capsid protein gene of the HPV-6 and HPV-11 samples in both RRP and genital wart samples. However no obvious repeated differences were observed between those of laryngeal biopsies and genital warts (Tables 4.7-4.10). The next candidate genes to sequence were the E6 and E7 oncogenes, since papillomata become malignant in around 1% of RRP patients.

Table 4.7: HPV-06a L1 Genetic Variations

Accession / Sample No.	Origin	Ref	5993	6105	6119	6218	6626	6722	7080	7283
NC001668	CA	(Hofmann, Cook et al. 1995)	a	c	a	a	c	a	g	t
AR169144	CA	(Hofmann et al. 1995)	-	-	-	-	-	-	-	-
L41216	CA	(Jansen 2001)	-	-	-	-	-	-	-	-
RRP-012	LP		-	-	g	-	-	-	-	c
GW-003	CA		g	a	-	t	a	g	c	c
GW-008	CA		g	a	-	t	a	g	c	c
a/a									431	
Genbank									E	
Variant									Q	

Table 4.8: HPV-06b L1 Genetic Variations

Accession / Sample No.	Origin	Ref	6598	6745	7099
X00203	CA	(Schwarz, Durst et al. 1983)	a	t	g
RRP-008	LP		t	a	a

Table 4.9: HPV-06_{vc} L1 Genetic Variations

Accession / Sample No.	Origin	Ref	6014	6014
AF092932	CA	(Kovelman, Bilter et al. 1999)	a	t
RRP-043	LP		t	g
RRP-058	LP		-	g
RRP-060	LP		-	g
a/a				219
Genbank				Y
Variant				D

- : As Genbank Sequence

Table 4.10: HPV-11 L1 Genetic Variations

Accession / Sample No.	Origin	Ref	6028	6106	6135	6379	6445	6484	6556	6700	6751	6760	6832	6988	7275*
M14119	LP	(Dartmann , Schwarz et al. 1986)	c	t	a	t	c	t	t	t	t	g	a	a	a
L36108	CT	(Fife 1994)	-	-	-	-	-	-	-	-	-	-	-	-	-
RRP-001	LP		t	-	-	-	-	-	-	-	-	-	-	-	g
RRP-003	LP		t	-	-	-	-	-	-	-	-	-	-	-	-
RRP-004	LP		t	-	-	c	-	-	-	-	-	-	-	-	-
RRP-007	LP		t	-	-	-	-	-	-	-	-	-	-	-	-
RRP-034	LP		t	-	-	-	-	-	-	-	-	-	-	-	g
RRP-039	LP		t	-	-	-	-	-	-	-	-	-	-	-	-
RRP-046	LP		t	-	-	-	-	-	-	-	-	-	-	-	-
RRP-061	LP		t	-	-	-	-	-	-	-	-	-	-	-	g
RRP-064			-	-	-	-	-	-	-	c	-	t	-	-	g
GW-001	CA		t	g	-	-	-	-	-	-	-	-	-	-	
GW-006	CA		t	-	-	-	-	c	-	-	-	-	-	-	
GW-013	CA		t	-	-	-	-	-	-	-	-	-	-	-	-
GW-014	CA		t	-	-	-	-	-	a	-	-	-	g	-	-
GW-016	CA		t	-	-	-	-	-	a	-	-	-	g	-	
GW-024	CA		t	-	-	-	-	-	-	-	-	-	-	t	-
GW-034	CA		t	-	-	-	-	-	-	-	-	-	-	-	g
RRP-049	LP		t	-	-	-	g	-	-	-	-	-	-	-	-
GW-012	CA		-	-	g	-	-	-	-	-	c	-	-	-	-
a/a					122										
Genbank					R										
Variant					K										

- : As Genbank Sequence

* : Stop Codon

Patient RRP-1: Progressed to lung involvement which leading to fatal malignancy

4.3.3 HPV-11 Oncogene Genetic Variations

Four homologous HPV-11 samples, two genital warts and two RRP samples shared four identical polymorphisms and two silent point mutations in the E6 and E7 genes when compared with the other HPV-11 genital wart and RRP samples (Table 4.11). In these samples polymorphisms and two silent point mutations were present in the E6 gene and two polymorphisms and one silent point muation in the E7 gene.

4.3.3.1 E6

The polymorphisms in E6 in these variants feature transversions at nucleotides 383 and 398; thymidine to guanine and adenosine to cytosine respectively. This leads to a substitution of neutral asparagine at position 94 for the larger and positively charged lysine in the variants, whilst the reverse is true at position 99.

4.3.3.2 E7

At nucleotide 662 within the E7 gene the four variants are in agreement with the Genbank sequence with a guanine present coding for alanine at position 45 of E7. In all other laryngeal and genital HPV-11 samples in the study there is a transversion to a thymidine resulting in a serine in this position. Another transversion at nucleotide 761, this time of a cytosine for an adenosine in the four variants leads to a lysine at a/a 78 in the variants and a glutamic acid in all other HPV-11 study samples and the Genbank sequences.

The HPV-11 Genbank reference sequences were M14119, a laryngeal wart (Dartmann et al. 1986) and L36108 a tongue cancer biopsy (Fife 1994). Only one sample in the study within the E6 and E7 genes exactly matches the HPV-11 Genbank sequences (Table 4.11).

Table 4.11: HPV-11 Oncogene Variations

		Gene	E6					E7	
Accession / Sample No.	Origin	Ref	137	380	383	392	398	662	761
M14119	LP	(Dartmann et al. 1986)	t	c	t	t	a	g	c
L36108	CT	(Fife 1994)	t	c	t	t	a	g	c
GW-020	CA		-	-	-	-	-	-	-
RRP-001	LP		c	t	-	-	-	t	-
RRP-003	LP		c	t	-	-	-	t	-
RRP-004	LP		c	t	-	-	-	t	-
RRP-007	LP		c	t	-	-	-	t	-
RRP-034	LP		c	t	-	-	-	t	-
RRP-039	LP		c	t	-	-	-	t	-
RRP-046	LP		c	t	-	-	-	t	-
RRP-061	LP		c	t	-	-	-	t	-
RRP-064	LP		c	t	-	-	-	t	-
RRP-081	LP		c	t	-	-	-	t	-
RRP-083	LP		c	t	-	-	-	t	-
RRP-084	LP		c	t	-	-	-	t	-
RRP-086	LP		c	t	-	-	-	t	-
GW-006	CA		c	t	-	-	-	t	-
GW-009	CA		c	t	-	-	-	t	-
GW-013	CA		c	t	-	-	-	t	-
GW-014	CA		c	t	-	-	-	t	-
GW-016	CA		c	t	-	-	-	t	-
GW-024	CA		c	t	-	-	-	t	-
GW-029	CA		c	t	-	-	-	t	-
RRP-049	LP		c	-	g	a	c	-	a
RRP-050	LP		c	-	g	a	c	-	a
GW-012	CA		c	-	g	a	c	-	a
GW-030	CA		c	-	g	a	c	-	a
a/a					94		99	45	78
Genbank					N		K	A	Q
Variant					K		N	S	K

-: As Genbank Sequence

CT: Tongue Cancer Biopsy

Patient RRP-1: Progressed to lung involvement which leading to fatal malignancy

Table 4.12: HPV-06a Oncogene Variations

		Gene	E6				E7		
Accession / Sample No.	Origin	Ref	157	351	474	480	646	709	792
NC001668	CA	(Hofmann et al. 1995)	a	t	g	t	a	g	g

AR169144	CA	(Hofmann et al. 1995)	a	t	g	t	a	g	g
L41216	CA	(Jansen 2001)	a	t	g	t	a	g	g
RRP-012	LP		-	-	a	-	-	-	-
RRP-038	LP		-	-	a	-	-	a	-
RRP-040	LP		-	-	a	-	-	-	-
RRP-045	LP		-	-	a	-	-	-	-
RRP-056	LP		-	-	a	-	g	a	-
RRP-085	LP		-	-	a	-	-	-	-
RRP-088	LP		-	-	a	-	-	-	-
GW-022	CA		-	-	a	-	-	-	-
GW-023	CA		-	-	a	-	-	-	-
GW-035	CA		-	-	a	y	-	-	-
GW-001	CA		t	-	a	c	-	-	a
GW-002	CA		t	-	a	c	-	-	a
GW-003	CA		t	-	a	c	-	-	a
GW-004	CA		t	-	a	c	-	-	a
GW-005	CA		t	-	a	c	-	-	a
GW-008	CA		t	-	a	c	-	-	a
GW-011	CA		t	-	a	c	-	-	a
GW-019	CA		t	-	a	c	-	-	a
GW-026	CA		t	-	a	c	-	-	a
GW-027	CA		t	-	a	c	-	-	a
GW-028	CA		t	-	a	c	-	-	a
GW-031	CA		t	-	a	c	-	-	a
GW-033	CA		t	-	a	c	-	-	a
GW-008	CA		t	g	a	c	-	-	a
GW-010	CA		t	g	a	c	-	-	a
GW-016	CA		t	g	a	c	-	-	a
a/a			19				39	60	88
Genbank			T				E	G	D
Variant			S				G	E	N

-: As Genbank Sequence

y = t & c

Table 4.13: HPV-06b Oncogene Variations

		Gene	E6	
Accession / Sample No.	Origin	Ref	221	473
X00203	CA	(Schwarz et al. 1983)	a	g
AF126428*	CA	(Ai, Toussaint and Roman 1999)	t	a
RRP-008	LP		t	a
RRP-011	LP		t	a
GW-038	CA		t	a

*E6 only

Table 4.14: HPV-06$_{vc}$ Oncogene Variations

		Gene	E6		E7
Accession / Sample No.	Origin	Ref	312	369	663
AF092932	CA	(Kovelman et al. 1999)	t	t	t
RRP-043	LP		-	-	-
RRP-044	LP		-	-	-
RRP-057	LP		-	-	-
RRP-058	LP		-	-	-
RRP-060	LP		-	-	-
RRP-087	LP		-	-	-
RRP-090	LP		-	c	-
RRP-091	LP		-	-	-
GW-007	CA		-	-	-
GW-017	CA		-	-	-
GW-015	CA		-	-	a
GW-021	CA		c	-	-
GW-025	CA		-	-	-
GW-032	CA		-	-	-
GW-034	CA		-	-	-
GW-036	CA		-	-	-
a/a					45
Genbank					S
Variant					T

-: As Genbank Sequence

4.3.3.4 HPV-6 Oncogene Genetic Variations

4.3.4.1 E6

At nucleotide 157 all but one of the HPV-6a genital warts in our study possess a thymidine giving a serine at a/a 19 (19S). The HPV-6a Genbank sequences NC001668 and AR169144 (also genital warts) and all RRP samples in our study (HPV-6, -6a, -6b and -11) had an adenosine at this position which results in a threonine at a/a 19 (19T). This very significant difference between laryngeal and genital warts is expressed statistically in Table 4.15.

Table 4.15: Polymorphism At E6 Residue 19 in Laryngeal and Genital Warts

E6 19S/T Polymorphism	Laryngeal Papillomata		Condyloma Acuminata	
Serine	0	0%	15	40%
Threonine	37	100%	20	60%

Fishers T test 2 Sided P Value: < 0.0001, Odds ratio: 48.911,

95% Confidence Interval: 2.784 to 859.17

4.3.4.2 E7

At nucleotide 792 all HPV-6a RRP samples in our study have a guanine which gives an aspartic acid at a/a 88 (88D) in the E7 gene. This 88D polymorphism is also present in the same HPV-6a positive genital warts that possessed the E6 19T variation. Each of the HPV-6a genital warts that possessed the E6 19T polymorphism have adenosine at this position giving an asparagine at a/a 88 (88N). However, unlike the E6 19S/T polymorphism, the laryngeal and genital wart HPV-6_{vc} -6b and -11 samples correspond to the majority of HPV-6a genital warts at this position and display the 88N variation. The Genbank HPV-6a sequences NC001668 and AR169144 (also genital warts) also possessed the 88D variation. The HPV-11 (laryngeal), -6b and -6_{vc} (genital)

Genbank sequences all displayed the 88N variation. It is seen in Table 4.16 that this polymorphism is not significant.

Table 4.16: Polymorphism At E7 Residue 88 in Laryngeal and Genital Warts

E7 88 D/N Polymorphism	Laryngeal Papillomata		Condyloma Acuminata	
Aspartic Acid	7	20%	4	11%
Asparagine	28	80%	33	89%

2 Sided P Value= 0.3378, Odds ratio= 0.4848,

95% Confidence Interval= 0.1285 to 1.830

In addition the HPV-6a positive sample RRP-056 possessed two more polymorphisms compared to the Genbank sequences in the E7 gene. Here a guanine and an adenosine at residues 646 and 709 respectively are transposed leading to a substitution in amino acids at a/a 39 and 60 of glycine and glutamic acid respectively. Sample RRP-38 also possessed the polymorphism at residue 709. One of the HPV-6_{VC} genital wart samples also varied slightly from the Genbank sequences in amino acid composition with a nucleotide transversion at residue 663. Here a thymidine in all other study laryngeal and genital biopsies becomes an adenosine in GW-15, resulting in a change from a serine to a threonine.

4.3.5 Potential Mechanism for Viral Escape in L1 Protein in RRP

4.3.5.1 HPV-11 L1

No polymorphisms or silent point mutations were observed in the p139 region of L1 HPV-11 positive samples when comparing those from genital warts and RRP biopsies to each other and the genital wart derived Genbank sequences. Figures 4.5 & 4.6 show this homology with the DRB1*0301 motif bordered in red and the anchoring residues highlighted in blue.

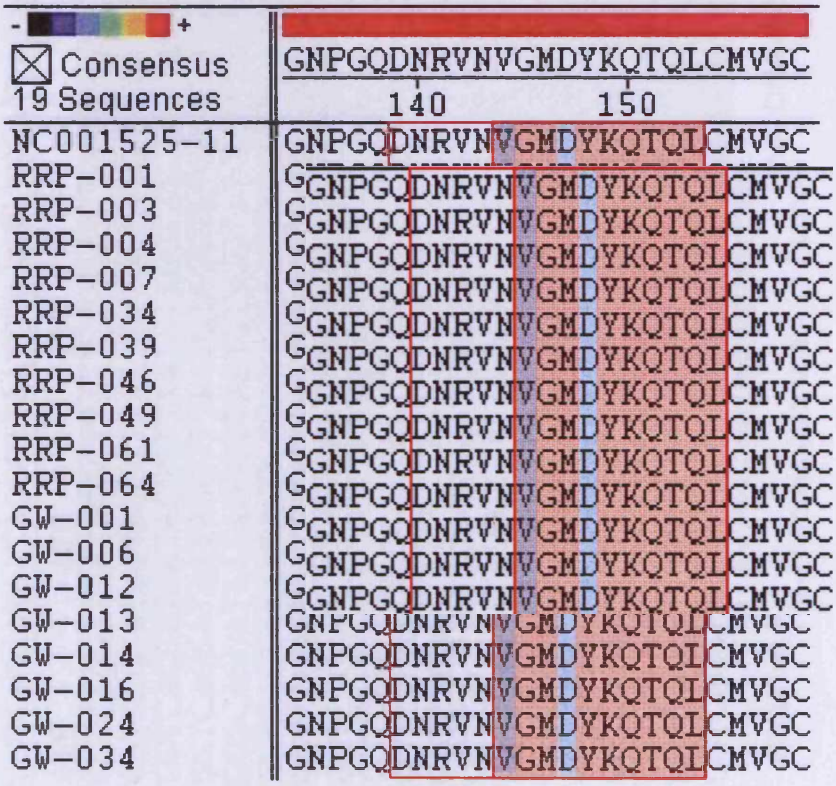


Figure 4.5: HPV-11 Immunodominant L1 Epitope Amino Acid Comparison

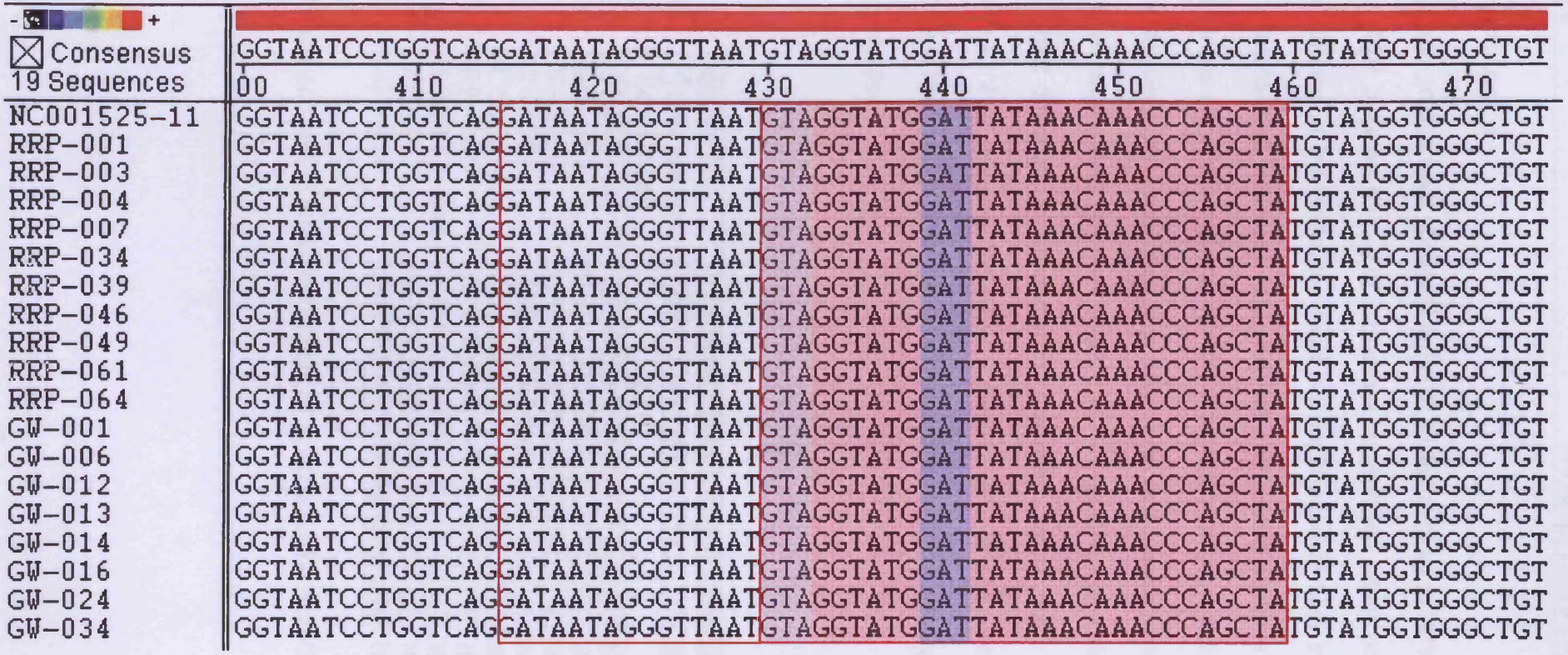


Figure 4.6: HPV-11 Immunodominant L1 Epitope DNA Sequence Comparison

4.3.6.2 HPV-06 L1

No polymorphisms were observed in the p139 region of L1 in HPV-6 positive genital warts or RRP biopsies (Fig. 4.6). However, in HPV-6a silent point mutation was observed in one of the anchoring residues (highlighted in blue in Fig 4.8). Here thymidine is observed in the two study HPV-6a genital wart samples sequenced whilst an adenosine is present in the study RRP HPV-6a sample sequenced and the HPV-6a (genital wart) Genbank Sequences (Fig. 4.8). Adenosine is also present at the same position both the HPV-6b Genbank (genital wart) sequences and study RRP HPV-6b sample, whilst thymidine is present in the Genbank (genital wart) HPV-6_{vc} and two study RRP HPV-6_{vc} samples (Fig 4.8). If these codon variations are rare in humans this may be a mechanism for the avoidance of host immune surveillance, as discussed in the introduction.

Consensus 9 Sequences	GNPGQDNRVNVGMDYKQTQLCMVGC 140 150
NC1668-6a	GNPGQDNRVNVGMDYKQTQLCMVGC
RRP-012	GNPGQDNRVNVGMDYKQTQLCMVGC
GW-003	GNPGQDNRVNVGMDYKQTQLCMVGC
GW-008	GNPGQDNRVNVGMDYKQTQLCMVGC
NC1355-6b	GNPGQDNRVNVGMDYKQTQLCMVGC
RRP-008	GNPGQDNRVNVGMDYKQTQLCMVGC
AF92932-6vc	GNPGQDNRVNVGMDYKQTQLCMVGC
RRP-043	GNPGQDNRVNVGMDYKQTQLCMVGC
RRP-058	GNPGQDNRVNVGMDYKQTQLCMVGC

Figure 4.7: HPV-6 Immunodominant L1 Epitope Amino Acid Comparison

Figure 4.8: HPV-6 of Immunodominant L1 Epitope DNA Sequence Comparison

4.3.6.3 Comparison of p139 Region of L1 HPV-11 and HPV-6

The adenosine observed in some of the HPV-6 samples in the anchoring residue is also present in all of the HPV-11 study samples and Genbank sequences. All HPV-6 subtypes and samples also display 3 additional silent point mutations compared to all HPV-11 samples. These are all shown in Figure 4.9 where the silent point mutations are highlighted in yellow.

