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Analysis of the role of human papillomaviruses and the p53/Rb tumour suppressor genes in the development of cutaneous squamous cell carcinoma

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Thesis submitted to the University of Dublin (Trinity College) for the degree of Doctor of Philosophy

Research was carried out in the Department of Pathology, Royal College of Surgeons in Ireland, under the supervision of Dr. Mohamed JEMF Mabruk

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March 2000

DECLARATION

I hereby certify that this thesis, submitted to the University of Dublin (Trinity College) for examination for the degree of Doctor of Philosophy, has not been submitted as an exercise for a degree at any other university. I also certify that all of the work recorded herein is my own.

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Darran Patrick O'Connor

March 2000

For my Mam and Dad

To know is to know,

Not to know is to "not to know",

That in itself is knowledge too.

Confucius, 551-478 B.C.

ACKNOWLEDGEMENTS

Firstly I would like to say a special "thank you" to my supervisor Dr Mohamed Mabruk for his constant support, encouragement, enthusiasm and great ideas. Without Mohamed I wouldn't be finished now. He arrived at a crucial point in this work and I cannot overstate the difference he made to the eventual outcome. So, once again, Thank you, not least for having to wade through the drafts of this thesis.

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Finally I'd like to thank all of my friends for putting up with me, my Mam and Dad and my sisters Clare and Emma for all of their love and support and last but not least Vicki for keeping me sane (matter of opinion), making me happy and helping me get through this.

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Chapter3: Immunohistochemical and molecular genetic analysis of p53 in skin lesions from renal transplant recipients and immunocompetent skin cancer patients

SUMMARY

Nonmelanoma skin cancers (NMSC) are the most frequent cancers in Caucasian populations. Renal transplant recipients (RTR) are at an increased risk of neoplasia, particularly skin cancer. Studies on anogenital cancer have highlighted a role for oncogenic human papillomaviruses (HPV) in tumourigenesis, however while HPV infection is necessary for the development of anogenital cancers, it is not sufficient and other factors must also be involved.

Though HPV are the most common viruses that infect human skin, they have until recently been largely overlooked as possible aetiological agents of NMSC. This study describes an analysis of the prevalence of various HPV types in benign and malignant skin lesions from RTR and immunocompetent skin cancer patients (ICP) in an attempt to identify a possible "high-risk" group of viruses that could be involved in the genesis of these cancers. The prevalence of these same HPV types in normal skin from the general population was also examined. Two polymerase chain reaction (PCR)-based strategies were employed to detect HPV types in viral warts, squamous cell carcinomas (SCCs) and normal skin from RTR and ICP and in normal skin from the general population. Viral typing was achieved by sequencing amplified PCR products and alignment with known HPV sequences deposited in the Genbank database.

The results of this study show that EV-associated HPV types predominate in SCCs from both renal transplant recipients and immunocompetent skin cancer patients. The viral warts analysed were found to also harbour EV-associated HPV types in addition to common cutaneous HPV types. The normal human skin analysed from both patient groups and individuals without skin cancer were also found to harbour EV-associated HPV types, albeit at a much lower frequency. Over all, 100% (19/19) of the

viral warts, 86% (18/21) of the SCCs, 22% (2/9) of the skin cancer patient normal skin and 15% (3/20) of the normal skin from individuals without skin cancer were deemed to be HPV-positive. A pilot study of HPV E6 gene expression in these lesions indicated that E6 is expressed in malignant tissue but not in benign lesions.

The oncogenic mucosal HPV types HPV16 and HPV18 encode proteins that interact with the p53 tumour suppressor protein and result in functional inactivation of the p53 pathway. It is not known whether the HPV types prevalent in skin lesions affect the p53 pathway in a similar manner. This study also describes an immunohistochemical evaluation of p53 expression in benign and malignant skin lesions from RTR and ICP. The effect of p53 gene mutations on the expression patterns observed was analysed by screening the p53 gene in cutaneous SCCs for mutations by single strand conformational polymorphism analysis followed by direct cycle-sequencing of products with altered band migration. The downstream effects of p53 expression on the expression of the Mdm2 oncoprotein and the p21 cyclin-dependent kinase inhibitor were also examined immunohistochemically in p53 positive SCCs.

The results of this study show that p53 expression in benign and malignant lesions differs markedly. In the viral warts examined, only 40% (6/15) demonstrated p53 expression, at low levels and in a small number of cells. Whereas in the SCCs analysed, 60% (9/15) demonstrated high levels of p53 expression and in a large number of cells. Two patterns of p53 expression were observed in the SCCs, diffuse expression throughout the invasive component of the tumour, or basal layer expression at the proliferating edge. The accumulated p53 appears to be wild type as only one of the SCCs was found to have a mutation by SSCP analysis. The accumulated p53 also appears not to elicit a normal p53 response as the downstream genes mdm2 and p21 were not expressed in p53-positive lesions.

The oncogenic mucosal HPV types HPV16 and HPV18 also encode a second oncoprotein that interacts with the Rb tumour suppressor protein and results in deregulation of cell-cycle control by Rb. There is no evidence that the HPV types found in cutaneous cancers inactivate Rb in the same manner. This study also describes an examination of the integrity of the *Rb* gene in cutaneous SCCs from RTR in an attempt to determine if another means of Rb inactivation might occur. Loss of heterozygosity (LOH) at the D13S153 locus (located in exon 2 of the *Rb* gene) and the D13S118 locus (distal telomerically to *Rb* in chromosomal band 13q14.3) was measured in RTR SCCs and matched normal controls. The effect of LOH on the expression of Rb in these tumours was also examined by immunohistochemistry.

The results of this study show that a high degree of chromosomal loss at 13q14.2-13q14.3 exists in cutaneous squamous cell carcinomas. Sixty four percent (7/11) of informative SCCs analysed demonstrated LOH at either the D13S153 or D13S118 locus. The chromosomal loss observed does not appear to affect Rb expression as only on of the SCCs found to exhibit LOH was found to be Rb-negative by immunohistochemistry and the expression of Rb in the SCCs analysed was found to be similar to the expression of Rb in benign viral warts.

Finally, a common polymorphism in the *p53* tumour suppressor gene at codon 72 has been proposed to be a risk factor for HPV-associated cancers. This study describes the distribution of p53 codon 72 genotypes in skin cancer patients (both RTR and ICP) and in an ethnically matched control population. A PCR-based strategy was used to amplify the different alleles present in both populations and the association between the development of skin cancer and p53 codon 72 genotype was examined.

The results of this study show that in a Celtic population, there appears to be no correlation between HPV infection, the p53 codon 72 polymorphism and the risk of developing skin cancer.

LOH	Loss of heterozygosity
mRNA	messenger ribonucleic acid
NGS	Normal goat serum
NHS	Normal human skin
NMSC	Nonmelanoma skin cancer
MAP	Mitogen activated protein
ORF	Open reading frame
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
RCSI	Royal College of Surgeons in Ireland
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase PCR
RTR	Renal transplant recipient
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulphate
SSCP	Single strand conformational polymorphism
SV40	Simian virus 40
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TBS	Tris-buffered saline
TE	Tris EDTA
TEMED	NNN'N' Tetramethylethylenediamine
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
UV	Ultraviolet
UVA	Ultraviolet A radiation
UVB	Ultraviolet B radiation
XP	Xeroderma pigmentosum
α-P^{32}dATP	P^{32}-labelled dATP

Chapter 1

Introduction

1.1 Skin cancers.

Primary neoplastic disease of the skin is frequent and increasing (Glass & Hoover 1989, Gallagher *et al.* 1990, Parker *et al.* 1996). The common skin cancers include melanoma and the non-melanoma skin cancers (NMSC), consisting of basal cell carcinomas (BCC) and squamous cell carcinomas (SCC). Non-melanoma skin cancers are the leading cause of cancer in Caucasian populations. Cutaneous SCC account for approximately 20% of cutaneous malignancies (Shiffman 1975). Recent estimates show that in the United States, there are approximately 62 cases of cutaneous SCC per 100,000 individuals each year (Parker *et al.* 1996). In Australia the rate is even higher with approximately 1035 cases per 100,000 males and 472 cases per 100,000 females each year (Marks *et al.* 1993, Green *et al.* 1996). The frequency of SCC metastasis varies according to sex, age, immune status and the site of the original tumour (Moller *et al.* 1979, Nixon *et al.* 1986, Rowe *et al.* 1992). Although the metastatic frequency of cutaneous SCC is low (0.3% to 3.7%), thus mortality rates are low, morbidity is high and the burden on healthcare is great (Breuninger *et al.* 1990).

1.1.1 SCC causation.

The aetiology of cutaneous SCC is multifactorial. Host factors such as skin type, immune status, age and genetic defects as in Xeroderma pigmentosum (XP) and Epidermodysplasia verruciformis (EV) contribute to the risk of developing SCC of the skin (reviewed in Salasche 2000). Environmental factors such as sun exposure and human papilloma viruses (HPV) may also play a co-carcinogenic role.

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1.1.2 Skin type, age and cutaneous squamous cell carcinomas.

The major constitutional risk factor for skin cancers is skin type. Host pigmentation, as shown from multiple epidemiological studies, greatly influences the susceptibility to skin cancer, with fair-skinned individuals at greatest risk (Giles *et al.* 1988). The reaction of the skin to sunlight may also play a minor role, for example individuals who tan but never burn tend to develop less skin cancers than those that burn but never tan (Evans *et al.* 1988). However, the incidence in individuals who never burn is still relatively high.

Increasing age is clearly associated with an increased risk of developing all skin cancers. However, migration studies have indicated that although individuals develop more skin cancer in old age, the critical time of UV-exposure appears to be childhood (Marks *et al.* 1990). Whether the skin is more sensitive to UV at a younger age or whether this is due merely to increased sun exposure during childhood has not been established.

1.1.3 Genodermatoses.

Inherited genetic disorders such as XP and EV place individuals at an increased risk of developing skin cancers. Xeroderma pigmentosum is an autosomal recessive disorder characterised by severe photosensitivity and an extremely high incidence of skin cancers. Patients with XP have a risk of developing skin cancer approximately 1000 times greater than that of the general population and the age of onset is up to 50 years earlier (Kraemer *et al.* 1994). Cells from XP patients have defective DNA excision

repair mechanisms and thus accumulate DNA damage caused by UV-irradiation (Friedberg *et al.* 1995). It has been suggested that excision repair deficiencies may also occur in normal individuals who develop BCCs at an early age (Wei *et al.* 1993, Moriwaki *et al.* 1996).

Epidermodysplasia verruciformis is a rare hereditary disease characterised by the development of persistent flat warts, often from a very early age. It is believed to be an autosomal recessive disorder (Orth 1987, Lutzner 1978), though there have been isolated cases indicating X-linked recessive inheritance (Androphy 1985) and in one family only females were affected (Rajagopolan 1972). Approximately one third of EV patients develop multifocal skin cancer, generally 25 years after the onset of verrucosis (Orth 1987). Benign EV lesions are associated with a wide variety of HPV types, whereas malignant tumours in EV patients are predominantly associated with HPV 5 and HPV 8 (Orth 1986). The tumours predominantly arise at sun-exposed sites, implying a co-carcinogenic role for UV radiation.

1.1.4 Immunobiology of skin cancers.

The immune system plays a key role in the development of cutaneous cancers. Organ transplant recipients (particularly renal or cardiac) exhibit an increased incidence of a wide range of cancers, skin cancer being the most common. In transplant patients there is an approximately 65-fold increase in the incidence of SCC and the duration and intensity of immunosuppressive therapy influences the rate at which skin cancers occur (McCann 1999). The increased risk is believed to be associated with impaired cell-

mediated immunity due to immunosuppressive drug therapy, thus affecting tumour development and viral clearance.

Patients with EV also exhibit impaired cell-mediated immunity. Viral persistence in EV lesions appears to be due to the inability of the patient's immune system to reject the cutaneous lesions, however the immunological defect that causes this remains unidentified (Majewski *et al.* 1997). Patients with EV do not appear to have an increased susceptibility to other HPV types (e.g. oncogenic mucosal HPV types), therefore it is proposed that the absence of an immune response to EV HPV may be due to inefficient antigen presentation locally in the skin-associated lymphoid tissue (Majewski *et al.* 1986). Normal antiviral and anticancer immunosurveillance mechanisms appear to be preserved in EV patients, however cell-mediated cytotoxicity to EV HPV-harbouring keratinocytes is reduced dramatically (Majewski *et al.* 1990). It has been suggested that polymorphisms in MHC genes could determine the host immune response to EV HPV types (Majewski *et al.* 1997).

Ultraviolet (UV)-radiation can also invoke local immunosuppression in the skin (Kripke 1990). UV-radiation alters antigen-presentation in the skin by directly affecting epidermal Langerhan's cells and also by inducing the release of immunomodulatory cytokines by keratinocytes (Nishigori *et al.* 1996). Skin type may also play a role in susceptibility to UV-induced immunosuppression as individuals with skin types I/II (sun sensitive/tan poorly) have been shown to exhibit a suppression of contact hypersensitivity following UV insult (Kelly *et al.* 2000). It is proposed that photo-immunosuppression decreases the immune response against HPV and in doing so may play a role in the development of non-melanoma skin cancers in addition to the effects of immunosuppression on tumour development (Vermeer and Bouwes-Bavinck 1998).

1.1.5 UV Radiation.

Ultraviolet-radiation of 290-320nm wavelength (UVB) is considered to have the highest carcinogenic activity of terrestrial UV-radiation (Urbach *et al.* 1997). UVA (320-400nm) has been implicated in the development of cutaneous SCC to a lesser extent, primarily in conjunction with photosensitising agents such as psoralen used in the treatment of psoriasis (Stern *et al.* 1988). The vast majority of cutaneous SCCs occur in sun-exposed sites (head, neck or hands). The frequency of occurrence increases with latitude, age and in outdoor workers indicating the cumulative risk associated with chronic UV-exposure (Stern 1999).

1.2 The papillomaviruses.

Papillomaviruses are the causative agents of the ubiquitous, benign tumours known commonly as warts. An accumulating body of evidence suggests that exceptional circumstances, such as allograft transplantation or a genetic predisposition, may in conjunction with various environmental and behavioural factors allow viral infection to result in malignant growth.

1.2.1 Papillomavirus taxonomy and phylogeny.

Papillomaviruses are primarily categorised according to the animal they infect, e.g. human papillomaviruses, bovine papillomaviruses, cottontail rabbit papillomaviruses. Human papillomaviruses are generally further classified as either "cutaneous" or "mucosal", depending on the original site of their isolation (see Tables 1.1 and 1.2). The segregation of HPV types into cutaneous, cutaneous involved in EV, cutaneous/mucosal and mucosal of high-risk and low-risk (oncogenic potential) is not clinically useful as mucosal types are often found at cutaneous sites and subtypes may have a different tissue tropism (de Villiers 1994, Orth 1994, Jablonska & Orth 1995). The various types and subtypes (of which there are currently about 80) are distinguished from one another on the basis of their nucleic acid. If two sequences show 90-98% homology, they are described as subtypes, whereas if the homology is less than 90%, they are considered to be different types. Isolates with greater than 98% homology are considered as variants (zur Hausen 1996). New HPV types are continually being described and novel HPV types are often referred to as being related to their nearest type (Chan *et al.* 1994, de Villiers 1994).

Analysis of sequence variation in the L1 and E6 genes has distributed the papillomaviruses into five distinct phylogenetic supergroups (Figure 1.1). Supergroup α contains, among others, the "high-risk" and "low-risk" mucosal HPVs (HPV16/18/6/11), together with common cutaneous types frequently isolated from mucosal sites (HPV2/27/57). It also includes HPV7, commonly associated with butcher's warts and oral lesions from HIV patients. Supergroup β comprises the EV-associated HPV types and some common cutaneous HPV types associated with warts in non-EV patients. Supergroup γ contains solely cutaneous HPV types. Supergroup δ contains the ungulate fibropapillomaviruses, while supergroup ε contains both the cutaneous HPV types HPV1/41/63 and cottontail rabbit HPV types. Three additional types cannot be assigned to any of the existing subfamilies (BPV4, MnPV and FPV1) (van Ranst *et al.* 1992, Chan *et al.* 1995, Myers *et al.* 1997).

Table 1.1 Low and High risk mucosal HPV types

Low-risk HPV types		High-risk HPV types	
HPV type	**Associated with**	**HPV Type**	**Associated with**
6	Genital mucosa	16	Anogenital cancer
11	Genital mucosa	18	Anogenital cancer
13	Oral mucosa (Heck's disease)	30	Anogenital, oral and laryngeal cancer
32	Oral mucosa (Heck's disease)	31	Anogenital cancer
34	Anogenital (intrepithelial neoplasia, BP)	33	Anogenital cancer
40	Anogenital (intrepithelial neoplasia)	35	Anogenital (intraepithelial neoplasia, cancer)
42	Anogenital (intrepithelial neoplasia, vulvar papilloma)	39	Anogenital (intraepithelial neoplasia, cancer)
43	Anogenital (intrepithelial neoplasia, vulvar hyperplasia)	45	Anogenital (intraepithelial neoplasia, cancer)
44	Anogenital (condylomas)	51	Genital (intraepithelial neoplasia, cancer)
53	Anogenital (normal mucosa)	52	Anogenital (intraepithelial neoplasia, cancer)
54	Genital (condylomata acuminata)	56	Anogenital (intraepithelial neoplasia, cancer)
55	Genital (BP)	58	Anogenital (intraepithelial neoplasia, cancer)
57	Anogenital (intrepithelial neoplasia), also found in cutaneous lesions	66	Anogenital cancer
59	Anogenital (intrepithelial neoplasia)	69	Anogenital (intraepithelial neoplasia, cancer)
61	Anogenital (intrepithelial neoplasia)		
62	Anogenital (intrepithelial neoplasia)		
64	Anogenital (intrepithelial neoplasia)		
67	Anogenital (intrepithelial neoplasia)		
68	Anogenital (intrepithelial neoplasia)		
69	Anogenital (intrepithelial neoplasia)		
70	Anogenital (vulvar papilloma)		
71	AIN		
72	Oral (papillomas in immunosuppressed indiviuals)		
73	Oral (papillomas in immunosuppressed indiviuals)		
74	AIN		

AIN anal intraepithelial neoplasia, BP bowenoid papulosis.

Table 1.2 Cutaneous and EV-associated HPV types.

Cutaneous HPV types		EV-specific HPV types	
HPV type	**Associated with**	**HPV Type**	**Associated with**
1	Myrmecia wart (v. plantaris)	5A/B/C	Benign lesions, EV cancer
2	Common wart (v. vulgaris)	8	Benign lesions, EV cancer
3	Plane wart (v. plana)	9	Benign lesions
4	Common wart (frequently palmar)	12	Benign lesions
7	Butcher's wart (proliferative hand warts)	14	Benign lesions, EV cancer
10	Plane or intermediate wart (EV-associated)	15	Benign lesions
26	Plane or intermediate wart (mostly in immunosuppressed patients)	17	Benign lesions, EV cancer
27	Plane or intermediate wart (mostly in immunosuppressed patients)	19	Benign lesions
28	Plane or intermediate wart	20	Benign lesions, EV cancer
29	Intermediate or common wart	22	Benign lesions
34	Bowen's disease (mainly genital)	23	Benign lesions
36	Actinic keratosis (mainly EV-HPV)	24	Benign lesions
37	Keratoacanthoma (single case)	25	Benign lesions
38	Malignant melanoma (single case) (EV-associated)	36	Benign lesions
41	Warts, SCC	46	Benign lesions
48	SCC (in immunosuppressed patients)	47	Benign lesions, EV cancer
49	Warts, premalignant lesions (in immunosuppressed patients) (EV-associated)	49	Benign lesions
57	Inverted maxillar papilloma (mainly genital)	59	Benign lesions
60	Epidermoid plantar cyst		
63	Myrmecia cystic wart		
65	Pigmented wart		
75	Common wart (in immunosuppressed patients)		
76	Common wart (in immunosuppressed patients)		
77	Common wart (in immunosuppressed patients)		

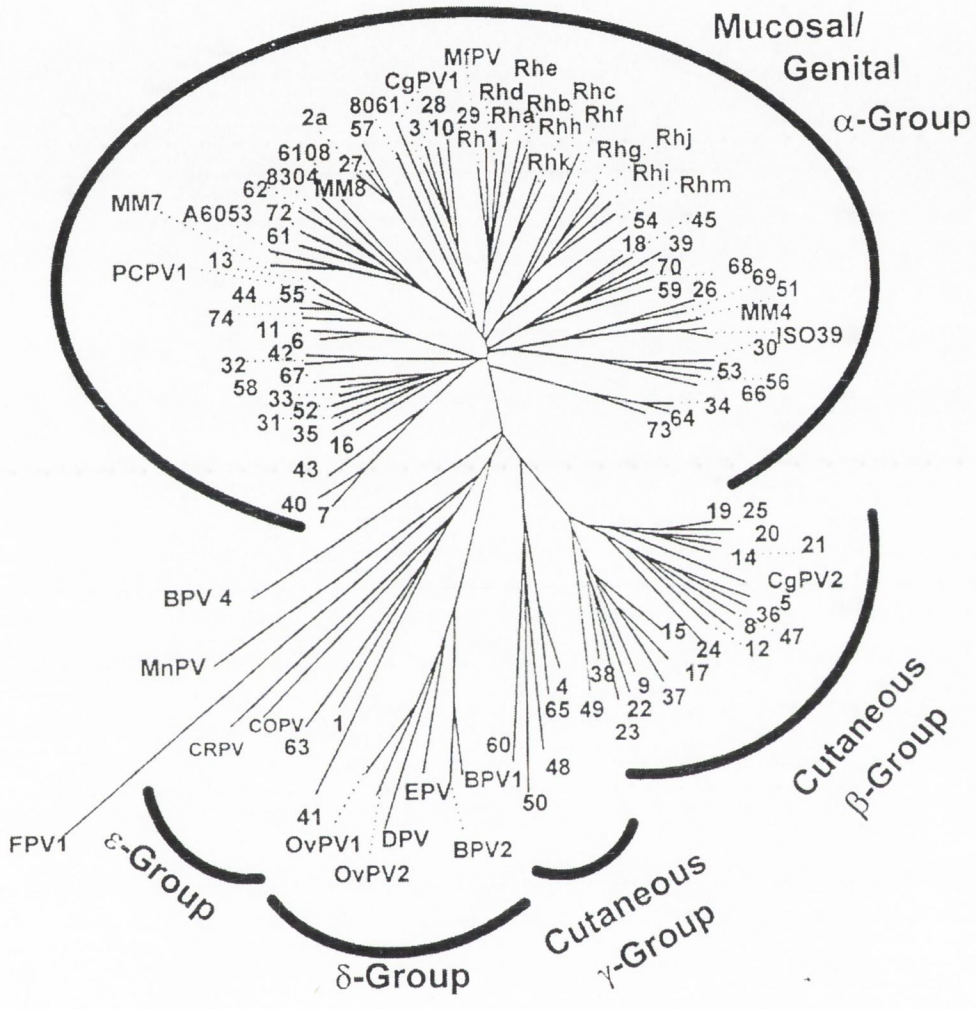


Figure 1.1 The evolutionary pedigree of the papillomavirus family. Adapted from Myer *et al.* 1997.

1.2.2 Papillomavirus structure and genomic organisation.

Papillomaviruses belong to the papovaviridae family, which also includes the oncogenic polyomavirus SV40. All papillomaviruses have an icosahedral, non-enveloped capsid with a diameter of approximately 50nm. The capsid is composed of 72 capsomers and contains two complimentary circular DNA strands, 7500-8000 nucleotides in length (Howley 1996). Analysis of the known papillomaviruses has shown that they all share the same genetic organisation (Figure 1.2)(Pfister and Fuchs 1994). Differential mRNA splicing allows papillomaviruses to produce 12-15 gene products from 9-10 open reading frames (Favre *et al.* 1997). The reading frames themselves are loosely designated early (E), consisting of the genes involved in regulating viral DNA replication, and late (L), comprising the structural genes responsible for the production of the viral capsid.

The L1 and L2 genes encode the major and minor capsid subunits. The L2 protein has been shown to bind to HPV16 DNA and appears to be essential for the production of functional virus particles as capsids composed solely of the L1 protein do not contain any viral DNA (Zhou and Frazer 1995). The E1 gene codes for a nuclear phosphoprotein with DNA-dependent ATPase and ATP-dependent helicase activity that is involved in the initiation of viral DNA replication (Chow and Broker 1994). The E2 gene product coordinates viral transcription and replication. Though classed as an early protein, the E4 gene encodes proteins that regulate viral maturation and the release of viral particles and as such is expressed late in the viral life-cycle. The entire E4 open reading frame (ORF) is contained within the E2 ORF and constitutes a multispecies family arising from different posttranslational modifications and multimerisations that aggregate both cytoplasmically and in nuclear inclusions (Doorbar 1996). The E5 gene

product is a membrane-associated hydrophobic protein and has been shown to exhibit some transforming activity though this is not fully characterised (Banks and Matlashewski 1996). The E5 gene product of bovine papillomaviruses type 1 is the predominant transforming factor, though in genital HPV types only weak transforming capabilities have been observed *in vitro* (Howley 1996).

The E6 and E7 genes encode the viral oncoproteins and are the major transforming proteins of genital HPV types. The E6 gene product is a small protein present in both the nucleus and in nonnuclear membranes that contains four zinc-binding Cys-X-X-Cys motifs characteristic of some transcriptional activation proteins (Howley 1996). The E7 gene encodes a zinc-binding nuclear protein (Farthing and Vousden 1994). The HPV E6 protein acts synergistically with the E7 protein to immortalise human keratinocytes (Farthing and Vousden 1994). Recently, an alternative splice product of the E2 gene that fuses a portion of the E2 ORF with a small ORF, designated E8, has been shown to be crucial for transient HPV replication in both normal and immortalised keratinocytes (Stubenrauch *et al.* 2000). The E8E2 fusion protein is believed to modulate viral replication through repression of E2-enhanced E1-dependent replication and by directly regulating viral gene expression.

A long control region (LCR) occupies about 10% of the HPV genome. The LCR of EV-associated HPV types is somewhat smaller than that of mucosal and cutaneous HPV types (Fuchs and Pfister 1996). The HPV LCR contains elements that interact with both viral and host transcription factors involved in regulating viral DNA replication and gene expression (Scheffner *et al.* 1994).