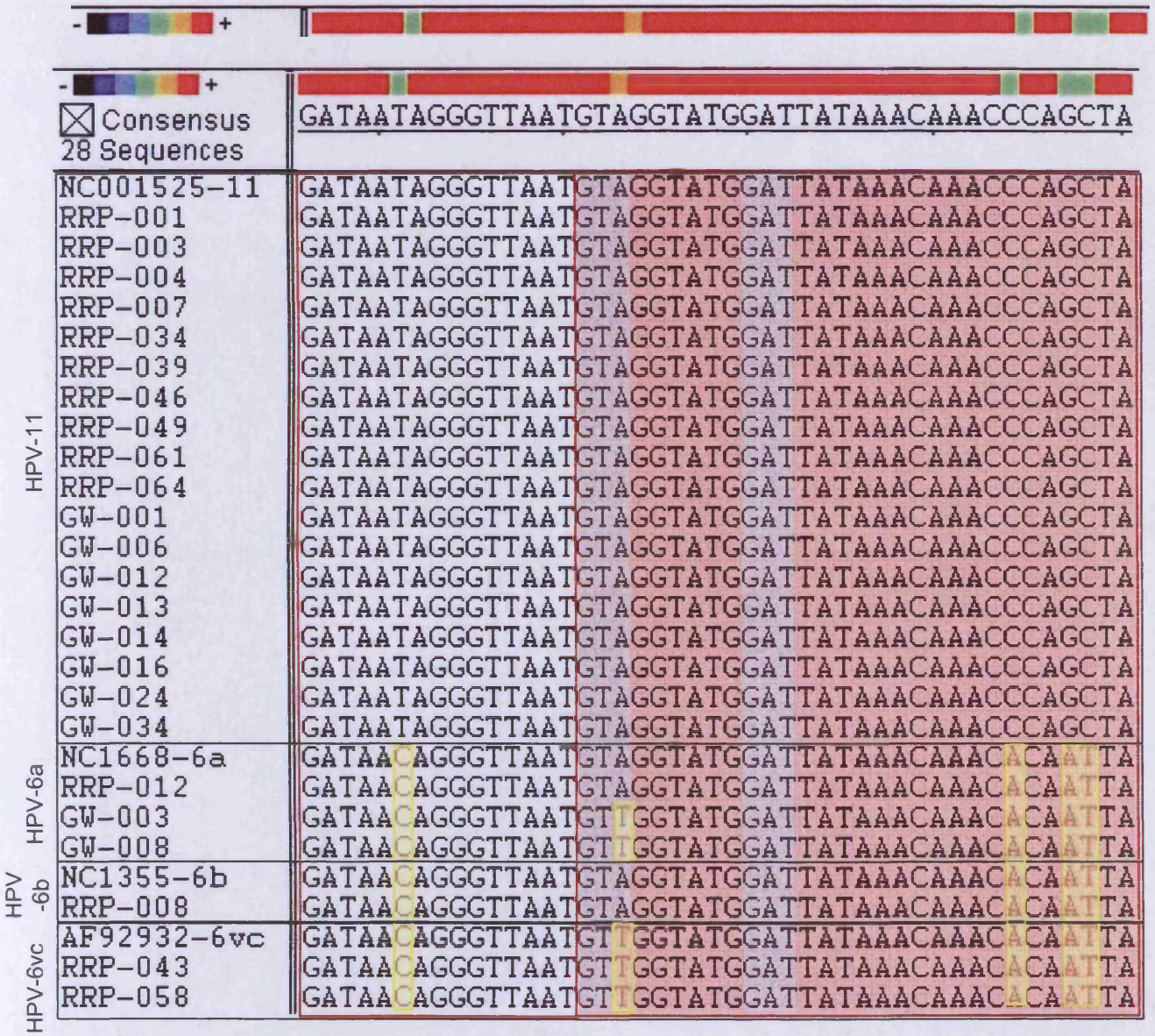


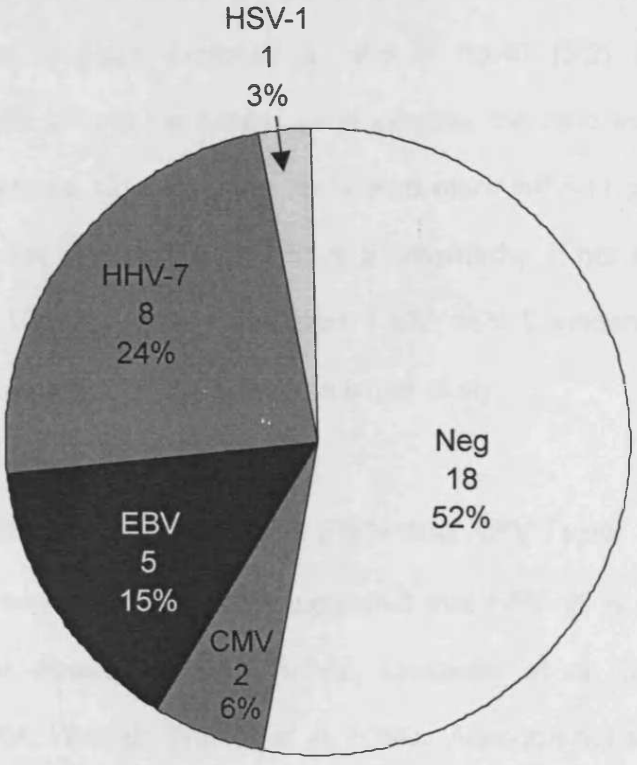
Figure 4.9: HPV-11 and -6 of Immunodominant L1 Epitope Sequence Comparison

4.3.6 Potential Herpes Virus Co-Factors in RRP

Table 4.17: Herpes Virus Prevalence in Laryngeal Biopsies

Virus	No.	% Total	Disease State		p Value*
			A	N/A	
Neg	18	60	12(80)	3(20)	
Pos	12	40	6(70	3(30)	1.000
Breakdown of Positives Inc. Co-Infections					
CMV	2	7	1		1.000
EBV	5	17	2	2	0.2722
HHV-6	0	0	-	-	
HHV-7	8	27	5	1	0.6349
HHV-8	0	0	-	-	
HSV-1	1	3	1	-	1.000
HSV-2	0	0	-	-	
VZV	0	0	-	-	

*Significance of disease state compared with negatives



4

Figure 4.4 : Percentage Prevalence of Herpes Viruses in Laryngeal Biopsies

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4 Discussion

4.4.0 Overview of Results

- Both HPV-6 & -11 found
- All 3 sub-types of HPV-6 found
- No Herpes viruses associated with disease severity
- No significant polymorphisms in L1
- Significant Polymorphism in the E6 and E7 oncoproteins between genital warts (malignancy: extremely rare) and laryngeal papillomata (malignancy: frequent)

4.4.1 HPV Type

4.4.1.1 Comparison of HPV Types in Laryngeal and Genital Warts

The laryngeal biopsies exhibited a ratio of 60:40 (3:2) of HPV-06 and -11 respectively, whilst among the genital warts samples the ratio was 74:26 (3:1). Thus amongst the laryngeal biopsies there are a third more HPV-11 positive samples than found in the genital wart samples. This is a noteworthy, if not statistically significant trend (2 Sided P Value: 0.2623, Odds Ratio: 1.838, 95% Confidence Interval: 0.7410 to 4.556) which should be studied further in a larger study.

4.4.1.2 Correlation of RRP Disease State and HPV Type

Previous studies have tentatively suggested that HPV-11 is more likely to cause more aggressive disease in RRP (Rabah, Lancaster et al. 2001; Obchinnikov Iu, Kiselev et al. 2004; Wiatrak, Wiatrak et al. 2004). Although not statistically significant, this appears to be the trend in this study (Table 4.18) with 89% of RRP patients positive for HPV-11 displaying aggressive disease compared to only 65% of HPV-06 positive patients.

Table 4.18: HPV Type Correlated Against RRP Aggression

HPV Type	n	Disease State			
		Aggressive		Non-Aggressive	
HPV-11	18	16	89%	2	11%
HPV-06	20	13	65%	7	35%
RRP Total	38	29	76%	9	14%

Fishers T Test 2 sided P value= 0.1305, Odds Ratio= 4.308

95% Confidence Interval= 0.7607 to 24.394

4.4.1.3 Correlation of RRP Disease State and HPV-06 Sub-Type

Table 4.19 shows that there is no significant association between disease state and HPV-06 subtype. Of the three HPV-06 sub-types only HPV-6a appears to show any trend towards a particular disease state with 78% of RRP patients shown to be positive of HPV-6a displayed aggressive disease albeit in a small cohort.

Table 4.19: RRP Disease State Correlated with HPV 6 Sub-Type

HPV Type	Disease State			
	Aggressive		Non-Aggressive	
HPV-06a	7	78%	2	22%
HPV-06b	1	50%	1	50%
HPV-06vc	4	57%	3	43%

χ^2= 1.036, P value= 0.5958 (2 Degrees of Freedom)

4.4.1.4 Correlation of RRP Onset and HPV Type

Despite 78% of AORRP patients being positive for HPV-06 (it must be noted not the same 78% that were positive for HPV-6a) there is no significant association in this comparison (Table 4.20).

Table 4.20: RRP Onset Correlated with HPV Type

Onset	HPV Type			
	11		6	
JORRP	19	46%	24	54%
AORRP	2	22%	7	78%
RRP Total	19	41%	27	59%

2 Sided P value: 0.2834, Odds Ratio: 2.771

95% Confidence Interval: 0.5148 to 14.915

4.4.1.5 Correlation of RRP Onset and HPV-06 Sub-Type

Table 4.21 shows that distribution of HPV-06 sub-type is not significantly associated with RRP onset however HPV-6a appears to be more common in JORRP and HPV-6vc more common in AORRP.

Table 4.21: RRP Onset Correlated with HPV 6 Sub-Type

Onset	HPV-06 Sub-Type					
	6a		6b		6vc	
JORRP	9	56%	1	6%	6	38%
AORRP	1	20%	1	20%	3	60%
RRP Total	7	41%	2	12%	8	47%

χ^2= 2.258, P value= 0.3234 (2 Degrees of Freedom)

4.4.3 HPV Polymorphisms

4.4.3.0 Overview

Several polymorphisms are observed in the E6 and E7 oncogenes of HPV-11 and -6 subtypes in laryngeal and genital biopsies some of which may have may have an immunogenic or structural or influence which are discussed in detail in the proceeding sub-sections. A recent study by Pou *et al* (Pou, Weems et al. 2004) found some of the oncogene silent point mutations and a polymorphisms that we also report. However, this study examined only thirteen RRP samples and did not include any comparisons with genital wart HPV DNA. The E6/7 polymorphims found by Pou *et al*, as well as ourselves, in RRP HPV-11 (Table 4.5) E6 are two silent point mutations at nucleotides 137 and 380 as well as a polymorphism from an alanine to a serine in HPV-11 E7. This conservative polymorphism, which has been found in all laryngeal and genital HPV-11 samples in this study (Table 4.7), lies within no known region of binding or structural significance. All DNA base discussed in this Chapter takes the origin of replication as base 1 and all amino acid residue discussion takes the first methionine of that protein as residue 1.

4.4.3.1. A Potential Viral Escape Mutant in RRP Oncogenes

Superficially the HPV-6a 19S/T mutation seems of little consequence as it is a conservative change between two neutral residues and does not lie within any known functional region of the E6 protein. Nevertheless we have found that HPV-6a is the most numerous HPV sub-type found in genital warts in this study with nearly half (49%) harbouring HPV-6a DNA. The 19S variant is found in 40% (82% of HPV-6a genital warts) of all genital warts in the study (including all HPV-6 subtypes and HPV-11) but in not one of the RRP biopsies, that all contain 19T. This is statistically significant (2 sided P value >0.0001, OR: 50.659, 95% CI:2.872 to 893.46). Residue 19T is part of a potential DRB1*0301 binding motif (LxxT), whilst this is not present in those samples with the 19S variation.

Thus, a potential epitope for HLA class II presentation which we have found to be significantly associated with RRP is present in the E6 gene of all RRP biopsies sequenced but only present in 60% of genital warts in our study. This epitope also appears to be unique to HPV-6 and -11 and is not present in the most common cutaneous (HPV-1,-2,-3, and -4) or cervical (HPV-16 and -18) HPV types. We have investigated the proliferation of PBMC's in healthy (non-RRP) individuals using thymidine incorporation (Manuscript in preparation). Stimulation was initiated by a peptide based upon HPV-11 which include the potential 19T DRB1*0301 motif. However we have not seen PBMC proliferation in response to the peptide which encompasses this region in healthy (non-RRP) donors (data not shown).

However, as discussed in the introduction to this Chapter, HPV may evade immune surveillance by the subverting the redundancy of the genetic code. Preferential use of codons, and concomitant tRNA, rarely used in humans ensures that only low levels of late proteins are presented to the host immune system (Zhou, 1999). Such a switch in codon / tRNA frequency from one frequently used in humans than in HPV to one used commonly in HPV early genes but significantly less so in humans is seen in the E6 19S/T polymorphism. Here in the samples with the serine in this position (40% of all genital warts in the study) the frequency of codon used is 4.2% in humans and is not seen at all in the Genbank HPV-6a sequences (Table 4.22). Conversely the codon seen in the threonine observed in all laryngeal papillomata in this study is used at a frequency of 7% in humans and at 5 times that frequency in HPV early genes (33%). The infrequent use of the serine codon compared to the threonine codon in humans and the reverse situation in HPV E6 suggests that the associated tRNA used by HPV at this residue in RRP patients will be in short supply. This has the potential resultant effect of limiting the replication of HPV in RRP with the consequence that the host immune system has less of a chance of detecting the virus.

Previous studies have found low levels of latent HPV in both RRP papillomata and laryngeal cancer biopsies and no detectable mRNA for E6 or E7 (Maran, Amella et al. 1995; Matzow, Boysen et al. 1998). A recent study has found that E6 and E7 transcripts are induced just after activation of infection and prior to the development of papillomata (Abramson, Nouri et al. 2004). Activation of latent papillomavirus infection thus appears to require the induction of moderate levels of E6 & E7 mRNA for the proteins before any lesion can be detected. Therefore, this reduced frequency of trachea papillomata may not due to a lower likelihood of infection or establishment of HPV infection as other factors may regulate HPV expression contribute to the lower frequency of tracheal disease. This polymorphism thus provides such a potential mechanism for latency of infection in RRP as a means of avoiding immune surveillance.

Another polymorphism is seen in the same HPV-6a polymorphisms in the E7 gene at amino acid 88 on E7. Here an aspartic acid is present in all the HPV-6a RRP samples and all the HPV-6a genital wart samples that possessed the E6 19T polymorphism. In all HPV-6a genital warts in which the E6 19S polymorphism was observed an asparagine is present at this position on E7 (Table 4.8). Unlike the E6 19T polymorphism the E7 88D polymorphism however is not present in HPV-6b, -6_{vc} or -11 laryngeal or genital biopsies in this study or on Genbank samples. Instead it is the 88N polymorphism that is seen at this position in all other RRP samples except for the HPV-6a positive samples. Although this asparagine to aspartic acid polymorphism in the E7 gene is only seen in HPV-6a RRP samples, it is a switch from a neutral to a negatively charged amino acid that may have effects upon three dimensional protein structure This is of particular interest as it lies within a zinc finger domain and thus has potential structural implications. This zinc finger is known to mediates E7 dimerisation

and interact with members of the AP-1 transcription family which as described in chapter one assist in E6/E7 mediated malignant transformation. The AP-1 factors which complex with this region of E7 are c-jun, jun-B, jun-D and c-fos and are also the most important factors involved in the transcription of HPV. Of considerable note also is that this polymorphism falls within a non-anchoring a/a of a potential DRB1*0301 motif. In addition the peptide based upon this region has been shown to induce PBMC proliferation in healthy donors (Personal Communication: P. Hillyer, Infection & Immunity, UWCM).

However like the E6 19 S/T polymorphism the E7 88 D/N polymorphism is a switch from a codon /tRNA used frequently in humans and rarely in HPV early genes to a codon / tRNA used frequently in HPV early genes and not in humans. In the majority of the HPV-6a genital warts and all the HPV-6b, -6vc & -11 samples the codon used for asparagine is used at a frequency of 23% in humans and 10% in HPV early genes. Whilst the codon for the aspartic acid seen in the HPV-6a RRP samples and minority of HPV-6a genital warts is used at a frequency of 29% in humans and 82% in HPV-6 early genes. Thus the concomitant tRNA for the codon used for the RRP samples with the E7 88D polymorphism is used at 3 times the frequency in HPV early genes than in humans. Thus again providing a potential mechanism for the limiting of HPV replication and resultant effects as described above however this time only in a minority, not all RRP samples.

Another this is a switch to a rare human codon / tRNA observed only in HPV-6a RRP, and not genital warts or in the other HPV type RRP samples is in L1. This polymorphism is observed residue 431 where the negatively charged glutamic acid is present in the Genbank and RRP HPV-6a L1 gene sequenced. Whilst at this position in the two genital wart HPV-6a sample L1 genes sequenced the neutral glutamine is

observed. However this is change in frequency is only just a doubling rather than the greater differences seen above.

4.4.3.2 E7 Polymorphism Within a Zinc Finger Motif

The change at residue 60 in the E7 gene of two of the HPV-6a RRP samples (Table 4.8) is of significance as it is the second x within a CxxC zinc finger motif, albeit without actually disrupting the motif itself. This transposition does however result in a change from a neutral amino acid with a small side chain (H) to a negatively charged amino acid with a much longer side chain (CH_2CH_2COOH) which, again, is likely to have consequences for three dimensional protein structure or binding of the AP-1 transcription factors. In addition a, discrete, polymorphism is observed only in RRP-038 at residue 39 of E7

4.4.3.3 HPV-11 Variants

As described in the results section of this chapter 2 RRP and 2 genital wart HPV-11 samples share a several polymorphisms and silent point mutations in the E6 and E7 genes (Table 4.7). None of the three polymorphisms are located within regions seen to be observed to stimulate PBMC proliferation (Personal Communication: P. Hillyer, Infection & Immunity, UWCM) or any known structural regions (Fig. 4.10). One of each of the RRP (RRP-049) and genital wart (GW-012) samples of these variants were successfully sequenced along the length of the L1 gene. However, although both possessed silent coding mutations, unlike the oncogenes there was no consistency between the two samples (Table 4.15). In addition GW-12 also possesses a polymorphism at residue 122 which is not within an immunogenic region of L1 (Williams, 2002). The silent point mutations seen in these variants are discussed in 4.4.4.

4.4.3.4 Further HPV-06 Polymorphisms

The intermittent E7 polymorphisms observed at residue 39 of the in RRP-056 (Table 4.8), and residue 45 in GW-015 (Table 4.10) are part of no known structural feature and are not within the single immunogenic epitope seen in the E7 protein (Personal Communication: P. Hillyer, Infection & Immunity, UWCM). However the E7 39E/G polymorphism is another switch to a rare human codon / tRNA but just more than double the frequency in this case (Table 4.22) and is only seen in one RRP sample.

At nucleotide 324 there is a polymorphism between the three HPV-6 sub-types that have the potential to provide SNP based HPV-6 sub-typing. HPV-6a has a cytosine, HPV-6b an adenosine and HPV-6vc a thymidine HPV-11 also possesses a thymidine at this position so this polymorphism cannot be exploited for SNP in samples with dual infection. However for the sub-typing of samples positive by PCR-ELISA for HPV-6 only this polymorphism has the potential to save the need for sequencing to establish subtype. This polymorphism has no effect upon amino acid composition and is conserved between genital and laryngeal warts in both study and Genbank samples.

In Tables 4.22 - 4.27 codon frequencies twice as high in the specific HPV type and gene than in humans (taken as significant) are indicated in bold. Human codon frequencies are from (Zhou, Liu et al. 1999), HPV codon frequencies were calculated by the Author.

Table 4.22: Codon Frequency Comparison in HPV Polymorphisms

HPV Type	Gene	Residue	Position		Codon	% Codon Frequency	
			DNA	Protein		Human*	HPV**
HPV-6a	E6	T	157	19	acg	6.7	**33.3**
		S			tcg	4.2	0
	E7	E	646	39	gaa	26.8	40.8
		G			gga	17.1	**40.8**
		G	709	60	gga	17.1	**40.8**
		E			gaa	26.8	40.8
		D	792	88	gac	29.0	**81.6**
		N			aac	22.6	10.2
	L1	E	7080	431	gaa	26.8	24.0
		Q			caa	11.1	**26.0**
HPV-6vc	E7	S	663	45	tca	9.3	**20.4**
		T			aca	14.1	**40.8**
	L1	Y	6444	219	tac	18.8	8.0
		D			gac	29.0	12.0
HPV-11	E6	N	383	94	aat	33.3	16.6
		K			aag	46.7	34.9
		K	398	99	aaa	22.2	33.3
		N			aac	22.6	26.7
	E7	A	662	45	gca	14.0	20.4
		S			tca	9.3	10.2
		Q	761	78	caa	11.1	**71.4**
		K			aaa	22.2	20.4
	L1	R	6135	122	aga	9.9	6.0
		K			aaa	22.2	34.0

* From (Zhou et al. 1999), ** Calculated by the Author

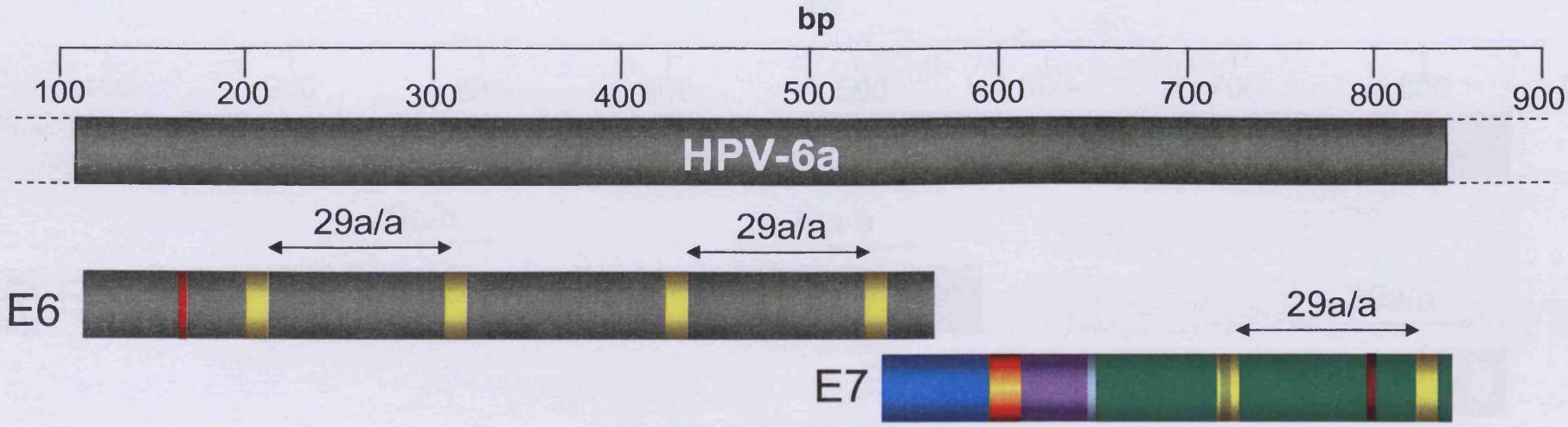

Figure 4.10: Gene Map of HPV-06a E6 and E7 Polymorphisms

Structural Features

- : Putative Zinc Finger CxxC Motif
- : Retinoblastoma Binding Region
- : E7 Conserved Domain 1 (CD-1)
- : E7 Conserved Domain 2 (CD-2)
- : E7 Conserved Domain 2 (CD-3)

Polymorphisms Found in This Study

- : a/a 19 - Threonine in all RRP, and some GW / Serine in most GW
- : a/a 39 - Glycine in RRP-056 / Glutamic Acid in all other samples
- : a/a 60 - Glutamic Acid in RRP-038 & RRP-056 / Glycine in all other samples
- : a/a 88 - Aspartic Acid in all RRP, and some GW / Asparagine seen in most GW

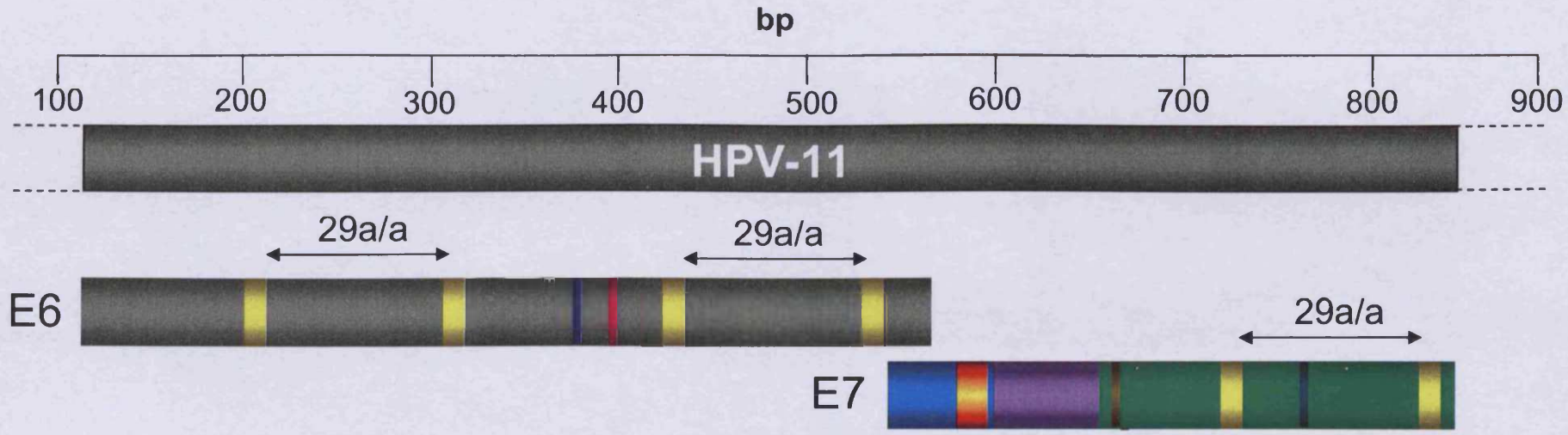

Figure 4.11: Gene Map of HPV-11 E6 and E7 Polymorphisms

Structural Features

- : Putative Zinc Finger CxxC Motif
- : Retinoblastoma Binding Region
- : E7 Conserved Domain 1 (CD-1)
- : E7 Conserved Domain 2 (CD-2)
- : E7 Conserved Domain 2 (CD-3)

Polymorphisms Found in This Study

- : a/a 94 - Lysine in 4 HPV-11 variant samples / Asparagine in all other samples
- : a/a 99 - Asparagine in 4 HPV-11 variant samples / Lysine in all other samples
- : a/a 45 - Serine in 4 HPV-11 variant samples / Alanine in all other samples
- : a/a 78 - Lysine in 4 HPV-11 variant samples / Glutamine in all other samples

4.4.4 Silent Point Mutations

Although silent point mutations do not affect amino acid and therefore protein composition, they may be changes to rare codons as seen in the polymorphisms and consequently potential immune evasion mechanisms. Silent point mutations are observed here in the E6 and L1 genes, but not the E7, gene in HPV-11 and all HPV-6 subtypes (Tables 4.22-4.24). Silent point mutations taken as being significant here are those where the where the codon frequency in the specific HPV type and gene is more than double the frequency of that codon in humans (Zhou, 1999). The complete calculated tables of gene frequencies are shown in the appendix to this chapter. Three such significant changes are observed from the E6 gene. One of these is in HPV-11 E6, where a single point mutation at base 97 results in an isoleucine codon switch in residue 97. Coding for this residue the Genbank samples and the majority of RRP and genital wart samples display a codon where the gene frequency is 15% in humans and 27% (1.8 times more in HPV) in HPV-11 E6 (Table 4.23). Whilst the four HPV-11 variant samples, two genital warts and two RRP samples, discussed in 4.4.3.4 show a switch to a codon where the frequency is 6% in humans and 33% in HPV-11 L1 (5.7 times more in HPV).

These four variants also exhibit two polymorphisms in both E6 and E7 as well as another insignificant silent point mutation in E6 (Table 4.7 & 4.21). Although the RRP sample and genital wart sample of these variants where the L1 gene was sequenced (RRP-49 & GW-12) again display variations from the majority of samples but none of these are consistent with each other (Table 4.16). However of silent point mutations they do display RRP-49 exhibits another switch to a codon rare in humans. RRP-49 displays a switch to a serine codon where the frequency is 13% in humans and 28% in HPV-11 L1 whilst all other samples and Genbank sequences display a codon that has a frequency of 18% in humans and only 4% in HPV-11 L1 (Table 4.16 & 4.24).

The other two changes in the E6 gene that are shifts from codon / tRNA frequent in humans but not in HPV to one frequent in HPV early genes but no in humans are both in protein 124 but in two different HPV-6 subtypes. In both HPV-6a and HPV-6b there is an alanine at this position the codon of which in both subtypes in the Genbank sequences is seen at a frequency of 7% in humans and 13% in all study samples. Whilst in both of these sub types in the study samples, both genital warts and laryngeal paplomata, there is a codon with a frequency of 16% in humans and 40% in HPV, 2.5 times the frequency in HPV than in humans (Table 4.23). However as this is seen in all study samples although this may be immune evasion this is likely to be a geographical restriction as none of the Genbank samples originate from the UK.

The potentially most interesting silent point mutation is that shared by the samples which exhibit the E6 19 / E7 88 polymorphisms. This mutation at base 480 is a cytosine in the E6 19S polymorphism samples (most of the HPV-6a genital wart samples) and thymidine in all RRP HPV-6a samples. However, rather than a switch to a codon more commonly used in humans there is a switch to a codon not seen at all in HPV-6a (Table 4.23).

The L1 gene in HPV-6b and -6vc also each display a significant switch to a codon rarely used in humans. Of the two HPV-6b samples found in this study RRP-08 was successfully sequenced the full length of the L1 gene. At residue 319 in the HPV-6b Genbank sequences a phenylalanine codon is seen with a frequency of 15% in humans and 12% in HPV-6b L1 (1.2 times more in humans). In RRP-08 this codon is switched to one with a frequency of 6% in humans and 18% HPV-6b L1 (3 times more in HPV) (Table 4.14 & 4.23). In the HPV-6vc L1 gene, the codon coding for proline at residue 75 is seen a frequency of 15% in humans and 18% in HPV-6vc L1 (1.2 times more in HPV) in the Genbank sequences and two of the three HPV-6vc RRP samples. In the other RRP sample there is a switch in this residue to a codon which has a

frequency of 16% in humans and 40% in HPV-6vc L1 (2.6 times more in HPV). Another silent point mutation switches a taa stop codon to a tga stop codon in the L1 gene of three HPV-11 RRP samples (table 4.15).

The HPV-11 variants thus not only share four polymorphisms but also a silent point mutation, with another in one of them, which demonstrate a considerable shift in codon availability. In HPV-6vc L1 a polymorphism and a rare codon switch silent point mutation are also seen. However the latter is only seen in one sample whilst the polymorphism is observed in all 3 RRP sample HPV-6vc L1 genes sequenced. None of the region of HPV-6 L1 observed to be immunogenic. Both the polymorphisms and rare codon switches may be mechanisms for escape of immune surveillance.

Table 4.23: E6 Silent Point Mutation Codon Frequency Comparison

HPV Type	Residue	Position		Codon	% Codon Frequency	
		DNA	Protein		Human*	HPV**
HPV-6a	A	351	83	gct	19.6	6.7
				gcg	7.2	13.3
	A	474	124	gcg	7.2	13.3
				gca	14.0	**46.7**
	F	480	126	ttt	15.8	**40.0**
				ttc	22.6	6.7
HPV-6b	T	221	40	aca	14.4	**33.3**
				act	12.7	6.7
	A	473	124	gcg	7.2	13.3
				gca	14.0	**46.7**
HPV-6vc	F	312	70	ttt	15.8	**33.3**
				ttc	22.6	6.7
	V	369	89	gtt	10.4	6.7
				gtc	16.3	6.7
HPV-11	S	137	12	tct	13.2	20.0
				tcc	17.7	6.7
	T	380	93	acc	23.0	20.0
				act	12.7	6.7
	I	392	97	att	14.9	26.7
				ata	5.8	**33.3**

* From (Zhou et al. 1999), ** Calculated by the Author

Table 4.24: HP-06 L1 Silent Point Mutation Codon Frequency Comparison

HPV Type	Residue	Position		Codon	% Codon Frequency	
		DNA	Protein		Human*	HPV**
HPV-6a	R	6105	106	cgg	10.4	8.0
				agg	11.1	14.0
	L	6119	110	tta	5.3	**26.0**
				ttg	11.0	**22.0**
	V	6218	143	gta	5.9	**20.0**
				gtt	10.4	**34.0**
	R	6626	279	cgc	11.3	10.0
				cga	5.4	2.0
	K	6722	311	aaa	22.2	34.0
				aag	34.9	26.0
	T	7283	498	act	12.7	16.0
				acc	23.0	2.0
HPV-6b	T	6598	292	aca	14.4	**34.0**
				act	12.7	16.0
	I	6745	319	att	14.9	12.0
				ata	5.8	**18.0**
	K	7099	437	aag	34.9	26.0
				aaa	22.2	34.0
HPV-6vc	P	6014	75	cca	14.6	18.0
				cct	15.5	**40.0**

* From (Zhou et al. 1999), ** Calculated by the Author

Table 4.25: HPV-11 L1 Silent Point Mutation Codon Frequency Comparison

Residue	Position		Codon	% Codon Frequency	
	DNA	Protein		Human*	HPV**
S	6028	86	tcc	17.7	4.0
			tct	13.2	**27.9**
V	6106	112	gtg	30.9	25.9
			gtt	10.4	**27.9**
L	6379	203	ggt	11.2	**37.9**
			ggc	25.4	10.0
V	6445	330	gtc	16.3	4.0
			gtg	30.9	25.9
P	6484	238	cct	15.5	**31.9**
			ccc	20	18.0
G	6556	262	ggt	11.2	**37.9**
			gga	17.1	16.0
L	6700	310	ctt	10.7	10.0
			ctc	19.9	2.0
F	6751	327	ttt	15.8	**41.9**
			ttc	22.6	0.0
V	6760	330	gtg	30.9	25.9
			gtt	10.4	**27.9**
S	6832	354	tca	9.3	16.0
			tcg	4.2	4.0
P	6988	406	cca	14.6	20.0
			cct	15.5	**31.9**

* From (Zhou et al. 1999), ** Calculated by the Author

4.4.5 Codon Switches Within the Immunodominant L1 Epitope

Within RRP linked DRB1*0301 epitope found to be immunodominant within the L1 protein (Gelder, 2003) another preferential use of codons is observed. The first anchoring residue of this motif is a valine which displays codon diversity between HPV-6 subtypes and HPV-11 and between the HPV-6 and laryngeal biopsy and genital wart where L1 was successfully sequenced. The HPV-6a sample, RRP-12, displays the same valine gta codon as the Genbank HPV-6a reference (genital wart), all study and Genbank HPV-11 samples as well as the Genbank and RRP (only) HPV-6 samples. The 2 genital wart samples HPV-6a samples, and the HPV-6vc samples (both RRP) and Genbank sample display the valine codon gtt (Fig. 4.9). The noteworthy difference is that, although there are low numbers, there is a difference in codons here between the HPV-6a laryngeal and genital biopsies and that the codon seen in laryngeal samples matches those seen in all HPV-11 samples.

Both the gta and gtt codons are significantly more commonly used in HPV L1 than in humans with both these codons observed at frequencies between 28%-34% in HPV-6 and -11 L1 (Table 4.26). However, whilst the gtt codon is observed at a frequency of 10.4% in humans, the frequency of the gta codon is only 5.9% in humans. Thus, the gtt codon is present in HPV L1 around 3 times more than in humans than, the gta codon is found between 5-6 times more in HPV L1 than humans. Again as seen above with E6 and E7 this implies the selection of codons / tRNA rarely found in humans and the implication of low HPV expression.

Table 4.26: Codon Frequencies in Anchoring Residue DRB1*0301 Epitope

Type or Subtype (Codon Distribution)	Valine Codon	% Codon Frequency	
		Human*	HPV L1**
HPV-11 (All)	gta	5.9	**27.9**
HPV-6a (RRP)			**34.0**
HPV-6b (RRP)			**32.0**
HPV-6a (GW)	gtt	10.4	**27.9**
HPV-6vc (All)			**34.0**

Table 4.27: Codon Frequencies in Non-Anchoring Amino Acid Variations Between HPV-11 & -6 Within DRB1*0301 Epitope

A/A	Position Within L1 Protein		Codon	% Codon Frequency	
				Human*	HPV-11 L1**
N	HPV-11	140	aat	16.6	**32.0**
	HPV-06a	139	aac	22.6	24.0
	HPV-06b				24.0
	HPV-06vc				24.0
T	HPV-11	151	acc	23.0	18.0
	HPV-06a	150	aca	14.4	**30.0**
	HPV-06b				**34.0**
	HPV-06vc				**30.0**
Q	HPV-11	152	cag	33.6	22.0
	HPV-06a	151	caa	**11.1**	**26.0**
	HPV-06b				**26.0**
	HPV-06vc				**26.0**
L	HPV-11	153	cta	6.2	6.0
	HPV-06a	152	tta	5.3	**26.0**
	HPV-06b				**28.0**
	HPV-06vc				**26.0**

4.4.6 Herpes Virus Co-Factors

The search for possible herpes virus involvement in RRP as viral co-factors showed a 60:40 Negative:Positive ratio and a high proportion of HHV-7 positive samples (27% of total positives). However neither the distribution of positives and negatives or HHV-7 positive samples and negatives showed any statistically significant correlation to disease (Table 4.5). However in order to make a true comparison we need to compare this data with levels of these viruses in the larynx of normal, non-RRP, individuals. However we were unable to obtain ethical permission to take biopsies from the larynxes of healthy individuals to provide such data and it is not available from other sources.

4.5 Concluding Remarks

All HPV-6a RRP samples sequenced were the specific variant with the polymorphism at residue 19 in the E6 gene, a silent mutation in the E6 at nucleotide 480 and a polymorphism in E7 at residue 88. The HPV-6a variant however, was present only in 16% (3/19) of HPV-6a positive genital warts (Fig. 4.1, Table 4.5). Indeed, the E6 polymorphism was observed in all RRP samples, regardless of HPV type but in only 59% of all genital wart samples. As this polymorphism also determines a putative DRB1*0301 epitope then it is possible that RRP can only progress if this epitope is present. Therefore if RRP is only caused by HPV-6 or -11 with around three quarters of genital warts are HPV-06 this may go some way towards explaining the rarity of RRP. However this is counter intuitive as one would expect the presence of a HLA motif to render the virus more, rather than less, liable to immune scrutiny. This enigma may be solved by further planned experiments involving looking at CD4+ T cell responses the alternate E6 19 polymorphism as well as functional protein experiments.

It is therefore seen that the most potentially critical polymorphism and is located on the E6 oncoprotein and that there is a much greater rate of both polymorphisms and silent point mutations in the E6 and E7 oncoproteins when compared to the L1 capsid protein. This is particularly significant as the L1 protein is twice the length of E6 and E7 combined. This use of rare codons and increase in genetic diversity in general in the oncoproteins in RRP compared to genital warts may also help to explain the apparent disparity between the potential for malignancy in RRP compared to genital warts. Crucially however no mutations were observed in any RRP samples of the glycine in amino acid 22 of E7. Other studies have shown that substitution of the glycine found in HPV-6 & -11 at this position for an aspartic acid associated with oncogenic types at this position leads to an increase in pRb binding affinity and consequently oncogenicity (Sang and Barbosa 1992).

It could be said that in the normal course of viral evolution minor point mutations in the HPV genome will occur. Sometimes however, minor genetic changes can have significant effects on protein structure and as a consequence, function. Recognition of the virus by the immune system may also be affected. We demonstrate here that in the HPV types which cause RRP, the E6 and E7 genes display polymorphisms which may have just such effects. Further investigations therefore will involve the effect of these mutations upon the E6 and E7 oncoproteins and their interactions with p53, Rb and AP-1 in vitro. This chapter thus demonstrates that it is crucial to further elucidate the role of the host immune system in RRP and two this end in the next two chapters this role is investigated in both HLA associations with RRP (Chapter 5) and cross reactive cytokine production in healthy HPV infected individuals.

Chapter Five:

Host Genetic Factors in RRP

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5.5 Concluding remarks

5.1 Introduction

5.1.1 Overview and Aims of this Chapter

In the previous Chapter an examination was made the role of HPV in host virus interactions in RRP, the obvious next step was to further examine the role of the host. Such is the range of diseases caused by low risk HPV types from verrucae to genital warts to RRP that host factors must play a major role in disease progression. Host genetic studies of RRP are constrained by the rarity of the disease. Our group has previously investigated a cohort of 60 RRP patients and found an association between RRP and the HLA class II alleles, DQB1*03, DRB1*14 and, most notably, DRB1*0301 as well as an association with the ancestral haplotype 8.1 (incorporating HLA A*0101, B*0801, Cw0701, DRB1*0301, DQB1*0201 and TNF α-2) (Gelder, Williams et al. 2003). Preliminary evidence in this original study had also suggested a tentative association between HLA DRB1*14 both overall and in the JORRP subset of patients. However this original study (Cohort I) was a biased towards JORRP patients and it was based on multiple comparisons. Therefore in order to validate these findings it was therefore important to repeat the study in a second independent cohort of donors. This chapter presents the results from an analysis of a second cohort of 50 RRP patients (Cohort II).

A recent US study (Bonagura, 2004) has made a secondary confirmation of the DRB1*0301 association in Afro-American and Caucasoid populations. However they failed to find the association with the AH 8.1 in either population. This is to be expected in the Afro-American population as AH 8.1 is preferentially found in the Caucasoid population. The Bonagura study has also found DRB*0602 to be negatively associated with aggressive RRP in Caucasians. Although no association was found between this allele and RRP and Cohort 1 (Fisher's 2 Sided P Value= 0.2345, OR= 0.6607, 95% CI= 0.8162 to 2.763), DQB1*0602 is examined in this Chapter to determine any association in Cohort II.

5.2 Methodology

5.2.1 Clinical Samples

A diagnosis of RRP was established by either the necessity of at least two recurrent surgical debulking procedures and distinctive papilloma histological changes, or both. Disease criteria aggression were based upon the Derkay scoring system shown in Fig. 5.1 (Derkay, 1998).

PATIENT INITIALS:____ DATE OF SURGERY_____ SURGEON_____
PATIENT ID #_____ INSTITUTION_____

1. How long since the last papilloma surgery? ____days, ___weeks, ___months,
   ___years,___ don't know,
   ____this is the child's first surgery
2. Counting today's surgery, how many papilloma surgeries in the past 12 months? _____
3. Describe the patient's voice today:
   normal__(0), abnormal__(1), aphonic__(2)
4. Describe the patient's stridor today:
   absent__(0), present with activity__(1), present at rest__(2)
5. Describe the urgency of today's intervention:
   scheduled__(0),elective__(1),urgent__(2),emergent(3)
6. Describe today's level of respiratory distress:
   none__(0), mild__(1), Mod__(2), severe__(3), extreme__(4)

Total score for questions 3-6=_____

FOR EACH SITE, SCORE AS: 0= NONE, 1= SURFACE LESION, 2=RAISED LESION, 3=BULKY LESION

LARYNX:
- Epiglottis
  - Lingual surface____ Laryngeal surface_____
- Aryepiglottic folds: Right____ Left____
- False vocal cords: Right____ Left____
- True vocal cords: Right____ Left_____
- Arytenoids: Right____ Left_____
- Anterior commissure_____
- Posterior commissure_____
- Subglottis _____

TRACHEA:
- Upper one-third_____
- Middle one-third_____
- Lower one-third_____
- Bronchi: Right___ Left____
- Tracheotomy stoma_____

OTHER:
- Nose____
- Palate_____
- Pharynx____
- Esophagus____
- Lungs_____
- Other_____

TOTAL SCORE ALL SITES: _____ TOTAL CLINICAL SCORE:_____

Figure 5.1: The Derkay RRP Scoring System

Individuals with RRP were recruited for this study by contacting all Ear, Nose and Throat surgeons throughout Wales and those in England known to be interested in RRP research. Blood samples were obtained, with informed consent, from patients receiving treatment for RRP at the UHW, Cardiff, as well as samples from: Sheffield Children's Hospital, Birmingham Children's Hospital, St Michaels Hospital, Bristol, Great Ormond St Hospital, London and the Royal Sussex Hospital, Brighton.

5.2.2 Control Subjects

The control data used has been well characterised and well validated to be representative of the general UK population (Bunce, Barnardo et al. 1996). This collection of control data was derived from 554 UK cadaveric organ donors which included children. We were unable to use sibling controls after preliminary studies raised some paternity issues, it is also impossible to prove that siblings are not affected and RRP is more common in the first born child.

5.2.3 Ethics

Written informed consent was obtained from patients. Ethical approval was obtained from the Multi Centre Research Ethics Committee for Wales, the Bro Taf Local Research Ethics Committee, and other appropriate Local Research Ethics Committees including the University Hospital of Wales (UHW) Research and Development Ethics Committee. All work was conducted in accordance with the Helsinki Declaration of 1975 as revised in 1983.

5.2.4 Genomic DNA Extraction

For fresh EDTA non-frozen blood samples Genomic DNA was extracted using the 'micro method'. For frozen and clotted specimens the 'salting out' extraction method was used. Both of these protocols are described in detail in Chapter 2.

5.2.5 HLA Class I and II Typing

HLA typing was also carried out using the PCR-SSP (Polymerase Chain Reaction – Sequence Specific Primer) method as described in Chapter 2 although some additional high resolution typing was performed by Dynal Biotech (Wirral UK) and the Welsh Blood Service (Pontyclun, Rhondda-Cynon-Taf, UK).

5.2.6 Statistical Analysis

All statistical analyses were carried out using Graphpad Instat Software (Graphpad Software Inc. San Diego CA. USA). For each calculation of statistical significance Fisher's exact test was used and the following calculated; the two sided P value (2 Sided P value), the odds ratio (OR) and the 95% confidence interval (95% CI). A two sided P value of less than 0.05 was considered significant.

5.3 Results

5.3.1 Associations with HLA Class II Allele DRB1*0301

Overall in Cohort II the DRB1*0301 allele was found again to be significant with 21/50 patients positive for this allele 2 Sided P value= 0.0108, OR= 2.226, 95% CI= 1.229 to 4.032) (Table 5.1). Two patients were homozygous for DRB1*0301, both of these patients had aggressive disease

Table 5.1: DRB1*0301 Association in Cohort II

DRB1*0301	RRP Patients n= 50		Control Group n= 554	
Positive	21	42%	136	25%
Negative	29	58%	418	75%

Fisher's Exact Test 2 Sided P Value= 0.0108, OR= 2.226, 95% CI= 1.229 to 4.032

5.3.2 RRP Associations with Ancestral Haplotype 8.1

Of the other alleles, apart from DRB1*0301 that comprise AH 8.1; the HLA class I allele B*08 and class II allele DQB1*02 are seen to be individually positively significantly associated with RRP. The B*08 allele is seen in 20/50 RRP patients compared with 133/554 controls 2 Sided P value= 0.0171, OR= 2.110, 95% CI= 1.160 to 3.840) whilst DQB1*02 2 Sided P value= 0.0361, OR= 1.917, 95% CI= 1.066 to 3.446). Overall the AH 8.1 is seen in 13/50 patients compared with 61/493 controls and is also significantly positively associated with RRP 2 Sided P value= 0.0052, OR= 2.840, 95% CI= 1.430 to 5.638) Table 5.6.