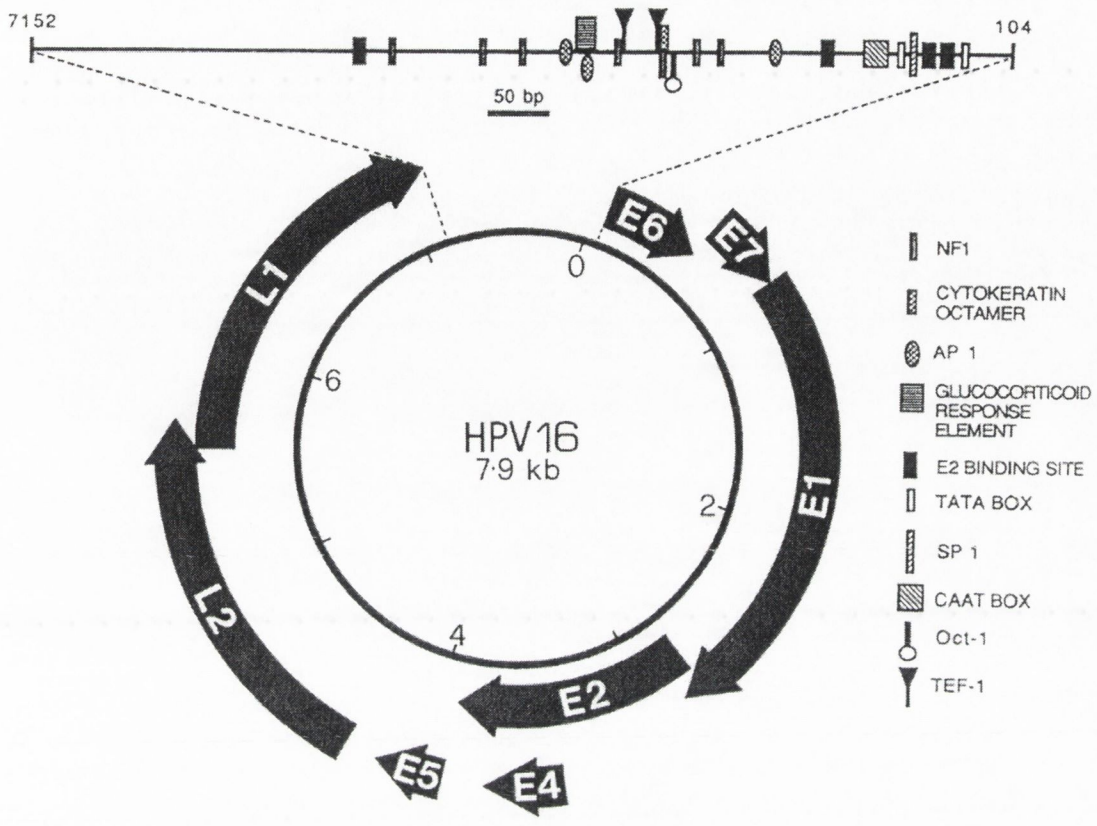


Figure 1.2 Organisation of the HPV 16 genome. The boxes show the viral open reading frames with arrows indicating the direction of transcription. The inset shows the viral upstream regulatory region and the location of known regulatory elements. Adapted from Storey *et al.* 1994.

1.2.3 Papillomavirus infection and life cycle.

Papillomaviruses are acquired through direct mucosal or cutaneous inoculation. It is assumed that basal cells are infected initially and that episomal viral DNA is translocated, together with the basal cell progeny, to the upper layers of the epithelium (Stoler *et al.* 1989, Shah and Howley 1996). There is a massive induction of viral gene expression and DNA replication once the viruses are resident in the terminally differentiated cells of the upper epithelium and it is believed that differentiation-specific signals initiate the process (Haller *et al.* 1995). It is proposed that the viral oncogenes stimulate the production of the cellular components required for DNA replication in these non-replicating cells and that the viral E1 and E2 proteins recruit these factors and coordinate the replication of the viral genome (Androphy 1994, Yang *et al.* 1993). Once replicated, the viruses are released into the environment as the epithelium undergoes its normal maturation process. As papillomaviruses are non-enveloped/non-lytic and are only released from the cell when the nuclear membrane disintegrates, they are not susceptible to the same environmental stresses as enveloped viruses and as such are extremely robust.

1.2.4 Detection of HPV in human cancers.

The association between HPV and particular human cancers is based upon the fact that HPV DNA is often detected in individual tumour cells. However, the detection method used may bias epidemiological and clinical data. Under-sensitive detection techniques lead to false negative results and the assumption that HPV is not associated with a particular tumour. Over-specific detection techniques also generate similar anomalous

results by failing to detect previously uncharacterised types, and given the heterogeneity of the papillomaviruses, this may be of paramount importance in both assigning a causal role for the virus in tumourigenesis and in determining the effectiveness of screening procedures.

Southern blotting has traditionally been used to detect HPV types in human tissues (Monk *et al.* 1994). This technique offers well characterised sensitivity levels, however, the results obtained are complicated by various other factors such as viral DNA deletion and rearrangement in malignant tumours, and technical problems, for example, cross-hybridisation with related HPV-types thus not allowing the correct typing of detected viruses.

In situ Hybridisation (ISH) has been used frequently to detect and type HPV DNA with variable success (Wagner *et al.* 1984). Some tumours fail to show any HPV DNA when analysed by this method, and others show up to 90% detection. Unfortunately, successful ISH depends upon the nature of the probes used. Only a limited amount of HPV-specific probes are available which limits the number of detectable types. Low-stringency hybridisation can be carried out in order to detect some novel types, however this can also result in non-specific hybridisation and false positive results. The main advantage of ISH is that tissue architecture is maintained and HPV DNA can be specifically located within a tissue section showing which cells in particular are infected, whereas other detection techniques merely confirm the presence of HPV DNA in the tissue as a whole.

More recently, the polymerase chain reaction (PCR) has been used to detect HPV DNA in a variety of human tissues. Various degenerate primer sets have been developed from published HPV sequences that amplify most known types (Manos *et al.* 1989, Shamanin *et al.* 1994, Berkhout et al. 1995). The majority of consensus primers

currently in use are based upon the conserved regions of the L1 and E1 genes. In an international study on cervical cancer, degenerate PCR detected HPV DNA in 92.3% of analysable tumours (Bosch *et al.* 1995). PCR is much more sensitive and specific than earlier methods of detection and novel related subtypes can be detected when degenerate primers are used. In a study carried out to compare the detection of HPV by PCR with Southern blotting, PCR detected HPV DNA in 60% of samples that had been classified as HPV-negative by Southern blot analysis (Monk *et al.* 1994). Variations on the PCR procedure may also be used to achieve more sensitive detection, for example nested-PCR and combinations of PCR primers can be used to broaden the range of detectable types (Berkhout *et al.* 1995, Surentheran *et al.* 1998). Using a pool of defined primers and avoiding the use of degenerate bases has also been shown to increase the sensitivity and specificity of PCR-based HPV detection (Gravitt *et al.* 2000). The major problem associated with PCR is sample contamination. Rigorous controls must be included in every PCR protocol to eliminate the possibility of non-specific amplification and false positive results.

Once HPV-specific DNA fragments have been amplified by PCR, there are numerous techniques available to determine the specific type isolated. Southern blotting and restriction fragment analysis have both been used, but sequencing the DNA is ultimately the most desirable option. Highly related subtypes may generate the same, or similar restriction patterns and thus may by indiscernible from one another. They may also hybridise to the same probe if typed by Southern blotting. Recently, a novel approach to genotyping HPV isolates has been described (Nelson *et al.* 2000). Fragments of the HPV L1 gene amplified with a combination of PCR primers with deoxyuridine incorporated into the amplified product in the place of deoxythimidine can be digested with uracil N-glycosylase and endonuclease IV to produce a fingerprint

unique to each HPV type. By comparing the fingerprints generated with the fingerprints of known HPV types it is possible to rapidly assign a genotype to an unknown HPV isolate.

In the case of genital HPVs, non-radioactive Hybrid Capture has proven a reliable and accurate method of HPV detection and typing (Schiffman *et al.* 1995). Interlaboratory studies have shown that excellent reproducibility is possible. However as with ISH, hybridisation based techniques are only as good as the probes available and with the growing number of HPV types this may limit the usefulness of the Hybrid Capture assay. Recently, positive results obtained by Hybrid Capture assay were found to be due to cross-hybridisation with HPV types not included in the probe cocktail, so while detection is possible, accurate typing may not be (Vernon *et al.* 2000).

The future of HPV detection and analysis of HPV-associated cancers may lie in *in situ*-PCR (IS-PCR). This technique offers the sensitivity and specificity of PCR, coupled with the spatial localisation of HPV associated with ISH. This technique could not only show the presence of HPV DNA in tumours, but it could specifically highlight which cells contain the viruses. It may also be important in deciphering the viral replication cycle as viral DNA is proposed to be maintained at low levels in lower layers of the epithelium and a single copy of viral DNA is well within the detection capabilities of IS-PCR, thus allowing confirmation of this theory.

1.2.5 HPV and cervical cancer.

Cervical cancer exhibits many of the traits associated with sexually-transmitted diseases (Franco 1995). Herpes simplex virus type 2 was initially perceived to be the most likely aetiological agent. Modern detection methods, however, have indicated that HPV, which in some cases has been isolated from over 90% of analysed tumours, may be the causative tumourigenic agent (Berumen *et al.* 1995).

More than 35 distinct HPV types are known to infect the genital tract, for example HPV16/18/6/11/31/33 (Bernard *et al.* 1994). Over 20 of these HPV types are believed to be cancer-associated. HPV 16 and 18 are considered "high-risk", HPV6 and 11 are perceived to be "low-risk", while the others are classed as "intermediate". HPV16 and HPV18 account for almost 70% of the HPV types isolated from cervical carcinomas, with HPV16 occurring twice as frequently as HPV18 (Lorincz *et al.* 1992). However each type is more commonly associated with particular types of cancer, for example, HPV18 is the predominant type found in adenocarcinomas and adenosquamous tumours (Tase *et al.* 1988). As cervical cancer is the second most common cancer in women and the fifth most common worldwide (Crook & Farthing 1993), the need for a comprehensive vaccination and screening programme is obvious, however the sheer diversity of this complex group of viruses and the number of different types that have been detected in cervical neoplasms, underlines the difficulties facing those attempting to tackle the problem.

1.2.6 Molecular events in HPV-associated neoplasia.

Papillomaviruses possess an intrinsic oncogenic potential due to the fact that they replicate in non-dividing cells and, as their genome is insufficient to encode all of the factors necessary for DNA replication, they must induce their expression in host cells. The viral E1 and E2 proteins are responsible for perverting normal cellular processes in order to reproduce the viral genome. The E6 and E7 proteins may function to remove potential blocks in DNA replication caused by p53 or Rb upon the detection of viral DNA and other genotoxic stresses. Under normal conditions, DNA replication in terminally differentiated cells does not result in uncontrolled cellular proliferation. However, malignancy occurs when viral oncogenes stimulate cell division in cells that have not yet become irreversibly converted to the non-replicating population.

The HPV E6 oncoprotein has been strongly implicated in the transformation of HPV infected cells (Crook & Vousden 1994, Crook *et al.* 1994, Burns *et al.* 1994, Pim *et al.* 1994, Storey *et al.* 1995). E6 has been shown to bind p53 *in vitro*. This binding results in the rapid breakdown of p53 via the ubiquitin pathway for protein degradation (Scheffner *et al.* 1990). An E6-associated protein (that is a member of the ubiquitin pathway) appears to be essential for degradation (Huibregste *et al.* 1993). Cells transformed by plasmids containing HPV18 E6 and an activated oncogene (EJ-*ras*), arrest in G_0/G_1 phase when E6 is removed, suggesting a role for E6 in regulating the cell-cycle. Subpopulations of E6/EJ*ras* transformed cell lines that have acquired spontaneous mutations in p53 do not arrest in G_0/G_1 upon removal of E6, suggesting that E6 functionally substitutes for dominant mutations in p53 and may have a novel cellular target that allows it to coordinately regulate the progression to cellular proliferation (Storey *et al.* 1995). The E6 protein also prevents the transcription of DNA

damage repair genes and cell-cycle regulatory genes that contain p53-responsive enhancers, e.g. *gadd*45, *p21* and *mdm*2 (Thomas *et al.* 1995). In addition, E6 also activates telomerase activity, interacts with calcium binding proteins and also with the cyclin-dependent kinase inhibitor $p16^{INK4}$, an association that apparently results in the functional impairment of E6 but leads to cellular immortalisation (Reznikoff *et al.* 1996, zur Hausen 1999).

The HPV E7 protein has also been implicated in cellular transformation (Morris *et al.* 1993), and has been shown to bind to Rb (Dyson *et al.* 1984), p107, p130, cyclin A and cyclin-dependent kinase 2 as well as other cell-cycle related proteins (Tommassino *et al.* 1993). The net result of the E7-Rb/p107/p130 associations is the release of the E2F transcription factor complexes from Rb/p107/p130 which then stimulate the transcription of proliferation-dependent genes (Vousden 1993). The fact that E7 also interacts with cyclin A and cyclin-dependent kinase 2 implies that E7 may also interfere at other stages in the cell-cycle. E7 has also been shown to bind and inactivate the cyclin-dependent kinase inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ (Funk *et al.* 1997, Jones *et al.* 1997). In $p21^{Waf1/Cip1}$ null hepatocytes, E7 expression correlates with increased DNA replication, an effect that is synergistically increased with the activation of the MAP kinase pathway. Loss of Rb function in the same p21 null cells allows mutant E7 (that has no Rb-binding activity) to produce the same effect. This suggests that E7 expression in conjunction with mitogen activation plays a key role in stimulating cellular proliferation when $p21^{Waf1/Cip1}$-mediated cell-cycle arrest is abrogated (Park *et al.* 2000).

Studies in mouse model systems have indicated that the E6 and E7 proteins act at different stages of tumour development. The E6 protein was found to act weakly at the promotion stage of carcinogenesis in the formation of benign tumours, but strongly

at the stage involved in malignant conversion. Conversely, the E7 protein was found to act strongly at the tumour promotion stage and weakly as the tumour became malignant. This presents a scenario whereby E7 promotes the formation of benign tumours and E6 accelerates the progression to malignancy (Song *et al.* 2000).

In the case of cervical carcinomas, viral integration into the host cell genome has been proposed as an activation mechanism for the progression from precancerous lesions to cancer (Schneider-Manoury *et al.* 1987). In benign cervical intraepithelial neoplasia lesions, HPV DNA exists episomally, however in HPV18-positive tumours, the majority of the viral DNA appears to be integrated (Cullen *et al.* 1991). Integration usually results in the disruption of the E1/E2 genes which leads to the overexpression of the E6/E7 oncogenes (Vousden 1993).

However in HPV16-positive cervical tumours, integration may vary from 30% to 70% (Cullen *et al.* 1991), and the E1/E2 genes are only disrupted in approximately 36% of cases (Berumen *et al.* 1994). The level of amplification of the HPV genome is also much greater in HPV16-positive tumours with intact E1/E2 genes than it is in HPV16/HPV18-positive tumours lacking the E1/E2 genes (Berumen *et al.* 1995). These observations could suggest that viral integration and subsequent inactivation of viral replication is not necessary for the development of tumours and that episomal overexpression of viral oncogenes may suffice.

Most of the experimental work to date aiming to elucidate the mechanism(s) of HPV-associated tumourigenesis has focused primarily on "high-risk" genital HPV types 16 and 18. The *in vitro* model cell systems used have varied significantly and it is likely that the infected cell type is an important factor in the ability of HPV to exert an effect on cellular proliferation/transformation. Viral oncogenes associated with specific HPV types and subtypes may exhibit distinct tumourigenic pathways and, in association with

the infected cell type, result in lesions of varying oncogenic potential. Indeed, there is no evidence to suggest that cutaneous HPV types follow the same oncogenic pathways as high-risk mucosal types.

Not all papillomavirus infections result in tumour development. Other factors are involved in the progression from cellular transformation to malignant growth. UV exposure and smoking may play significant roles in the development of HPV-associated cancers (Herrington 1995) and other genetic changes undoubtedly occur, for example, chromosomal abnormalities, particularly on chromosomes 1 and 11, are observed in human cells that are transformed by HPV E6 and H-*ras* (Matlashewski *et al.* 1988). The high frequency at which HPV is detected in various human cancers implies a causal role, however the precise mechanism of oncogenesis remains to be determined.

1.2.7 Epidermodysplasia verruciformis and HPV.

Epidermodysplasia verruciformis was the first known example of HPV-associated skin cancer and as such represents a good model to study the role of both viruses and host factors in the development of HPV-induced carcinogenesis (Majewski & Jablonska 1995). HPV5 and HPV8 are associated with 90% of carcinomas in EV patients (Pfister 1992) and to a lesser extent HPV14, HPV17, HPV20 and HPV47 (Orth 1994, Adachi *et al.* 1996, Orth 1987, Yutsudo *et al.* 1994). The HPV genome is maintained episomally in EV tumours and tumour progression has been shown to be associated with increased expression of the E6 oncogene of HPV5 and 8 (Favre 1997). Deletion of portions of the HPV5 and 8 genomes encompassing the late regions and some of the LCR have been observed in EV tumours, however the role these deletions play in tumourigenesis is yet to be determined (Deau *et al.* 1991).

The E6 proteins of HPV5 and 8 do not appear to bind p53 and only show weak transforming activity in human keratinocytes compared with high-risk mucosal types (Steger and Pfister 1992). The E7 proteins of HPV5 and 8 do not appear to have any transforming activity (Androphy 1994). In contrast to sporadic skin cancers in the general population, p53 mutations are relatively rare in EV tumours and those that do harbour mutations do not have UV-induced mutational signatures (Brash *et al.* 1991). Wild-type p53 is found to accumulate in EV tumours and a lack of expression of *bcl*2 and *bax* has also been observed suggesting a different oncogenic mechanism to the high-risk mucosal HPV types (Majewski *et al.* 1997).

1.2.8 HPV and non-melanoma skin cancer in immunosuppressed and immunocompetent hosts.

Although HPV are the most common viruses that infect human skin, they have, until the last decade, largely been overlooked as aetiological agents of skin cancer. Recent evidence suggests, however, that they may be important in the onset of the disease. Whereas the oncogenic properties of high-risk mucosal HPV types are well documented, molecular studies investigating a role for cutaneous HPV types in skin cancers are only beginning.

EV-associated HPV types are frequently isolated from cutaneous warts of long-term immunosuppressed patients (Benton *et al.* 1992, Shamanin *et al.* 1994, Stark *et al.* 1994, van der Leest *et al.* 1987). In renal transplant patients these cutaneous warts often precede cutaneous carcinomas, SCC (Tieben *et al.* 1994, Euvrard *et al.* 1993). EV-associated HPV types are also frequently detected in SCCs of renal transplant recipients. In a number of studies, approximately 80% of RTR SCCs were found to

harbour predominantly EV-associated HPV (Berkhout *et al.* 1995, de Villiers *et al.* 1997, Harwood *et al.* 1999a). EV-HPV types found in renal allograft recipients are also frequently co-detected with multiple common cutaneous HPV types in benign viral warts and to a lesser extent with mucosal HPV types in SCCs (Harwood *et al.* 1999a). Other studies have reported lower rates of detection (up to 40%) and a broad spectrum of HPV types (low-risk mucosal HPV types, high-risk mucosal HPV types and common cutaneous HPV types) (Shamanin *et al.* 1994, Shamanin *et al.* 1996, Mansat-Krzyzanowska *et al.* 1997). Combining the methodologies used in these studies has proven a more reliable method of defining the prevalence of various HPV types in benign and malignant skin lesions of renal transplant recipients (Surenthuran *et al.* 1998).

Immunocompetent skin cancer patients also demonstrate HPV in both benign and malignant skin lesions (Shamanin *et al.* 1996). Most studies estimate the prevalence of HPV in NMSC of immunocompetent patients to be less than 40% (Shamanin *et al.* 1996, Proby and Harwood 1998, Astori *et al.* 1998). The HPV types detected include EV-associated HPV types, common cutaneous HPV types and genital HPV types. EV-associated HPV DNA has also been detected in the normal skin of immunocompetent host and renal transplant recipients at a much higher frequency than might be expected (Boxman *et al.* 1997, Astori *et al.* 1998, Boxman *et al.* 1999).

It is difficult to define an active role for HPV in cutaneous malignancies, given that a wide range of types appear to be prevalent and that within individual lesions a number of different types may be present. It is more probable that, in contrast to genital HPV types, which are necessary but not sufficient to induce cancer, cutaneous HPV types may act as cofactors or promoters of malignant conversion. A broad-spectrum detection approach that could accurately define the viral aetiology of cutaneous

malignancies might identify putative HPV candidates for further molecular virological studies. In addition to the possible role of HPV, the role of other factors in NMSC development should also be examined. Alterations in cell-cycle regulation and the normal response to UV-induced genotoxic stress, chromosomal abnormalities and other risk factors may all contribute to the development of primary neoplastic skin diseases.

1.3 Tumour suppressor genes.

Loss of function of a number of cellular genes plays a critical role in the development of cancers. Such genes, negative regulators of neoplastic disease, have been termed cancer-susceptibility genes, recessive cancer genes, recessive oncogenes and tumour suppressor genes. The first such gene to be identified was the retinoblastoma-susceptibility gene, *Rb*. Following the isolation of *Rb*, a growing list of tumour suppressor genes have been identified. Loss of function of many of these genes contributes to the development of both inherited and sporadic forms of cancer.

1.3.1 The *p53* tumour suppressor gene.

Maintenance of genomic stability is central to cancer prevention. As a regulator of normal cellular responses to genotoxic stress and DNA damage, the *p53* gene plays a key role in preserving the integrity of the genome.

Initially it was thought that *p53* was an oncogene, as levels of p53 protein were increased in SV40-transformed cells and it appeared that increased expression correlated with cellular transformation. Further analysis showed that the accumulated p53 was in fact mutated and when the wild-type gene was isolated it was shown to have no transforming potential (Cooper 1995). In addition, overexpression of wild-type p53 was found to inhibit transformation, thus *p53* was classed as a tumour suppressor gene (Finlay *et al.* 1989, Eliyahu *et al.* 1989).

The human *p53* gene is comprised of eleven exons, spanning approximately 20kb of DNA. A little over 50% of human cancers contain mutations in this gene. The mutations are primarily clustered in four regions of the gene, between codons 120 and

290. Approximately half of the characterised *p53* mutations affect five specific codons (175, 248, 249, 273 and 282) (Nigro *et al.* 1989, Hollstein *et al.* 1991, Sidransky *et al.* 1991). Phenotypically, these mutations result in the functional inactivation of *p53* and affect the expression of a wide range of genes involved in cell cycle arrest, DNA repair and apoptosis.

1.3.2 p53-regulated responses to genotoxic stresses: Upstream events.

Under normal conditions, p53 is maintained latent at low concentrations in the cell, probably due to its short half-life. However, once the cell is placed under genotoxic stress, a complex pattern of modifications occur that result in the stabilisation and activation of p53. The nature of the modifications that occur seems to depend on the nature of the stress, for example, UV-radiation does not elicit the same stabilisation and activation responses as ionising radiation.

The regulation of the p53 response is due mainly to post-translational modifications of the p53 protein, thus the rate of transcription of the *p53* gene only plays a minor role. However, increased *p53* gene expression is observed in cells upon serum stimulation (Reich and Levine 1984). It may seem paradoxical that a negative regulator of the cell cycle should be induced by the growth factors in serum, however, an increased abundance of *p53* gene transcripts merely places the cell at an higher state of readiness to deal with any DNA damage accrued during extensive proliferation. If no DNA damage occurs, the p53 remains latent and is subsequently degraded, however should any damage occur, the higher levels of p53 ensure a rapid response (Oren 1999). Enhanced translation of p53 mRNA may contribute to the overall increase in p53 protein levels observed after genotoxic stress as ionising radiation has been shown to

increase p53 translation via stimulation of its 3' untranslated region (Fu and Benchimol 1997). However, the relative importance of increased translation versus increased p53 protein stabilisation in eliciting a p53 response in stressed cells remains to be clarified.

That *de novo* gene transcription is apparently not necessary for an effective p53 response is beneficial to cells with severely damaged genomes. In response to genotoxic stress, p53 concentrations in the cell increase markedly, probably due to increased stabilisation. In addition to the stabilisation of the protein, it also appears to be converted from a latent form to an active form. The regulation of p53 is tightly linked to the protein product of a cellular oncogene, *mdm*2. Under normal conditions, p53 has a half-life of approximately 5-20 minutes in most cell types (Giaccia and Kastan 1998). Mdm2 binds p53 and interferes with the recruitment of basal transcription machinery thus preventing transactivation of downstream genes and also targets p53 for ubiquitin-mediated degradation (Lu and Levine 1996, Thut *et al.* 1997). p53 itself binds specifically to the *mdm*2 gene and stimulates its transcription, thus creating a negative-feedback loop (Figure 1.3)(Barak *et al.* 1993). The function of this autoregulatory loop is presumably to terminate the p53 response once the triggering stress has been removed. In some cases the expression of Mdm2 is seen after the expression of other p53-regulated genes, thus providing a window whereby the cell can halt the cell cycle, repair any damage and then Mdm2-mediated p53 degradation occurs allowing the cell to re-enter the cell cycle (Wu and Levine 1997).

The Mdm2 protein is believed to facilitate ubiquitin-mediated degradation of p53 by acting as a p53-specific E3 ubiquitin-protein ligase that covalently attaches ubiquitin groups to p53 (Honda and Yashuda 1999). Whether Mdm2 acts alone or is part of a wider E3 complex is not yet known. Control of p53 autoregulation via Mdm2 may involve a number of different mechanisms (Figure 1.4). Modifications of p53 upon

the detection of genotoxic stress, particularly phosphorylation at key residues involved in the interaction with Mdm2, could block Mdm2-mediated p53 degradation and thus lead to the stabilisation of p53 (Shieh *et al.* 1999, Unger *et al.* 1999). Alterations in Mdm2 itself may also achieve the same result (Mayo *et al.* 1997). Modification of Mdm2 or interaction with other proteins (e.g. ARF) that result in inactive ubiquitin ligase activity but not p53-binding activity may also result in stabilised p53 (Zhang *et al.* 1998, Kubbutat *et al.* 1999).