Table 5.2: RRP Association with the HLA Class I allele A*01

A*01	RRP Patients n= 50		Control Group n= 554	
Positive	22	44%	172	31%
Negative	28	57%	382	69%

Fisher's Exact Test 2 Sided P value: 0.0808, OR= 1.745, 95% CI= 0.970 to 3.138

Table 5.3: RRP Association with the HLA Class II allele; B*08

B*08	RRP Patients n= 50		Control Group n= 554	
Positive	20	40%	133	24%
Negative	30	60%	421	76%

Fisher's Exact Test 2 Sided P value= 0.0171, OR= 2.110, 95% CI= 1.160 to 3.840

Table 5.4: RRP Association with the HLA Class II allele Cw*07

Cw*07	RRP Patients n= 50		Control Group n=554	
Positive	12	61%	248	45%
Negative	7	39%	306	55%

Fisher's Exact Test 2 Sided P value= 0.1586, OR= 2.115, 95% CI= 0.820 to 5.455

Table 5.5: RRP Association with the HLA Class II allele; DQB1*02

DQB1*02	RRP Patients n= 50		Control Group n= 554	
Positive	29	58%	232	42%
Negative	21	42%	322	58%

Fisher's Exact Test 2 Sided P value= 0.0361, OR= 1.917, 95% CI= 1.066 to 3.446

Table 5.6: RRP Association with the Ancestral Haplotype 8.1

AH 8.1	RRP Patients n= 50		Control Group n= 554	
Positive	13	26%	61	11%
Negative	37	74%	493	89%

Fisher's Exact Test 2 Sided P value = 0.0052, OR= 2.840, 95% CI= 1.430 to 5.638

5.3.3 Associations with HLA Class II Allele DQB1*03

The HLA class II allele DQB1*0301 is found in 23/50 patients compared with 364/554 individuals in the control group (Table 5.7). It is thus seen to be negatively associated with RRP (2 Sided P value= 0.0083, OR= 0.4446, 95% CI= 0.2481 to 0.7968).

Table 5.7: DQB1*03 Association with Cohort II

DQB1*03	RRP Patients n= 50		Control Group n= 554	
Positive	23	46%	364	66%
Negative	27	54%	190	34%

Fisher's Exact Test 2 Sided P value= 0.0083, OR= 0.445, 95% CI= 0.248 to 0.797

5.3.4 Associations with HLA Class II Allele DRB1*14

In Cohort II the HLA Class II allele DRB1*14 only observed in 1/50 RRP patients compared with 25/554 control subjects (Table 5.8). No significant association to RRP (2 Sided P value= 0.7134, OR= 0.4408, 95% CI= 0.05842 to 3.326).

Table 5.8: RRP Association with the HLA Class II allele; DRB1*14

DRB1*14	RRP Patients n= 50		Control Group n= 554	
Positive	1	2%	25	5%
Negative	49	98%	529	95%

Fisher's Exact Test 2 Sided P Value= 0.7134, OR= 0.441, 95% CI= 0.058 to 3.326

5.3.5 Associations with HLA Class II Allele DQB1*06

Overall in Cohort II the HLA class II allele DQB1*06 is seen in 18/44 RRP patients compared 108/446 control subjects (Table 5.9). This is a positive association with RRP (2 Sided P value= 0.0100, OR= 2.323, 95% CI= 1.256 to 4.295).

Table 5.9: DQB1*06 Association with Cohort II

DQB1*03	RRP Patients n= 50		Control Group n= 554	
Positive	18	36%	108	19%
Negative	44	64%	446	81%

Fisher's Exact Test 2 Sided P value= 0.0100, OR= 2.32, 95% CI= 1.256 to 4.295

5.4 Discussion

5.4.0 Summary of Results

- Significant positive association between RRP and HLA class II alleles DRB*0301, & DQB1*06
- Significant negative association between RRP and HLA class II allele DQB1*03
- Significant positive association between RRP and AH 8.1 and certain of its constiuient alleles: DQB1*02, A*01 & B*08

5.4.1 Analysis of RRP Associations with HLA Allele in Cohort II and Comparisons with Cohort I and Other Studies

*5.4.1.1 DRB1*0301*

In Cohort II, although still significant, the association between the DRB1*0301 allele and RRP has a greater 2 sided P value (2 Sided P value= 0.0108, OR= 2.226, 95% CI= 1.229 to 4.032) than that seen between Cohort I and DRB1*0301 (2 Sided P value= <0.0001, OR= 3.76, 95% CI= 2.18-6.47). However the odds ratios for this allele from both cohorts are similar so the difference in P value may me due to the difference in overall numbers between the two studies (Cohort I n=60, Cohort II n=50). However unlike in the original study where there was a significant association with the non-AH 8.1 patients positive for the DRB1*0301 allele this is not the case with Cohort II. Of the patients where the full AH 8.1 is not present only 8/37 (22%) are positive for DRB1*0301 compared to 75/432 (17%) controls (Two-sided P value= 0.3452, OR= 1.537, 95% CI= 0.677 to 3.493) (Table 5.10). Whilst in the original study this figure was: 16/43 (37%) (Two-sided P value= 0.0009, OR= 3.303, 95% CI= 1.698 to 6.426). Therefore within Cohort II alone, the association between RRP and DRB1*0301 is only significant in association with the AH 8.1 and not independently. Thus it is possible that there is a difference in demographics of Cohort II compared to Cohort I. The original association with DRB1*0301 has subsequently been confirmed by Bonagura *et al* (Bonagura, Vambutas et al. 2004) in a US population.

Table 5.10: Significance of Non-AH 8.1 DRB1*0301 Positive Patients

	Non- AH 8.1 Patients				2 Sided P Value	Odds Ratio	95% Confidence Interval
Cohort	Positive		Negative				
Control	75	15 %	418	85 %			
I	16	37 %	27	63 %	0.0009	3.303	1.698 to 6.426
II	8	22 %	29	78 %	0.3452	1.537	0.677 to 3.493

When a comparison of the geographical distribution of DRB1*0301 in Cohorts I & II it is observed that in Cohort I DRB1*0301 positives and negatives are roughly equal, whilst in Cohort II some imbalances are observed. This is particularly noticeable in the Great Ormond Street Hospital (GOS) subset where there are three times the number of DRB1*0301 negative patients than there are positive. A positive negative imbalance is also seen in the Bristol and Newcastle subsets, the latter in favour of DRB1*0301 positive patients but these both have relatively low numbers. However the GOS subset does appear to skew the trend. This hospital treats a relatively large number of overseas patients and thus may not be representative of the UK population.

Table 5.11: Comparison of Patient Location Correlated with DRB1*0301 in Cohorts I & II

Location	Cohort I				Cohort II			
	DRB1*0301				DRB1*0301			
	Pos		Neg		Pos		Neg	
Bristol	4	12%	2	7%	0	-	2	7%
Birmingham	-	-	-	-	2	10%	3	10%
Brighton	-	-	-	-	6	29%	8	28%
London (GOS)	-	-	-	-	4	19%	12	41%
London (RF)	4	12%	4	15%	-	-	-	-
Middlesbrough	1	3%	1	4%	-	-	-	-
Newcastle	-	-	-	-	4	19%	1	3%
Sheffield	10	30%	9	33%	5	24%	1	3%
Wales	14	42%	11	41%	0	-	2	7%

GOS= Great Ormond Street Hospital

RF= Royal National Ear, Nose & Throat Hospital

5.4.1.2 The Ancestral Haplotype 8.1

The HLA DRB1*0301 allele is often inherited as a component of an ancestral haplotype (AH), a highly conserved, evolutionarily stable, battery of genes. The extended ancestral haplotype 8.1 also incorporates the HLA Class II allele DQB1*02 as well as the Class I alleles; A*0101, Cw*0701 and B*0801.

Overall the in this study, the AH 8.1 association with RRP is significant (2 Sided P value= 0.0052, OR= 2.840, 95% CI= 1.430 to 5.638). A*01 and Cw*07 were not found to be individually significant. Individual significant associations were found not only with the DQB1*02 allele (2 Sided P value= 0.0361, OR= 1.917, 95% CI= 1.066 to 3.446) and the B*08 allele (2 Sided P value= 0.0171, OR= 2.110, 95% CI= 1.160 to 3.840). However as was seen in this study with the DRB1*0301 allele neither the DQB1*02 or the B*08 alleles are significant independently when the non-AH 8.1 population are examined only within the AH 8.1 association (Tables 5.12 & 5.13). Of the patients where the full AH 8.1 is not present 16/37 (43%) are positive for DQB1*02 compared to 171/554 (35%) controls (Two-sided P value= 0.291, OR= 1.435, 95% CI= 0.729 to 2.822) (Table 5.13). In the B*08 positive patients where the full AH 8.1 is not present only 7/37 (19%) are positive for B*08 (Two-sided P value= 0.473, OR= 1.364, 95% CI= 0.577 to 3.224) (Table 5.12).

Table 5.12: Significance of Non-AH 8.1 B*08 Positive Patients

	Non- AH 8.1 Patients				2 Sided P Value	Odds Ratio	95% Confidence Interval
Cohort	Positive		Negative				
Control	72	15 %	421	85 %			
I	4	9%	39	91 %	0.493	0.600	0.208 to 1.730
II	7	19 %	30	81 %	0.473	1.364	0.577 to 3.224

Table 5.13: Significance of Non-AH 8.1 DQB1*02 Positive Patients

Cohort	Non- AH 8.1 Patients				2 Sided P Value	Odds Ratio	95% Confidence Interval
	Positive		Negative				
Control	171	35 %	322	65 %			
I	19	44 %	24	56 %	0.2449	1.491	0.794 to 2.799
II	16	43 %	21	57 %	0.2909	1.435	0.729 to 2.822

In the original study of the individual HLA alleles that comprise AH 8.1 only in A*01 (2 Sided P value= 0.04, OR= 1.82, 95% CI= 1.06–3.12) and DQB1*02 (2 Sided P value= 0.009, OR= 2.08, 95% CI= 1.21–3.59) were individually significant associations with RRP observed. However, only DRB1*0301 was seen to be individually associated with RRP outside of the AH 8.1 associated sample subset. Overall, in the original study AH 8.1 was found to be significantly associated with RRP 2 Sided P value= 0.0007, OR= 3.20, 95% CI= 1.72–5.95). Bonagura *et al* failed to find the AH 8.1 association with RRP in their US cohort (Bonagura et al. 2004) (this is discussed later).

*5.4.1.3 DQB1*03*

In Cohort I, DQB1*03 allele was found to be negatively associated RRP (2 Sided P value= 0.0045, OR= 0.4567, 95% CI= 0.2670 to 0.7812). This negative association is maintained in Cohort II with a very similar statistical relationship to that seen in Cohort I with 23/50 (46%) of patients positive for this compared to 364/554 (66%) in the control group (P = 0.0083, OR= 0.4446, 95% CI= 0.2481 to 0.7968). Conversely, a positive significant association between this allele and RRP was previously found by Bonagura *et al* (Bonagura, Siegal et al. 1994). This study of 16 patients found 12 (75%) to be positive for DQB1*03 compared to only 22% within their control group (2 Sided P Value= <0.0001). However, the DQB1*03 frequency in the control group used by this study (22%) is three times less than that observed in the control group used in our study where 364/554 (66%) individuals were positive for this allele. The Bonagura

1994 study used as a control population HLA frequencies derived from the 11th International Histocompatibility Workshop's Caucasian database. The DQB1*03 allele displays wide fluctuations in frequency between amongst Caucasoid populations (Marsh, Parham and Barber 2000). Thus it is possible that the Bonagura 1994 study may not have suitably matched their control population whilst this study was compared to a pool of unrelated organ donors (Bunce et al. 1996) that has been demonstrated to be representative of the UK population from which all our patients are drawn.

In addition the Bonagura 1994 study is on a very small population compared to our study of 50 samples and HLA studies of less than 28 individuals are not statistically significant (Professor Ken Welsh, Imperial College of Science, Technology and Medicine, Immunity, Personal Communication). For instance, as discussed earlier the first study into the immunogenetics of RRP carried out by our group (Cohort I) (Gelder et al. 2003) found a tentative but significant association between DRB1*14 and RRP (2 Sided P Value= 0.01) and in particular the JORRP subset of patients (2 Sided P Value= 0.008). However, this allele was found in this study to not be significant (2 Sided P Value= 0.7134). DRB1*14 is found rarely in the control population 25/554 (5%) and the 7/60 (12%) RRP patients in Cohort I found to be positive for this allele, although few, were enough to make this allele appear significant. Although this study was nearly four times the size (60 patients) of the of the Bongura 1994 study (16 patients) this illustrates the danger of basing associations upon with in a small pool of samples. As the larger the study HLA study the more significant the results, in this study for the correlation of results with patient epidemiology the results from both Cohorts I & II have been pooled to greatly increase the statistical power of the data (5.4.4).

*5.4.1.4 DQB1*06*

No association was found in Cohort I between RRP and the DQB1*06 allele. However, Bonagura *et* al (Bonagura, 2004) have found DRB*06 to be negatively associated with aggressive RRP in Caucasians. Conversely DQB1*06 was found in Cohort II to be positively significantly associated with RRP (2 Sided P value= 0.0100, OR= 2.323, 95% CI= 1.256 to 4.295). It therefore appears that the Bonagura positive association with RRP may be a chance finding due to multiple comparisons.

5.4.1 Epidemiology and Demographics of Cohort I Vs Cohort II

Several differences are observed between the two Cohorts. The most noticeable of these being that the DRB1*0301, AH 8.1 and DQB1*03 alleles in Cohort II display a reversal of the onset associations observed in Cohort I. In order to determine any reason for this switch the epidemiology and demographics of the two cohorts are examined. Here we examine the differences between these two cohorts on the bases of; disease onset, disease aggression, gender and location.

The two cohorts have approximately equivalent percentages of JORRP and AORRP patients with a roughly 60:40 split in favour of JORRP in both cohorts although Cohort I has 6% more JORRP than Cohort II (Table 5.14). In terms of gender Cohort II has a marginally greater percentage of male patients than Cohort I with a 60:40 ratio compared with the 53:47 split in Cohort I (Table 5.15). An examination of the patient locations (Table 5.16) shows that nearly three quarters of Cohort I to originate from two locations Wales (42%) and Sheffield (32%). In contrast Cohort II has a slightly more widespread geographical distribution, but a large number of samples greatest number samples in Cohort II originate from two locations London (38%) and Brighton (26%).

Table 5.14: Comparison of Disease Onset in Cohorts I & II

HPV Type	n	Onset			
		JORRP		AORRP	
Cohort I	60	37	62%	23	38%
Cohort II	50	24	56%	19	44%
RRP Total	110	61	59%	42	41%

Table 5.15: Gender Differences between Cohorts I & II

HPV Type	n	Gender			
		Male		Female	
Cohort I	60	32	53%	28	47%
Cohort II	50	30	60%	20	40%
RRP Total	109	62	61%	48	39%

Table 5.16: Patient Location Correlated with DRB1*0301 in Cohort II

Location	DRB1*0301			
	Positive		Negative	
Bristol	0	-	1	100%
Birmingham	2	40%	3	60%
Brighton	6	43%	8	57%
London	4	25%	12	75%
Newcastle	4	80%	1	20%
Sheffield	5	83%	1	17%
Wales	0	-	2	100%

Table 5.17: Comparison of Patient Location in Cohorts I & II

Location	Onset					
	Cohort I		Cohort II		RRP Total	
Bristol	6	10%	1	2%	7	6%
Birmingham	0	-	5	9%	5	4%
Brighton	0	-	14	26%	14	12%
London	8	13%	20	38%	28	25%
Middlesbrough	2	3%	0	0%	2	2%
Newcastle	0	-	5	9%	5	4%
Sheffield	19	32%	6	11%	25	22%
Wales	25	42%	2	4%	27	24%

Thus only major difference between the cohorts appears to be geographical distribution. When the geographic distribution of DRB1*0301 positive and negative patients within each cohort is examined (Tables 5.16 & 5.17) whilst all the locations in Cohort I have a roughly 50:50 positive:negative balance whilst in Cohort II a number of contrasts are seen. The greatest of these contrasts is seen in the samples from London in Cohort II which were sourced from Great Ormond Street (GOS) hospital (all JORRP) whereas the Cohort I London samples which originated from the Royal Free Hospital (mostly AORRP). The GOS samples display three time as many patients negative for DRB1*0301 whilst the samples from Sheffield and Newcastle (although the numbers are lower than GOS overall) display 5:1 and 4:1 positive:negative ratios respectively. So it seems that the entirely JORRP group of patients from GOS is bucking the trend and decreasing the overall DRB1*0301.

5.4.4 Analysis of Pooled Cohorts

5.4.4.0 Overview

In order to study the data subsets such as age of onset of disease or disease aggression it is necessary to pool Cohorts I & II to increase statistical power. For instance there are now sufficient adults in the pooled cohort to examine age of onset. Here these pooled findings are presented.

*5.4.4.1 DRB01*0301*

The onset split in this allele shows a strong significant association with both JORRP with 29/61 (48%) positive for this allele (2 Sided P value= 0.0004, OR= 2.785, CI= 1.625 to 4.773) and AORRP with 21/42 (50%) (2 Sided P value= < 0.0001, OR= 4.418, CI= 2.268 to 8.608) positive (Table 5.18). This is also true of the gender split but the significance is less similar with the female subset (2 Sided P value= < 0.0001, OR= 3.952, CI= 2.164 to 7.217) displaying a much lower P value and an odds ratio nearly twice that of the male subset (2 Sided P value= 0.0022, OR= 2.371, CI= 1.384 to 4.061) (Table 5.20). In terms of disease aggression there is a far more clear cut difference with no significance in the non-aggressive disease subset of patients (2 Sided P value= 0.0699, OR= 2.113, CI= 0.957 to 4.665) but a significant relationship between the patients with aggressive disease and the DRB1*0301 allele (2 Sided P value= < 0.0001, OR= 4.501, CI= 2.475 to 8.927) (Table 5.19). Thus as seen in the original study, the DRB1*0301 allele is associated with aggressive disease in RRP with an extremely high odds ratio of 4.5. Unlike the original study, where only the adult subset of patients was seen to be significantly associated with DRB1*0301, due to insufficient numbers, in the combined study both JORRP and AORRP are significantly associated with this allele (Table 5.18). This allele is also marginally more associated with females than males and this is not just due to the lower number of females in the study as the odds ratio for females is more than twice that of males (Table 5.20).

Table 5.18: Disease Onset Associations with DRB01*0301 Allele

Onset	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	136	25%	418	75%			
JORRP	60	29	59%	32	41%	< 0.0001	4.418	2.268 to 8.608
AORRP	43	21	50%	21	50%	0.0004	2.785	1.625 to 4.773

Table 5.19: Disease State Associations with DRB01*0301 Allele

Disease State	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	136	25%	418	75%			
Aggressive	43	26	60%	17	40%	< 0.0001	4.701	2.475 to 8.927
Non-Agg.	27	11	40%	16	60%	0.0699	2.113	0.957 to 4.665

Table 5.20: Gender Associations with DRB01*0301

Gender	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	136	25%	418	75%			
Male	62	27	44%	35	56%	0.0022	2.371	1.384 to 4.061
Female	48	27	56%	21	44%	< 0.0001	3.952	2.164 to 7.217

*5.4.4.2 DQB1*03*

The negative association with the DQB1*03 allele is seen to be significant in the AORRP subset of patients (2 Sided P value= 0.0002, OR= 0.2900, CI= 2.475 to 8.927) but not the JORRP subset patients (2 Sided P value= 0.0899, OR= 0.6152, CI= 0.361 to 1.049) (5.20). There is also a noticeable difference between the male and female subsets with a significant association with seen in the male subset (2 Sided P value= < 0.0001, OR= 0.3078, CI= 0.179 to 0.531) but no significant association was observed

in the female subset (2 Sided P value= 0.3440, OR= 0.7308, CI= 0.401 to 1.332) (Table 5.23). A significant negative association was observed with this allele in non-aggressive disease subset (2 Sided P value= 0.0371, OR= 0.4176, CI= 0.1912 to 0.910) but not in aggressive disease subset of patients (2 Sided P value= 0.0679, OR= 0.5468, CI= 0.2932 to 1.020) (Table 5.22).

Table 5.21: Disease Onset Associations with DQB01*03 Allele

Onset	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	364	66%	190	34%			
JORRP	61	33	54%	28	66%	0.0899	0.6152	0.361 to 1.049
AORRP	42	15	36%	27	64%	0.0002	0.2900	0.151 to 0.558

Table 5.22: Disease State Associations with DQB01*03 Allele

Disease State	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	364	66%	190	34%			
Aggressive	43	22	51%	21	49%	0.0679	0.5468	0.293 to 1.020
Non-Agg.	27	12	44%	15	56%	0.0371	0.4176	0.1912 to 0.910

Table 5.23: Gender Associations with DQB01*03

Gender	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	364	66%	190	34%			
Male	62	23	37%	39	63%	< 0.0001	0.3078	0.179 to 0.531
Female	48	28	58%	20	42%	0.3440	0.7308	0.401 to 1.332

*4.4.3 AH 8.1 (A*01, B*08, Cw*07, DRB1*03, DQB1*02)*

As with the DRB1*0301 allele, the overall AH 8.1 is seen to be significantly associated with both onset subsets of RRP and again as with DRB1*0301 the overall AH 8.1 was seen in Cohort I alone to be only significantly associated with JORRP only again possibly due to low numbers of AORRP patients. In the pooled Cohort, there are 17/61 (26%) positive patients in the JORRP subset (2 Sided P value= 0.0008, OR= 3.123, CI= 1.680 to 5.804) and in the AORRP subset 13/42 (31%) (2 Sided P value= 0.0008, OR= 3.623, CI= 1.788 to 7.342) compared to 61/554 (11%) in the control group. Also as seen in the DRB1*0301 allele there a much stronger significant association with female patients, 17/48 (35%) (2 Sided P value= < 0.0001, OR= 4.432, CI= 2.317 to 8.479), than male patients, 13/62 (21%) (2 Sided P value= 0.0364, OR= 2.144, CI= 1.101 to 4.178) (Table 5.26). The very strong significant association with aggressive RRP and not with non-aggressive RRP seen in DRB1*0301 is also maintained in the overall AH 8.1. This group of alleles is seen 17/43 (40%) aggressive patients (2 Sided P value= < 0.0001, OR= 5.284, CI= 2.713 to 10.295) and in only 6/27 (22%) patients with non-aggressive disease (2 Sided P value= 0.1119, OR= 2.309, CI= 0.897 to 5.945) compared with 61/554 (11%) in the control pool.

Bonagura et al (Bonagura et al. 2004) found weaker statistical significance and only in causasoids with DRB1*0301-DQB1*02 (aggressive subset = 13/33 (39%), controls= 284/1632 (17%)) (2 Sided P Value= 0.004, Odds Ratio= 3.1, 95% CI= 1.4 to 6.6) but, as mentioned failed to find the association with the full AH 8.1. This may be due to an insufficient sample size as they only had 33 caucasoid patients in this aggressive subset.

Table 5.24: Disease Onset Associations with AH 8.1

Onset	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	61	11%	493	89%			
JORRP	61	17	26%	44	74%	0.0008	3.123	1.680 to 5.804
AORRP	42	13	31%	29	69%	0.0008	3.623	1.788 to 7.342

Table 5.25: Disease State Associations with AH 8.1

Disease State	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	61	11%	493	89%			
Aggressive	43	17	40%	26	60%	< 0.0001	5.284	2.713 to 10.295
Non-Agg.	27	6	22%	21	78%	0.1119	2.309	0.897 to 5.945

Table 5.26: Gender Associations with AH 8.1

Gender	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	61	11%	493	89%			
Male	62	13	21%	49	79%	0.0364	2.144	1.101 to 4.178
Female	48	17	35%	31	65%	< 0.0001	4.432	2.317 to 8.479

*4.4.4 A*01*

Individually the A*01 allele is significantly associated with the JORRP subset (21/42 (50%) (2 Sided P Value= 0.0159, OR= 2.221, 95% CI= 1.181 to 4.175) but not with the AORRP subset (25/61 (41%) both compared to 172/554 (31%) positive controls (Table 5.27). This allele is also significantly associated with aggressive disease with 21/43 (49%) of patients with aggressive disease positive for this allele (2 Sided P Value= 0.0265, OR= 2.120, 95% CI= 1.135 to 3.959) with no association with the non-aggressive disease subset (Table 5.28). There is also no significant association between this allele and either gender, although in both cases they do approach

significance (Table 5.29).

Table 5.27: Disease Onset Associations with A*01 Allele

Onset	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	172	31%	382	69%			
JORRP	42	21	50%	21	50%	0.0159	2.221	1.181 to 4.175
AORRP	61	25	41%	36	59%	0.1475	1.542	0.898 to 2.650

Table 5.28: Disease State Associations with A*01 Allele

Disease State	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	172	31%	382	69%			
Aggressive	43	21	49%	22	51%	0.0265	2.120	1.135 to 3.959
Non-Agg.	27	11	41%	16	59%	0.2945	1.527	0.694 to 3.360

Table 5.29: Gender Associations with A*01 Allele

Gender	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	172	31%	382	69%			
Male	62	27	44%	35	56%	0.0617	1.713	1.005 to 2.921
Female	48	22	46%	26	54%	0.0521	1.879	1.036 to 3.410

*4.4.5 B*08*

Again significant associations with both the AORRP and JORRP subsets are seen with B*08 allele alone, (Table 5.30) (JORRP 22/61 (36%) (2 Sided P value= 0.0442, OR= 1.786, CI= 1.022 to 3.120) AORRP (17/42 (33%) (2 Sided P value= 0.0256, OR= 2.152, CI= 1.128 to 4.108) compared to 133/334 (24%) controls). In Cohort I alone, this allele was not associated with either disease onset subset. Also once again, this allele is associated with the aggressive patient subset, 18/43 (42%) aggressive patients

(2 Sided P value= 0.0165, OR= 2.279, CI= 1.206 to 4.308) and not associated with the non-aggressive subset (Table 5.31). This allele is also associated significantly with female patients and not with male patients, with 21/48 female patients positive for this allele (2 Sided P value= 0.0051, OR= 2.462, CI= 1.347 to 4.499) (Table 5.32).

Table 5.30: Disease Onset Associations with B*08 Allele

Onset	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	133	24%	421	76%			
JORRP	61	22	36%	39	64%	0.0442	1.786	1.022 to 3.120
AORRP	42	17	33%	25	67%	0.0256	2.152	1.128 to 4.108

Table 5.29: Disease State Associations with B*08 Allele

Disease State	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	133	24%	421	76%			
Aggressive	43	18	42%	25	58%	0.0165	2.279	1.206 to 4.308
Non-Agg.	27	7	35%	20	65%	0.8190	1.108	0.458 to 2.678

Table 5.32: Gender Associations with DRB01*0301

Gender	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	133	24%	421	76%			
Male	62	21	34%	41	66%	0.0913	1.621	0.9251 to 2.841
Female	48	21	44%	27	56%	0.0051	2.462	1.347 to 4.499

*4.4.6 DQB1*02*

Significant associations with both the AORRP and JORRP subsets are seen the DQB*02 allele alone (Table 5.33) (JORRP 37/61 (61%) (2 Sided P value= 0.0063, OR= 2.140, CI= 1.246 to 3.675) AORRP (25/42 (60%) (2 Sided P value= 0.0347, OR= 2.041, CI= 1.077 to 3.867) both compared to 133/334 (24%) controls). It is also strongly associated with the aggressive patient subset, seen in 31/43 (72%) of aggressive patients (%) (2 Sided P value= 0.0002, OR= 3.585, CI= 1.803 to 7.131) (Table 5.34). A similar but weaker association has been made by Bonagura et al (Bonagura et al. 2004) in a caucasoid aggressive subset of patients with DQB1*02 present in 15/33 (45%) compared to 499/1632 (30%) in their control group (2 Sided P value= 0.08, Odds Ratio= 1.9, 95% CI= 0.9 to 4.0). It is also significantly associated with both male (37/62 (60%) (2 Sided P value= 0.0099, Odds Ratio= 2.054, 95% CI= 1.203 to 3.507) and female (30/48 (62%) (2 Sided P value= 0.0063, Odds Ratio= 2.313, 95% CI= 1.259 to 4.250) patient subsets (Table 5.35).

Table 5.33: Disease Onset Associations with DQB1*02 Allele

Onset	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	232	42%	322	58%			
JORRP	61	37	61%	24	39%	0.0063	2.140	1.246 to 3.675
AORRP	42	25	60%	17	40%	0.0347	2.041	1.077 to 3.867

Table 5.34: Disease State Associations with DQB1*02 Allele

Disease State	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	232	42%	322	58%			
Aggressive	43	31	72%	12	28%	0.0002	3.585	1.803 to 7.131
Non-Agg.	27	12	44%	15	56%	0.8430	1.110	0.510 to 2.417

Table 5.35: Gender Associations with DQB1*02 Allele

Gender	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	232	42%	322	58%			
Male	62	37	60%	25	40%	0.0099	2.054	1.203 to 3.507
Female	48	30	62%	18	38%	0.0063	2.313	1.259 to 4.250

*5.4.4.7 DQB1*06*

In the combined pool the DRB1*06 allele is seen in 16/42 (38%) of the AORRP patients where a positive association is observed (2 sided P value: 0.0089, OR= 2.541, CI= 1.317 to 4.904) compared to 108/554 (19%) of the control group (Table 5.36). It therefore appears that DRB1*06 allele was not seen as significant in Cohort I as there were fewer AORRP patients (24/60 (38%)) in the original study than in Cohort II (19/50 (44%)) (Table 5.15). A significant positive association is also seen between this allele and the male subset of patients (Table 5.38) where 21/61 (34%) were positive (2 sided P value: 0.0089, OR= 2.541, CI= 1.317 to 4.904). No significant associations were observed in the JORRP subset or the female subset of patients (Tables 5.36 & 5.38). Most interestingly of all there is a positive association with the non-aggressive subset of patients where 11/27 (41%) patients were positive for this allele (2 sided P value: 0.0089, OR= 2.541, CI= 1.317 to 4.904). This is in stark contrast with Bonagura *et al* (Bonagura et al. 2004) who found this allele to be negatively associated with caucasoid RRP patients with aggressive disease. The Bonagura group found this allele in 7/56 (12%) (2 sided P value: 0.04, OR= 0.4, CI= 0.2 to 1.0) of their caucasoid subset compared to 407/1632 (25%) within the control population they compared to (Gjertson and Lee 1998). This is not due to a patient population biased towards adult or female patients which, as seen above would skew this association, as both male AORRP subsets are actually in the majority in the Bonagura 2004 study. This may therefore be due to multiple comparisons within a low sample number, which as we have seen in our non pooled cohorts can skew associations.

Table 5.36: Disease Onset Associations with DQB1*06 Allele

Onset	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	108	19%	446	81%			
JORRP	61	15	25%	46	75%	0.3982	1.347	0.725 to 2.502
AORRP	42	16	38%	26	62%	0.0089	2.541	1.317 to 4.904

Table 5.37: Disease State Associations with DQB1*06 Allele

Disease State	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	108	19%	446	81%			
Aggressive	43	9	21%	34	79%	0.8420	1.093	0.509 to 2.348
Non-Agg.	27	11	41%	16	59%	0.0130	2.839	1.281 to 6.294

Table 5.38: Gender Associations with DQB1*06 Allele

Gender	n	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
Controls	554	108	19%	446	81%			
Male	62	21	34%	41	66%	0.0128	2.115	1.200 to 3.727
Female	48	13	27%	35	73%	0.2581	1.534	0.784 to 2.999

5.5 Concluding remarks

It was observed that in this study the DRB1*0301 unlike in the original study is not seen to be significantly associated with RRP outside of the association with the AH 8.1. However a secondary study in a US population has shown that this allele is separately associated with RRP. It was also shown earlier that this reduced frequency may be due to a geographical subset where this allele is underrepresented. However in the pooled cohorts, not only is there a significant association between DRB1*0301 and RRP (2 Sided P Value= < 0.0001, OR= 2.964, 95%= 1.945 to 4.515) (Table 5.39) but also that this association is seen outside of the AH 8.1 association (2 Sided P Value= 0.0022 OR= 2.964, 95%= 1.395 to 4.090) (Table 5.41). However, this individual association outside of the AH 8.1 is not seen with the B*08 or the DQB1*02 alleles.

A strong significant positive association is also maintained with the AH 8.1 overall (2 Sided P Value= < 0.0001, OR= 3.031, 95% CI= 1.844 to 4.982) (Table 5.39) and a strong negative association was observed with the DQB1*03 allele (2 Sided P Value= 0.0002, OR= 2.389, 95% CI= 0.298 to 0.683). Also the novel positive association between DQB1*06 and RRP observed in this study but not in the original study is maintained overall (Table 5.39).

Table 5.39: Overall Associations with Class II Alleles and AH 8.1

Allele	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
DRB1*0301	54	49%	56	51%	< 0.0001	2.964	1.945 to 4.515
DQB1*03	51	46%	59	54%	0.0002	0.4512	0.298 to 0.683
DQB1*06	34	31%	76	69%	0.0106	1.847	1.171 to 2.914
AH 8.1	30	27%	80	73%	< 0.0001	3.031	1.844 to 4.982

Table 5.40: Pooled Cohort Significant Individual AH 8.1 Allele Associations with RRP

Within the AH 8.1 Association

Allele	Positive		Negative		2 Sided P Value	Odds Ratio	95% Confidence Interval
DQB1*02	67	61%	43	39%	0.0003	2.163	1.423 to 3.287
B*08	42	38%	68	62%	0.0029	1.955	1.270 to 3.010

Table 5.41: Pooled Cohort Individual AH 8.1 Allele Associations with RRP Outside of AH 8.1 Association

	All Patients				Non- AH 8.1 Patients				2 Sided P Value	Odds Ratio	95% Confidence Interval
Allele	Positive		Negative		Positive		Negative				
DRB*0301	54	49 %	56	51 %	24	30 %	56	70 %	0.0022	2.389	1.395 to 4.090
DQB*02	65	59 %	45	41 %	35	44 %	45	56 %	0.1319	1.465	0.907 to 2.365
B*08	41	37 %	69	63 %	11	14 %	69	86 %	1.0000	0.9322	0.471 to 1.847

Bonagura *et al* had already made an association between DQB1*03 and RRP but this was a positive association (Bonagura et al. 1994), this study however as shown above was essentially flawed in sample size and design. In Chapter 7 a discussion of HLA associations with cervical cancer is made and the DQB1*03 alelle is seen to be associated, in a wide population spectrum, with progression to cervical cancer (Wank and Thomssen 1991). Therefore DQB1*03 appears to play a role in disease mediation in more than one HPV associated disease even if those effects are contrary to each other. In the next chapter an examination is made of Cytokine responses to peptides based upon cutaneous and genital HPV types in healthy immunologically normal HPV infected individuals. In light of the findings and extended associations made in this chapter particular examination shall be made of how these responses relate to HLA Class II alleles.

Chapter Six:

Potential HPV Cross Reactivity in $CD4^+$ T Cells

In Response to HPV L1 Derived Peptides

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6.5 Concluding Remarks

6.1 Introduction

6.1.1 Host Virus Interactions In RRP – A HPV Mediated Disease

In Chapter 5 it was established that aberrant HLA class II immune responses may form the foundation of RRP pathogenesis. The majority of HPV infections are transient in nature with most cleared by the immune system within 1-3 years (Moscicki, Palefsky et al. 1993; Ostor 1993; Parkin, Pisani and Ferlay 1993, Chua, 1996 #125; Bosch 1995; Burd 2003). Infiltrating $CD4^+$ T cells have been observed in spontaneously regressing warts (Coleman, Birley et al. 1994; Hong, Greer et al. 1997; Opaneye 1999) and as discussed in Chapter 3, individuals immunosuppressed for example by HIV/AIDS are more likely to develop cervical cancer (Feingold, Vermund et al. 1990; Lefevre, Hankins et al. 2004, Baay, 2004 #64; Moscicki, Ellenberg et al. 2004; Moscicki, Ellenberg et al. 2004).

Our group has also previously demonstrated a negative correlation between the clinical status of RRP patients and the strength of $CD4^+$ T-cell proliferative responses in tritiated thymidine incorporation experiments (Gelder, Williams et al. 2003). Also that the primary cytokine response by ELISpot in patients with the DRB1*0301, shown to be associated with RRP, is from IFN-γ (Gelder et al. 2003). Bonagura *et al* (Bonagura, Hatam et al. 1999) have examined the effect upon cytokine mRNA levels of *in vitro* exposure of T-cells isolated from autologous papillomata when exposed to autologous papillomata. They found in these T-cells significant mRNA levels of the Th1 cytokine IFN-γ and the Th2 cytokines IL-4 and IL-10 in individuals with mild to moderate disease. However, although patients with severe disease displayed significant levels of IL-4 and IL-10 mRNA, minimal IFN-γ mRNA was observed. Therefore as a mainly Th2, and thus humoral, response is observed to papilloma tissue in RRP patients with severe disease they may be incapable of mounting a suitable and competent HPV-specific T-cell response.

6.1.2 Cross-Reactivity between Mucosal and Cutaneous HPV Types in Normal Healthy Donors

Our group has also previously established that $CD4^+$ T-cell responses are cross reactive between HPV types (Williams, Hart et al. 2002), dispelling the previously held paradigm that T-cell responses to HPV were extremely type specific (Shepherd, Rowe et al. 1996; Rudolf, Nieland et al. 1999). Our group found that greater than 80% of twenty five normal, healthy volunteers tested displayed $CD4^+$ T-cell proliferative responses to the HPV-11 L1 VLP. The pentameric L1 outer capsid protein is produced late in the life cycle of the virus but is the major capsid component forming 95% of the HPV viron. Production of L1 in bacteria results in self-assembly into a VLP the shape of which and immune response to mirror that of the HPV viron itself (Hagensee, Yaegashi and Galloway 1993).

A battery of peptides were designed based upon commonly recognised HPV-11 L1 epitopes (p30, p139, p186, p243, p304, p412) in three of the most common mucosal types (HPV-6, -16, and –18) and four of the most common cutaneous HPV types (HPV-1, -2, -3, and –4) (Tables 6.2-6.7) (Williams et al. 2002). In tritiated thymidine incorporation experiments with HPV-11 L1 VLP specific lines at least one of these peptides was recognised. The responses were inversely proportional to the variation between the HPV-11 peptides and the HPV type upon which they were based (Williams et al. 2002). Additionally, between 88-99% of the HPV responses were derived from the CD45RA low / CD45RO high (RO^+) memory T-cell population as opposed the CD45RA high / CD45RO low ($CD45\ RA^+$) naïve T-cell population (Williams et al. 2002). Most individuals are infected by low risk HPV types at some stage, often in childhood, be it a verruca, a or a simple skin wart. We thus conclude that the high rate of responses to the genital type HPV-11 is likely to be due to cross reactive T-cell memory responses to HPV antigens as discussed with regard to vaccine design in Chapter 3.

6.1.3 Cytokine Production in HPV Infection

As discussed in Chapter 1, HPV like most viruses has been found to induce a Th1 pattern of cytokine expression with the production of IFN-γ and reduced production of IL-4 preceding viral clearance (Scott, 1999 #12). It has been demonstrated in RNA viruses such as HIV that immune responses may be modulated by escape mutations in key epitopes which may even lead to a diminished or even absent response even to other viral clades (Soudeyns, Paolucci et al. 1999; Allen, Altfeld et al. 2004; Feeney, Tang et al. 2004). However, HPV is a DNA virus and although as seen in Chapter 4 mutations do occur, it is comparatively far more genetically conserved than RNA viruses. The emergence of novel viral clades during infection is therefore unlikely. However the majority of the population are constantly exposed to cutaneous HPV types in the course of every day life (Welters, de Jong et al. 2003). It is therefore possible that this prior exposure to HPV may moderate cytokine production in response to peptides based upon genital HPV types such as HPV-11, -16 & -18 in a manner comparable that achieved by the novel HIV clades. Alternately it may be that RRP patients are rare individuals whose initial encounter HPV is in the trachea with no prior cutaneous exposure.

Whilst cervical $CD4^+$ and $CD8^+$ T cells from subjects infected with HPV-16 and non-infected individuals have been found *in vitro* to express both IFN-γ and IL-4 in response to control stimulation, the infected group express IFN-γ in response to HPV-16 L1 stimulation (Passmore, Burch et al. 2002). In order to further examine the previously established cross-reactivity in HPV infected individuals, quantitative data on cytokine production, particularly IFN-γ, in response to this battery of peptides based upon genital and cutaneous HPV types is crucial. To this end Cytometric Bead Array (CBA) experiments were carried out on the same (apart from one different individual) healthy, non-related, non-RRP, HPV infected individuals used in the original experiments. These were all adults with 3 males and 3 females.

6.2 Methodology

6.2.1 Isolation of Peripheral Blood Mono-nuclear Cells

PBMC were isolated from whole blood by the histopaque density centrifugation for the isolation of PBMC detailed in Chapter 2.

6.2.2 Cellular Staining and Sorting

PBMC were sorted into CD45RA high / CD45RO low (CD45 RA$^+$) and CD45RA low / CD45RO high (RO$^+$) populations using a MoFlo cell sorter (Cytomation, Freiburg, Germany). The preparatory method and protocol for this are detailed in Chapter 2. All sorts were checked by FACS analysis and were >98% pure in all cases.

6.2.3 Tritiated thymidine Incorporation (Proliferation) Assay

Tritiated thymidine incorporation assays were carried out using 15mer peptides representing epitopes on the HPV L1 outer capsid protein for which the particular donor had previously been found to respond (Williams et al. 2002). Peptides corresponding to each of these epitopes were derived from a cross section of HPV types both cutaneous (HPV-1, 2, 3 and 4) and genital (HPV-11, 16 & 18). These peptides are shown in Tables 6.2-6.7 with the region of each peptide believed to lie within the HLA class II peptide binding groove indicated in bold. The Genbank accession numbers for the sequences of HPV types upon which the peptides are based are shown in Table 6.1. After cell sorting the CD45 RO$^+$ memory T-cell population and CD45 RA$^+$ naïve T-cells were resuspended separately and in complete media (see Chapter 2) and transferred to 48 well plates at 0.7 x 10^6 cells per well. HPV-11 L1 VLP (10μg/ml), influenza haemagglutinin (A/Beijing/32/92) (H3N2) (0.1μg/ml), tetanus toxoid (1.2IU/ml) and PPD (200 U/ml) were used as positive controls with three empty wells serving as negative controls. Cells were harvested and assayed on days 7 & 8 of the experiment; the full method for the tritiated thymidine incorporation is detailed in Chapter 2.

Table 6.1: Genbank Accession Numbers for HPV Types Used in Peptide Design

HPV Type	11	6b	1	2	3	4	16	18
Genbank Acc. No.	NC001525	NC001355	NC001356	NC001352	NC001588	X70827	AF125673	NC001357

Table 6.2: Peptide p30 (30-45)

HPV Type	Sequence														
HPV-11	T	N	I	F	Y	H	A	S	S	S	R	L	L	A	V
HPV-6b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HPV-1a	-	-	L	-	-	-	-	T	-	E	-	-	-	L	-
HPV-2a	-	-	V	Y	-	-	G	G	-	-	-	-	-	T	-
HPV-3	-	-	-	Y	-	Y	-	G	-	-	-	-	-	T	-
HPV-4	-	S	L	Y	F	-	-	G	T	E	-	-	-	T	-
HPV-16	-	-	-	Y	-	-	-	G	T	-	-	-	-	-	-
HPV-18	-	S	-	-	-	-	-	G	-	-	-	-	-	T	-

Table 6.3: Peptide p139 (139–144)

HPV Type	Sequence														
HPV-11	D	N	R	V	N	V	G	M	D	Y	K	Q	T	Q	L
HPV-6b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HPV-1a	-	S	-	Q	-	T	A	F	-	A	-	-	-	-	M
HPV-2a	-	G	-	E	-	I	S	-	-	-	-	-	-	-	-
HPV-3	-	S	-	D	-	I	S	V	-	N	-	-	-	-	-
HPV-4	-	-	-	Q	D	-	S	L	-	P	-	-	-	-	M
HPV-16	-	-	-	E	C	I	S	-	-	-	-	-	-	-	-
HPV-18	-	V	-	D	-	-	S	V	-	-	-	-	-	-	-

Table 6.4: Peptide p186 (186–201)

HPV Type	Sequence														
HPV-11	E	L	I	T	S	V	I	Q	D	G	D	M	V	D	T
HPV-6b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HPV-1a	Q	M	-	E	-	-	-	E	-	-	-	-	M	-	I
HPV-2a	Q	F	T	N	T	T	-	E	-	-	-	-	-	E	-
HPV-3	-	-	-	-	A	P	-	-	-	-	-	-	-	-	-
HPV-4	-	-	V	N	-	Y	-	-	-	-	-	-	C	-	I
HPV-16	-	-	-	N	T	-	-	-	-	-	-	-	-	-	-
HPV-18			K	N	T		L	E							

Table 6.5: Peptide p243 (243–258)

HPV Type	Sequence														
HPV-11	L	F	F	Y	L	R	K	E	Q	M	F	A	R	H	F
HPV-6b	-	-	-	F	-	-	-	-	-	-	-	-	-	-	-
HPV-1a	M	-	-	F	A	-	R	-	-	-	Y	T	-	-	-
HPV-2a	M	-	-	S	-	-	R	-	-	-	-	T	-	-	-
HPV-3	M	-	-	-	-	-		-	-	L		-	-	-	-
HPV-4	-	-	-	F	G	-	R	-	-	L	Y	-	-	-	-
HPV-16	-	-	-	-	-	-	R	-	-	-	-	V	-	-	L
HPV-18	M	-	-	C	-	-	R	-	-	L	-	-	-	-	-

Table 6.6: Peptide p304 (304–319)

HPV Type	Sequence														
HPV-11	F	N	K	P	Y	W	L	Q	K	A	Q	G	H	N	N
HPV-6b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HPV-1a	-	-	R	S	-	-	-	-	R	C	-	-	Q	-	-
HPV-2a	-	-	-	-	-	-	-	R	R	-	-	-	-	-	-
HPV-3	-	-	-	-	-	-	-	R	R	-	-	-	-	-	-
HPV-4	-	-	R	-	-	-	-	N	R	-	-	-	T	-	-
HPV-16	-	-	-	-	-	-	-	-	R	-	-	-	-	-	-
HPV-18	-	-	-	-	-	-	-	H	-	-	-	-	-	-	-

Table 6.7: Peptide p412 (412–417)

HPV Type	Sequence														
HPV-11	E	D	T	Y	R	Y	V	Q	S	Q	A	I	T	C	Q
HPV-6b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HPV-1a	-	-	Q	-	-	F	L	G	-	S	L	A	A	K	C
HPV-2a	Q	-	-	-	-	-	L	-	-	-	-	-	-	-	-
HPV-3	-	-	-	-	-	F	L	T	-	S	-	-	-	-	-
HPV-4	-	-	Q	-	-	F	L	-	-	R	-	T	R	-	P
HPV-16	-	-	-	-	-	F	-	T	-	-	-	-	A	-	-
HPV-18	V	-	-	-	-	F	-	-	-	V	-	-	-	-	-

6.2.4 Cytometric bead array

In order to quantify cytokine production in response to these batteries of peptides CBA was used. This is a multiplexed assay which essentially replicates a sandwich ELISA on a bead. A battery of such beads with distinct fluorescence spectra capture and quantitate specifically the soluble target. This target is then measured and quantified by fluorescence detection and flow cytometric analysis and plotting the results against known standards on a standard curve. The Th1/Th2 array (BD biosciences) used quantifies the analysis of the Th1 cytokines; IFN-γ, TNF-α, and IL-2 and the Th2 cytokines; IL-4, IL-5 and IL-10. To determine the optimum days at which to sample each of a 28 day time course was carried on a healthy donor. PBMC were sorted into CD45 RA^+ and CD45 RO^+ populations (detailed in Chapter 2) stimulated in separate wells of 48 well plates with; HPV-11 L1 VLP (10μg/ml), in order to confirm an immune response to HPV, tetanus toxoid (1.2IU/ml) and PPD (200 U/ml) with three empty well used as a negative control. On each of the 28 days an aliquot of

supernatant from each well was taken and transferred to separate cryovials (Greiner), snap frozen in liquid nitrogen and stored at -90°C ready for the CBA assay.