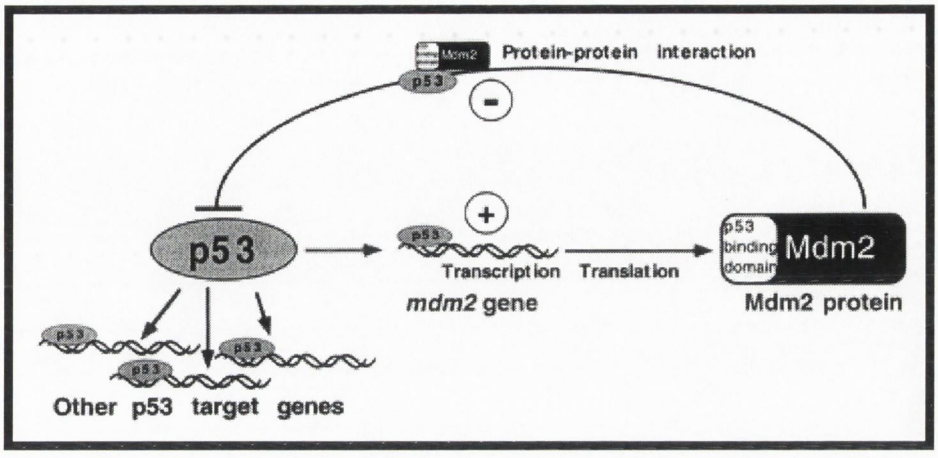


Figure 1.3 The p53-Mdm2 autoregulatory loop. The p53 protein binds to the *mdm2* gene and activates its transcription. The Mdm2 protein generated binds to p53 and blocks its activity. Adapted from Oren 1999.

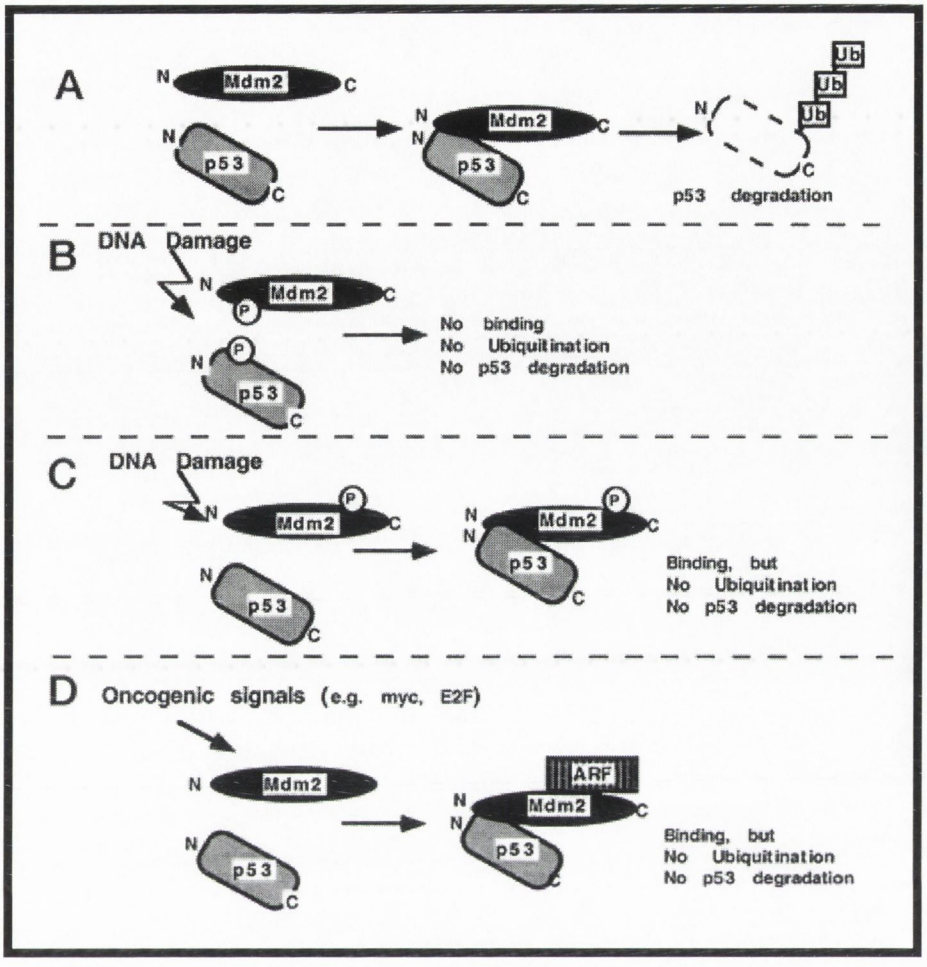


Figure 1.4 Regulation of p53-mediated p53 degradation. **A** Mdm2 binding to p53 promotes p53 ubiquitination and degradation. **B** DNA damage induces a covalent modification of p53 or Mdm2 resulting in an inhibition of Mdm2 binding and activation of p53. **C** p53 degradation may also be blocked by covalent modification of Mdm2 in regions required for its enzymatic activity even if Mdm2 p53 binding activity is preserved. **D** Deregulated oncoproteins induce the synthesis of ARF which binds Mdm2 and prevents its action. Ub = ubiquitin monomer. Adapted from Oren 1999.

A number of other models of p53 stabilisation and activation have been proposed, involving phosphorylation, acetylation, glycosylation and binding to regulatory proteins. One plausible candidate for stress signalling to p53 is the DNA-dependent protein kinase (DNA-PK) (Figure 1.5). DNA-PK is activated by double-strand breaks in DNA and it has been shown to phosphorylate residues 15 and 37 of the p53 protein (Gottlieb and Jackson 1993). Phosphorylation of serine 15 affects the transactivation and growth arrest functions of p53 in some cells (Fiscella *et al.* 1993). However, cells lacking in DNA-PK can still elicit a p53 response suggesting alternative pathways of p53 activation must also exist (Huang *et al.* 1996).

Other protein kinases may also directly or indirectly affect the phosphorylation status of p53 and thus may modulate its activity. The ATM protein, which is defective in patients with ataxia telangiectasia (AT), has been proposed to be involved in an alternative model of p53 activation (Figure 1.6). In this model, phosphorylated p53 is dephosphorylated by an ATM-dependent phosphatase at serine 376. The removal of a phosphate at this residue is proposed to allow 14-3-3 proteins to bind to the carboxy terminus of p53, thus activating the DNA-binding and transcriptional activity of p53 (Woo *et al.* 1998). However, both of these models are questioned by the fact that mutation of all known phosphorylation sites on p53 does not result in a complete abrogation of the p53 response to DNA damage (Lane 1998).

Whatever the mechanism of activation, it is clear that either different genotoxic stresses activate p53 by different pathways, or that compensatory pathways of activation exist. Loss of *ATM* gene function slows the p53 response to ionising radiation but not to UV radiation (Lu and Lane 1993). In addition to serine 376 dephosphorylation, ATM phosphorylates p53 at serine 15 in response to ionising radiation (Shieh *et al.* 1997). Interestingly, p53 in *ATM*-deficient cells also becomes phosphorylated at serine 15 after

UV irradiation, suggesting that a different kinase is induced by UV, but that it targets the same site on p53 as ATM (Giaccia and Kastan 1998).

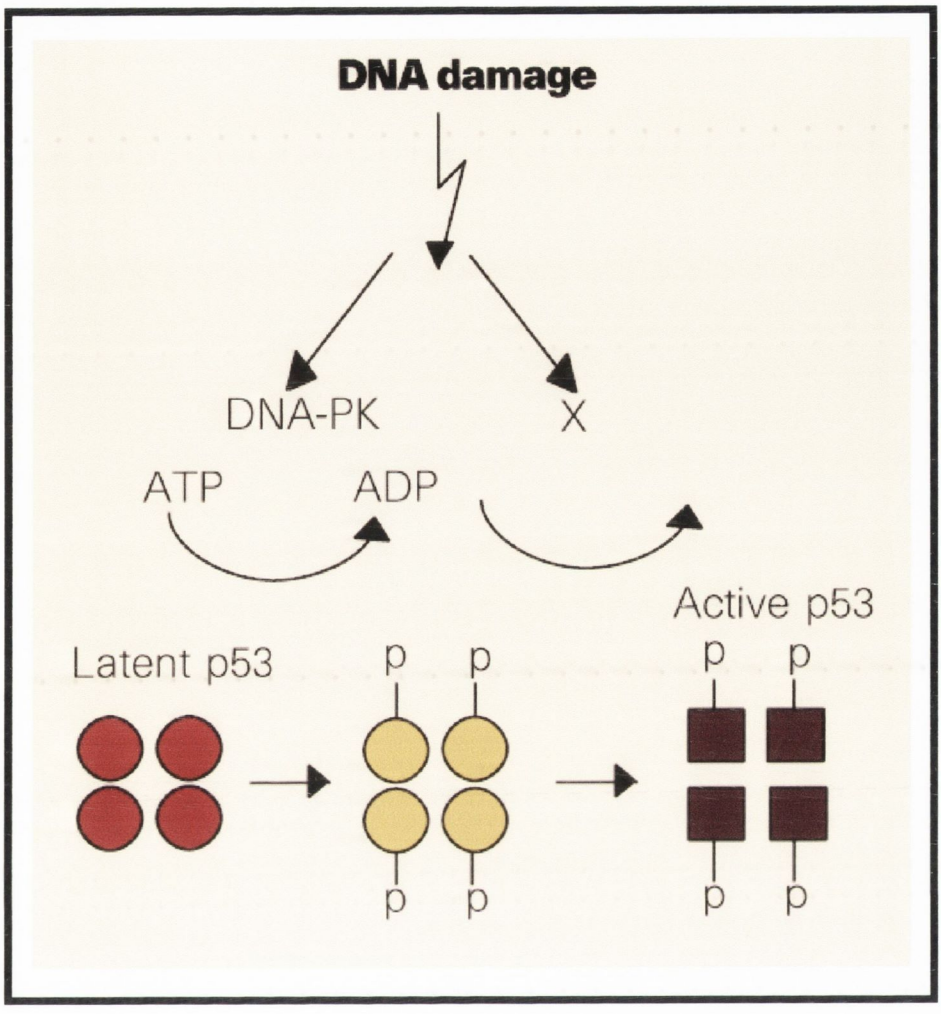


Figure 1.5 Proposed model of p53 activation by the DNA-dependent protein kinase (DNA-PK). DNA-PK phosphorylates p53 and in association with another factor (X) activates DNA binding and transcription by p53. Adapted from Lane 1998.

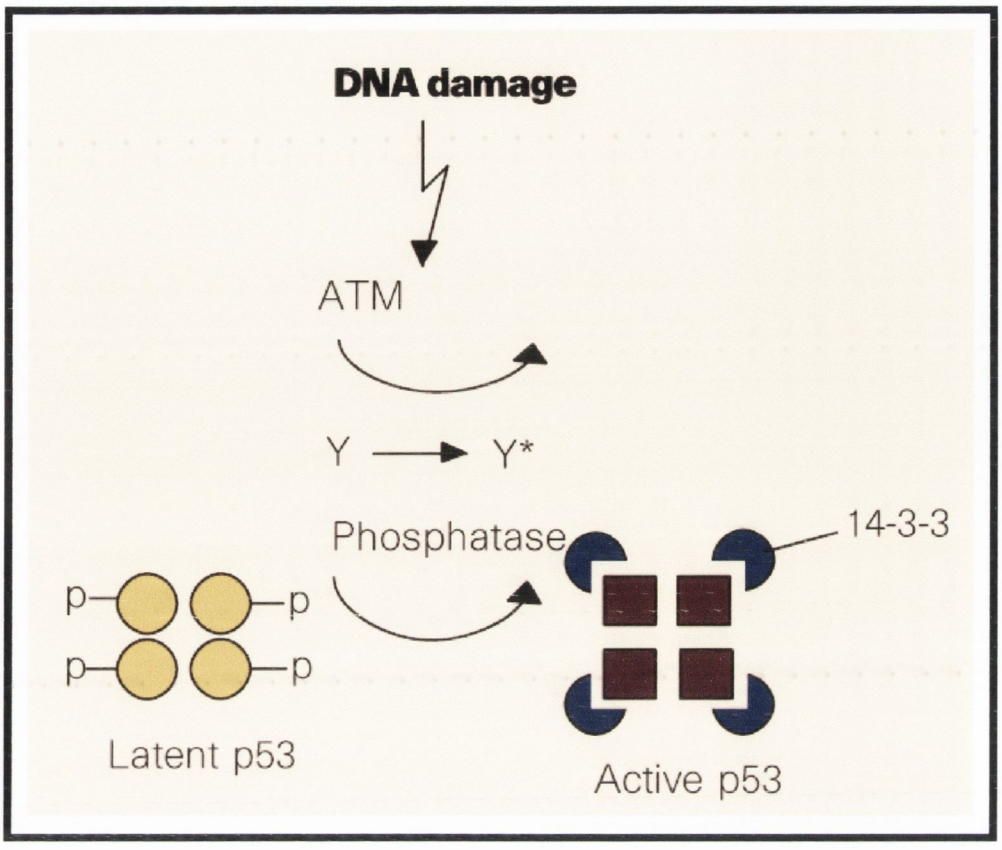


Figure 1.6 Proposed model of p53 activation by the ATM protein kinase. p53 is made in an inactive latent state . It is phosphorylated but remains inactive until DNA damage activates the ATM kinase. ATM activates a p53-specific phosphatase (Y) that removes phosphate from serine 376, allowing 14-3-3 proteins to bind p53 and activate DNA binding and transcription. Adapted from Lane 1998.

1.3.3 p53-regulated responses to genotoxic stresses: Downstream events.

The activation of p53 results in the expression of a number of downstream genes involved in cell cycle arrest, DNA repair and apoptosis. The *p16-cyclin D_1-cdk4-Rb* pathway plays a key role in the regulation of the G_1-S phase transition. One of these four genes is mutated or altered in nearly all known cancers (Hall and Peters 1996, Sherr 1996). $p16^{INK4}$ is a negative regulator of cyclin D_1-cdk4 and is inactivated in many forms of cancer (Levine 1997). Cyclin D_1 amplification and overexpression and *cdk*4 mutants that no longer respond to p16 have been reported in a number of tumours (Levine 1997). The Rb protein is the major target of cyclin D_1-cdk4 in cell cycle regulation. Rb regulates the E2F transcription factor complex (E2F-1, 2, and 3) which in turn induces the expression of a number of proliferation-dependent genes. Phosphorylation of Rb by cyclin D_1-cdk4 results in the release of the E2F complex and cell cycle progression (reviewed in Ewen 1998). In response to DNA damage, tetrameric p53 induces the expression of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ (El-Deiry *et al.* 1993). The net result of $p21^{Waf1/Cip1}$ expression is the hypophosphorylation of Rb, thus preventing the release of the E2F transcription factor complex and cell cycle arrest at the G_1 boundary (Agarwal *et al.* 1998). $p21^{Waf1/Cip1}$ also binds PCNA and prevents DNA replication but not DNA repair (Tournier *et al.* 1996, Levine 1997).

The function of p53-mediated cell cycle arrest is to allow the cell to repair any DNA damage. To this end, p53 also induces the expression of *GADD*45, a growth arrest and DNA damage-dependent gene involved in DNA repair (Fan *et al.* 1997). Coupling of cell cycle checkpoints and DNA repair by p53 may also involve other factors such as the basal transcription factor complex TFIIH, which contains the repair-associated

proteins ERCC2 and ERCC3 (Seroz *et al.* 1993). p53 has been shown to bind to TFIIH *in vitro* and recent evidence suggests that other proteins involved in regulating cell cycle checkpoints may have dual roles in DNA repair, however whether p53 targets any of these factors is as yet unknown (Leveillard *et al.* 1996).

In addition to its protective responses, p53 can also induce apoptosis in some cell types. The induction of apoptosis by p53 does not require p53 to be transcriptionally active because inhibition of transcription by actinomycin D does not affect p53-mediated cell death (Caelles *et al.* 1993). In addition, inhibition of protein phosphatases induces p53-mediated apoptosis in the absence of transactivation (Yan *et al.* 1997). However, transcription of pro-apoptotic genes such as *bax* are induced by p53 and play a key role in initiating p53-mediated cell (Agarwal *et al.* 1998). DNA damage induces growth arrest in some cell types and apoptosis in others. The mechanisms that decide whether p53 elicits a protective or apoptotic response are largely unknown, however, deletion of $p21^{Waf1/Cip1}$ in cells that would otherwise undergo cell cycle arrest results in cell death (Polyak *et al.* 1996).

The p53 signalling pathway connects with oncogenes and tumour suppressor genes that influence control of the cell cycle (Figure 1.7). Alteration of the p53 pathway, either upstream or downstream of p53 may result in an outcome similar to inactivation of p53 itself, leading to deregulation of the cell cycle, genomic instability and possibly cancer.

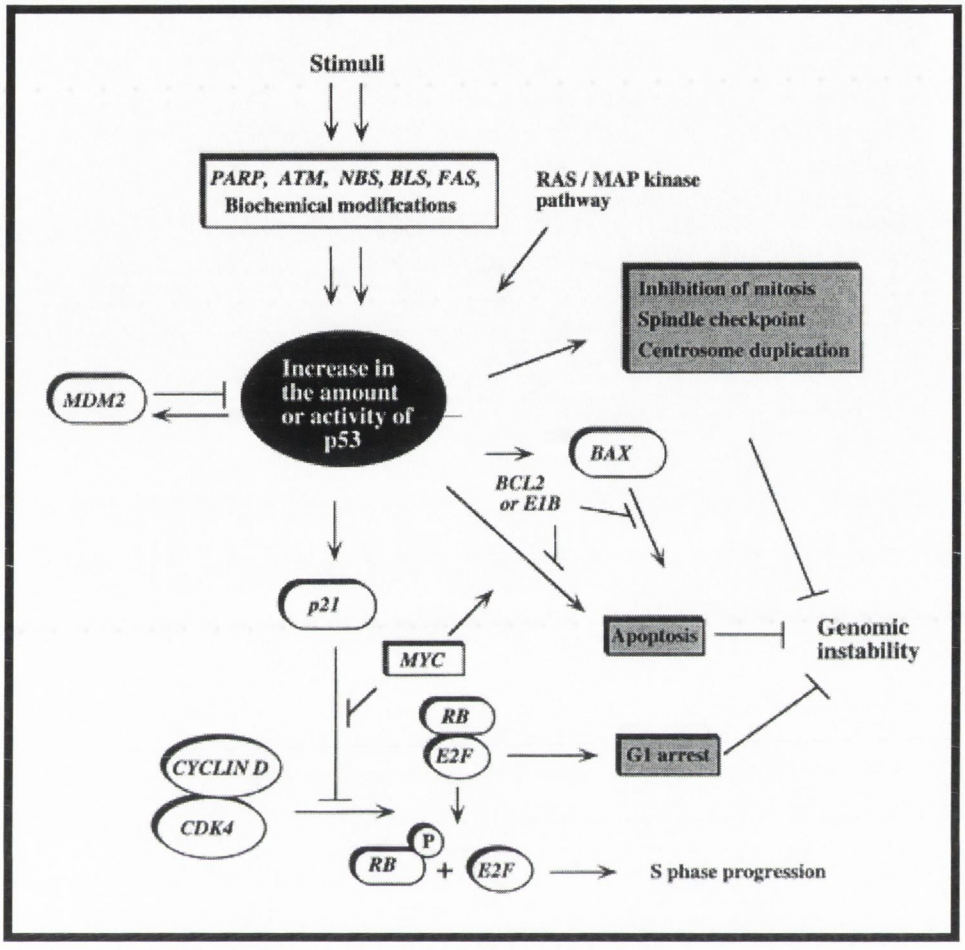


Figure 1.7 Components of p53 signalling pathways. p53 accumulate and is modified and activated in response to signals generated by variety of genotoxic stresses. The RAS-MAP kinase pathways may als play a role in establishing basal levels of p53. Some of the cellula functions of p53 can be deregulated or compromised by the expressio of Myc, Bcl2, or E1B. Control of p53 activity includes the Mdm autoregulatory loop. The intact set of p53-dependent pathways preserv genomic integrity by arresting cells via p21 induction, or by promotin apoptosis via Bax. p53 also helps to regulate entry into mitosis, spindl formation and cell cycle checkpoints that are likely to involve DN damage prevention. Adapted from Agarwal *et al.* 1998.

1.3.4 The *Rb* tumour suppressor gene.

The retinoblastoma susceptibility gene, *Rb*, located on the long arm of chromosome 13 is involved in regulating cell cycle progression at the G_1-S boundary. Its gene product is negatively regulated by cell cycle-dependent phosphorylation catalysed by cyclin-dependent kinases (CDKs) in the late G_1 cell cycle phase. Rb functions through interaction with cellular target molecules such as the E2F transcription factor complex and is crucially involved in S-phase commitment (Figure 1.8). Two Rb homologues, p107 and p130, function in a broadly similar manner to Rb and are regulated by a similar set of protein kinases. Deregulation of the Rb pathway is seen as a major mechanism underlying many cancers.

1.3.5 Rb, p107 and p130 in cell cycle control: Upstream events.

Cell cycle progression is mediated by the coordinated action of a number of CDKs, which are themselves regulated by their association with and activation by cyclins. Cell cycle progression through the G_1 boundary is regulated by D-type cyclins, which activate CDK4 and CDK6 and cyclin E which activates CDK2 (Ewen 1998). When Rb is hypophosphorylated, the interaction with E2F is maintained. Inactivation through hyperphosphorylation results in the release of E2F and cell cycle progression. Cyclin D-dependent kinases have been shown to phosphorylate Rb and thus inactivate its ability to cause G_1 arrest (Kato *et al.* 1993). The expression of $p16^{INK4}$, a CDK4/6-specific cyclin-dependent kinase inhibitor, results in Rb-dependent cell cycle arrest in G_1 (Medema *et al.* 1995). Cyclin E can operate in both an Rb-dependent and Rb-independent manner. Similar to cyclin D expression, cyclin E can induce the

hyperphosphorylation of Rb and induce cell cycle progression (Hinds *et al.* 1992). However, in cells lacking functional Rb, cyclin E is essential for S phase entry (Hofmann and Livingston 1996). It is proposed therefore, that cyclin E controls a different step during G_1 progression to cyclin D. This is supported by the fact that $p21^{Waf1/Cip1}$, a CDK-inhibitor that can inhibit the action of both cyclin D- and cyclin E-dependent kinases, induces cell cycle arrest in G_1 in cells lacking functional Rb (Sherr and Roberts 1995).

The function of p107 and p130 is also controlled by their phosphorylation state in a manner similar to that of Rb. Cyclin D-mediated hyperphosphorylation inactivates the growth suppressive activity of both p107 and p130 (Beijersbergen *et al.* 1995, Claudio *et al.* 1996). Cyclin E does not appear to effect to function of p107, however both cyclin E and cyclin A can override p130-mediated cell cycle arrest (Beijersbergen *et al.* 1995, Claudio *et al.* 1996).

1.3.6 Rb, p107 and p130 in cell cycle control: Downstream events.

The E2F family of transcription factors are the most extensively studied downstream targets of the Rb tumour suppressor family. Ectopic expression of E2F1 has been shown to override Rb-induced cell cycle arrest (Qin *et al.* 1995). Similarly, E2F1 can reverse $p16^{INK4}$-induced G_1 arrest (DeGregori *et al.* 1995). The E2F transcription factors form dimers with the members of another family of transcription factors termed DPs (Johnson *et al.* 1993). Five different E2Fs (E2F-1, 2, 3, 4, 5) and three DPs (DP-1, 2, 3) have been identified (Sardet *et al.* 1995). Complexes containing E2F-1, 2 and 3 have been shown to associate with Rb but not p107 (Dyson *et al.* 1993). E2F5 binds

preferentially to p130 and E2F4 interacts with Rb, p107 and p130 (Hijmans *et al.* 1995, Moberg *et al.* 1996).

Rb, p107 and p130 complex formation with the E2F transcription factors varies according to the phase of the cell cycle. During quiescence (G_0) the major E2F species is p130-E2F4, which is thought to repress the activity of the *E2F1* and *cdc*2 genes and maintain a state of non-cycling (Vairo *et al.* 1995, Johnson 1995, Tommasi and Pfiefer 1995). Rb-E2F complexes are found in mid- to late G_1 phase and are maintained into the S and G_2 phases (Ewen 1998). p107-E2F complexes are generally found in S phase (Shirodkar *et al.* 1992).

Repression of transcription by the interaction of Rb with the E2F complex is not completely defined. One possibility is that the Rb-E2F complex might interfere with other transcription factors thus blocking their interaction with the basal transcription machinery (Weintraub *et al.* 1995). Alternatively, further proteins may interact with Rb or different domains of the Rb protein itself may interact to promote transcriptional repression (Sellers *et al.* 1995, Chow and Dean 1996). In addition, the Sp1 transcription factor has been shown to interact with E2F1, 2 and 3 to activate transcription, thus sequestration of the E2F complex by Rb may impede Sp1-mediated transcription (Lin *et al.* 1996).

The Rb pathway plays a critical role in the control of cell cycle progression in addition to other functions in cell growth and differentiation, however, loss of function and tumour development may centre on how the Rb pathway interacts with the p53 pathway.

1.3.7 p53/Rb and apoptosis versus G_1 arrest.

The interaction of tumour suppressor genes, oncogenes and the response to DNA damage in relation to G_1 arrest or induction of apoptosis suggests an important role for Rb/p107/p130 in p53-mediated regulation of the G_1-S phase transition. Signalling to p53 appears to control the decision between these two outcomes (Levine 1997). Rb acts downstream to p53 in cell cycle arrest in response to DNA damage as p53 induces the expression of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ which inhibits Rb phosphorylation (Levine 1997). Deregulation of the Rb pathway may be one of the signals that results in p53-induced apoptosis, thus Rb may act upstream of p53 in this instance. In essence, p53 monitors the Rb-mediated G_1-S phase transition. This hypothesis is supported by the fact that p53-mediated apoptosis occurs in cells with overexpressed E2F1 and in cells with ectopically expressed adenovirus E1A protein which binds and functionally inactivates Rb (Debbas and White 1993, Qin *et al.* 1994). SV40 T antigen and HPV E7 binding to Rb also result in p53-mediated cell death and inactivation of p53 in these systems results in tumour development (Saenz Robles *et al.* 1994, Howes *et al.* 1994). Thus, the products of the *p53* and *Rb* tumour suppressor genes interact to prevent cellular transformation and tumour development as evidenced by the fact that oncogenic DNA viruses (HPV, SV40 and adenovirus) target both of the proteins in order to maintain cellular proliferation and inhibit cell death.