On days 7, 8 and 9 cells were plated out in triplicate and used in a tritiated thymidine incorporation proliferation experiment (detailed in Chapter 2). The proliferation assays showed that the T-cell response to L1 peaked at day 8 for this individual (Fig. 6.1). CBA assays were carried out on the CD45 RO^+ memory T-cell aliquots produced in response to PPD and tetanus toxoid from days 1-14 (as detailed in Chapter 2). Plots of the resulting cytokine responses over time (Fig. 6.2) show that TNF-α and IL-2 peak around days 2/3, whilst IFN-γ and IL-5 responses plateaued at days 6/7. Consequently experimental CBA aliquots were taken on days 2 and 7.

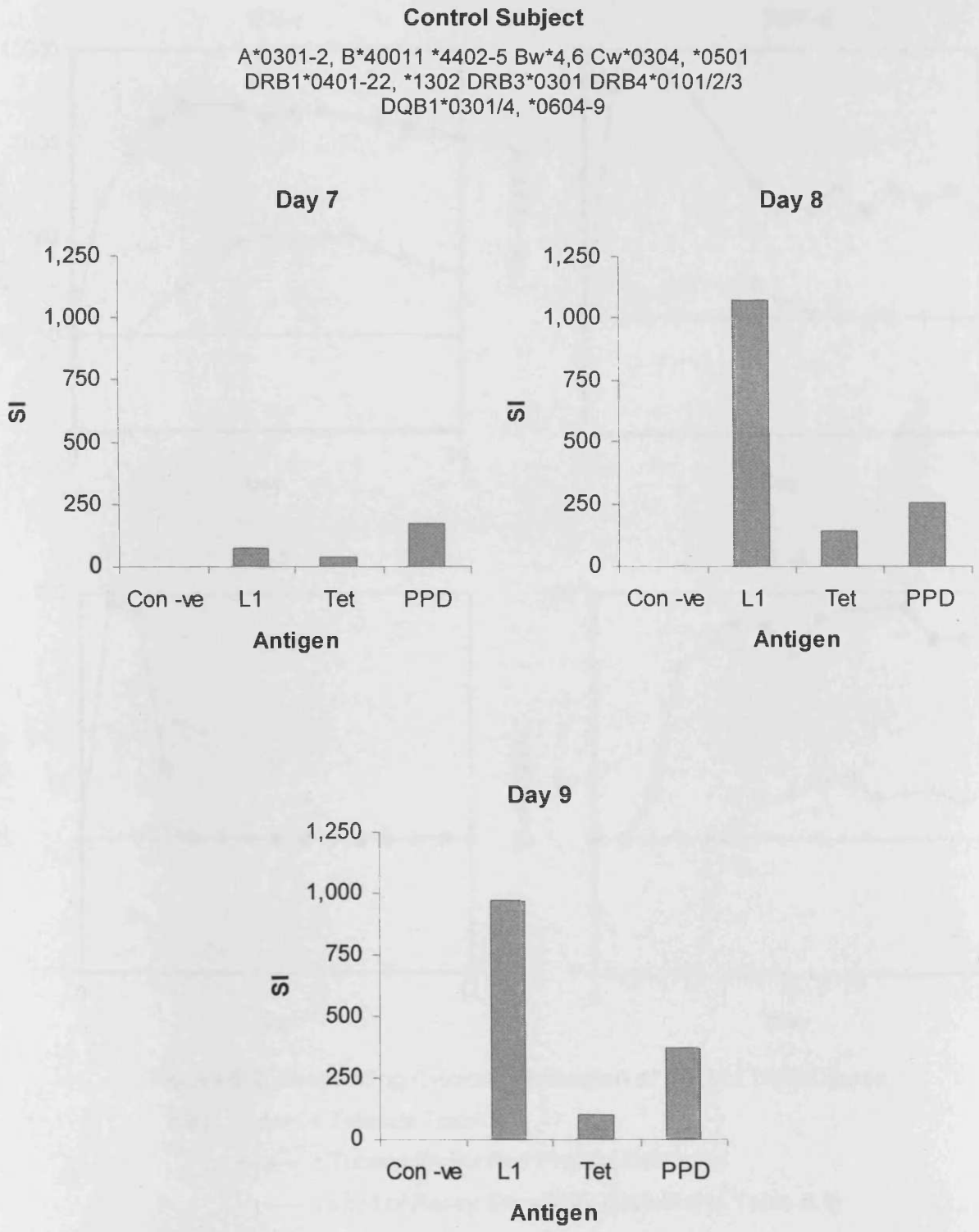

Figure 6.1: Proliferative T-Cell Responses of Control Time Course

$CD45RO^+$ populations stimulated in separate wells of 48 well plates with; HPV-11 L1 VLP, tetanus toxoid, PPD and negative control. On days 7, 8 and 9 cells were plated out in triplicate and used in a tritiated thymidine incorporation proliferation experiment.

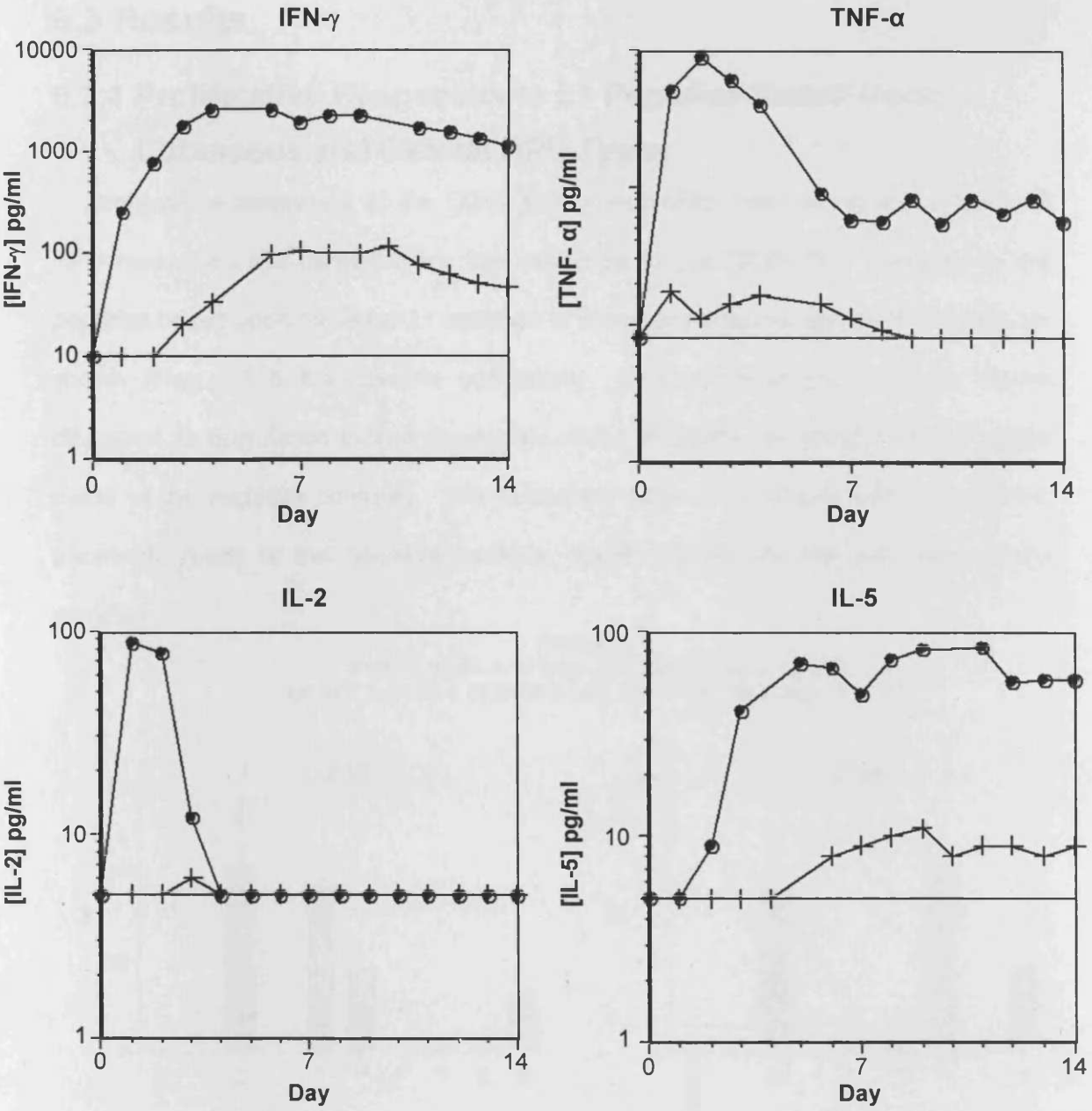

Figure 6.2: Responding Cytokine Production of Control Time Course

Key: —+— : Tetanus Toxoid

—●— : Tuberculin Purified Protein Derivative

——— : Limit of Assay Sensitivity (Detailed in Table 6.8)

Table 6.8: Detectable Limits of CBA Assay

Cytokine	IFN-γ	TNF-α	IL-10	IL-5	IL-4	IL-2
CBA Detectable Limit (pg/ml)	4.9	9.0	19.1	5.05	9.7	12.2

6.3 Results

6.3.1 Proliferative Responses to L1 Peptides Based Upon Cutaneous and Genital HPV Types

Proliferative responses of the CD45 RO+ populations, from which the virtually all HPV responses are derived (very little response in the CD45 RO+ fraction), to the peptides based upon the listed L1 epitopes in the cutaneous and genital HPV types are shown (Figs 6.3 & 6.4) as this population,. These are pooled triplicate results displayed as stimulation indices (geometric mean of counts per minute divided by the mean of the negative controls). The horizontal dotted line indicates three times the geometric mean of the negative controls, results above this line are taken to be significant.

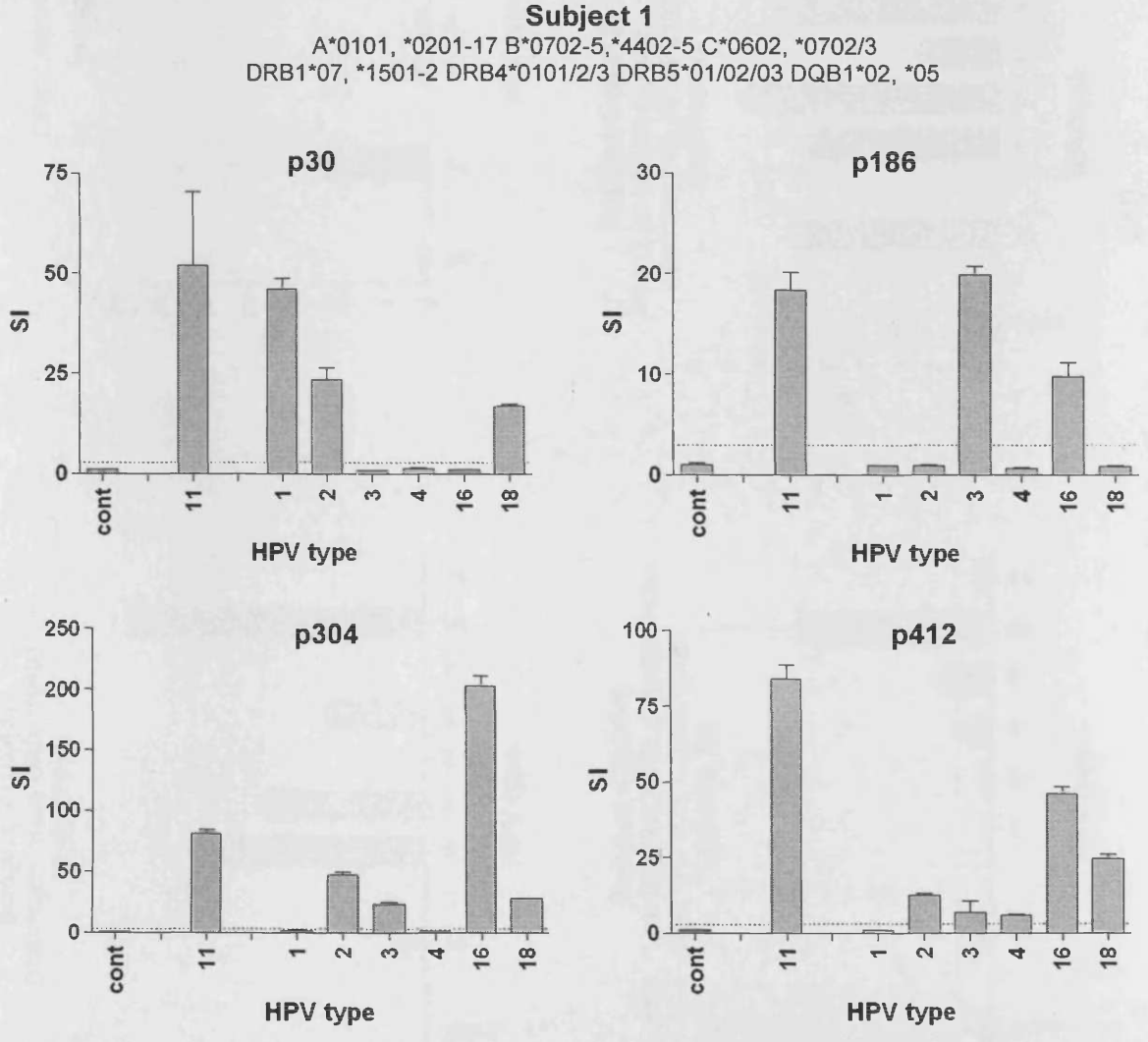

Figure 6.3: Subject: 1 Proliferative Responses to Cutaneous & Genital HPV Peptides

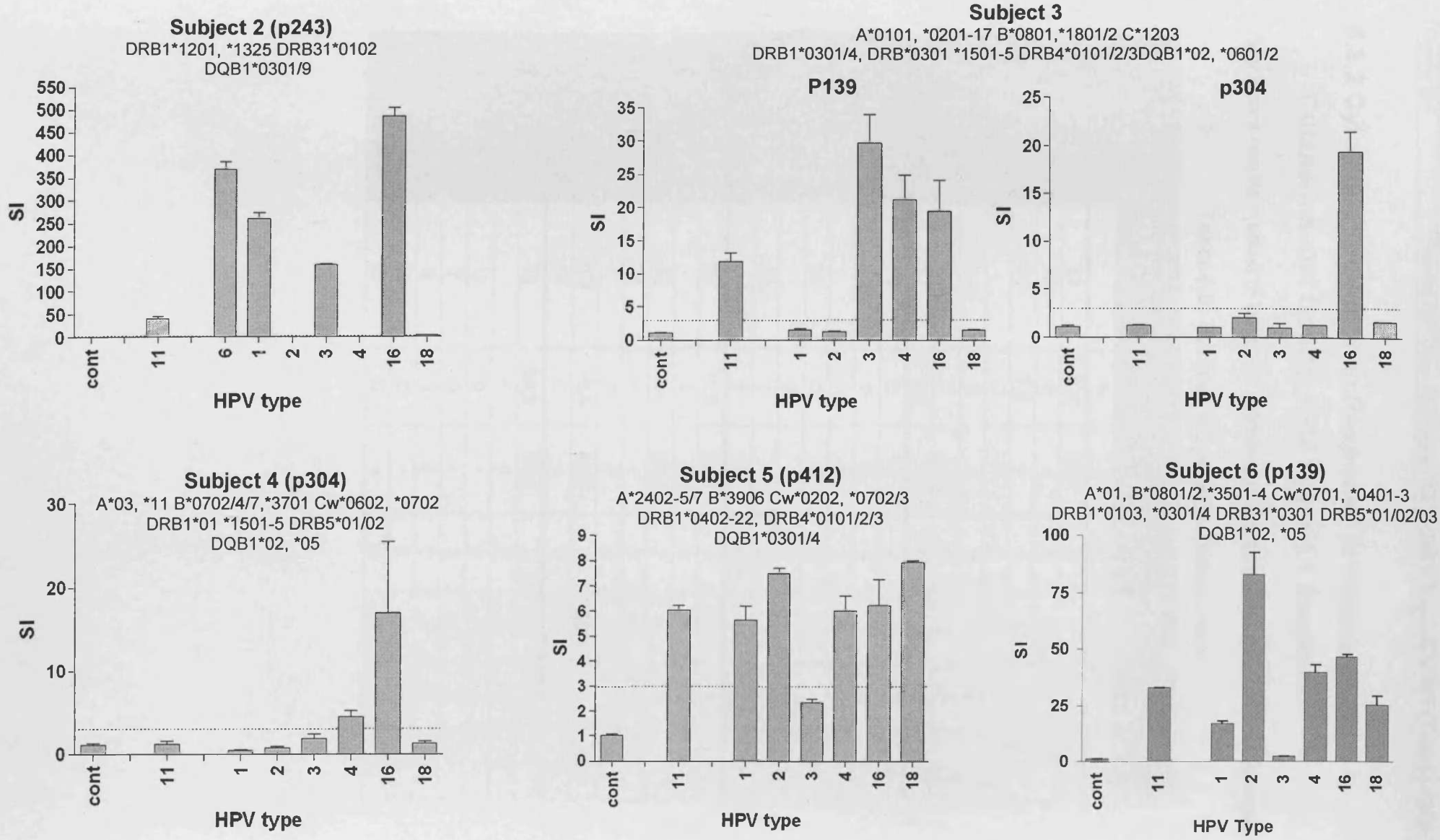

Figure 6.4: Proliferative Responses to Cutaneous & Genital HPV Peptides

6.3.2 Cytokine Production in Response in Response to Cutaneous and Genital HPV Types L1 Peptides

Significant results marked in bold, + = cytokines detectable, but at lower limit of assay

Table 6.9: Subject 1 Cytokine Responses

Peptide	HPV Type	Proliferation SI	Cytokine Production pg/ml					
			IFN-γ	TNF-α	IL-10	IL-5	IL-4	IL-2
	Control		+	+	0	0	0	+
30	HPV-11	52	**8.6**	**22.1**	0	+	0	0
	HPV-1	46	0	+	0	0	0	+
	HPV-2	23	**7.4**	+	0	0	0	+
	HPV-3	-	0	+	0	0	+	0
	HPV-4	-	0	+	0	0	+	0
	HPV-16	-	0	0	0	0	0	0
	HPV-18	17	**8.2**	+	0	0	0	0
186	HPV-11	18	0	0	0	0	0	0
	HPV-1	-	0	0	0	0	0	0
	HPV-2	-	0	0	0	0	0	0
	HPV-3	20	0	0	0	+	+	0
	HPV-4		0	+	0	0	+	0
	HPV-16	10	0	+	0	0	0	0
	HPV-18	-	0	0	0	0	+	0
304	HPV-11	82	**11.3**	**20.1**	0	+	+	+
	HPV-1	-	0	0	0	+	+	+
	HPV-2	47	**7.4**	**11.4**	0	+	0	+
	HPV-3	24	0	+	0	+	0	+
	HPV-4	-	0	0	0	0	0	0
	HPV-16	203	**35.5**	**31.4**	0	**35.4**	+	**15.1**
	HPV-18	28	0	+	0	0	0	+
412	HPV-11	84	**25.0**	**16.6**	0	0	0	+
	HPV-1		0	0	0	0	0	0
	HPV-2	12	0	+	0	+	0	+
	HPV-3	7	0	+	0	0	0	+
	HPV-4	6	0	0	0	0	0	0
	HPV-16	46	0	+	0	+	0	0
	HPV-18	25	0	+	0	0	0	0

Table 6.10: Subject 2 Cytokine Responses to p243

HPV Type	Proliferation S.I.	Cytokine Production pg/ml					
		IFN-γ	TNF-α	IL-10	IL-5	IL-4	IL-2
Control		18.6	+	+	+	16.4	+
HPV-11	41	11.6	+	0	0	10.2	0
HPV-6	371	**69.2**	**35.8**	0	**11.4**	0	+
HPV-1	261	**28.7**	**18.0**	+	+	0	+
HPV-2	-	17.7	0	+	+	0	0
HPV-3	159	**66.2**	**15.8**	**28.1**	0	+	0
HPV-4	-	21.3	0	0	0	0	+
HPV-16	489	**65.3**	**26.6**	+	+	0	**15.1**
HPV-18	-	9.0	0	0	0	0	0

Table 6.11: Subject 3 Cytokine Responses to p139

HPV Type	Proliferation S.I.	Cytokine Production pg/ml					
		IFN-γ	TNF-α	IL-10	IL-5	IL-4	IL-2
Control		0	0	0	+	+	+
HPV-11	12	0	+	0	+	0	+
HPV-1	-	0	0	0	+	+	+
HPV-2	-	0	0	0	+	0	+
HPV-3	30	+	0	0	+	0	+
HPV-4	21	0	+	0	+	+	+
HPV-16	19	0	+	0	+	+	+
HPV-18	-	+	+	0	+	0	+

Table 6.12: Subject 3 Cytokine Responses to p304

HPV Type	Proliferation S.I.	Cytokine Production pg/ml					
		IFN-γ	TNF-α	IL-10	IL-5	IL-4	IL-2
Control		0	0	0	+	+	+
HPV-11	-	+	0	0	+	+	+
HPV-1	-	0	0	0	+	0	+
HPV-2	-	+	+	0	+	+	+
HPV-3	-	0	0	0	+	+	+
HPV-4	-	0	0	0	+	+	+
HPV-16	19	0	0	0	+	+	+
HPV-18	-	+	+	0	+	0	+

Table 6.13: Subject 4 Cytokine Responses to p304

HPV Type	Proliferation S.I.	Cytokine Production pg/ml					
		IFN-γ	TNF-α	IL-10	IL-5	IL-4	IL-2
Control		+	+	+	+	+	+
HPV-11	-	+	+	+	+	**9.9**	+
HPV-1	-	0	+	0	0	0	+
HPV-2	-	+	+	+	0	+	+
HPV-3	-	**7.1**	+	+	+	+	+
HPV-4	4	+	+	+	+	**14.1**	+
HPV-16	17	**7.5**	+	+	**5.3**	+	+
HPV-18	-	**8.1**	+	+	+	+	+

Table 6.14: Subject 5 Cytokine Responses to p412

HPV Type	Proliferation S.I.	Cytokine Production pg/ml					
		IFN-γ	TNF-α	IL-10	IL-5	IL-4	IL-2
Control		+	+	+	+	+	+
HPV-11	22	**65.9**	**42.5**	+	**79.7**	+	+
HPV-1	12	**28.4**	**18.2**	+	**20.5**	+	+
HPV-2	28	**52.1**	**42.5**	+	**124.6**	+	+
HPV-3	-	+	+	+	+	+	+
HPV-4	20	**31.1**	**18.0**	+	**27.1**	0	+
HPV-16	4	+	+	+	0	0	+
HPV-18	27	**96.0**	**40.1**	+	**182.5**	0	+

Table 6.15: Subject 6 Cytokine Responses to P139

HPV Type	Proliferation S.I.	Cytokine Production pg/ml					
		IFN-γ	TNF-α	IL-10	IL-5	IL-4	IL-2
Control		5.5	+	+	+	13.4	+
HPV-11	33	5.1	+	+	+	+	+
HPV-1	17	**6.5**	+	+	+	**30.7**	+
HPV-2	83	**16.4**	**12.9**	+	+	+	+
HPV-3	-	5.5	+	+	0	+	+
HPV-4	40	**10.0**	+	+	+	13.8	+
HPV-16	46	**10.4**	+	+	+	+	+
HPV-18	25	+	+	+	+	0	+

6.4 Discussion

6.4.0 Overview of Results

- Wide range of proliferative responses to both cutaneous and genital HPV types
- Cytokine responses generally follow proliferative responses in both magnitude an HPV type responses
- Consistently significant Th1 response
- Some Th2 responses in some donors but only consistent in one individual
- IFN-γ is dominant cytokine expressed

6.4.1 Proliferative Responses In Healthy Donors in Response to Cutaneous and Genital HPV Types L1 Peptides

Subject 1 is the individual in this study that responded to the greatest number of HPV peptides; p30, p186, p304 and p412. This subject mounted a CD45 RO^+ T cell response to all four of the HPV-11 peptides (Fig. 6.3). A substantial number peptides based upon the other genital types also provoked responses. All HPV-16 peptides, except p30, and all HPV-18 peptides, except p186, responded. The cutaneous types also induced responses but not from all peptides. There was a response from; p304 and p412 of HPV-2, p186, p304 & p412 of HPV-3 and p412 only from HPV-4. Subject 2, was found to respond to p243 of HPV-11, -6, -1, -3 and -16 (Fig. 6.4). Subject 3, displayed proliferation to; p139 and p304 of HPV-11, p139 of HPV-3, -4 and -16 and to p304 of HPV-16 (Fig. 6.4). Subject 4, who also responded to p304, displayed proliferation to HPV-16 and HPV-4 (Fig.6.4). Subjects 5 and 6 responded to Peptides 186 and 139 respectively and both mounted proliferative responses to HPV-11, -1, -2, -4, -16 and -18 (Fig 6.4).