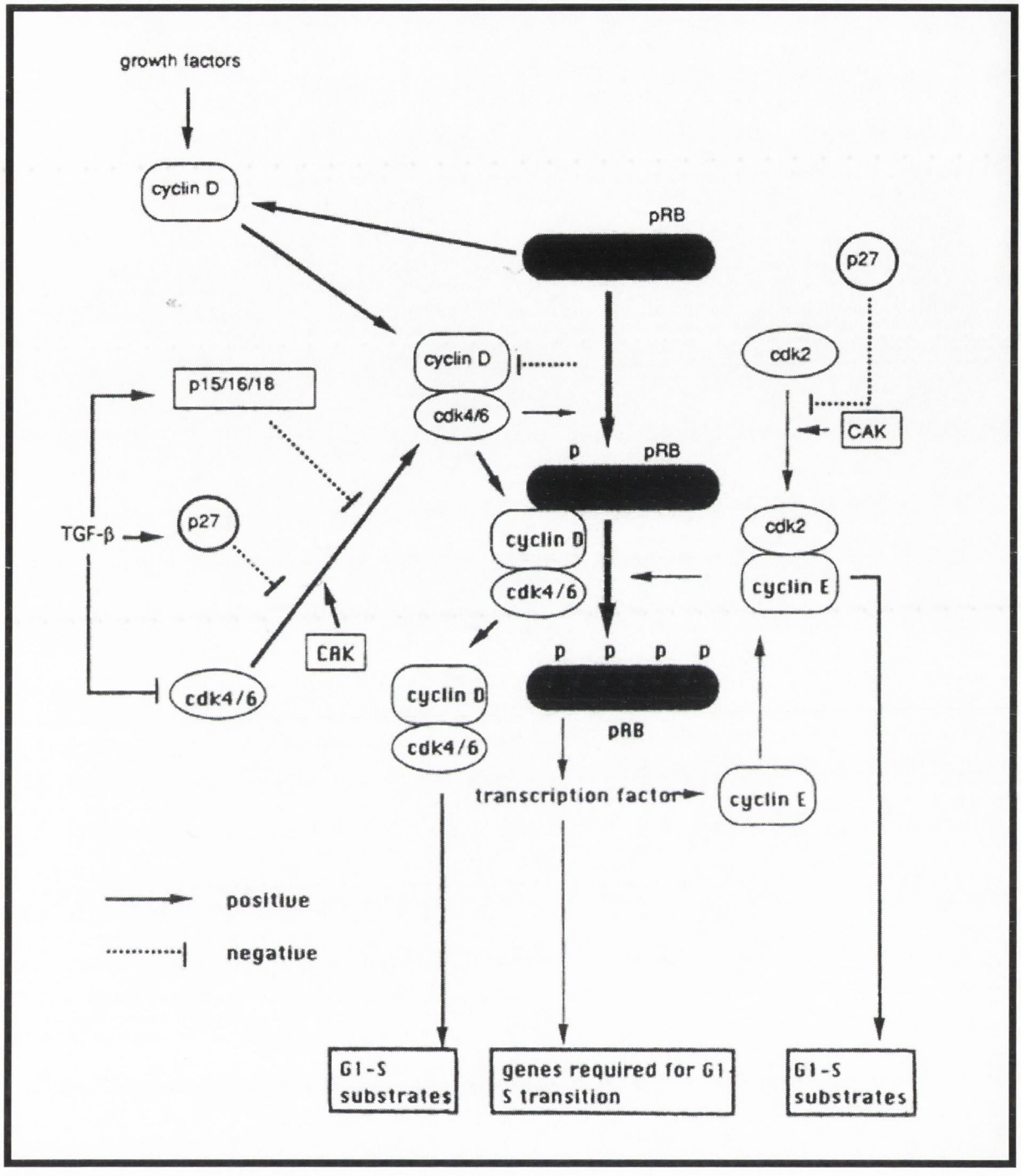


Figure 1.8 The functional relationship between the Rb tumour suppressor and cell cycle regulators. Adapted from Hatakeyama and Weinberg 1995.

1.4 Aims.

The oncogenic potential of mucosal HPV types is well documented. Knowing the high-risk HPV types involved has facilitated many molecular studies that have contributed to the understanding of how HPV plays a tumourigenic role in anogenital carcinomas. In the case of non-melanoma skin cancers, however, similar high-risk types are not known, indeed it is currently debatable as to whether HPV play a role in the genesis of cutaneous malignancies.

In an effort to determine if such a high-risk group of HPV types exist in cutaneous squamous cell carcinomas, this work describes an analysis of the prevalence of various types and subtypes of HPV in benign and malignant skin lesions from renal transplant recipients and immunocompetent skin cancer patients and in normal skin from the general population.

Deregulation of the p53 pathway is central to the genesis of many cancers. In HPV-positive anogenital cancers, p53 inactivation involves the viral E6 oncoprotein. It is not known whether HPV infection in cutaneous malignancies affects the p53 pathway. This work describes an analysis of the expression of p53 and other components of the p53 pathway in benign and malignant skin lesions from renal transplant recipients and immunocompetent skin cancer patients, firstly to determine if the pathway is inactivated and secondly to examine possible reasons why.

In addition to inactivation of the p53 pathway, high-risk mucosal HPV types also target the Rb cell cycle control pathway. There is no evidence to suggest that the HPV types found in cutaneous malignancies act in a similar manner. However, as the *p16-cyclin D_1-cdk4-Rb* pathway is altered in virtually all cancers, this work describes an

examination of the integrity of the *Rb* tumour suppressor gene in an effort to determine the means of deregulation of this pathway in cutaneous squamous cell carcinomas.

Finally, a common polymorphism of the *p53* tumour suppressor gene results in the substitution of an arginine and a valine residue at codon 72. It has recently been suggested that arginine homozygous individuals are at greater risk of developing HPV-associated cancers. This work describes an examination of the distribution of p53 codon 72 genotypes in skin cancer patients and an ethnically matched control population and correlates the genotypes present with the risk of developing HPV-associated skin cancer.

Chapter 2

The prevalence of human papillomaviruses in skin lesions from renal transplant recipients, immunocompetent skin cancer patients and in normal human skin from the general population

2.1 Introduction.

Non-melanoma skin cancers (NMSC) are the most frequent cancers in Caucasian populations. In Ireland, 15% of males develop skin cancer by the age of 75 (Irish National Tumour Register). In renal transplant recipients (RTR) the risk of neoplasia, particularly skin cancer, is greatly increased (London *et al.* 1995). The prevalence of post-transplant skin cancers is related to latitude, for example in Australia 70% of RTR develop skin cancer within 20 years of transplantation. In a recent study in the UK, 40% of RTR were found to develop a neoplastic lesion within 10 years post-transplant (Leigh and Glover, 1995). In RTR, squamous cell carcinomas (SCC) occur more frequently than basal cell carcinomas (BCC). The reverse is true of the general population (London *et al.,* 1995; Shiel *et al.,* 1985). The majority of tumours arise on sun-exposed sites implicating ultraviolet (UV) radiation in the oncogenic pathway. RTR also develop skin cancer 20-30 years earlier than immunocompetent patients (ICP), therefore immunosuppression is thought to play a pivotal role (Sequeira and Cutler, 1992). Though human papillomaviruses (HPV) are the most common viruses to infect human skin, they have until recently been largely overlooked as possible additional aetiological agents of skin cancer.

While UV-induced mutation of tumour suppressor genes undoubtedly occurs in cutaneous malignancies, not all SCCs display these features, therefore other oncogenic mechanisms (possibly involving HPV) are likely to play an important role. The following is a study to ascertain if any correlation could be established between a range of skin lesions and the presence of specific HPV types in both renal transplant recipients and immunocompetent skin cancer patients. The prevalence of HPV in normal human skin from both patient groups and the general population was also examined.

2.2 Methods.

2.2.1 Buffers, Solutions and Media.

Lysis Buffer

50 mM Tris-HCl (pH 8.5)

1 mM EDTA

0.5% Tween 20

H_2O 10ml

Luria Bertani (LB) Broth

10g Tryptone

5g Yeast extract

10g NaCl

H_2O 1000ml

LB agar

10g Tryptone

5g Yeast extract

10g NaCl

15g Agar

H_2O 1000ml

Long Ranger gel

6ml 10X TBE

6ml Long Ranger gel solution

27g Urea

H_2O to 60ml

350µl 10% APS

35µl TEMED

2.2.2 Tissue samples.

Forty-nine benign and malignant lesions comprising viral warts (19), SCCs (21) and normal human skin (NHS) (9) from 16 renal transplant recipients (11 viral warts, 9 SCCs and 5 NHS) and 18 immunocompetent skin cancer patients (8 viral warts, 12 SCCs and 4 NHS) were examined to determine the HPV types present (see figure 2.1 for examples of the lesions analysed in this study). Twenty NHS samples from the general population were also examined for HPV. All warts and SCCs were histologically proven. All RTR and ICP viral warts and SCCs were excised from sun-exposed sites (Figure 2.1). All RTR and ICP NHS was upper inner arm skin taken as a 6mm punch biopsy. NHS from the general population was a mixture of autopsy material and perilesional skin from routine excisions in the dermatology clinic. Autopsy material was taken from sun-exposed sites, primarily the back of the neck and perilesional skin was also taken from sun-exposed sites. Ethics committee approval was obtained for this study.

2.2.3 Tissue handling, sectioning and DNA extraction.

All tissue samples (viral warts, SCCs and NHS) were frozen in liquid nitrogen immediately after excision. They were sectioned at -20°C and a representative section was stained with haematoxylin and eosin for microscopic analysis. In the case of SCCs and viral warts, normal tissue was microdissected out and 3-5 10μm sections were taken for DNA extraction. One hundred microlitres of lysis buffer and 2μl of proteinase K (20mg/ml) were added to the tissue sections. The sections were incubated at 37°C

overnight. Following incubation, the samples were heated to 100°C for 10 min and spun in a microcentrifuge for 30s. A 3µl sample of the supernatant was taken for analysis by Polymerase Chain Reaction (PCR).

2.2.4 Amplification of p53 exon 7 from total genomic DNA extracted from human skin lesions.

At first, the supernatants were checked to confirm the presence of total genomic DNA. This was achieved by amplifying exon 7 of the p53 gene using the PCR primers 7PS/7PA (for primer sequences and cycling conditions see tables 2.1 and 2.2). An 8µl sample of the amplified DNA product was taken and analysed by agarose gel electrophoresis, followed by staining with ethidium bromide and visualisation under UV light.

2.2.5 Degenerate PCR amplification of HPV L1 gene fragments from total genomic DNA extracted from human skin lesions.

Once samples were shown to contain total genomic DNA a degenerate PCR with the primers MY09/MY11 was used to selectively amplify a 450bp fragment of the HPV L1 gene, which codes for the protein coat of the virus (for primer sequences and cycling conditions see tables 2.1 and 2.2). MY09/MY11 are generic HPV PCR primers, however they do not amplify EV types with good efficiency. Eight microlitre samples were taken and analysed as before.

2.2.6 Nested PCR amplification of HPV L1 gene fragments.

All samples were also analysed by a nested PCR using CP65/CP70 as external primers, and CP66/CP69 as internal primers to amplify a fragment of the HPV L1 gene of approximately 400bp (for primer sequences and cycling conditions see tables 2.1 and 2.2). Using both the degenerate MY09/11 primers and the EV-specific nested CP65-70 primer sets for the detection of HPV was recently shown to be much more effective than using either in isolation (Surentheran *et al.* 1998). Eight microlitre samples were taken and analysed as before. All PCR protocols contained positive, negative and contamination controls. Positive controls comprised HPV16 and HPV 20 clones (provided by G. Orth, Pasteur Institute), negative controls comprised total genomic DNA extracted from human blood and contamination controls comprised the PCR master mix without the addition of any target DNA. Initially, all PCR protocols were carried out using *Taq* polymerase. However, the cloning strategy used prior to sequencing of the PCR products required blunt-ended fragments and as *Taq* polymerase adds an adenine residue to the end of all amplified products, all positive samples were reamplified with *Pfu* polymerase, which generates blunt-ended PCR products, to facilitate cloning. When *Pfu* polymerase was used for the initial PCR amplification directly from the clinical samples, a significantly greater number of non-specific bands were observed.

2.2.7 Purification of HPV-specific gene fragments amplified by degenerate PCR.

All *Pfu*-generated PCR products were purified by TAE agarose (2%w/v) gel electrophoresis, subsequent excision of the correct band and recovery of the amplified DNA with the Wizard^{TM} PCR prep kit. The kit was used according to the manufacturer's recommendations with the following exceptions, i) the gel slice was incubated with the resin at room temperature rather than at 65°C and ii) the columns were spun for 1min rather than 30s. This resulted in a far greater percentage recovery of purified DNA.

2.2.8 Cloning of purified HPV L1 gene fragments into pCR Script AMP SK(+).

All purified fragments were then cloned into pCR Script AMP SK(+) in Epicurian Coli XL-1 Blue MRF' Kan supercompetent cells. Cloning and PCR screening of cloned inserts were carried out according to the manufacturer's instructions. All purified *Pfu*-generated PCR products were precipitated in ethanol and resuspended in the ligation mix. Positive clones were subcultured onto LB agar master plates containing 50µg/ml ampicillin, grown overnight at 37°C and screened by PCR for insert size. Using a sterile tip, a sample of each colony was taken and added to 50µl dH_2O, vortexed and subsequently boiled for 5min. The denatured samples were then spun for 2min in a microcentrifuge and 2µl of the supernatant was taken for analysis by PCR.

2.2.9 Purification of plasmid DNA.

Clones containing inserts of the correct size were grown overnight at 37°C in LB broth containing 50μg/ml ampicillin and plasmid DNA was recovered using the Wizard™ *Plus* SV miniprep kit as described by the manufacturers.

2.2.10 Cycle-sequencing of cloned HPV L1 gene fragments.

Due to both the degeneracy of the primers used to detect HPV in complex clinical samples, the highly sensitive nature of the nested PCR strategy used and the fact that cutaneous squamous cell carcinomas frequently harbour multiple HPV types, the sequencing of amplified fragments was difficult to achieve. After PCR, what appeared to be a discrete band was actually found to contain multiple DNA species. The net result of this was a high level of background sequencing when directly sequenced and analysed on a fluorescent sequencer.

To alleviate this problem numerous cloning and sequencing strategies were followed, all with varying levels of success. Initially, a direct cycle-sequencing strategy was attempted. PCR primers with attached M13 sequences were used to amplify target sequences, followed by cycle-sequencing with universal primer. This resulted in a large amount of background sequencing due to the fact that many of the samples demonstrated co-infection with multiple HPV types. Using the degenerate and nested primers directly for sequencing generated the same result. In order to separate the different types present in each lesion, the PCR bands were cloned. At first, a T-vector was used, however, many of the clones were found to contain no inserts, so a second

cloning strategy using the pCR Script vector was used. The final strategy used is a combination of the various techniques that were attempted.

Plasmid DNA was subjected to repeated primer extension sequencing using the Thermosequenase fluorescent labelled primer sequencing kit and analysed on an ALFexpress™ sequencer. Approximately 1µg of plasmid DNA was added to 10pmol of Cy-5 labelled universal M13 primer and dH_2O to a final master mix volume of 25µl. Two microlitres of the A, C, G and T reagents from the Thermosequenase kit were added to separate PCR tubes. Six microlitres of the master mix was added to each tube. A drop of mineral oil was placed on top of each reaction and the tubes were placed in the thermal cycler and subjected to 30 cycles of 94°C for 45s, 55°C for 45s and 72°C for 1 min. This generated approximately 8µg of finished product. When the cycling was finished, 4µl of formamide stop solution were added to each tube. Eight microlitres of each of the A, C, G and T reactions were loaded on the automated sequencer. On average 3 clones per sample were sequenced.

2.2.11 Database homology search of HPV sequences.

All sequences generated as detailed above were aligned with known HPV sequences in the Genbank Database using the Basic Local Alignment Search Tool (BLAST)(http:\\www.ncbi.nlm.nih.gov\BLAST)(Altschul *et al.,* 1990).

2.2.12 Analysis of HPV gene expression in RTR SCCs and a normal human skin sample.

Total RNA was extracted from 2 frozen SCCs and a frozen normal human skin sample using TRIzol™ reagent according to the maufacturer's instructions. Total RNA was then reverse transcribed to cDNA using Superscript II™ reverse transcriptase. Total RNA (0.5-2μg) was added to a microcentrifuge tube and the total volume made up to 10μl with DEPC-treated water. Random hexamers (0.5μg) were added to the tube and then heated to 70°C for 10 min and then cooled on ice. A master mix of the following was prepared: 1μl of DEPC-treated water, 0.5μl of Rnasin (38 units), 0.5μl of 10mM dNTPs, 4μl of RT buffer, 2μl of DTT and 1μl of Superscript II™ reverse transcriptase. Nine microliters of the master mix was then added to the tube containing the RNA and the random hexamers and incubated at 37°C for 16h. The reverse transcriptase was then inactivated by heating to 70C for 15 min and the cDNA stored at –20°C. Once the RNA was reverse transcribed, HPV 20 E6 and HPV 17 E6 sequences were amplified with the primers HPV20E6UP/LOW and HPV17E6UP/LOW (for primer sequences and cycling conditions see Table 2.1). An 8μl sample was taken and anaysed as described before. Positive controls of HPV 20 and HPV 17 clones were used in addition to negative and contamination controls as described in section 2.2.6.

2.3 Results.

2.3.1 PCR analysis of viral warts, SCCs and normal human skin.

After amplification with MY09/MY11, 78% of the RTR viral warts analysed (7/9) displayed a 450bp band (Figure 2.2A). All of the RTR warts were also analysed by nested PCR analysis with CP65/70 and CP66/69 and in this case 100% (11/11) displayed a band of approximately 400bp (Figure 2.2B). None of the ICP viral warts analysed displayed any bands after amplification with MY09/MY11 (Figure 2.2C). However, after analysis with the nested CP65-70 PCR, 100% (8/8) of the samples analysed demonstrated a possible HPV infection as indicated by the presence of a band of approximately 400bp (Figure 2.2D).

After the MY09/MY11 PCR, only two of the RTR SCCs (F2ET and F2GT) demonstrated 450bp bands (Figure 2.3A). In the case of the other RTR SCCs, while feint bands were visible, cloning and subsequent sequencing of the PCR products was impossible. All of RTR SCCs were also analysed by the more sensitive nested CP65-70 PCR. At this stage 89% (8/9) of the RTR SCCs analysed demonstrated a band of approximately 400bp indicative of the possible presence of HPV (Figure 2.3B). Only one RTR SCC (F25AT) was deemed to be HPV-negative following both PCR analyses. None of the ICP SCCs demonstrated any bands after analysis with the MY09/MY11 PCR (Figure 2.3C). However, after the nested CP65-70 PCR 83% (10/12) were positive (Figure 2.3D). Only two ICP SCCs (FT2 and FT5) were deemed to be HPV-negative following PCR analysis with both strategies.

Normal human skin from RTR and ICP was also analysed by both PCR methods. In the case of the MY09/MY11 amplification no bands were visible (Figure

2.4A), however, after amplification by the nested CP65-70 PCR, 33% (3/9) of the samples (N17A, FN1 and FN5) showed bands of approximately 400bp (Figure 2.4B). Normal human skin (perilesional skin and autopsy material) from the general population was also analysed by both PCR methods. After the MY09/MY11 PCR, none of the skin samples demonstrated any bands (Figure 2.5A and 2.5B). However, after the nested CP65-70 PCR, 15% (3/20) of the samples (NHS 8, NHS 9 and NHS12) were found to be positive (Figure 2.5C and 2.5D).

2.3.2 Fluorescent semi-automated sequencing of cloned HPV L1 gene fragments.

All of the samples that were positive after PCR amplification were cloned, sequenced, and analysed on the Pharmacia ALFexpress gene sequencer. Sequence data were only accepted if there were no ambiguities and an arbitrary minimum sequence length of 100 bases was chosen. All sequences generated were aligned with known HPV sequences in the Genbank sequence database.

The sequencing results for the RTR viral warts (Table 2.3) showed that all of the warts were HPV-positive and contained both cutaneous HPV types normally associated with common warts in the general population and EV-associated or novel EV-related HPV types when analysed with both PCR strategies. In the case of the ICP viral warts, all were found to be positive for HPV and were again found to harbour EV-associated HPV types or novel EV-related HPV types (Table 2.3). However, only one viral wart (WN1A) was found to harbour a HPV type normally associated with common warts (HPV2a).

In the case of the RTR SCCs analysed (Table 2.4), all of the HPV-positive samples were found to harbour EV-associated HPV types or novel EV-related HPV types and many also demonstrated infection with multiple HPV types (see Figure 2.6 for sample sequence and Figure 2.7 for sample alignment). The HPV-positive ICP SCCs again harboured HPV types normally associated with EV or novel EV-related HPV types and also frequently harboured multiple HPV types (Table 2.4).

The HPV-positive normal skin samples from both RTR and ICP were also found to contain EV-associated types or novel EV-related HPV types (Table 2.5) as were the HPV-positive normal human skin samples from the general population (Table 2.6). One NHS sample (Sample number 12) from the general population was also found to harbour an unknown HPV type.

Overall, 100% (19/19) of the viral warts and 86% (18/21) of the SCCs analysed were HPV-positive. Thirty-three percent (3/9) of the skin cancer patient's (RTR and ICP) NHS was HPV-positive and 15% (3/20) of the NHS from the general population harboured HPV. All of the HPV-positive samples were found to harbour EV-associated HPV types or novel EV-related HPV types and the RTR viral warts were also found to harbour common cutaneous HPV types. The most frequent HPV type detected was a novel HPV23-related type that was detected in 47% (9/19) viral warts (6 RTR and 3 ICP) and in 50% (5/10) HPV-positive ICP SCCs. A novel HPV38-related type was detected in 50% (4/8) of HPV-positive RTR SCCs. Together these two types were detected in 50% (9/18) of HPV-positive SCCs.

2.3.3 HPV gene expression in RTR SCCs and a normal human skin sample.

Following RT-PCR analysis, both of the tumour samples analysed demonstrated a 183bp band indicating the expression of HPV20 E6 (figure 2.8). The normal human skin sample analysed did not demonstrate any band, indicating that there was no expression of HPV17 E6 in that sample (figure 2.8). Both sets of primers readily amplified HPV E6 sequences from positive controls and the nature of the amplimers was confirmed by cloning and sequencing as described in sections 2.2.8-2.2.11.

Table 2.1 PCR primers used for detection of HPV and HPV gene expression in skin lesions of RTR and ICP and in NHS from the general population.

Primer	Sequence	Reference
7PA	5' TCTCCCCAAGGCGCACTG 3'	Bennett et al. 1996
7PS	5' AAGAGGTGGGCCCAGGGG 3'	Bennett et al.1996
MY09	5 'CGTCCMARRGGAWACTGATC 3'	Manos et al. 1989
MY11	5' GCMCAGGGWCATAAYAATGG 3'	Manos et al. 1989
CP65	5' CARGGTCAYAAYAATGGYAT 3'	Berkhout et al. 1995
CP66	5' AATCARMTGTTTRTTACWGT 3'	Berkhout et al. 1995
CP69	5' GWTAGATCWACATYCCARAA 3'	Berkhout et al. 1995
CP70	5' AAYTTTCGTCCYARAGRAWATTGRTC 3'	Berkhout et al. 1995
HPV20E6UP	5' ACTAAGATACCAACCGCACCC3'	This work
HPV20E6LOW	5' TTGGCAGCCTTACATTTCCTC 3'	This work
HPV17E6UP	5' ACCTTGTGTATTCCATTAGT 3'	This work
HPV17E6LOW	5' CTTGTGCTCTATTTCCTCTA 3'	This work

M=A or C, R=A or G, W=A or T, Y=C or T.

Table 2.2 Optimised PCR conditions for the detection of HPV and HPV gene expression in skin lesions from RTR and ICP and in NHS from the general population.

Primer pair	Cycling conditions	Product Size	[$MgCl_2$]	[dNTP]	[Primer]
7PS/7PA	95°C for 2 min, 61°C for 2 min, and 72°C for 2 min, 35 cycles	229bp	1.5 mM	0.25mM	30pmol/ 50µl rxn
MY09/11	95°C for 30s, 55°C for 30s, and 72°C for 1 min, 30 cycles.	450bp	1.5mM	0.25mM	30pmol/ 50µl rxn
CP65/70	95°C for 1 min, 50°C for 1.5 min, and 72°C for 2 min, 5 cycles 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, 35 cycles.	Approx. 400bp	3.4mM	0.25mM	30pmol/ 50µl rxn
CP66/69	95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, 30 cycles.	Approx. 400bp	3.4mM	0.25mM	30pmol/ 50µl rxn
HPV20E6UP/LOW	95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, 30 cycles	183bp	1.5mM	0.25mM	30pmol/ 50µl rxn
HPV17E6UP/LOW	95°C for 1 min, 48°C for 1 min, and 72°C for 2 min, 30 cycles	216bp	1.5mM	0.25mM	30pmol/ 50µl rxn

Table 2.3 HPV types detected in RTR and ICP viral warts.

Patient	Sample	Immune Status	MY09/MY11	CP65-70
1	W2A	RTR	HPV2a/HPV57	HPV23-rel
2	W14A	RTR	HPV57	HPV38-rel
	W14B		HPV10	HPVx14b
3	W37A	RTR	HPV10	RTRX5/RTRX6
4	W46A	RTR	HPV17	HPV23-rel
5	W50A	RTR	HPV10	HPV2a/HPV23-rel/RTRX10
6	W55A	RTR	nd	HPV23-rel
	W55B		nd	RTRX10
7	W56A	RTR	-ve	RTRTX10-rel
8	W57A	RTR	HPV57	HPV 23-rel
9	W58A	RTR	-ve	HPV23-rel
17	WN1A	ICP	-ve	HPV2a/HPV10-rel
18	WN2A	ICP	-ve	HPV20
	WN2B		-ve	HPV5-rel/HPV15-rel
19	WN5A	ICP	-ve	HPV23-rel
	WN5B		-ve	HPV23-rel
	WN5C		-ve	HPV20/HPV38-rel
20	WN6A	ICP	-ve	HPVx2b
21	WN7A	ICP	-ve	HPV23-rel

See appendix I for alignments of HPV types detected with known HPV types deposited in the Genbank database. The suffix –rel denotes novel HPV types and their most closely related known type.

Table 2.4 HPV types detected in RTR and ICP squamous cell carcinomas.

Patient	Sample	Immune Status	MY09/MY11	CP65-70
1	F2BT	RTR	-ve	HPV15
	F2CT	RTR	-ve	HPV36/RTRX1
	F2ET	RTR	HPV17/HPV38-rel	HPV36
	F2GT	RTR	HPV22	RTRX1/HPV38-rel
10	F23AT	RTR	-ve	HPV38-rel
11	F24AT	RTR	-ve	HPV38-rel
12	F25AT	RTR	-ve	-ve
13	F26AT	RTR	-ve	HPVx20
14	F27AT	RTR	-ve	HPV15
22	WN3A	ICP	-ve	HPV20
24	FT2	ICP	-ve	-ve
25	FT3	ICP	-ve	HPV20
26	FT4	ICP	-ve	HPV21
27	FT5	ICP	-ve	-ve
28	FT6	ICP	-ve	HPVx2b
29	FT7	ICP	-ve	HPV 23-rel
30	FT8	ICP	-ve	RTRX1
31	FT9	ICP	-ve	HPV 23-rel
32	FT10	ICP	-ve	HPV23-rel
33	FT11	ICP	-ve	HPV23-rel
34	FT13	ICP	-ve	HPV 23-rel

See appendix II for alignments of HPV types detected with known HPV types deposited in the Genbank database. The suffix –rel denotes novel HPV types and their most closely related known type.

Table 2.5 HPV types detected in RTR and ICP normal human skin.

Patient	Sample	Immune Status	MY09/MY11	CP65-70
1	N2A	RTR	-ve	-ve
2	N14A	RTR	-ve	-ve
4	N46A	RTR	-ve	-ve
15	N4A	RTR	-ve	-ve
16	N17A	RTR	-ve	RTRX9/HPV37/ HPV15-rel
23	FN1	ICP	-ve	HPV24/HPV17
24	FN2	ICP	-ve	-ve
25	FN3	ICP	-ve	-ve
26	FN5	ICP	-ve	HPV7

See appendix III for alignments of HPV types detected with known HPV types deposited in the Genbank database. The suffix –rel denotes novel HPV types and their most closely related known type.

Table 2.6 HPV types detected in the general population.

Volunteer	MY09/MY11	CP65-70
1	-ve	-ve
2	-ve	-ve
3	-ve	-ve
4	-ve	-ve
5	-ve	-ve
6	-ve	-ve
7	-ve	-ve
8	-ve	HPV38-rel/ HPV23-rel
9	-ve	HPV23
10	-ve	-ve
11	-ve	-ve
12	-ve	Unknown HPV
13	-ve	-ve
14	-ve	-ve
15	-ve	-ve
16	-ve	-ve
17	-ve	-ve
18	-ve	-ve
19	-ve	-ve
20	-ve	-ve

See appendix III for alignments of HPV types detected with known HPV types deposited in the Genbank database. The suffix –rel denotes novel HPV types and their most closely related known type.

Table 2.7 Summary of HPV types detected in skin lesions of RTR and ICP and in normal human skin from the general population.

Clinical Diagnosis	Histological Diagnosis	Percentage Positive	Predominant HPV types
Clinically "common warts and plane warts " (n=19)	Non-invasive squamous lesions	100% (19/19)	EV-associated types and common cutaneous types.
Normal Human Skin from skin cancer patients (n=9)	Normal Human Skin	33% (3/9)	EV-associated types
Normal human skin from the general population (n=20)	Normal Human Skin	15% (3/20)	EV-associated types
Clinically SCC (n=21)	Invasive SCC	86% (18/21)	EV-associated types

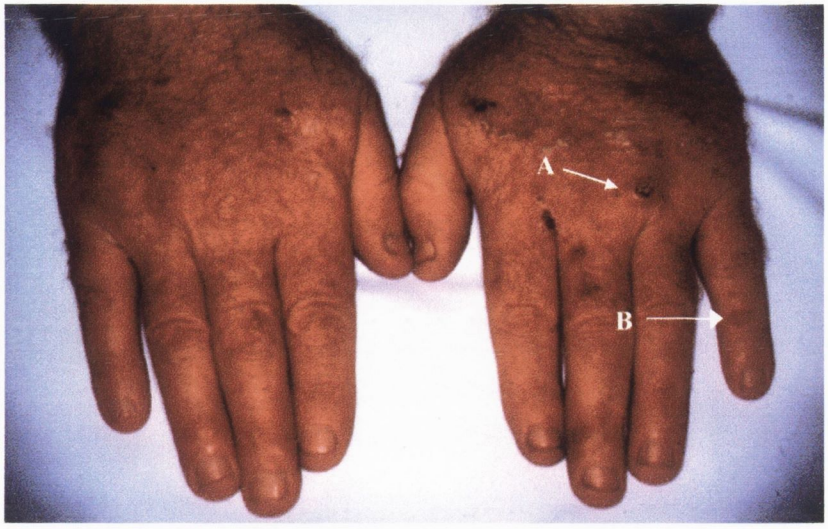


Figure 2.1 Photograph of the hands of a renal transplant recipient depicting examples of a cutaneous squamous cell carcinoma (A) and a viral wart (B) similar those analysed in this study.

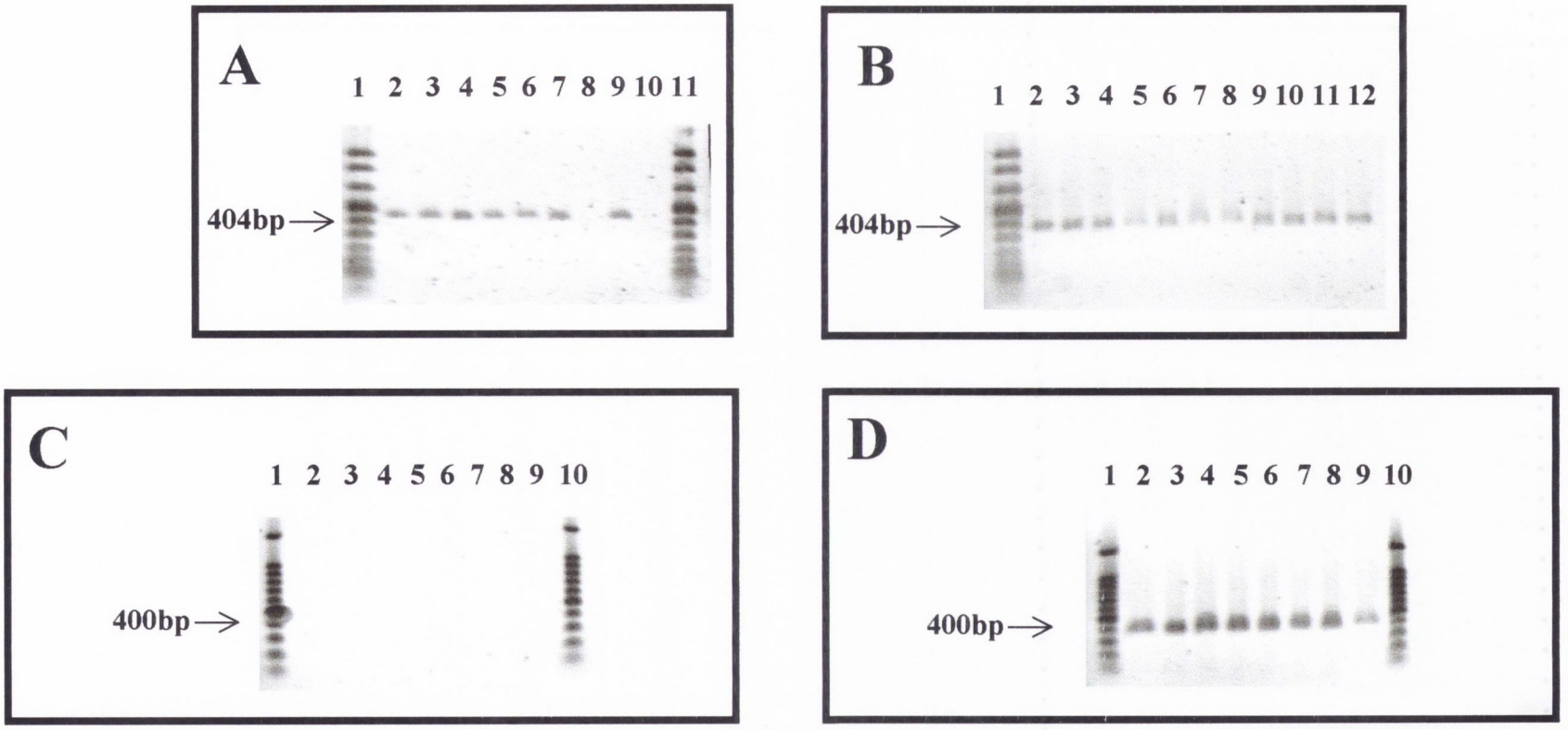


Figure 2.2. A 1%(w/v) agarose gel depicting the analysis of HPV in RTR viral warts by MY09/MY11 PCR. Positive samples show a distinct 450bp band. Lane 1 Molecular weight marker (HaeIII-digested pBluescript), Lane 2 W2A, Lane 3 W14A, Lane 4 W14B, Lane 5 W37A, Lane 6 W46A, Lane 7 W50A, Lane 8 W56A, Lane 9 W57A, Lane 10 W58A, Lane 11 Molecular weight marker(HaeIII-digested pBluescript). **B** 1% agarose gel depicting the analysis of HPV in RTR viral warts by CP65-70 PCR. Positive samples show a band of approximately 400bp. Lane 1 Molecular weight marker (HaeIII-digested pBluescript), Lane 2 W2A, Lane 3 W14A, Lane 4 W14B, Lane 5 W37A, Lane 6 W46A, Lane 7 W50A, Lane 8 W55A, Lane 9 W55B, Lane 10 W56A, Lane 11 W57A, Lane 12 W58A. **C** 1%(w/v) agarose gel depicting the analysis of HPV in ICP viral warts by MY09/MY11 PCR. Positive samples show a distinct 450bp band. Lane 1 Molecular weight marker (100bp ladder), Lane 2 WN1A, Lane 3 WN2A, Lane 4 WN2B, Lane 5 WN5A, Lane 6 WN5B, Lane 7 WN5C, Lane 8 WN6A, Lane 9 WN7A, Lane 10 Molecular weight marker (100bp ladder). **D** 1% agarose gel depicting the analysis of HPV in ICP viral warts by CP65-70 PCR. Positive samples show a band of approximately 400bp. Lane 1 Molecular weight marker (100bp ladder), Lane 2 WN1A, Lane 3 WN2A, Lane 4 WN2B, Lane 5 WN5A, Lane 6 WN5B, Lane 7 WN5C, Lane 8 WN6A, Lane 9 WN7A, Lane 10 Molecular weight marker (100bp ladder). All gels were stained with ethidium bromide and visualised under UV light.

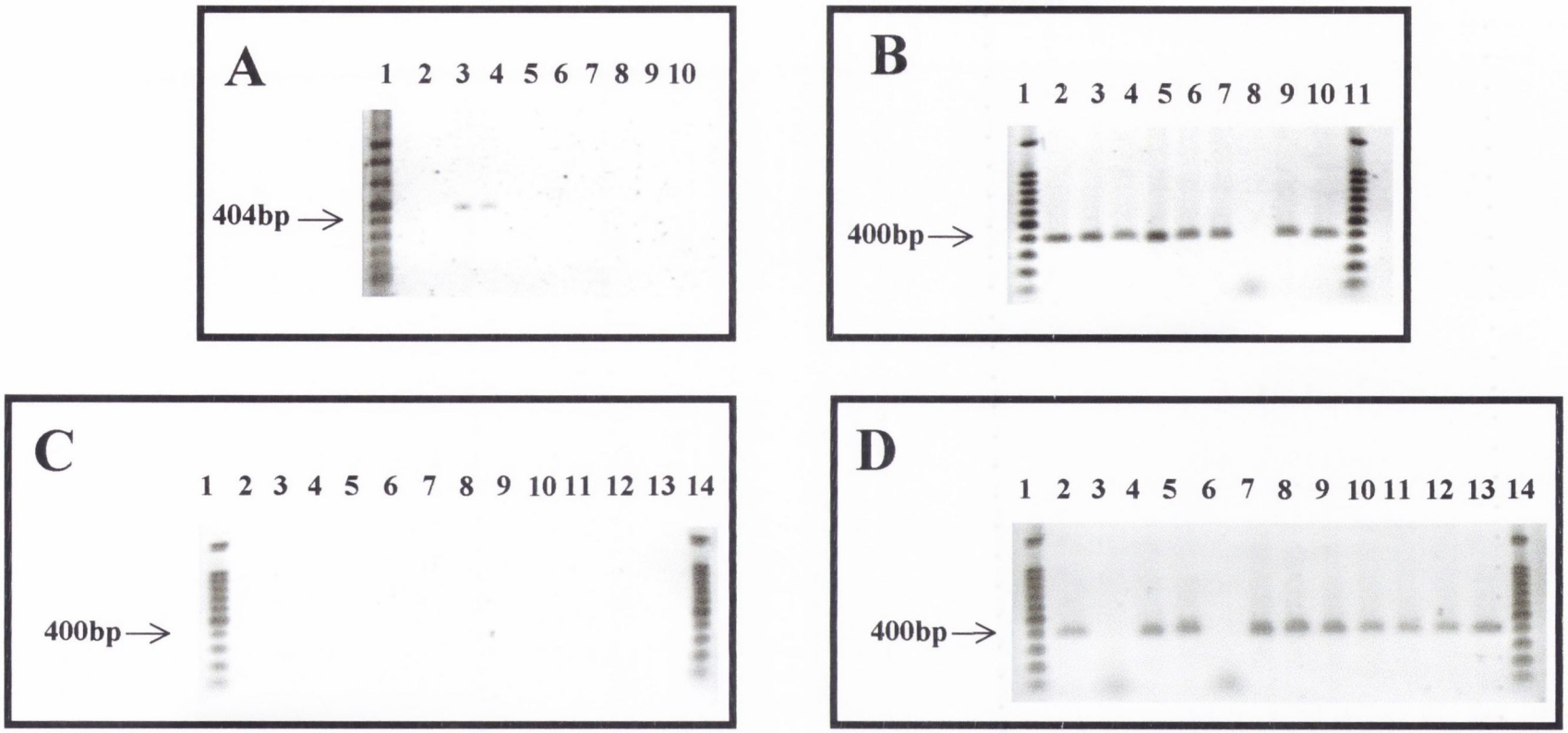


Figure 2.3. **A** 1%(w/v) agarose gel depicting the analysis of HPV in RTR SCCs by MY09/MY11 PCR. Positive samples again show a distinct 450bp band. Lane 1 Molecular weight marker (HaeIII-digested pBluescript), Lane 2 F2BT, Lane 3 F2CT, Lane 4 F2ET, Lane 5 F2GT, Lane 6 F23AT, Lane 7 F24AT, Lane 8 F25AT, Lane 9 F26AT, Lane 10 F27AT, Lane 11 Molecular weight marker (HaeIII-digested pBluescript). **B** 1% agarose gel depicting the analysis of HPV in RTR SCCs by CP65-70 PCR. Positive samples again show a distinct band of approximately 400bp. Lane 1 Molecular weight marker (100bp ladder), Lane 2 F2BT, Lane 3 F2CT, Lane 4 F2ET, Lane 5 F2GT, Lane 6 F23AT, Lane 7 F24AT, Lane 8 F25AT, Lane 9 F26AT, Lane 10 F27AT, Lane 11 Molecular weight marker (100bp ladder). **C** 1%(w/v) agarose gel depicting the analysis of HPV in ICP SCCs by MY09/MY11 PCR. Positive samples again show a distinct 450bp band. Lane 1 Molecular weight marker (100bp ladder), Lane 2 WN3A, Lane 3 FT2, Lane 4 FT3, Lane 5 FT4, Lane 6 FT5, Lane 7 FT6, Lane 8 FT7, Lane 9 FT8, Lane 10 FT9, Lane 11 FT10, Lane 12 FT11, Lane 13 FT13, Lane 14 Molecular weight marker (100bp ladder). **D** 1%(w/v) agarose gel depicting the analysis of HPV in ICP SCCs by CP65-70 PCR. Positive samples again show a distinct band of approximately 400bp. Lane 1 Molecular weight marker, Lane 2 WN3A, Lane 3 FT2, Lane 4 FT3, Lane 5 FT4, Lane 6 FT5, Lane 7 FT6, Lane 8 FT7, Lane 9 FT8, Lane 10 FT9, Lane 11 FT10, Lane 12 FT11, Lane 13 FT13, Lane 14 Molecular weight marker (100bp ladder). All gels were stained with ethidium bromide and visualised under UV light.

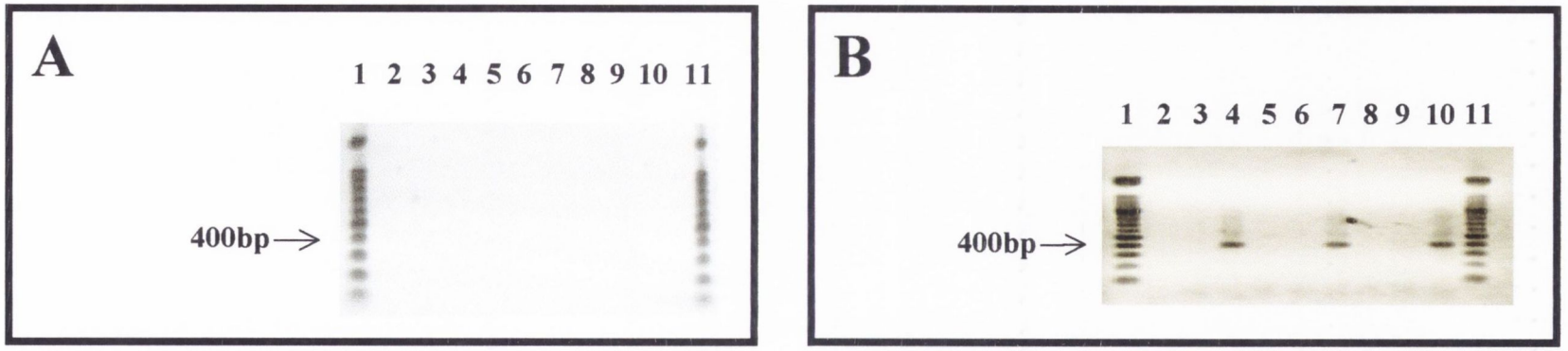


Figure 2.4. A 1%(w/v) agarose gel depicting the analysis of HPV in RTR and ICP normal human skin warts by MY09/MY11 PCR. Lane 1 Molecular weight marker (100 bp ladder), Lane 2 N2A, Lane 3 N4A, Lane 4 N17A, Lane 5 N14A, Lane 6 N46A, Lane 7 FN1, Lane 8 FN2, Lane 9 FN3, Lane 10 FN5, Lane 11 Molecular weight marker (100 bp ladder). **B** 1%(w/v) agarose gel depicting the analysis of HPV in RTR and ICP normal human skin warts by MY09/MY11 PCR. Positive samples show a distinct band of approximately 400bp. Lane 1 Molecular weight marker (100 bp ladder), Lane 2 N2A, Lane 3 N4A, Lane 4 N17A, Lane 5 N14A, Lane 6 N46A, Lane 7 FN1, Lane 8 FN2, Lane 9 FN3, Lane 10 FN5, Lane 11 Molecular weight marker (100 bp ladder). All gels were stained with ethidium bromide and visualised under UV light.

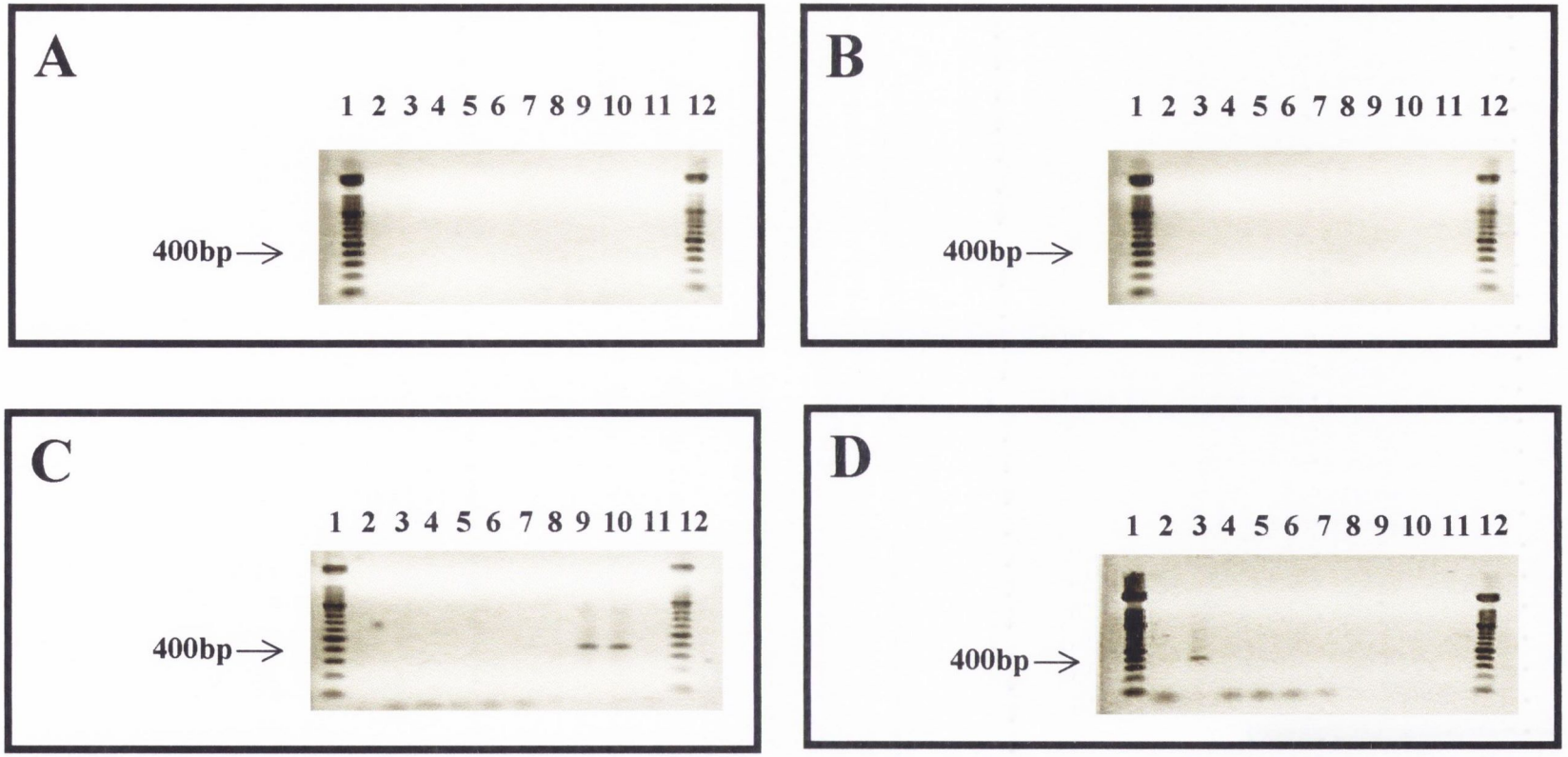


Figure 2.5. A 1%(w/v) agarose gel depicting the analysis of HPV in normal human skin from the control population by MY09/MY11 PCR. Lane 1 100 bp ladder Molecular weight marker, Lane 2 NHS1, Lane 3 NHS2, Lane 4 NHS3, Lane 5 NHS4, Lane 6 NHS5, Lane 7 NHS6, Lane 8 NHS7, Lane 9 NHS8, Lane 10 NHS9, Lane 11 NHS10, Lane 12 100 bp ladder Molecular weight marker. **B** 1% agarose gel depicting the analysis of HPV in normal human skin from the control population by MY09/MY11. Lane 1 100 bp ladder Molecular weight marker, Lane 2 NHS11, Lane 3 NHS12, Lane 4 NHS13, Lane 5 NHS14, Lane 6 NHS15, Lane 7 NHS16, Lane 8 NHS17, Lane 9 NHS18, Lane 10 NHS19, Lane 11 NHS20 Lane 12 100 bp ladder Molecular weight marker. **C** 1%(w/v) agarose gel depicting the analysis of HPV in normal human skin from the control population by CP65-70 PCR. Positive samples show a distinct band of approximately 400bp. Lane 1 100 bp ladder Molecular weight marker, Lane 2 NHS1, Lane 3 NHS2, Lane 4 NHS3, Lane 5 NHS4, Lane 6 NHS5, Lane 7 NHS6, Lane 8 NHS7, Lane 9 NHS8, Lane 10 NHS9, Lane 11 NHS10, Lane 12 100 bp ladder Molecular weight marker. **D** 1% agarose gel depicting the analysis of HPV in normal human skin from the control population by CP65-70 PCR. Positive samples again show a distinct band of approximately 400bp. Lane 1 100 bp ladder Molecular weight marker, Lane 2 NHS11, Lane 3 NHS12, Lane 4 NHS13, Lane 5 NHS14, Lane 6 NHS15, Lane 7 NHS16, Lane 8 NHS17, Lane 9 NHS18, Lane 10 NHS19, Lane 11 NHS20, Lane 12 100 bp ladder Molecular weight marker. All gels were stained with ethidium bromide and visualised under UV light.