6.4.2 Cytokine Production In Healthy Donors in Response to Cutaneous and Genital HPV Types L1 Peptides

6.3.2.1 Subject 1

p30

The dominant proliferative response of T-cells observed from Subject 1 to p30 is to HPV-11 where there is significant production of the Th1 cytokines IFN-γ and TNF-α (Table 6.9). IFN-γ production at a similar level to those seen to in response to the HPV-11 p30 peptide is also seen in response to the HPV-2 and -18 p30 peptides. These three peptides are those from which T-cell proliferation was observed,

additionally proliferation of a similar magnitude to that seen in response to HPV-11 was observed in response to HPV-1 p30 (Fig. 6.8), to which no significant cytokine response was mounted. The other Th1 cytokine examined IL-2 also is also produced but at the lower detectable limit of the assay (Table 6.8 & 6.9).

As discussed in 6.1.1 predominantly Th1 cytokine response is observed to HPV and it is the Th1 cellular mediation of HPV that is the predominant response in all of these individuals.

p186

The p186 peptides did not stimulate any significant cytokine production from the T-cells of Subject 1 despite a number of proliferative responses (Fig. 6.9). However the scale of these proliferative responses was significantly less overall than those seen in response to the other peptides and of approximately the same magnitude of the responses in the other peptides that also produced no significant cytokine responses.

p304

A number of strong cytokine responses were observed to the p304 peptides of three different HPV types, as with p30 there was significant IFN-γ and TNF-α production in response to the HPV-11 and HPV-2 peptides. Several strong responses from the Th1 cytokines; IFN-γ, TNF-α, IL-2 and the Th2 cytokine IL-5 were induced by p304. This is reflected by the proliferation data where the dominant response to the p304 peptide is to the HPV-16 peptide, with lesser but still high T-cell proliferation to the HPV-11 and HPV-2 peptides (Fig. 6.10). T-cell proliferation is also observed to the HPV-3 and HPV-18 peptides, however no significant cytokine production is observed in response to these two peptides.

p412

Again, high levels of IFN-γ and TNF-α are produced in response to stimulation by the p412 peptide of HPV-11 which is by far the dominant proliferative response of the p412 peptides. Again IL-2 displays a response to HPV-11 at the lower limit of the assay (Fig. 6.11).

6.3.2.2 Subject 2 (p243)

T-cells from Subject 2 displayed a predominantly Th1 cytokine response to the p243 peptides with high levels of IFN-γ and TNF-α production stimulated by the HPV-6, -1, -3, -16 peptides (Table 6.10). In response to the HPV-16 peptide, in addition to production of these cytokines there was also significant production of IL-2. These HPV peptides are also those which exhibited exceptionally elevated levels of T-cell proliferation (Fig. 6.12), there was also significant production of the Th2 cytokines; IL-5 and IL-10 to the HPV-6 and -3 peptides respectively.

6.3.2.3 Subject 3 (p139 & p304)

No significant cytokine production was observed from Subject 3 to either the p139 peptides (Table 6.11) or the 304 peptides (Table 6.12). With regard to the p304 peptides this reflects a dearth of T-cell proliferative responses to these peptides (Fig. 6.14) with the only proliferative T-cell response being to the HPV-16 peptide. There was however, a significant proliferative T-cell response to the p139, HPV-11, -2, -3, -4, peptides.

6.3.2.4 Subject 4 (p304)

Subject 4 interestingly displayed similarly low level Th1 and Th2 responses to the p304 peptides (Table 6.13). The Th-2 cytokine IL-4 is produced in significant levels in response to both the HPV-4 and HPV-11 peptides. The HPV-3, -16 and -18 peptides

all stimulate similar IFN-γ responses with the HPV-16 peptide also stimulating production of the Th2 cytokine IL-5. Only the HPV-16 and HPV-4 peptides stimulate significant T-cell proliferative responses, with the former by far the dominant response and latter only just over the level of significance (Fig. 6.15).

6.3.2.5 Subject 5 (p412)

In response to the p412 peptides the T-cells of Subject 5 produced a uniform pattern of cytokine responses (Table 6.14). Each of the HPV type peptides that elicited significant proliferative responses (Fig. 6.16); HPV-11, -1,-2, -4, -16 and -18 all stimulated the production of significant levels of IFN-γ, TNF-α and more intriguingly the Th-2 cytokine IL-5. IL-5 promotes the eosinophyl proliferation, B-cell differentiation and the synthesis of IgA. Subject 5 thus appears to exhibit a significant Th1 and Th2 response to a broad range of HPV types. This additional Th2 like humoral response may be the reason for the relatively reduced proliferative responses observed in this individual.

6.3.2.6 Subject 6 (p139)

Subject 6 displayed mainly low level IFN-γ production to the p139 peptides from the cutaneous HPV types HPV-1, -2, -4 and the genital type HPV-16. The HPV-2 peptide demonstrated the dominant proliferative response and also stimulated the highest IFN-γ production along with the production of TNF-α. The Th2 cytokine IL-4 was also produced in at a significant level in response only to the HPV-1 peptide but against an unusually high IL-4 negative control response.

6.4.3 Th1 Cytokine Production in Healthy Donors in Response to Cutaneous and Genital HPV Types L1 Peptides

Across the range of the most common UK cutaneous and genital HPV types examined, the production of cytokines in response to the HPV based peptides approximately follows the corresponding proliferative responses. In all responding subjects there is cytokine production in response to peptide from at least one cutaneous and one genital HPV type. In virtually all instances where a significant proliferative response was observed to a specific peptide then IFN-γ was produced, frequently coupled with TNF-α production. As reported by other studies into cytokine production in response to HPV (Passmore, 2002 #15;Scott, 1999 #12), a predominantly Th1 response was observed , in 4/5 of the subjects who exhibited significant cytokine production, with IFN-γ the dominantly expressed cytokine.

6.4.4 Th2 Cytokine Production in Healthy Donors in Response to Cutaneous and Genital HPV Types L1 Peptides

The production of Th2 cytokines was also observed but, apart from Subject 5, there was no consistent repeated pattern of Th2 cytokine production. Subject 5 displayed a significant Th1 and Th2 response against all but two of the HPV type peptides. This is a dissimilar response though to that observed in mild to medium severity RRP patients by Bonagura *et al* (Bonagura et al. 1999) . In the Bonagura study the Th1/Th2 response was from IFN-γ and IL-4 whereas in Subject 5 the balance is between IFN-γ and IL-5. However, although both IL-4 and IL-5 are Th2 cytokines that induce a humoral response, as described in Chapter 1 these two cytokines have very different specific effects very different. IL-4 promotes Th2 differentiation and stimulates T-cell growth. IL-6 acts as a T-cell activation factor and, in conjunction with IL2, stimulates the differentiation of mature and immature T-cells into cytotoxic T-cells. In addition the proliferation assay for Subject 5 (Fig. 6.16) shows that this balanced cytokine

production is against a high background response with relatively low peptide responses. It is therefore likely that this shift to a Th2 response is an *in vitro* artefact.

6.4.5 Potential HPV Type Cross Reactivity

Of the six control subjects examined only two possessed the HLA class II allelle DRB1*0301 and ancestral haplotype 8.1 these were Subjects 3 and 6. It was seen in Chapter 5 that both of these are significantly associated with RRP (Gelder, 2003 #4). In order to confirm the validity of these results in a control subject with AH 8.1, and as no significant cytokine from, proliferation experiments and CBA assays were repeated on Subject 6. These were carried out at intervals of eight months and one year after the initial experiments and produced very similar results to those seen here (P. Hillyer, Infection & Immunity UWCM, Personal Communication).

Subject 6 has previously exhibited a substantial cutaneous papilloma and by far the dominant proliferative and cytokine response in this subject is to the peptide of the cutaneous HPV type HPV-2 (Fig. 6.17 & Table 6.16). Therefore, it is possible that this papilloma was caused by HPV-2. Consequently, as discussed above, this cutaneous type may have primed the subject's immune system to HPV, and thus the lower level HPV responses seen to other cutaneous (HPV-1,& -4) and genital types (HPV-11, -16 & -18) may be due to HPV cross reactivity. However, it is also possible that this individual may have come into contact with the other responsive HPV types, so this is not conclusive.

6.5 Concluding Remarks

As expected a largely Th1 response was observed with IFN-γ the predominant cytokine expressed and cytokine production reflected PBMC proliferation. One subject also produced a consistent combination of Th1 and Th2 responses. Thus cross reactive $CD4^+$ T-cell responses induced by previous exposure to cutaneous HPV types may prime the immune response which has been demonstrated here to dominated by IFN-γ. This therefore raises the interesting possibility that previous exposure to cutaneous HPV might offer some protection against genital HPV infection. The role of host immunogenetics factors in HPV induced cytokine production is discussed in Chapter 7.

Chapter Seven:

General Discussion

Contents

7.3 Comparison of Findings in RRP & Cervical Cancer

7.0 Host-Virus Interactions in HPV Mediated Disease

As we have seen in Chapter 1 and throughout this thesis the diseases mediated by HPV are widespread and varied, ranging from cutaneous warts to cervical cancer. However, the majority of HPV infections are transient in nature and cleared by the immune system (Hildesheim, Schiffman et al. 1994), however some infections persist and go on to manifest as disease. There appears to be a balance of interactions between the virus and the host keeping the HPV in check and halting the progression to disease. When this balance is upset, or host mechanisms that contribute to this process are inhibited or missing altogether then this can lead to serious, possibly life threatening disease. This thesis has investigated such host and viral contributions to both normal clearance and to the disease states. Specifically it has examined two diseases mediated by HPV.

Cervical cancer is a common disease with a high mortality rate that has been lowered in Western industrialised nations due to an understanding of HPV distribution and cervical screening. A key question is whether the same findings, such as HPV prevalence and type distribution apply to ethnically different populations where prophylactic vaccines against the HPV virus itself have the potential to lower the cervical cancer mortality rate.

RRP is a rare disease mediated by low risk RRP but is life threatening if untreated as multiple papillomata may occlude the airway. A key question in the study of this disease is whether some of the findings that have helped reduce death from cervical cancer such as HPV type distribution and relationship with host immunogenetics can be applied to this disease.

7.1 Host-Virus Interactions in Low Risk HPV Mediated Disease

7.1.0 Overview

As discussed throughout this thesis, most individuals are thought to be infected with a cutaneous HPV type in the course of their lives. Genital HPV-6 & -11 infection is also common with genital warts being observed in around 1% of the sexually active population (Koutsky 1997; Maw 2004). Oral HPV-6 & -11 infection also appears to be common with rates of 20% reported in one study (Miller and Johnstone 2001), yet RRP remains a rare disease. Furthermore, not only are there differences between RRP and gential warts in disease frequency, there are differences in the disease course, in particular 1 % of RRP patients develop squamous carcinomas (Reidy, Dedo et al. 2004), while malignant transformation is exceptionally rare in genital warts (Personal Communication: Dr Humphrey Birley, Genitourinary Consultant, Genitourinary Medicine, Cardiff Royal Infirmary). Indeed one of the patients in this study (patient RRP-001) died of squamous cell lung cancer at the age of 22. These differences between two diseases, caused by the same HPV types, could conceivably be related to host or viral factors.

Our group has previously found a significant association between RRP and the HLA class II allele DRB1*0301 which has been extended in this thesis (Gelder, Williams et al. 2003). However, this is only present in around 50% of RRP patients and therefore cannot be the basis of progression to disease RRP alone. Viral factors cannot on their own be the cause of RRP, as no reported case of the disease has been reported in patients immunosuppressed by HIV infection, unlike cervical cancer which, as discussed in Chapter 3, is increased in HIV infected individuals (Feingold, Vermund et al. 1990; Lefevre, Hankins et al. 2004, Baay, 2004; Moscicki, Ellenberg et al. 2004;

Moscicki, Ellenberg et al. 2004), along with the many other rare opportunist diseases. Thus RRP is an ideal disease for the study of HPV mediated disease as host-virus interactions appear to be critical to disease progression.

7.1.1 Viral Factors in RRP

7.1.1.1 RRP Association with Viral Type

In Chapter 4 it was confirmed that both HPV-6 & -11 are found in the laryngeal papillomata of RRP patients with a 60:40 split in favour of HPV-6, no HPV types other than these two types were found. Two other studies have found laryngeal papillomata infected with HPV-16, -31, -33, -35, & –39, these are isolated findings and in ethnically different populations (Mexico and Hong Kong) to that studied here (Dickens, Srivastava et al. 1991; Penaloza-Plascencia, Montoya-Fuentes et al. 2000). Other more recent studies agree with the findings observed here of only HPV-6 & -11 being associated with RRP (Obchinnikov Iu, Kiselev et al. 2004; Wiatrak, Wiatrak et al. 2004). Potential co-factor associations between RRP and herpes viruses reported by another other study (Pou, Rimell et al. 1995) were also examined with no significant association.

7.1.1.2 RRP Disease Associations with Viral Type

It was also shown in Chapter 4 that HPV-11 displayed a much stronger association (89%) with aggressive RRP than HPV-6 (65%). This is in line with associations in other studies (Rabah, Lancaster et al. 2001; Obchinnikov Iu et al. 2004; Wiatrak et al. 2004) but the first time such a definitive association has been found. It was also seen that whilst there was a roughly even split between HPV-6 & -11 with the JORRP subset there was an 80:20 split in favour of HPV-11 in the AORRP subset. However this latter finding should be treated with caution as much fewer AORRP samples were typed compared to JORRP samples. The reason for this lower number of AORRP biopsies being available is seen in Table 7.7 where it is seen that JORRP causes more aggressive disease, and as a direct consequence fewer AORRP biopsies are available.

7.1.1.3 Sequencing of L1 Gene

Our group had previously found an immunodominant epitope within the L1 outer capsid protein in RRP patients with the DRB1*0301 allele (Gelder et al. 2003). There is thus potential for evasion of immune surveillance if there are mutations within this epitope. This gene was sequenced in RRP patients to examine if such mutations exist. Some polymorphisms and silent point mutations were found within L1 but no consistent polymorphisms were found. However one of the silent point mutations, located in an anchoring residue of the DRB1*0301 epitope is a switched to a codon rarely used in humans and as a consequence the concomitant tRNA is likely to be less available within the host cell. This may result in a lower level of transcription of HPV L1 and consequently less chance of the virus being detected by the immune system, possibly explaining the lack of immune response to L1 in RRP patients. However as this silent point mutation is seen in both genital warts and RRP it may represent a "UK variant" and have no influence over disease aggression.

7.1.1.4 Sequencing of the E6 & E7 Oncogenes

As mentioned above RRP papillomata may undergo malignant transformation mediated, as described in Chapter 1, by the E6 and E7 oncoproteins. It was thus important to sequence the ORF of these proteins in laryngeal papilliomata and genital warts. The latter had to be studied because the HPV 11 reference strain in Genbank was obtained from an individual with RRP, thus to find differences between RRP and genital wart HPV-11 clades we had first to establish the "normal" HPV-11 sequence in genital warts. In addition detailed sequencing of genital wart material allowed us to differentiate between UK HPV-6 sub type genetic variation due to "geographical variation" from those "specific" for RRP. The most interesting genetic variation found in these genes was a polymorphism at amino acid 19 of the E6 gene. This was found in all RRP samples, regardless of HPV type but in only 59% of all genital wart samples.

As this polymorphism also determines a putative DRB1*0301 epitope then it is possible that RRP can only progress if this epitope is present. However no proliferative T-cell response to stimulation with a peptide (containing the polymorphism seen in RRP) representing this region of E6 has been observed in either RRP patients or healthy controls (P. Hillyer, S. Wall, & C.M.Gelder - Unpublished observations) Although such an experiment with a peptide containing the alternate polymorphism has yet to be performed.

However, an alternative explanation for the role of this polymorphism in RRP pathogenesis is also possible as the polymorphism at amino acid 19 of the E6 gene present in all RRP papillomata causes a switch to a codon / tRNA rarely used in humans. This is potential mechanism for immune evasion by limiting the expression of E6. This reduced frequency provides a potential mechanism for latency of infection in RRP and may well be a fundamental mechanism by which HPV avoids immune surveillance.

Overall, when compared to the L1 capsid protein, there is a much greater rate of both polymorphisms and silent point mutations in the E6 and E7 oncoprotein which when combined are still only half the length of L1. So this high rate of mutations in RRP HPV in compared with to the (non-RRP 'normal') genital wart is particularly significant. As described above RRP papillomata have the potential for malignant transformation whilst this has never been reported in genital warts. The relatively great variation in the oncoproteins of HPV in these two diseases may help to explain this difference in oncogenic potential between the two diseases. However these viral differences do not explain why this disease is so rare, so host interactions were examined further.

7.1.2 Host Factors in RRP

7.1.2.1 HLA Associations with RRP

In Chapter 5 the significant positive association between RRP and HLA class II allele DRB*03 and the AH 8.1 as well certain of its other constituent alleles seen in the original study (Gelder et al. 2003) were confirmed in this second population. A significant negative association between RRP and HLA class II allele DQB1*03 also observed in the original study was also maintained in this study. The positive association with the HLA class II allele DQB1*06 observed in this study had not been seen in the original study. Indeed DQB1*06 has been negatively associated with aggressive RRP in a small subset of caucasoid patients by another group (Bonagura, Vambutas et al. 2004). The previous study by this group of RRP was limited by a low number of AORRP patients. When combined with results from this study we now have sufficient numbers to examine the significance these HLA alleles within of epidemiological subsets, such as disease onset or aggression. Within the pooled cohorts the DRB1*03 displays significant positive association with RRP (Fishers exact test 2 Sided P Value= < 0.0001, OR= 3.0, 95% CI= 1.9 to 4.5). Such a positive significant association is also seen with the AH 8.1 overall (Fishers exact test 2 Sided P Value= < 0.0001, OR= 3.0, 95% CI= 1.8 to 5.0). It is observed that both of these positive associations have odds ratios showing that they are 3 times more likely to occur than not occur in RRP. A negative association with the DQB1*03 allele was also observed (2 Sided P Value= 0.0002).

7.1.2.2 HLA Associations with RRP Epidemiological Subsets

When examining the relationship between the RRP subsets and the HLA alleles most closely associated with RRP an appreciable difference between the epidemiological spread of the DRB1*03 and the DQB1*03 alleles is noted (Table 7.2). With the DRB1*03 link AH 8.1 as a comparison it can be seen that whilst both class

two alleles are similarly associated with AORRP only DRB1*03 is associated with JORRP. In terms of gender both DRB1*03 and DQB1*03 are similarly associated with female patients whilst the latter shows no significant association with male patients. Most significantly there is an even greater differentiation between these two alleles when disease state is examined. DRB1*03 being associated with aggressive disease whereas is DQB1*03 associated with non-aggressive disease (Table 7.1).

Table 7.1: Disease Onset HLA Associations

Association	Onset		Disease State		Gender	
	JORRP	AORRP	Agg.	Non-Agg.	Male	Female
DRB1*03	++++	+++	++++	-	+++	++++
DQB1*03	-	+++	-	++	-	++++
AH 8.1	+++	+++	++++	-	+	++++

(2 Sided P Value) -: Not Signifcant, +: <0.05, ++: < 0.01, +++: < 0.001, +++: < 0.001

As discussed above, JORRP patients are significantly more likely to display aggressive disease than AORRP patients. The gender association between these alleles can be seen in Table 7.2. The general the general trend in this study is of a greater association of female RRP patients with aggressive disease. Thus the HLA linkage with disease state demonstrated above can be seen to be a differential between DRB1*03 and DQB1*03. Patients possessing the DRB1*03 allele appear to be more susceptible to aggressive disease whilst the DQB1*03 allele appears to have a protective effect. The DRB1*03 allele appears to be the dominant as of patients with both alleles, 9 (82%) displayed aggressive disease and 2 (18%) displayed non-aggressive disease. This is supported by the observation that all RRP patients in this study homozygous for DRB1*03 have aggressive disease, whilst 4 out of 5 patients homozygous for DQB1*03 display non-aggressive disease. In addition, none of the patients homozygous for DRB1*03 were positive for DQB1*03 and vice versa. This

raises the possibility that aggressive and non-aggressive RRP may be two separate disease response mechanisms modulated in part by these two alleles.