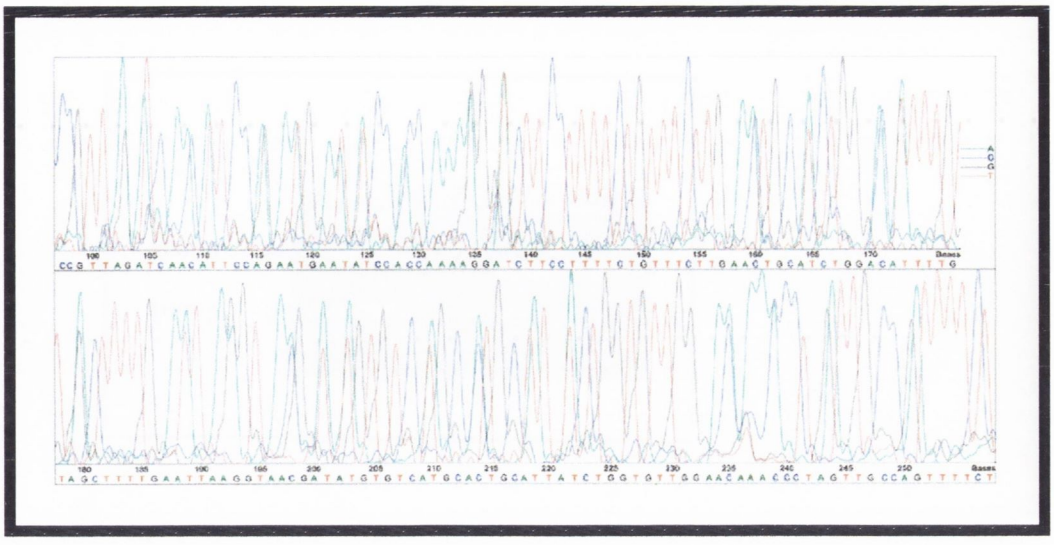


Figure 2.6 Sample sequence of HPV L1 PCR product generated on the ALFexpress semi-automated fluorescent sequencer from an RTR SCC (F2BT).

```
>gb:HPV15 Human papillomavirus type 15 genomic DNA.
          Length = 7412

  Plus Strand HSPs:

 Score = 1199 (331.3 bits), Expect = 1.0e-91, P = 1.0e-91
 Identities = 243/247 (98%), Positives = 243/247 (98%), Strand = Plus / Plus

F2BT :      2 AATCAGCTGTTTGTTACTGTAGCAGATAACACAAGGAATACAAATTTTACTATTAGTGTT 61
              ||||||| |||||| |||||||| ||||||||||||||||||||||||||||||||||||
HPV15:   6711 AATCAGATGTTTATTACTGTTGCAGATAACACAAGGAATACAAATTTTACTATTAGTGTT 6770

F2BT :     62 ACCTCTGATGGTAATGCCATAAATGAATATAATTCACAAGATATCAGAGAATTTTTAAGA 121
              |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
HPV15:   6771 ACCTCTGATGGTAATGCCATAAATGAATATAATTCACAAAATATCAGAGAATTTTTAAGA 6830

F2BT :    122 CATGTGGAAGAATATCAGTTATCTATTATTTTGCAATTGTGTAAAATACCTTTAAAAGCT 181
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV15:   6831 CATGTGGAAGAATATCAGTTATCTATTATTTTGCAATTGTGTAAAATACCTTTAAAAGCT 6890

F2BT :    182 GAGGTATTAACACAAATTAATGCTATGAATTCAGGTATTTTAGAAGACTGGCAACTAGGG 241
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HPV15:   6891 GAGGTATTAACACAAATTAATGCTATGAATTCAGGTATTTTAGAAGACTGGCAACTAGGG 6950

F2BT :    242 TTTGTTC 248
              |||||||
HPV15:   6951 TTTGTTC 6957
```

Figure 2.7 Sample alignment of generated sequence with known HPV sequence in the Genbank database using the BLAST homology search program.

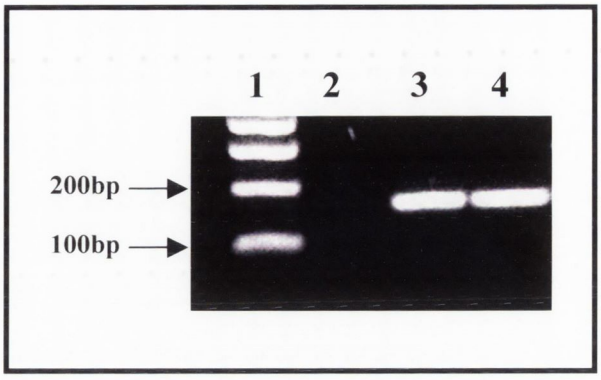


Figure 2.8 1% (w/v) agarose gel depicting the analysis of HPV E6 gene expression in two SCCs and a normal human skin sample. Lane 1 100bp ladder, Lane 2 normal human skin, Lane 3 SCC1, Lane 4 SCC2. Gel was stained with ethidium bromide and visualised under UV light. The presence of a 183bp band in the two tumour samples indicates the expression of HPV20 E6 in these samples. The absence of a 216 bp band in the normal human skin sample indicates that HPV17 E6 is not expressed in this sample.

2.4 Discussion.

Warts are ubiquitous in the general population. Immunity to HPV infection and the mechanisms underlying the regression of warts are poorly defined. However, cell-mediated immunity (CMI) is generally regarded as a major mechanism of defence against HPV and the clearance of warts. Thus any condition that results in a depletion of CMI might cause the incidence and severity of warts to increase, as well as the predilection for malignant conversion, should warts be a precursor for cancer. Epidermodysplasia verruciformis (EV) is a rare genetic disorder characterised by widespread cutaneous warts with an increased incidence of SCCs on sun-exposed sites. EV-patients also quite frequently demonstrate an impairment of helper T-cell function (Orth, 1987). In the case of the skin cancer patients analysed in this study, all of the tumours were isolated from sun exposed sites (predominantly the hands and forehead) and UV radiation is known to induce local immunosuppression (Kripke, 1984). In RTRs, chronic systemic immunosuppression coupled with local UV-induced immunosuppression places these patients at an even greater disadvantage. A depleted immune response is probably an important cofactor for tumour development in the face of viral persistence.

The evidence supporting a role for "high-risk" HPV types 16 and 18 in genital neoplasia is now compelling (Herrington, 1995), as is the association between HPV 5/8 and malignancy in EV (Orth, 1987; Pfister, 1992). The role of HPV in cutaneous cancers of renal transplant recipients and immunocompetent patients is, however, far from clear. Early Southern hybridisation and *in situ* hybridisation based studies often underestimated the prevalence of HPV in cutaneous lesions due to the specificity of the probes used (Barr *et al.,* 1989; Rudlinger, Grob, 1989). More recent polymerase chain

reaction (PCR) based studies have also generated varying results, which can again be attributed to the specificity and also the sensitivity, of the detection techniques employed (Smith *et al.,* 1993; Stark *et al.,* 1994b; Tieben *et al.,* 1994). In an effort to widen the range of detectable HPV types, Shamanin *et al* used 16 different PCR primer combinations to examine cutaneous SCCs from RTRs and ICPs. They demonstrated HPV DNA in 62% of RTR SCCs and in 32% of ICP SCCs. Multiple mucosal types were found in addition to the predicted cutaneous types, indicating that latent or subclinical infection with other HPV types may exist (Shamanin *et al.,* 1996).

Using a more sensitive nested PCR approach, HPV DNA has been detected in about 80% of RTR SCCs and EV-associated types (both known and novel EV-like HPV) were found to predominate (Berkhout *et al.,* 1995; de Jong-Tieben *et al.,* 1995; Hopfl *et al.,* 1997). Combinations of both EV-specific primers and degenerate mucosal/cutaneous primers have further widened the range of HPV types detectable in NMSC. Numerous studies have demonstrated the prevalence of HPV in RTR SCCs to be between 80% and 91% with EV-associated HPV types predominating and the prevalence of mucosal HPV types much lower than in the study by Shamanin *et al.* (de Villiers *et al.*, 1997; Surenthuran *et al.*, 1998; Harwood *et al.*, 1999a and Harwood *et al.*, 1999b). Indeed when the skin lesions analysed by Shamanin *et al.* were re-examined with EV-specific primers, most demonstrated coinfection with EV-associated HPV types. Harwood *et al.* also demonstrated EV-associated HPV in 75% of PUVA-associated NMSCs (Harwood *et al.* 1998).

The findings of this study are summarised in Table 2.7 and show that EV-associated HPV types predominate in SCCs and in viral warts (from both RTR and ICP). They also demonstrate the presence of EV-associated types in normal human skin from both patient groups and individuals without skin cancer albeit at a much lower

frequency. Based on these results, the conclusion that a single viral type represents a "high risk" type for cutaneous tumours cannot be drawn. The "high risk" types, if indeed HPV have a role to play in the development of skin cancer, may be the EV-associated HPV types as a group. If HPV are involved in malignant conversion and also if warts can progress to malignancy, based on these results, the warts most likely to undergo malignant conversion are those that harbour EV-associated HPV types. Clinical observations appear to support this hypothesis as clinically dysplastic lesions are often seen to progress to invasive SCC.

The absence of common cutaneous HPV types in all but one of the ICP viral warts analysed in this study, compared with the greater prevalence in RTR viral warts, may reflect a bias in sampling rather than a real difference in the viral aetiology of warts in immunocompetent individuals. Viral warts are routinely excised in transplant patients whereas in immunocompetent individuals they are treated with cryotherapy. The ICP viral warts in this study were excised because they clinically resembled SCCs, however, histologically they show no evidence of invasiveness and retain all of the features of viral warts. The atypical clinical appearance of the ICP viral warts may simply reflect the viral types they harbour.

The detection of EV-associated HPV types in the NHS analysed in this study is consistent with the findings of other researchers. Recent results suggest that EV-types can be detected in hair follicles of RTR and immunocompetent patients (Boxman *et al.*, 1997). EV-associated HPV types have also been found in both the general population and in skin cancer patients at a much higher frequency than was previously expected, indicating that a reservoir of these viruses may exist in a latent or subclinical state (Astori *et al.*, 1998; Harwood *et al.*, 1998 and Forslund *et al.* 1999). HPV 5, an EV-associated HPV type considered to be “high-risk” for cutaneous malignancies, has

recently been demonstrated to be prevalent in both immunosuppressed and immunocompetent individuals. The prevalence of HPV 5 was similar in both individuals with and without NMSC indicating that the virus is commonly found in the general population (Boxman *et al.* 1999). Forslund *et al.* recently demonstrated EV-associated HPV types in 75% of skin surface swabs taken from healthy volunteers and skin cancer patients (Forslund *et al.* 1999).

The detection of HPV in NHS that this study and others have shown questions the putative role of the EV-associated HPV types as co-carcinogens in NMSC. In an attempt to address this apparent contradiction a pilot study of EV-associated HPV E6 gene expression in SCCs and NHS was undertaken. Due to the relatively small quantities of total RNA available from the clinical samples that were HPV-positive, reverse transcriptase (RT)-PCR was chosen as the appropriate method to detect HPV E6 gene expression. The predominant HPV types detected in the SCCs in this study were only approximately 80% related to known EV HPV types and as such were not good candidates for an RT-PCR based study of HPV gene expression. This allied to the general heterogeneity of the papillomaviruses greatly hampered the study. Initial attempts to design a degenerate primer set for the E6 gene of all of the EV-associated HPV types were unsuccessful. The consensus/degenerate primer designing software CODEHOP could not generate any functional PCR primers for the EV HPV genes (http:\\bioinformatics.weizmann.ac.il\blocks\codehop.html) (Rose *et al.* 1998). Using the Clustal W multiple sequence alignment program, consensus regions in a large group of EV-related HPV types were identified (http:\\www2.ebi.ac.uk\clustalw)(Thompson *et al.* 1994). However, upon analysis with the Oligo 4.0 PCR primer design software (National Biosciences Inc., Plymouth MN, USA) none of the consensus regions contained any putative PCR primers. For these reasons, the expression of the HPV20

and HPV17 E6 genes alone were analysed as HPV20 was detected in two of the SCCs and HPV17 was detected in one of the NHS samples.

RT-PCR analysis of HPV20 E6 gene expression in the SCCs and HPV17 E6 gene expression in the NHS sample indicated that the E6 gene is expressed in the SCCs but not in the NHS. However, the logistical constraints of having to design separate PCR primers and optimise individual PCR protocols for each sample resulted in the discontinuation of the experiment. Should the observed differential expression of HPV genes in benign and malignant skin lesions be true, it may partially resolve the paradox of HPV-positive normal skin without malignancy.

The possible reasons why HPV should express E6 in malignant tissue but not in benign tissue are manifold. Firstly, the normal human skin sample analysed was from a transplant patient and was upper-inner arm skin and as such was not sun-exposed. Recently, a promoter of a novel HPV (HPV77) found only in cutaneous lesions was found to be UV responsive via a p53-dependent mechanism (Purdie *et al.* 1999) implicating UV as an important co-carcinogen (all of the SCCs analysed in this study were from sun-exposed sites, further illustrating this). Also, the expression of the E6 oncogene of "high-risk" mucosal HPV types is regulated by host-cell factors resulting in their down-regulation in replicating normal cells (zur Hausen 1989). Different papillomaviruses also demonstrate differential transcription activity in different cell types (Sailaja *et al.* 1999) and experimentally, different viral promoters demonstrate differential activity in benign and malignant human cells of skin origin (Artuc *et al.* 1995). Whatever the reason(s) governing differential HPV gene expression in benign and malignant skin cells, the weight of evidence implicating HPV in cutaneous NMSC is growing. Recent observations that mucosal HPVs inhibit Bak-induced apoptosis via their E6 oncoproteins (Thomas and Banks 1998) and that the ability to do so is

conserved among "high-risk" and "low-risk" mucosal HPV types (Thomas and Banks 1999), has since been shown to also apply to cutaneous HPV types (Jackson and Storey, unpublished data).

Chapter 3

Immunohistochemical and molecular genetic analysis of p53 in skin lesions from renal transplant recipients and immunocompetent skin cancer patients

3.1 Introduction.

Renal transplant recipients frequently develop numerous benign and malignant skin lesions (London *et al.* 1995). In chapter 2, the viral aetiology of these skin lesions was determined suggesting that EV-HPV may be involved in the evolution of cutaneous malignancies in both RTR and immunocompetent skin cancer patients. Studies on anogenital cancers have highlighted the potential role of HPV in human carcinogenesis however, clinical and experimental data imply that HPV alone is not sufficient to induce cancer.

The p53 tumour suppressor gene is the most frequently mutated gene in a wide range of human cancers (reviewed in Levine 1997). Under normal circumstances, the p53 gene product regulates the response of the cell to genotoxic stresses such as UV-radiation (reviewed in Giaccia & Kastan 1998 and Amundson *et al.* 1998). A decrease in p53 function (be it through mutation or otherwise) may lead to uncontrolled cellular proliferation, the accumulation of DNA damage and ultimately cancer.

Immunohistochemistry has been used to detect modified p53 proteins, since many of the mutations in the p53 coding region result in a structurally altered, inactive protein that is more stable than its wild type counterpart, resulting in high levels of protein detectable by antibody (Bartek *et al.* 1990, Rodrigues *et al.* 1990). However, inactivation of p53 can occur by other means such as mutation in the regulatory or non-coding regions of the p53 gene or by changes in other genes that affect the activation and stabilisation of the p53 protein.

Mutations due to direct absorption of UV light by DNA are predominantly C → T transitions at dipyrimidine sites (including CC → TT double base mutations) (Maher 1979, Miller 1985, Brash *et al.* 1987, McGregor *et al.* 1991). Characteristic UV-induced

mutations in the p53 gene have been observed in 8-48% of RTR SCCs and in up to 72% of sporadic skin tumours in the general population depending on technique used and population examined (Brash *et al.* 1991; Bennett *et al.* 1996; McGregor *et al.* 1997; Stark *et al,* 1994a).

The following describes an immunohistochemical study to evaluate the expression of p53 in benign and malignant skin lesions from renal transplant recipients and immunocompetent skin cancer patients. The effect of p53 mutations on the expression patterns observed was examined by PCR-SSCP analysis and direct cycle-sequencing. The expression of the p53-regulated cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ and Mdm2, the p53-induced protein involved in targeting p53 for ubiquitin-mediated degradation, were also examined in p53-positive lesions.

3.2 Methods.

3.2.1 Buffers and solutions.

SSCP sample loading buffer

95% formamide

20mM EDTA (pH 8.5)

0.1M NaOH

0.025% bromophenol blue

0.025% xylene cyanol

SSCP gel fixing solution

H_2O /ethanol/acetic acid (89.5/10/0.5 v/v)

SSCP gel silver stain

0.2g $AgNO_3$

200ml H_2O

SSCP gel developer solution

4.5g NaOH

30mg sodium borohydride

1.2ml formaldehyde (37%)

300ml H_2O

Wash Buffer A

100ml 20x SSC

10ml 10% SDS

890ml H_2O

Wash Buffer B

25ml 20X SSC

10ml 10% SDS

965ml H_2O

Wash Buffer C

5ml 20X SSC

10ml SDS

985ml H_2O

Tris Buffer 0.05M, pH 7.6

6.1g Tris

40ml 1M HCl

H_2O to 1000ml

Tris Buffered Saline

500ml Tris Buffer

38.25g NaCl

125µl Tween 20

H_2O to 5000ml

Hydrogen Peroxide Solution

9ml Hydrogen peroxide (30% w/v)

300ml H_2O

3.2.2 Samples for immunohistochemical analysis of p53/p21/Mdm2 expression.

A total of 15 SCCs (10 from RTR and 5 from ICP) and 15 viral warts (11 from RTR and 4 from ICP) were examined immunohistochemically for p53 expression. All p53-positive SCCs were examined for Mdm2 and p21 expression. All cases were histologically confirmed. The Positive control for p53 expression was a cutanoues squamous cell carcinoma with a known p53 mutation. A normal human skin biopsy taken 24h post-UV irradiation was used a a positive control for Mdm2 expression and a viral wart previously shown to be positive for p21 was used as a positive control for p21 expression.

3.2.3 Tissue section cutting of samples for immunohistochemical analysis of p53/p21/Mdm2 expression.

Paraffin embedded cutaneous squamous cell carcinomas, viral warts and NHS were sectioned on a microtome. To prevent any cross-contamination, the microtome blade was cleaned with ethanol prior to cutting the first section and in between each case. Gloves were worn throughout the sectioning process. Two 3μm sections were cut in each case and placed on Vectabond-treated glass slides. The slides were incubated at 55°C overnight to ensure adequate adhesion. Tissue section cutting was carried out by members of the RCSI Histopathology laboratory. The sections were dewaxed in two changes of xylene, cleared in two changes of alcohol and brought to water. Endogenous peroxidase activity was blocked by incubating the sections in hydrogen peroxide solution for 15 min. No antigen retrieval method was necessary to detect p53 expression. For the detection of p21 the sections were subjected to Pronase treatment (0.05% in Tris Buffered Saline (TBS) pH7.2) for 6 min at room temperature. For the detection of Mdm2 the sections were subjected to microwave pre-treatment in 0.01M sodium citrate solution (pH 6.0) for 22 min at full power (850W). The pre-treatment protocols and dilutions used for each antibody were previously determined in the Histopathology laboratory at Beaumont Hospital and are used for routine diagnostic immunohistochemistry.

3.2.4 Immunohistochemical detection of p53/p21/Mdm2 expression.

Following blocking of endogenous peroxidase and any antigen retrieval that was necessary, the slides containing the tissue sections were placed under running water for 5 min. The slides were then placed in incubation trays and the sections were covered in TBS pH 7.2 and incubated for 10 min. Excess TBS was then drained away and the area around the sections was dried with a clean tissue. The sections were then covered with normal goat serum (NGS) diluted 1/10 in TBS and incubated for 10 min. The NGS was then drained off and any excess wiped away. The upper section on each slide was then covered with p53/p21 or Mdm2 mouse monoclonal antibody at the appropriate dilution (see table 3.1 for antibody descriptions and dilutions) and incubated for 40 min. The lower (negative control) section on each slide was covered with TBS alone. The sections were then washed twice with TBS (4 min each wash). The TBS was then drained off, any excess wiped away and the sections were covered with goat antimouse secondary antibody (10µl reagent C from the Dako Duet kit mixed with 10µl NGS made up to 1ml with TBS) and incubated for 30 min. The sections were then washed twice with TBS (4 min each wash). The TBS was drained off and any excess was wiped away. The sections were then covered with streptavidin/biotinylated horseradish peroxidase (10µl reagent A and 10µl reagent B from the Dako duet kit made up to 1ml with TBS) and incubated for 30 min. The sections were then washed twice with TBS (4 min each wash). The TBS was drained off and any excess wiped away. The sections were then covered in approximately 300µl of DAB reagent, which was prepared according to the manufacturer's instructions. The sections were incubated with DAB reagent until brown nuclear staining was visible and then they were washed in running water. The sections were counterstained with Harris haematoxylin, dehydrated in two

changes of alcohol, cleared and coverslipped using a Tissue-Tek coverslipping machine. All immunohistochemistry slides were photographed using a Nikon FX 35mm camera at original magnification x 100 on a Nikon BIOPHOT microscope.

3.2.5 Samples for SSCP mutation screening.

A previous study of p53 mutation frequency in SCCs of RTR and ICP was carried out in our laboratory (Bennett *et al.* 1997). Six of the 15 SCCs examined for p53/p21/Mdm2 expression in this study were part of the previous study and therefore were not re-analysed. The remaining 9 SCCs were analysed by PCR-SSCP to determine their p53 status. In addition, 5 peripheral blood samples from RTR were analysed as normal controls. The SCC F25AT was previously shown to have point mutations in exons 5 and 7 of p53 and was used as a positive control for detection of mutations in exons 5 and 7. DNA extracted from the cell line SW680 known to have a mutation at codon 273 in exon 8 of the p53 gene was used as a positive control for the detection of mutations in exon 8. No positive control was available for exon 6.

3.2.6 PCR amplification of p53 exons 5-8.

Total genomic DNA was extracted from tumour and normal samples as described in section 2.2.3. Exons 5-8 of the p53 tumour suppressor gene were amplified using the primers described in table 3.2 under the conditions described in table 3.3. Typically,

100ng of total genomic DNA was used as template for all amplifications using *Taq* Polymerase (1.5U per 50µl reaction). Contamination and negative controls were included in all amplifications. PCR products were analysed by agarose gel (2% w/v) electrophoresis.

3.2.7 Southern blotting of PCR amplified p53 exons 5-8.

Following PCR amplification, two sample PCR products representing each exon were run on agarose gels (2% w/v). Following electrophoresis, gels were trimmed and denatured for 45min in 1.5M NaCl/0.5M NaOH with gentle agitation. The gels were then rinsed in deionised water and neutralised by soaking for 30 min in 1M Tris (pH 7.4)/1.5M NaCl with gentle agitation. The neutralisation solution was then changed and the gels further neutralised for 15 min, again with gentle agitation. Following neutralisation, DNA from the gels was transferred onto Zeto probe nylon filter membranes as described by Sambrook *et al.* Transfer was allowed to proceed for 18-24h, following which the nylon membranes were removed from the gels and neutralised by soaking in 0.5M Tris (pH 7.2)/1M NaCl for 15 minutes. The membranes were removed from the neutralising solution and allowed to dry on paper towels for approximately 30 min. When air-dried, the membranes were placed between two pieces of Whatman 3MM filter paper and baked for 2h at 80°C.

3.2.8 Random-primer labelling of p53 probe.

The radiolabelled p53 probe was generated using the Prime-a-gene random primer labelling system. Approximately 2μg of plasmid DNA was added to dH_20 to a final volume of 30μl. The DNA was denatured by heating to 100°C for 2 min and immediately quenched on ice. In a separate tube, the following components were added in order; 5μl of labelling buffer, 2μl of unlabelled dNTPs (dNTPs were prepared by mixing 1μl of each of dCTP/dGTP/dTTP to yield a 3μl solution), 1μg of denatured DNA, 2μl of BSA, 5μl of α-^{32}dATP, dH_20 to a final volume of 50μl and 1μl (5 units) of Klenow enzyme. The reaction was mixed gently and incubated at room temperature for 1h. The reaction was terminated by heating to 100°C for 2 min and adding 1μl of 1M EDTA. Unincorporated nucleotides were removed from the labelled probe by passing the reaction mixture through a Sephadex G-50 Nick column. The probe was eluted from the column with 2 x 400μl TE buffer, retaining the 2nd elution. The probe was heated to 100°C and kept on ice to keep the DNA single-stranded.

3.2.9 Hybridisation of radiolabelled p53 probe to Southern blotted p53 exons 5-8.

Radiolabelled p53 probe was hybridised to Southern blotted exons 5-8 in Rapid-hyb buffer. The Rapid-hyb buffer was prewarmed to 65°C. The nylon membranes were then completely immersed in the buffer and prehybridised for 1h at 65°C in cylindrical hybridisation bottles in a rotary hybridisation oven. Eighty microlitres of the nick column-purified probe was added and hybridisation was carried out at 65°C overnight.

Following hybridisation, the membranes were washed in wash buffer A at room temperature for 15 min. Wash buffer A was then discarded and the membranes were washed in wash buffer B again for 15 min at room temperature. Wash buffer B was discarded and the membranes were finally washed with wash buffer C for 15 min at 65°C. Following washing, the membranes were exposed to Cronex 10S x-ray film using Quanta rapid intensifying screens overnight at –70°C.

3.2.10 SSCP analysis of exons 5-8 of the p53 tumour suppressor gene.

A 4µl aliquot of each PCR product was mixed with 5µl of loading buffer and boiled for 15 min. Following boiling, all samples were immediately quenched on ice and then eletrophoresed for 24h at room temperature and 4°C (20mA) on 12% acrylamide gels containing 10% glycerol. Following electrophoresis, gels were fixed for 5min, stained in silver solution for 30 min and developed until the bands became clear (generally 5-20 min). Gels were visualised on a light box and photographed with a Polaroid camera. This protocol has been routinely used in the Biochemistry laboratory, RCSI for SSCP screening over the last decade.

3.2.11 Sequencing of SSCP bands with altered migration.

Sequencing of SSCP bands with altered migration was carried out by MWG-Biotech, Milton-Keynes, England.

3.3 Results.

3.3.1 p53 expression profiles in benign and malignant skin lesions of renal transplant recipients and immunocompetent skin cancer patients.

A squamous cell carcinoma with a known mutation in its *p53* gene was used as a positive control for the immunohistochemical study of p53 expression. The intensity and extent of staining in this lesion was classified as strong and in >80% of cells. The level and extent of p53 staining in all other lesions analysed in this study were compared with this positive control.