Table 7.2: Correlation of RRP Onset and Disease State

Disease State	Male		Female	
Aggressive	19	49%	26	74%
Non-Aggressive	20	51%	9	26%

Fisher's Exact Test 2 Sided P value= 0.0326, OR= 2.32, 95% CI= 0.12 to 0.88

7.1.3 Host Immunogenetics Vs Viral Type in RRP

When the DRB1*03 and DQB1*03 alleles are correlated with HPV type it is observed that there are no significant association observed (Tables 7.3 & 7.4). There is marginally greater number of patients infected with HPV-11 that are DRB1*03 positive, however this difference is marginal and not significant.

Table 7.3: Correlation of HPV-11 with Host Immunogenetics

	HPV-11			
Allele	Positive		Negative	
DRB1*03	12	60%	8	40%
DQB1*03	10	50%	10	50%

Fisher's Exact Test 2 Sided P value= 0.7512, OR= 1.500, 95% CI= 0.43 to 5.25

Table 7.4: Correlation of HPV-6 with Host Immunogenetics

	HPV-11			
Allele	**Positive**		**Negative**	
DRB1*03	13	43%	17	57 %
DQB1*03	13	43%	17	57 %

Fisher's Exact Test 2 Sided P value= 1.2052, OR= 1.000, 95% CI= 0.36 to 2.78

7.1.4 Host Virus Interactions in the Proposed Genital-Laryngeal Transfer of HPV

It was seen in Chapter 4 that the laryngeal biopsies displayed a third more HPV-11 positive samples than found in the genital wart samples. As HPV in the respiratory tract is, almost certainly, contracted from the genital tract it might therefore be assume that the same distribution of HPV-6 and -11 would be present in both. However within the AORRP cohort there is a very similar HPV-6: HPV-11 ratio to that is seen in genital warts (76:24). It is the JORRP cohort, with its almost even 54:46 split, which inflates the prevalence of HPV-11 in the overall RRP data set. The obvious difference between the two forms of the disease is the proposed manner of infection. AORRP is believed to be contracted from males and females whilst as JORRP is only believed to be passed vertically from mother to infant it is therefore only contracted from females (if we discount infant/child abuse as a disease mechanism). It could therefore be postulated that the higher levels of HPV-11 be gender based as similarly high levels are observed when adult females are compared to adult males. However not one HPV-11 positive female adult was seen in the study. Therefore there must be some other factor(s) in JORRP leads to higher levels of HPV-11 than in AORRP and genital warts. It also suggests that AORRP and JORRP may not just be two distributions of the same disease but two separate diseases.

Also in Chapter 4 it was demonstrated that HPV-11 is more common in JORRP and

that HPV-11 appears to be more likely to cause aggressive disease than HPV-6. In addition, the older the individual at the point at which HPV-6 or -11 are orally contracted the more likely the individual to mount a cross reactive immune response to the virus. Therefore it may be that the cross-reactivity between HPV types demonstrated both in Chapter 6 and previously by our group, and the consequent immune response that is the primary influence upon disease progression.

As has been found in previous studies (Lindeberg and Elbrond 1989; Gabbott, Cossart et al. 1997), an almost 1:1 male:female distribution is observed in the JORRP patients (Table 7.6). However, this ratio in AORRP was 3:1 in favour of males which is in line with another study (Doyle, Gianoli et al. 1994). This may be due to the fact that male genital warts are more visible, and their presence decreases the likelihood of oral-genital intercourse from females to males reducing the rate of AORRP in females.

Table 7.6: RRP Onset Correlated with Patient Gender

Gender	AORRP		JORRP	
Male	31	74%	30	46%
Female	11	26%	35	54%

Fisher's Exact Test 2 Sided P value= 0.0054, OR= 0.3041, 95% CI= 0.1309 to 0.7069

As discussed above disease state distributions between JORRP and AORRP subsets mirror each other (Table 7.7). In JORRP there are three times as many individuals displaying aggressive disease as there are non-aggressive, whilst in AORRP the reverse is true. If we consider that as HPV cannot cross the placental barrier, the immature immune system of JORRP patients will not have encountered HPV before it is contracted in the birth canal. Thus in terms of HPV, prenatally RRP patients of individuals are immunologically naïve until birth as confimed by studies showing low rates of JORRP in those born by caesarean section (Shah, Stern et al.

1998). AORRP patients however, are likely to have encountered at least one HPV at some point in their life. We have shown in Chapter 6 and previously (Williams, Hart et al. 2002) that there is cross-reactivity between HPV types. Therefore the older the patient at age of onset the more likely they are to have encountered HPV and, consequently, increase the possibility that their immune system is able to mount a defence against the virus

Table 7.7: Correlation of RRP Onset and Disease State

Disease State	AORRP		JORRP	
Aggressive	7	30%	38	75%
Non-Aggressive	16	70%	13	25%

7.1.5 Host Cytokine Production Correlated with HLA Type

As discussed above of particular interest are the responses from subjects who possessed the DRB1*03 or the DQB1*03 alleles seen to be associated with aggressive and non-aggressive RRP respectively. It has been previously shown by our group that IFN-γ is the dominant cytokine produced in RRP patients possessing the DRB1*0301 allele (Gelder et al. 2003). Only two of the test subjects possessed DRB1*0301; Subject 3 and Subject 6. Bonagura *et al* have also examined cytokine mRNA in RRP patient T-cells stimulated with HPV-11 E6 and correlated this to patient HLA class II alleles (Bonagura et al. 2004). They found raised IFN-γ production in RRP patients to be associated with DRB1*01, *02 & *06 and DQB1*01, whilst DRB1*03, *04, *05 & *07 and DQB1*02 & *03 alleles were associated with lowered IFN-γ production. Table 7.8 shows the DRB1 and DQB1 types of the control subjects examined in our study correlated with the magnitude of IFN-γ production.

Table 7.8: Correlation of DRB1 & DQB1 alleles and Magnitude of IFN-γ Production

Subject	HLA Class II Alleles				Magnitude of IFN-γ Response
	DRB1		DQB1		
1	*07	*15	*02	*05	+
2	*12	*13	*03		+++
3	*03	*15	*02	*06	-
4	*01	*15	*05	*06	+
5	*04		*03		++++
6	*01	*03	*02	*05	+

Key for Table 7.8: Mean IFN-γ Production Above Control Response

Range (pg/ml)	0	1-15	16-30	31-45	46-60
	-	+	++	+++	++++

It is observed that neither of the two subjects possessing the DRB1*0301 expressed high levels of IFN-γ. Indeed one of the DRB1*0301 individuals was the only subject where no significant cytokine production was observed at all. However, Subjects 2 & 5 display by far the greatest magnitude of IFN-γ response (Table 7.8). These are the only two subjects who possess the DQB1*0301 allele, moreover they are both homozygous for this allele. Thus in this healthy population of HPV responders a clear correlation is apparent between DQB*03 and increased IFN-γ production. It has been shown here that IFN-γ production is higher in individuals with the DQB1*03 allele stimulating a Th1 immune response against HPV which is protective in both healthy individuals and RRP patients. As discussed above not only does a significant negative association exists between DQB1*03 and RRP but this allele when present in RRP patients is significantly associated with non-aggressive disease.

.7.1.6 Host-Virus Interactions in RRP - Conclusions

This study has confirmed the association our group has previously found between aggressive RRP and the HLA class II allele DRB1*03 (Gelder et al. 2003) within a significantly large and balanced population of patients. Additionally, it has been demonstrated here that the DQB1*03 allele appears to implicated in the modulation of immune response to RRP. It is significantly and negatively associated with RRP and in patients with RRP it is significantly associated with non-aggressive disease. It has also been demonstrated, in experiments examining the cytokine responses by CBA (as used in Chapter 6) in RRP patients with the DQB1*03 allele also displayed raised IFN-γ production as observed here in non-RRP HPV infected individuals (Personal Communication - Dr P Hillyer, Infection & Immunity, UWCM).

Thus the DQB1*03 allele appears to offer protection to RRP due to increased levels of IFN-γ production against HPV in both RRP patients and individuals with HPV infection but no RRP. IFN-α has long been used in the treatment of RRP leading to clearance of disease, at least temporarily, in around 50% of patients and is the most commonly used pharmaceutical supplement to surgery in US patients (Kashima, Leventhal et al. 1988; Kimberlin 2004). As an interferon response is effective against RRP or at the very least reducing the aggression of the disease a vaccine which elicits a Th1 response is more likely to induce disease remission than a vaccine designed to elicit an antibody response against HPV surface coat proteins such as the L1 VLP which have recently been employed successfully in prophylactic trials against cervical metaplasia (Harper, Franco et al. 2004). One option would be to use a vaccine containing modified HPV-11 E6 and or E7 genes (modified to inactivate these proteins) in a vaccina vector. Indeed the modified vaccinia virus Ankara (MVA), the present 'gold standard' vaccinia vector has been successfully shown to invoke a Th1 like immune response in animal models (Kovarik, Gaillard et al. 2001; Weidinger, Ohlmann et al. 2001; Ober, Bruhl et al. 2002).

7.1.7 Further Investigation of Host-Virus Interactions in RRP

Further studies will involve investigation into the mechanisms by which DQB1*03 appears to be a protective allele and the related IFN-γ response. For instance, a recent study (Lai, Chang et al. 2005) has found an association between a polymorphism of the IFN-γ gene and individual susceptibility to cervical carcinogenesis. Further study may include investigating this polymorphism in RRP patients as possible mediator of RRP aggression or marker for progression to malignancy. A further examination will be made of the effect of the polymorphisms observed here upon the E6 and E7 oncoproteins and their interactions with p53, Rb and AP-1 in vitro.

7.2 Host HPV Interactions in High Risk HPV Mediated Disease in a Rural Non-Caucasoid Population

7.2.1 Implications of Gambian Study on Vaccine Development

HPV-16 is the HPV type most predominantly associated with cervical cancer worldwide and as such it is been the almost exclusive target of prophylactic HPV vaccine research. However, as discussed in Chapter 3, on a local basis, other high risk HPV types can have a greater association (Bosch, Manos et al. 1995; Herrero, Hildesheim et al. 2000; Castellsague, Menendez et al. 2001; Mayaud, Gill et al. 2001; Xi, Toure et al. 2003). The primary HPV protein targets in vaccine research are the E6, E7 oncoproteins and the L1 outer capsid protein (Koutsky, Ault et al. 2002, Peng, 2004; Wlazlo, 2004; Zhou, 2004). A comparison between these three genes in the reference HPV-16 (16R) and the variant of African type 1 (Af1) observed in this study is seen in Table 7.9. It can be seen that in these three proteins there is little overall difference between the reference strain variant of HPV-16, upon which vaccine research is based, and the variant of the HPV-16 Af1 found exclusively amongst HPV-16 in this study.

When taking into account that HPV-16 is the most common type it would seem that a vaccine based upon the reference Genbank sequence (HPV-16R) may well be effective in the Gambia.

The E7 gene, as we shall see, may be a better choice as the candidate upon which to base an effective, potentially cross-reactive vaccine. Not only are the reference and Af1 variant homologous on the E7 protein level (Table 7.9) but HPV-16 E7 is highly conserved and rarely are DNA changes seen that lead to amino acid changes (Icenogle, Sathya et al. 1991).

Table 7.9: Divergence of E6, E7 and L1 Between HPV-16R and HPV-16 Af1 DNA

Level	% DNA Divergence			
	E6	E7	L1	Genome
DNA	2.6	0.7	1.3	1.5
Protein	2.0	0.0	1.1	-

Peptides based upon the E7 oncoprotein of HPV-16 have been found *in vivo* to elicit a significant cellular response to HPV correlating to regression of CIN in a study where 36% of the participants were of African-American extraction (Kadish, Timmins et al. 2002). A more recent study in a murine model showed priming with the DNA vaccines to E7 reduced the efficacy of a viral vector expressing an identical antigen (Wlazlo, Deng et al. 2004). Another approach is the use of the TA-HPV vaccine, a recombinant vaccinia virus which expresses the HPV-16 and -18 E6 and E7 proteins (Borysiewicz, Fiander et al. 1996). This Vaccine, which induces a HLA class I restricted specific response, has been proven safe in in-vivo trials. (Kaufmann, 2002).

Of the common oncogenic types found in this study six of the seven most prevalent are from HPV group A, this group includes HPV-16, -35, - 33, -58, -31, -52 and -67

(Fig. 7.1). These are also relatively homologous to each other within the E7 protein (Fig.7.2). All HPV types of Group A were found in this study and this group makes up 70% of all high oncogenic risk HPV types and 62% of all HPV infections found in this study. It is therefore possible that a vaccine targeting HPV-16 may well generate a cross reactive response to other members of the group especially HPV-35 which is not only the most closely related HPV type to HPV-16 (Fig 7.1) but the second most dominant HPV type in the Gambian study. These types also constitute all but 2 of the types that are found in the HGSIL lesions that we have histology data on, the other 2 types are HPV-18 and HPV-45 which both belong to HPV group C.

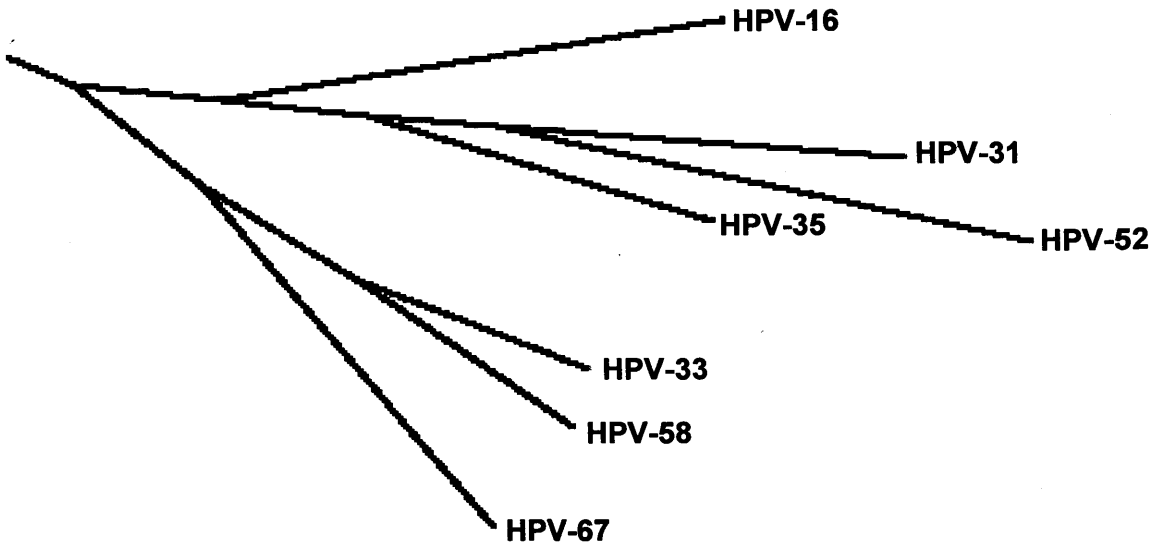

Figure 7.1: Genomic Phylogenetic Tree of HPV Group A

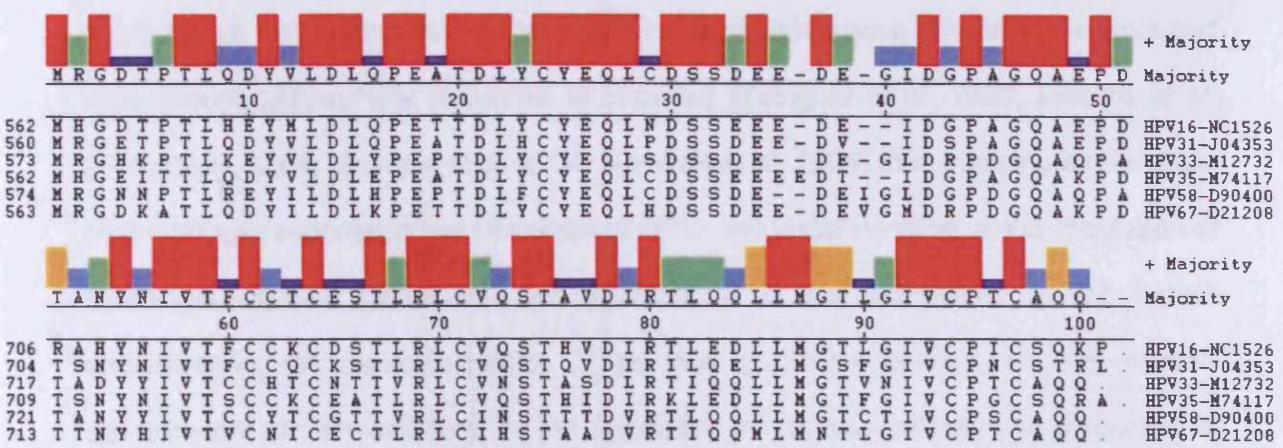

Figure 7.2: Alignments of HPV Group A E7 Proteins

In a study where VLPs based upon HPV-6, -11, -18, -31, -33, -35, -39, and -45 were used to study potential cross-reactive ability to prevent HPV-16 infection of cultured cells in immortalised human keratinocytes, inhibition was only observed by HPV-33 VLP (White, Wilson et al. 1998). However the HPV VLP only consists of the L1 outer capsid protein so it is still possible that the E6 & E7 oncoproteins may elicit cross reactivity. Around 20-40% of HPV-16 positive patients with cervical cancer produce no antibodies to the E6 and E7 oncoproteins (Nindl, Zumbach et al. 2000). Nindl and co-workers have found that this lack of response is independent of HPV-16 variant, having tested a number of common variants including African Type 2 (Nindl et al. 2000). Thus, as seven out of the eight most prevalent types may cross react with HPV-16 E7 and the other most prevalent type is HPV-18 also expressed by the TA-HPV vaccine, a suitable vaccine strategy for The Gambia would be the use of the TA-HPV vaccine.

7.2.2 HLA Interactions with HPV in Cervical Cancer and Its Implications for The Gambia

As discussed in Chapter 1, most HPV infections are transient in nature with around 90% of cervical changes being cleared by the immune system within 1-3 years (Moscicki, Palefsky et al. 1993; Ostor 1993; Parkin, Pisani and Ferlay 1993, Chua,

1996; Bosch 1995; Burd 2003). However in individuals where HIV/AIDS has induced immunosuppression HPV clearance is impeded (Feingold et al. 1990; Lefevre et al. 2004, Baay, 2004; Moscicki et al. 2004; Moscicki et al. 2004) thus implying a role of the host immune response in the clearance of HPV. An understanding of the regulation of host–virus immune interactions are therefore of crucial importance in the further understanding of cervical cancer. This has particular bearing on the rational development of HPV vaccines for the prevention of cervical cancer. As discussed in Chapter 1 numerous studies have found a strong linkage between CIN and the HLA class II allele DQB1*03. This was originally found in a US study (Wank and Thomssen 1991) but has also been found in a diverse range of populations and ethnicities in studies in; the UK (David, Taylor et al. 1992; Mehal, Lo et al. 1994; Odunsi, Terry et al. 1996; Cuzick, Terry et al. 2000), Norway (Helland, Borresen et al. 1992; Helland, Borresen et al. 1994), Spain (Montoya, Saiz et al. 1998), Sweden (Sanjeevi, Hjelmstrom et al. 1996), Japan (Nawa, Nishiyama et al. 1995), Honduras (Ferrera, Olivo et al. 1999), as well as US studies of African-American (Gregoire, Lawrence et al. 1994) and Hispanic (Apple, Erlich et al. 1994) populations. In all of these studies where the DRB1*1301-5 alleles were examined it was found to confer a protective effect against cervical cancer.

A study from Senegal (Lin, Koutsky et al. 2001) found that, although DQB1*03 was more common in cervical cancer cases than in controls, no significant association was observed. As Senegal, is the only nation with which The Gambia shares a border and most of its ethnic groups it is likely that this is the case in The Gambia. A study of class II alleles in the Gambia has found the DRB1*1304 allele to be particularly enriched in the study population (Hill, Allsopp et al. 1992). This study compared the HLA class II diversity of The Gambia with that of Malawi in South Eastern Africa and found the frequency of DRB1*1304 to be more than 9 times higher in The Gambia than in

Malawi. The same group has also found a correlation between the DRB1*1302 allele and hepatitis B virus (HBV) clearance as well as a protective effect against severe malaria (Hill, Allsopp et al. 1991; Thursz, Kwiatkowski et al. 1995). Intriguingly, Malawi is a nation that almost dissects Mozambique and shares a border with Tanzania. Studies in these nations have shown HPV prevalence rates more than double those found in this study (Mayaud et al. 2001, Mayaud, 2003, Castellsague, 2001).

7.2.3 HPV in Cervical Cancer in The Gambia - Conclusions

When the Gambian HPV prevalence study was devised, HPV immune responses were believed to be type specific. Therefore it was considered essential to check for variation amongst types associated with HGSIL in the Gambian population. The sequencing of HPV-16 in this study showed no protein variation from the reference strain upon which vaccines will be based. However HPV-16 is only associated with around 20% of HGSIL in rural Gambia. More recent work by our group (Williams et al. 2002), as discussed in detail in Chapter 6, has demonstrated a cross reactive response to HPV by $CD4^+$ T-Cells. As described in Chapter 3, HIV is a co-factor in the development of cervical cancer and the main effect of HIV on the immune system is $CD4^+$ depletion. Therefore $CD4^+$ T-cells are likely to play a role in the immune response against HPV in cervical cancer.

7.2.4 Further Work on Cervical Cancer in The Gambia

It is possible that the lower HPV prevalence figure found in this study compared to South East African studies is due to the combined protective effect of the DRB1*1304 allele and possible low frequency of the DQB1*03 allele. Further work on this study should therefore include an urgent study of the HLA types of the Gambian population studied and a correlation with cytology and HPV status. Additional further study in the Gambia should involve more in depth sequencing of the other types associated with

HGSIL especially those of HPV Group A to examine the potential for cross reactivity. This should include sequencing of the E6 and E7 oncogenes which, as shown may be better candidates for basing vaccines upon. Further work should also include HLA typing as discussed and an examination of $CD4^+$ T-cells responses to peptides based upon Gambian HPV variants to examine the potential for cross reactive vaccines.

7.3 Comparison of Findings in RRP & Cervical Cancer

It has been demonstrated here that both RRP and cervical cancer are complex diseases where multiple host-factor interactions are involved in disease pathogenesis. Factors such as HPV type and the specific viral and host genetics are fundamental determinants in the modulation of such host-virus interactions. One such interaction, and a key finding of this thesis, is the relationship between RRP and DQB1*03 and it's apparent modulation of IFN-γ as a protective response against HPV. As described above this is in sharp contrast to the associations made by other studies with DQB1*03 and cervical cancer where this allele is conversely a risk factor in progression to cervical cancer. These differing relationships with the same allele in diseases mediated by different types of the same virus underlines the importance of host-virus interactions in HPV mediated disease.

To conclude, I believe in this thesis I have contributed towards the better understanding of both of RRP and cervical cancer, by increasing the knowledge available about these devastating diseases.

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