Two distinct expression patterns were observed in the viral warts examined (Table 3.4). Firstly, 60% (9/15) viral warts showed no expression of p53 either in the lesion itself or in the adjacent normal skin (Figure 3.1). The remaining 40% (6/15) showed weak to moderate staining in a few isolated p53-positive cells (<10%). Expression was basal or suprabasal in the lesion and in adjacent normal skin in 83% (5/6) of p53-positive cases (Figures 3.2 and 3.3), however one case (WN1A) showed no staining in the lesion itself but isolated foci of basal layer p53 expression in adjacent normal skin.

The expression of p53 in the SCCs analysed was distinctly different from the benign viral warts (Table 3.5). Three broad expression profiles were observed. Thirty-three per cent (5/15) of the SCCs showed no expression of p53 either in the invasive tumour or in adjacent normal skin (Figure 3.4). Thirty-three per cent (5/15) of the SCCs showed diffuse expression of p53 throughout the invasive component of the tumour. The numbers of positive cells (>30% to>80%) showed a striking increase compared with the viral warts and the intensity of staining was moderate to strong as opposed to

weak to moderate in the p53-positive viral warts. Sixty per cent (3/5) of the SCCs that showed diffuse p53 expression showed no expression of p53 in adjacent normal tissue (Figure 3.5) and the remaining 40% (2/5) showed focal basal layer expression in the adjacent tissue (Figures 3.6 and 3.7). A further 33% (5/15) of the SCCs showed basal layer expression of p53 (Figure 3.8). Eighty per cent (4/5) of the SCCs expressing p53 in the basal layer showed expression both in the tumour and in adjacent normal tissue and 20% (1/5) showed no expression in the tumour itself but focal basal layer expression in adjacent normal tissue. Again the numbers of positive cells ranged from >30% to >80% and the intensity of staining was moderate to strong.

Overall, 66% (10/15) of the SCCs analysed demonstrated p53 expression. The numbers of p53-positive cells ranged from >30% to >80% and the intensity of staining was moderate to strong.

3.3.2 PCR-SSCP and direct cycle-sequencing analysis of p53 mutations in SCCs from renal transplant recipients and immunocompetent skin cancer patients.

Following amplification with the primer combinations described in Table 3.2, all of the samples demonstrated bands of the correct size for each exon (Figure 3.9). Two random PCR products representing exons 5-8 of p53 were analysed by Southern blotting prior to SSCP analysis to confirm that the amplified product was p53-derived (Figure 3.9). The results of the PCR-SSCP analysis are shown in table 3.6. Only one of the 9 SCCs analysed in this study (F27AT) showed altered band migration when analysed by PCR-SSCP (Figure 3.10B). The altered migration suggested a mutation in exon 5 of p53 in this sample. The previously known mutations in exons 5 and 7 of p53 in the SCC

F25AT were detected (Figures 3.10A and 3.10D) as was the mutation in exon 8 of p53 in the SW680 cell line (Figure 3.10E). No altered migration of exon 6 bands was seen for any of the samples analysed (Figure 3.10C). Subsequent direct cycle-sequencing of bands with altered migration confirmed the mutations in F25AT and SW680. However, despite sequencing both strands in either direction, no mutation could be found in exon 5 of p53 in F27AT.

3.3.3 Expression of Mdm2 and $p21^{Waf1/Cip1}$ in cutaneous SCCs expressing wild type p53.

The expression of the Mdm2 oncoprotein in p53-positive SCCs was examined immunohistochemically. Previous studies in our laboratory have indicated that Mdm2 is expressed in normal sun-exposed skin downstream of the p53 response. No mdm2 expression could be detected in any of the tumours that demonstrated diffuse expression of p53, either in the invasive tumour or in the adjacent normal tissue. Mdm2 expression was also absent in the tumours that demonstrated basal layer expression of p53, again both in the invasive tumour and in the adjacent normal tissue (Figure 3.11). A positive control of a UV-irradiated normal skin biopsy taken 24h post-irradiation was included and Mdm2 expression was clearly demonstrated, indicating that the lack of expression detected was not false negativity (Figure 3.12).

The expression of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ was also examined in the p53-positive tumours by immunohistochemistry. A previous study in our laboratory indicated that $p21^{Waf1/Cip1}$ expression was co-localised with p53 expression in UV-irradiated normal skin. No $p21^{Waf1/Cip1}$ expression was detected in any of the tumours that exhibited diffuse p53 staining, either in the invasive tumour or in the

adjacent normal tissue. The expression of $p21^{Waf1/Cip1}$ was also absent in the tumours that demonstrated basal layer expression of p53, again in both the invasive tumour and in the adjacent normal tissue (Figure 3.13). A positive control of a viral wart known to express $p21^{Waf1/Cip1}$ was included and clearly demonstrated the expression of $p21^{Waf1/Cip1}$, indicating that the lack of expression detected was not due to false negativity (Figure 3.14).

Table 3.1 Antibodies used for the immunohistochemical analysis of p53/p21/Mdm2 expression in benign and malignant lesions from renal transplant recipients and immunocompetent skin cancer patients.

Antibody	Target	Working dilution	Antigen retrieval	Source
DO-7	p53	1/160	None	Dako
P21 (187)	p21	1/25	Pronase	Santa Cruz
Mdm2 (SMP7)	Mdm2	1/4000	Microwave	Santa Cruz

Table 3.2 PCR primers used for the amplification of p53 exons5-8 for SSCP analysis.

Primer	Sequence	Reference
5PS	5' TTC TTT GCT GCC GTG TTC CAG T 3'	Bennett *et al.* 1997
5PA	5' GTC ATG TGC TGT GAC TGC TTG TA 3'	Bennett *et al.* 1997
5*PS	5' TGT GCA GCT GTG GGG TTG ATT 3'	Bennett *et al.* 1997
5*PA	5' TCC ACT CGG ATA AGA TGC TG 3'	Bennett *et al.* 1997
6PS	5' CTG GGG CTG GAG AGA CGA 3'	Bennett *et al.* 1997
6PA	5' CCA CTG ACA ACC ACC CTT AA 3'	Bennett *et al.* 1997
7PS	5' TCT CCC CAA GGC GCA CTG 3'	Bennett *et al.* 1997
7PA	5' AAG AGG TGG GCC CAG GGG 3'	Bennett *et al.* 1997
8PS	5' GCT TCT CTT TTC CTA TCC 3'	Bennett *et al.* 1997
8PA	5' TGG TGT TGT TGG GCA GTG 3'	Bennett *et al.* 1997

Table 3.3 Optimised PCR conditions for amplification of p53 exons 5-8.

Primer pair	Cycling conditions	Product Size	[$MgCl_2$]	[dNTP]	[Primer]
5PS/PA	95°C 2 min, 45°C 2 min, 72°C 2 min, 32 cycles.	192bp	1.5mM	200µM	0.6µM
5*PS/PA	95°C 2 min, 58°C 2 min, 72°C 2 min, 32 cycles.	229bp	1.5mM	200µM	0.6µM
6PS/PA	95°C 2 min, 47°C 2 min, 72°C 2 min, 32 cycles.	221bp	1mM	200µM	0.6µM
7PS/PA	95°C 2 min, 61°C 2 min, 72°C 2 min, 32 cycles.	221bp	1.5mM	200µM	0.6µM
8PS/PA	95°C 2min, 50°C 2 min, 72°C 2 min, 32 cycles.	254bp	1.5mM	200µM	0.6µM

Table 3.4 p53 expression patterns in viral warts.

Sample	Lesion	Adjacent	Number of +ve cells	Pattern
W46A	++	++	<10%	Basal lesion/basal adjacent
WN1A	-ve	++	nd	Basal adjacent
W56A	+	+	<10%	Basal lesion/basal adjacent
W14B	+	+	<10%	Suprabasal lesion/suprabasal adjacent
W58A	+	+	<10%	Basal lesion/basal adjacent
W55A	+	+	<10%	Basal lesion/basal adjacent
WN2A	-ve	-ve	N/A	N/A
WN2B	-ve	-ve	N/A	N/A
WN4A	-ve	-ve	N/A	N/A
W50A	-ve	-ve	N/A	N/A
W37A	-ve	-ve	N/A	N/A
W14A	-ve	-ve	N/A	N/A
W57A	-ve	-ve	N/A	N/A
W55B	-ve	-ve	N/A	N/A
W2A	-ve	-ve	N/A	N/A

+ = Weak expression
++ = Moderate expression
nd = not determined

Table 3.5 p53 expression patterns in cutaneous squamous cell carcinomas.

Sample	Tumour	Adjacent	Number of +ve cells	Pattern	p53 Mutant	Mutation detection
F25AT	+++	-ve	>80%	Diffuse	YES	Bennett *et al.* 1997
F27AT	++	-ve	>30%	Diffuse	NO	This work
FT1	+++	-ve	>30%	Diffuse	NO	This work
F2DT	++	+++	>50%	Diffuse in tumour/basal adjacent.	NO	Bennett *et al.* 1997
F24AT	+++	+++	>80%	Diffuse in tumour/basal adjacent	NO	This work
FT3	-ve	-ve	N/A	N/A	NO	This work
F2GT	-ve	-ve	N/A	N/A	NO	Bennett *et al.* 1997
F23AT	-ve	-ve	N/A	N/A	NO	This work
FT2	-ve	-ve	N/A	N/A	NO	This work
FT4	-ve	-ve	N/A	N/A	NO	This work
F2ET	++	++	>80%	Basal tumour/basal adjacent	NO	Bennett *et al.* 1997
F2FT	++	++	<30%	Basal tumour/basal adjacent	NO	Bennett *et al.* 1997
F2AT	+++	+++	nd	Basal tumour/basal adjacent	NO	Bennett *et al.* 1997
FT5	++	++	>50%	Basal tumour/basal adjacent	NO	This work
F2CT	-ve	+++	nd	Focal basal expression in adjacent	NO	This work

++ = Moderate expression
+++ = Strong expression
nd = not determined

Table 3.6 SSCP analysis of p53 exons 5-8

Sample	Lesion	Exon 5	Exon 6	Exon 7	Exon 8
F2DT	SCC	WT	WT	WT	WT
F24AT	SCC	WT	WT	WT	WT
F25AT	SCC	MUT	WT	MUT	WT
F27AT	SCC	WT	WT	MUT	WT
FT1	SCC	WT	WT	WT	WT
FT2	SCC	WT	WT	WT	WT
FT3	SCC	WT	WT	WT	WT
FT4	SCC	WT	WT	WT	WT
FT5	SCC	WT	WT	WT	WT
B2	NHS	WT	WT	WT	WT
B24	NHS	WT	WT	WT	WT
B25	NHS	WT	WT	WT	WT
B28	NHS	WT	WT	WT	WT
B30	NHS	WT	WT	WT	WT

WT – wild-type
MUT – mutant (altered band migration)
SCC – Squamous cell carcinoma
NHS – Normal human skin

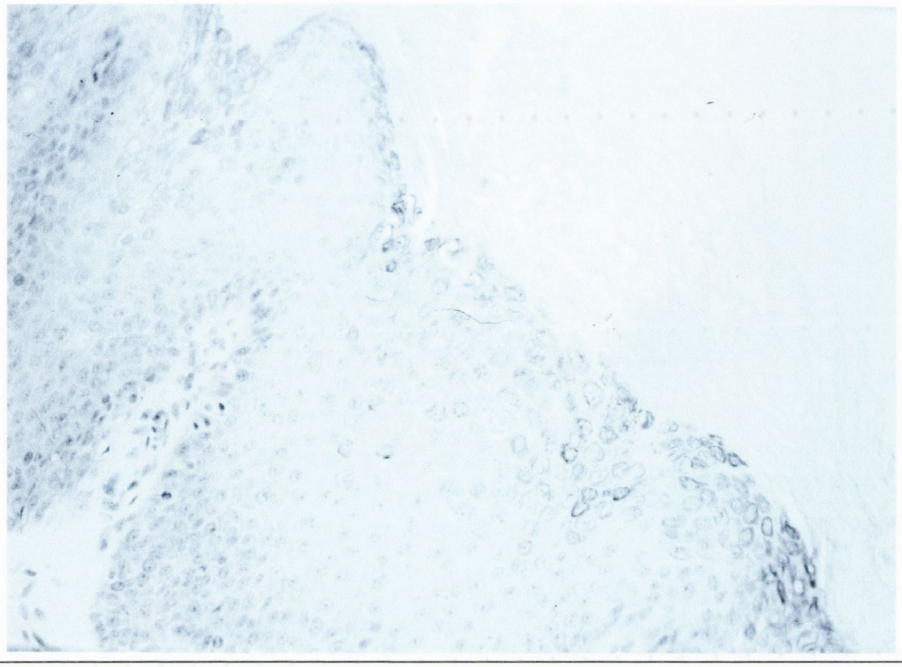
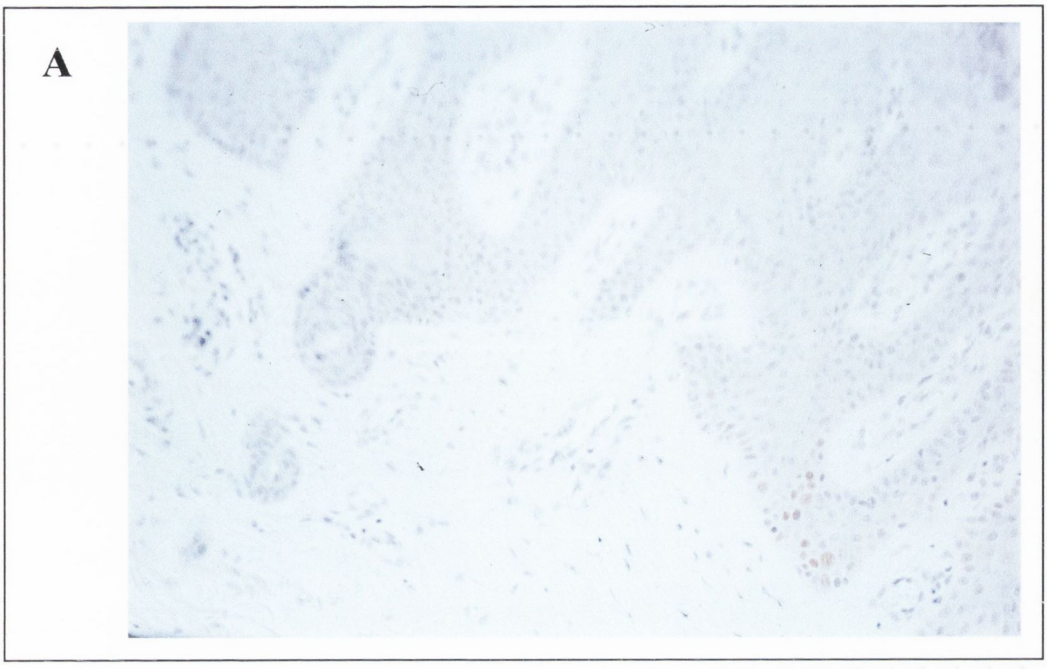


Figure 3.1 p53 immunostaining with the DO-7 antibody depicting negative p53 expression in the viral wart W50A.



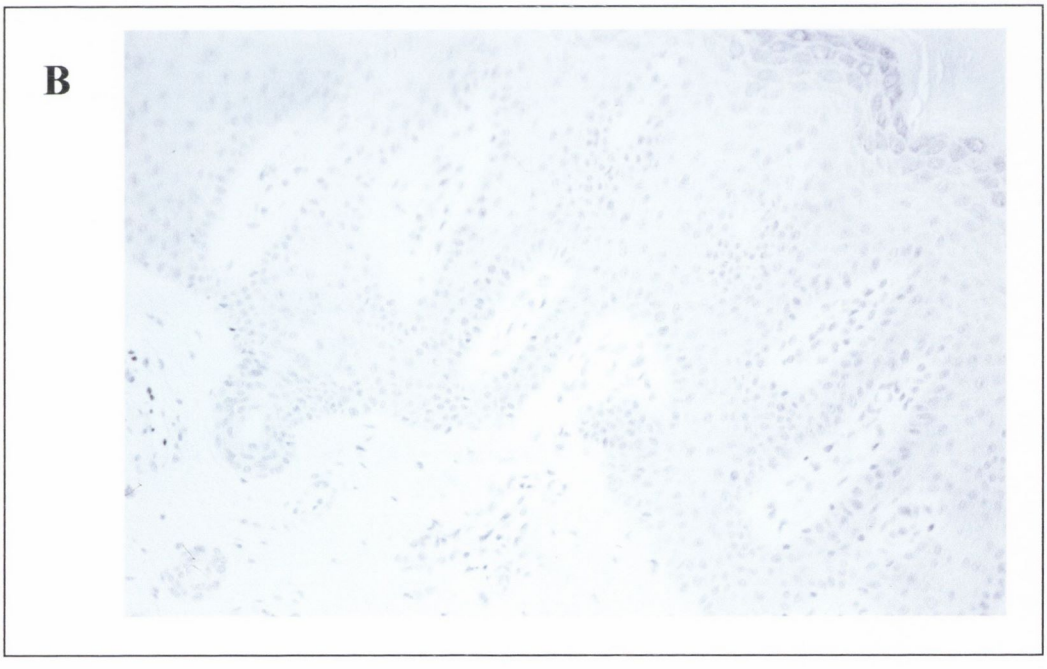
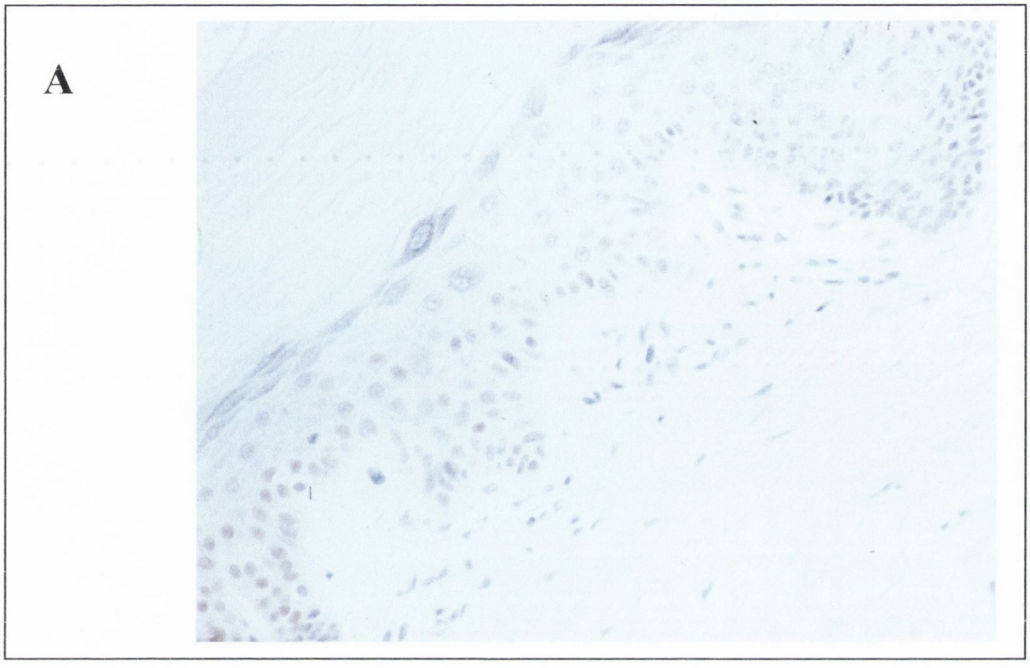


Figure 3.2 **A** p53 immunostaining with the DO-7 antibody depicting basal layer expression of p53 in the viral wart W46A. **B** Negative control of the above.



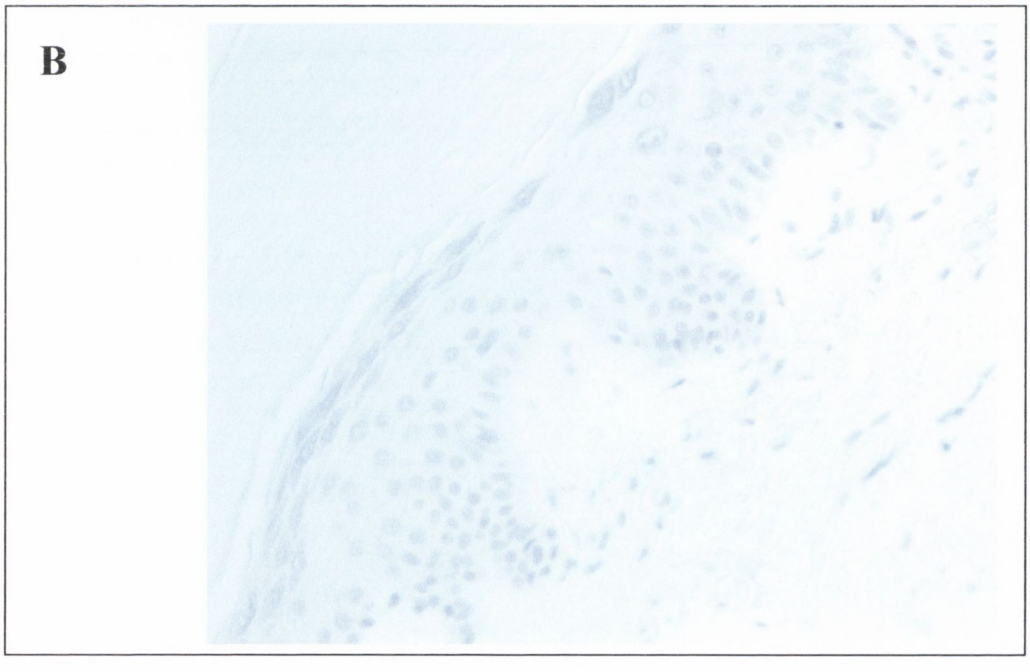


Figure 3.3 A p53 immunostaining with the DO-7 antibody depicting basal layer p53 expression in adjacent normal tissue of viral wart W46A. **B** Negative control of the above.

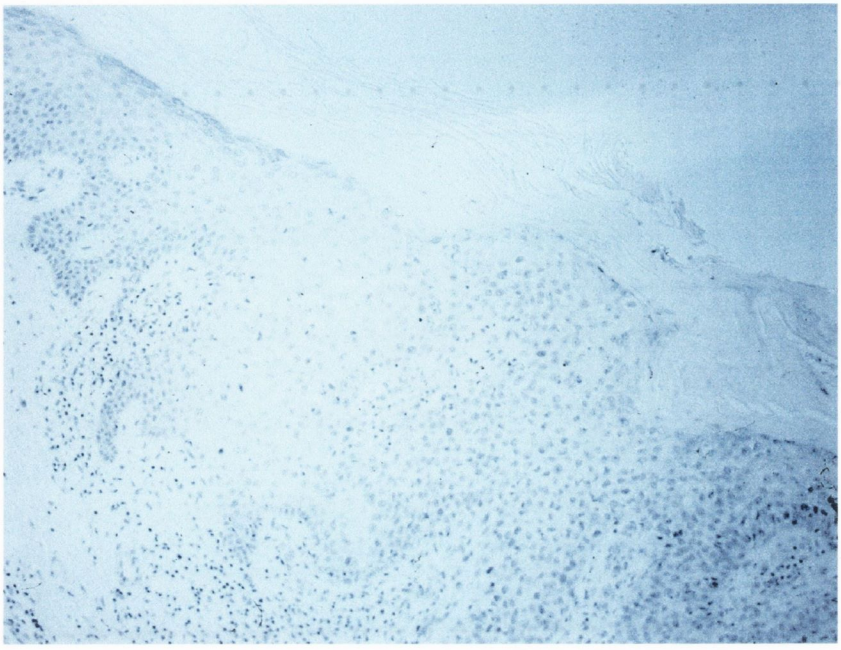


Figure 3.4 p53 immunostaining with the DO-7 antibody depicting negative p53 expression in the SCC FT2.

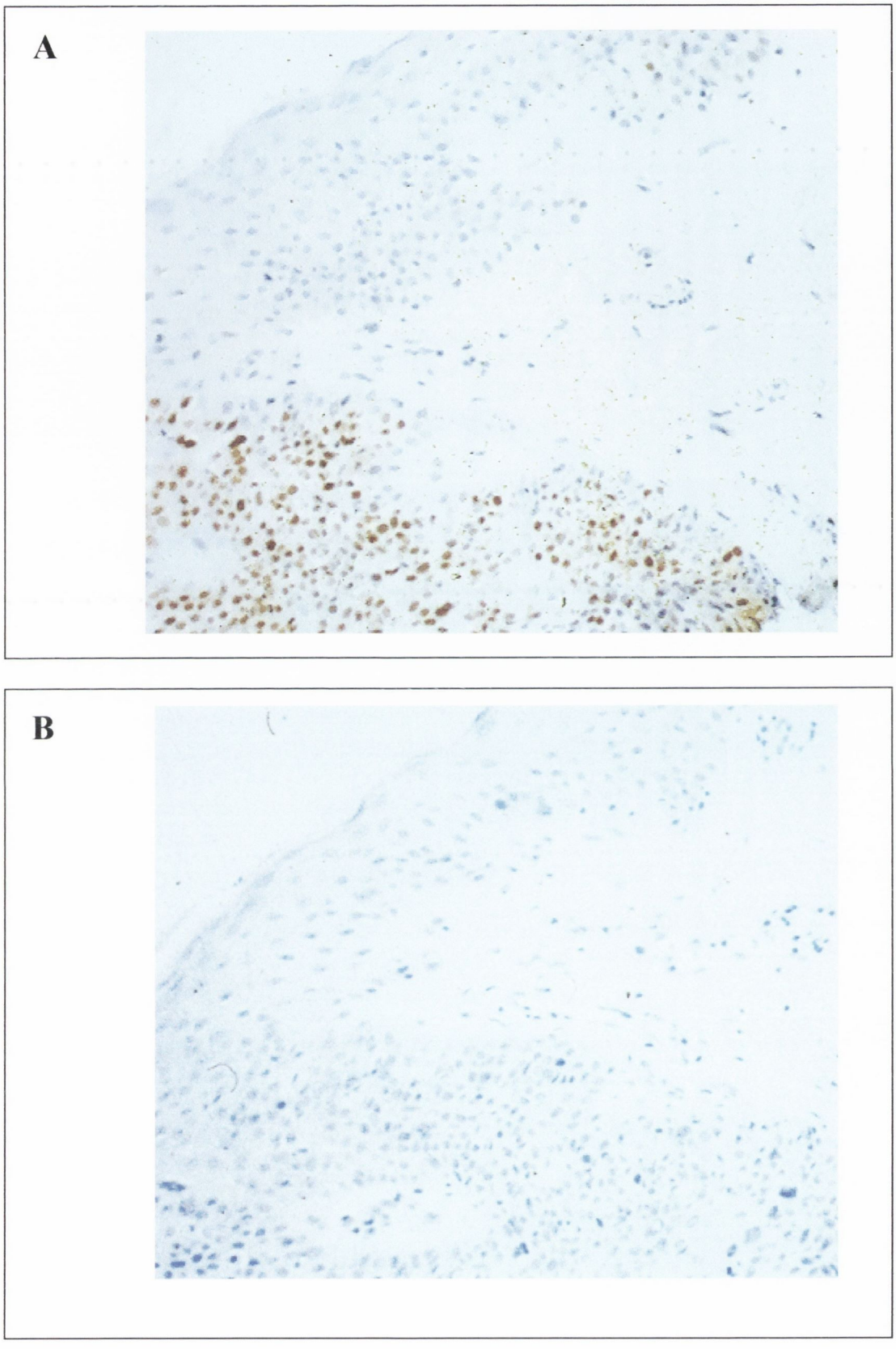
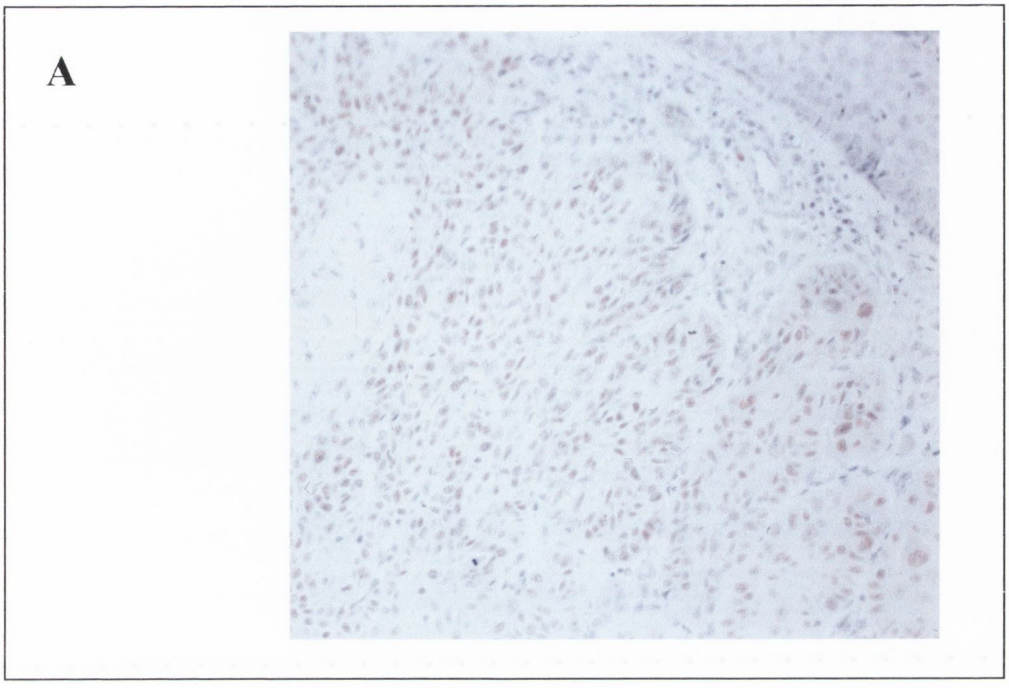


Figure 3.5 **A** p53 immunostaining with the DO-7 antibody depicting diffuse expression of p53 in the tumour F25AT and negative expression of p53 in adjacent normal tissue. **B** Negative control of the above.



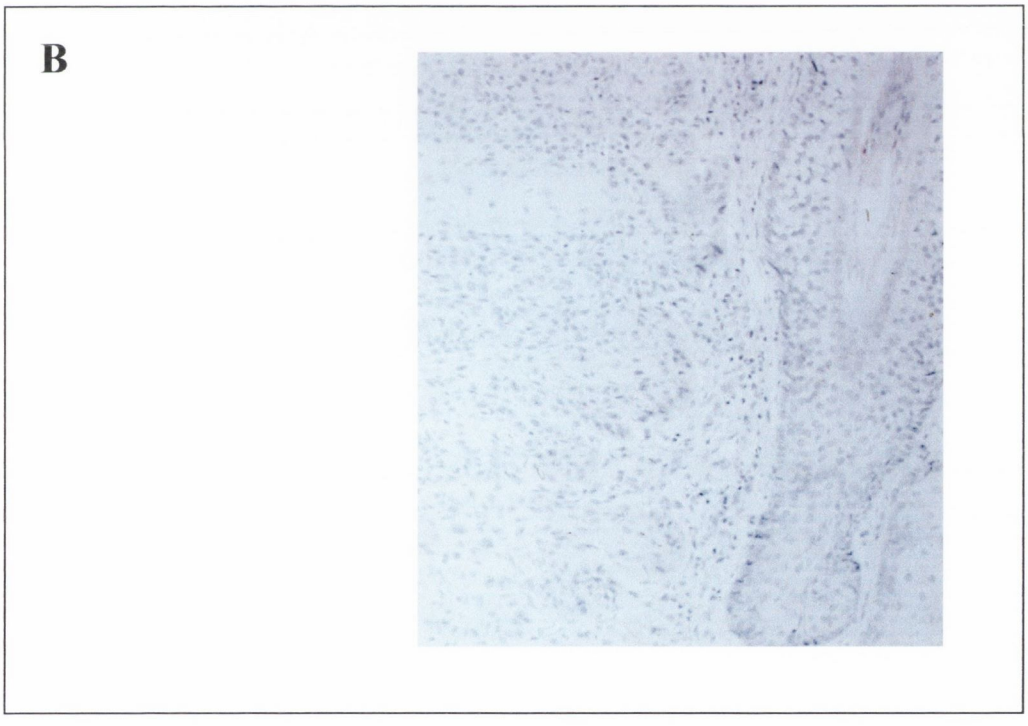


Figure 3.6 A p53 immunostaining with the DO-7 antibody depicting diffuse p53 expression in the SCC F2DT. **B** Negative control of the above.

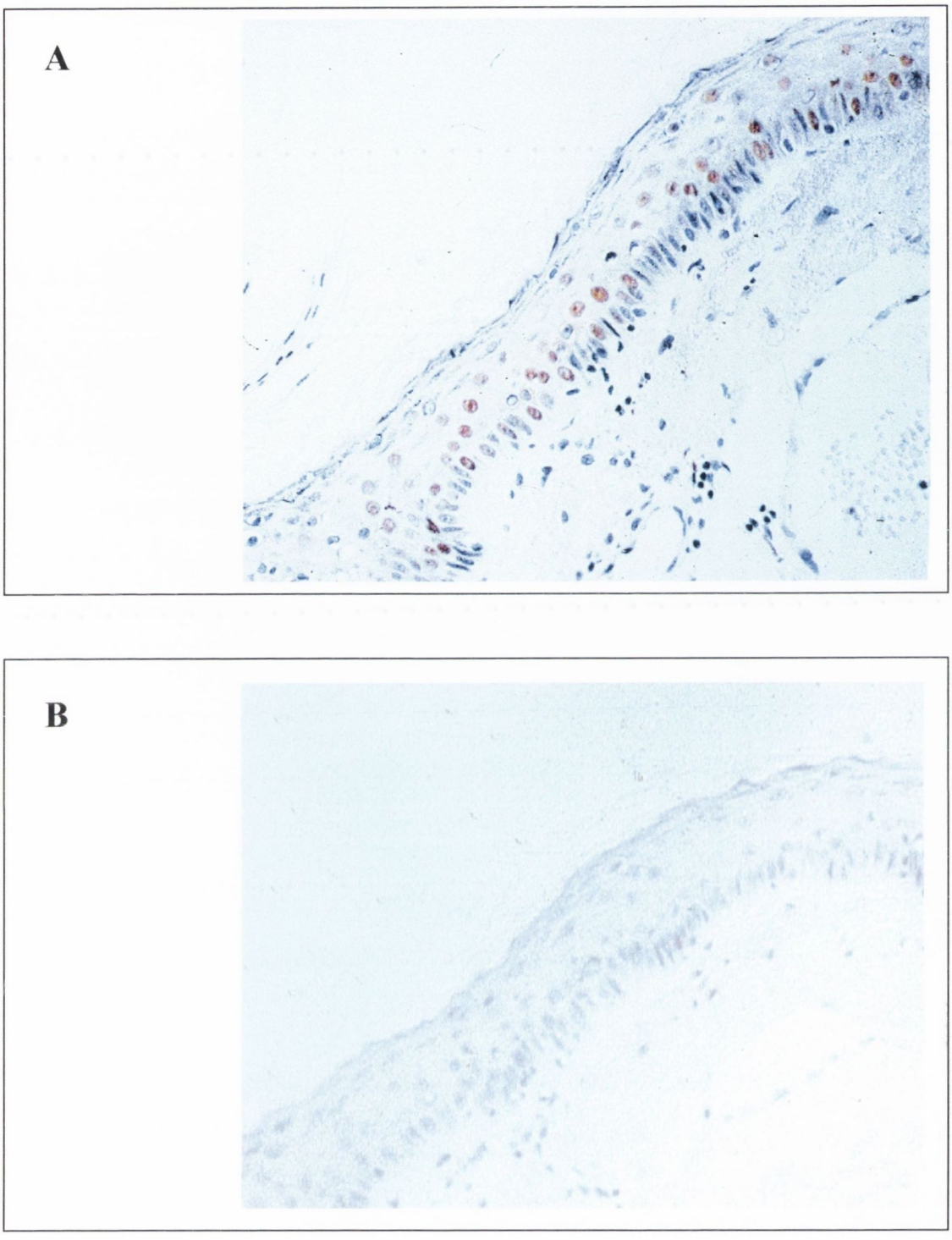


Figure 3.7 **A** p53 immunostaining with the DO-7 antibody depicting positive p53 expression in adjacent normal tissue in SCC F2DT. **B** Negative control of above.

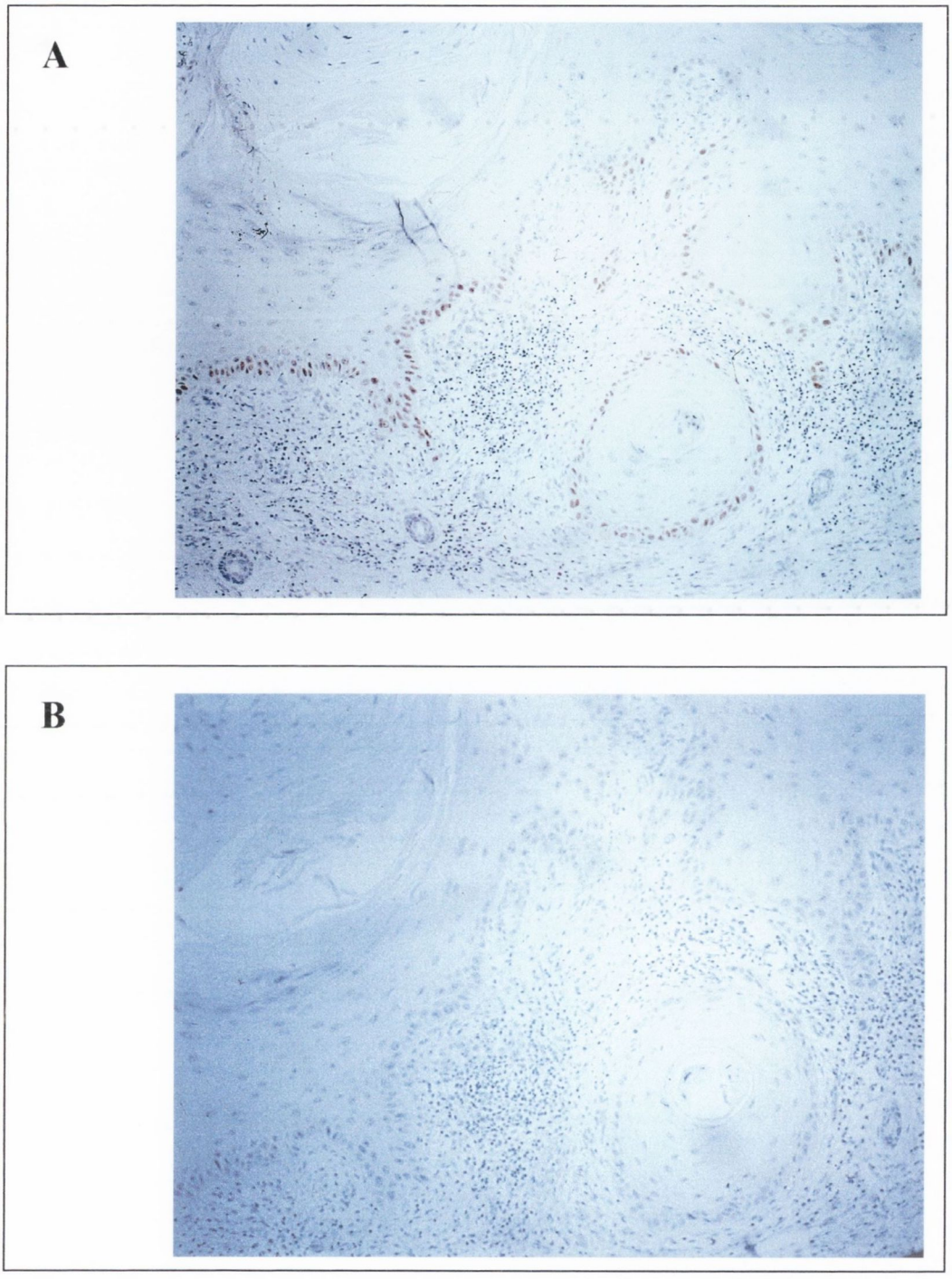


Figure 3.8 **A** p53 immunostaining with the DO-7 antibody depicting basal layer p53 expression in the SCC F2ET. **B** Negative control of the above.

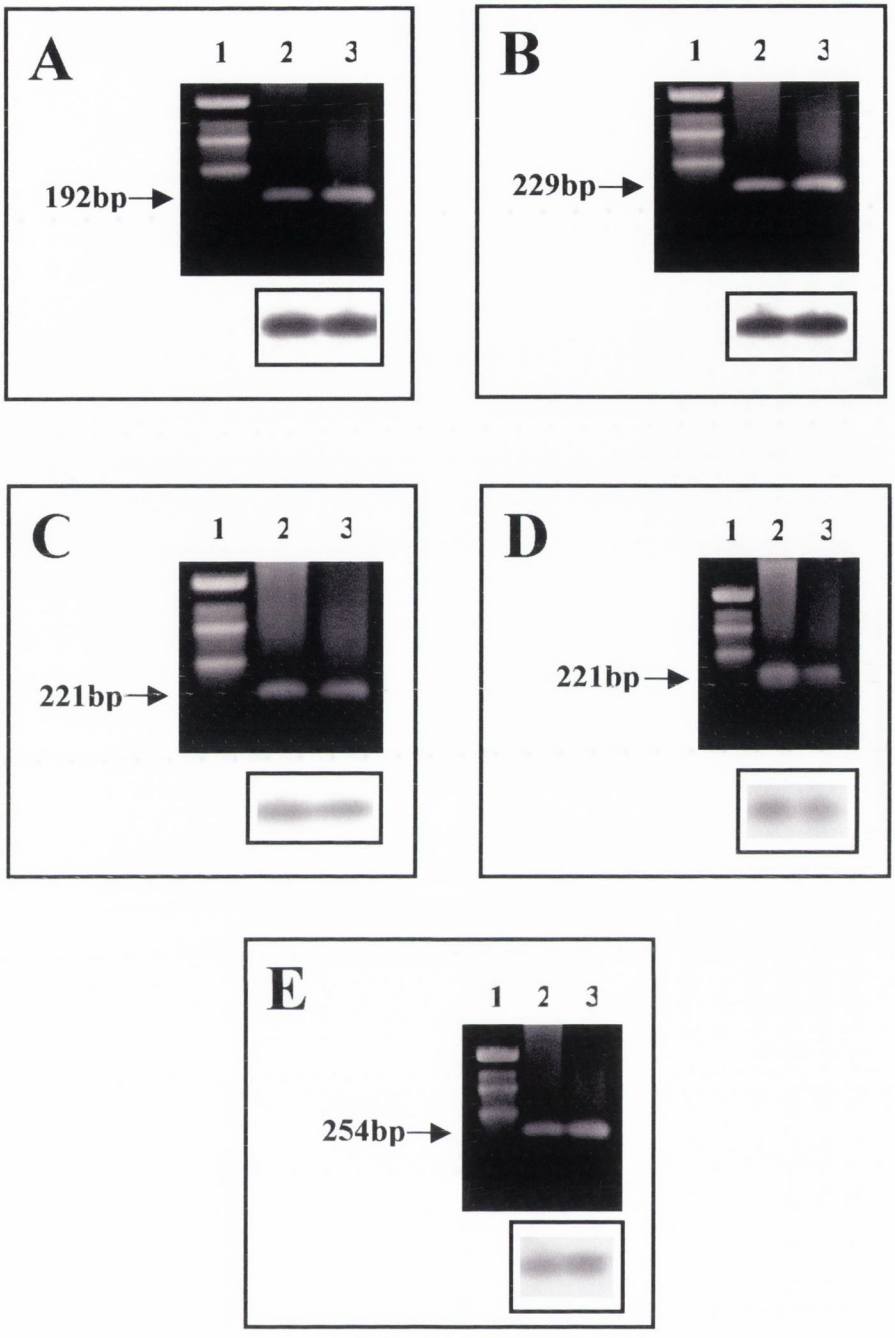


Figure 3.9 A Upper panel 1% (w/v) agarose gel depicting PCR amplified fragments of p53 exon 5 from two random SCCs. Lane 1, Hae III-digested ϕX174 DNA molecular weight marker, lanes 2 and 3, random SCCs showing amplification of a 192bp band of p53 exon 5 amplified with the primers 5PA/5PS. **Lower panel** Southern blot analysis of gel depicted in upper panel, probed with radiolabelled p53. **B Upper panel** 1% (w/v) agarose gel depicting PCR amplified fragments of p53 exon 5* from two random SCCs. Lane 1, Hae III-digested ϕX174 DNA molecular weight marker, lanes 2 and 3, random SCCs showing amplification of a 229bp band of p53 exon 5 amplified with the primers 5*PA/5*PS. **Lower panel** Southern blot analysis of gel depicted in upper panel, probed with radiolabelled p53. **C Upper panel** 1% (w/v) agarose gel depicting PCR amplified fragments of p53 exon 6 from two random SCCs. Lane 1, Hae III-digested ϕX174 DNA molecular weight marker, lanes 2 and 3, random SCCs showing amplification of a 221bp band of p53 exon 6 amplified with the primers 6PA/6PS. **Lower panel** Southern blot analysis of gel depicted in upper panel, probed with radiolabelled p53. **D Upper panel** 1% (w/v) agarose gel depicting PCR amplified fragments of p53 exon 7 from two random SCCs. Lane 1, Hae III-digested ϕX174 DNA molecular weight marker, lanes 2 and 3, random SCCs showing amplification of a 221bp band of p53 exon 7 amplified with the primers 7PA/7PS. **Lower panel** Southern blot analysis of gel depicted in upper panel, probed with radiolabelled p53. **E Upper panel** 1% (w/v) agarose gel depicting PCR amplified fragments of p53 exon 8 from two random SCCs. Lane 1, Hae III-digested ϕX174 DNA molecular weight marker, lanes 2 and 3, random SCCs showing amplification of a 254bp band of p53 exon 8 amplified with the primers 8PA/8PS. **Lower panel** Southern blot analysis of gel depicted in upper panel, probed with radiolabelled p53.

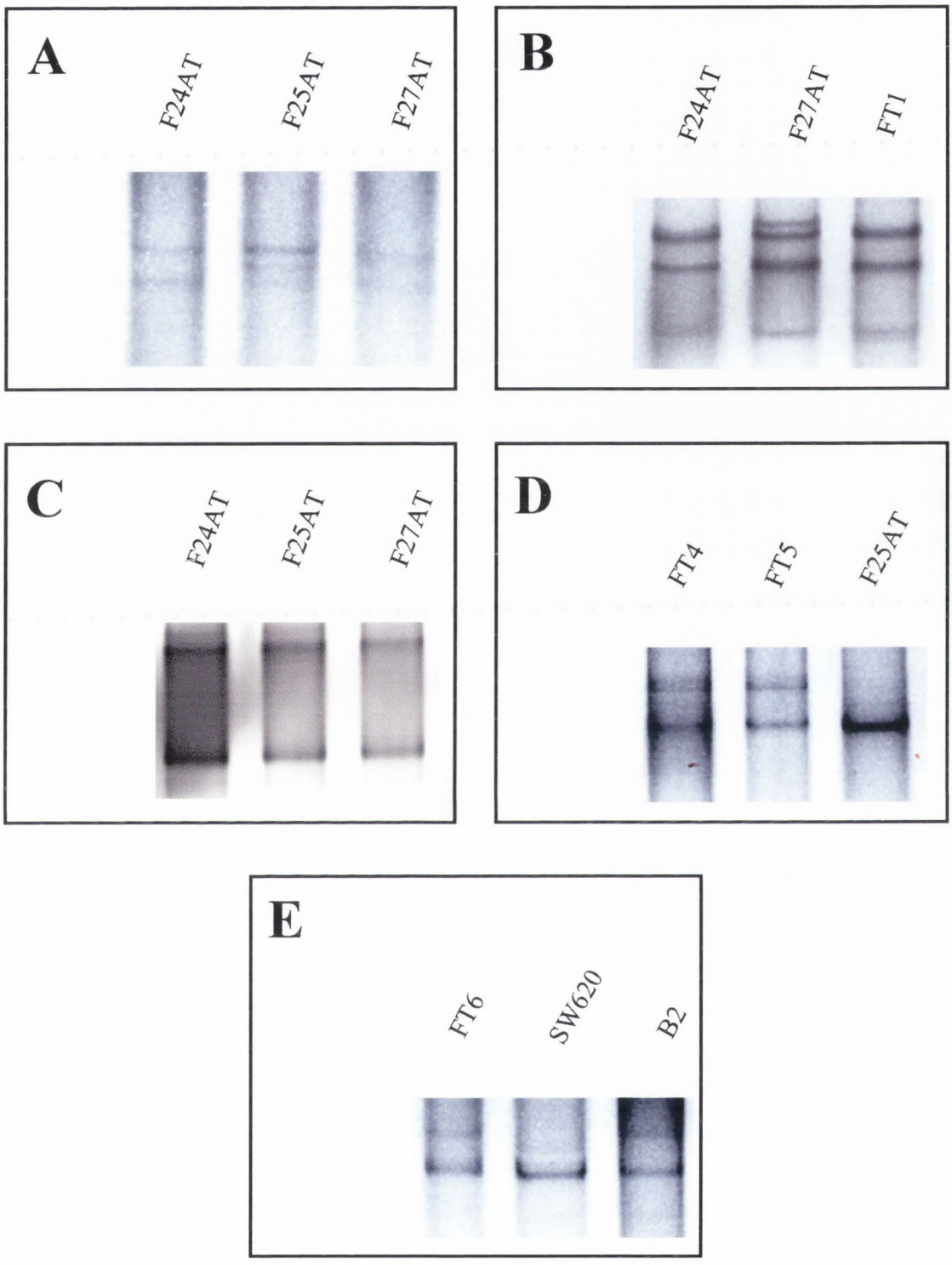
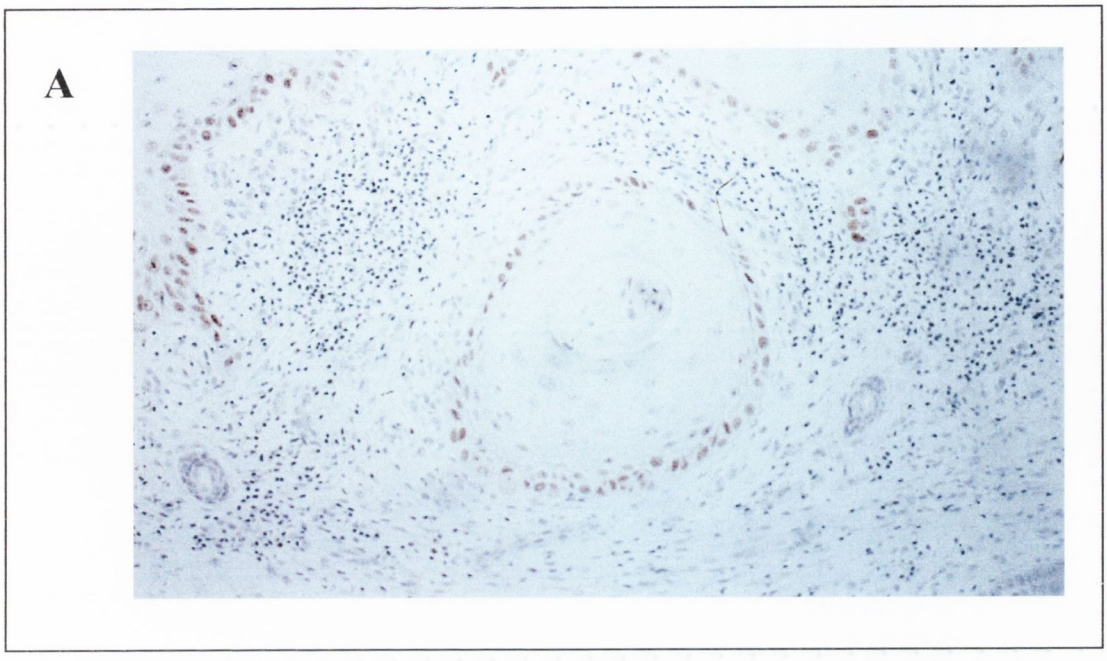


Figure 3.10 A PCR-SSCP analysis of p53 exon 5 depicting altered band migration in the SCC F25AT compared with normal band migration in F24AT and F27AT. F25AT was shown in a previous study to have a point mutation at codon 161 of exon 5. **B** PCR-SSCP analysis of p53 exon 5* depicting altered band migration in the SCC F27AT compared with normal band migration in F24AT and FT1. Despite sequencing both strands in either direction, no mutation could be found in this sample. **C** PCR-SSCP analysis of p53 exon 6 depicting normal band migration. No altered band migration was found in any of the samples analysed. **D** PCR-SSCP analysis of p53 exon 7 depicting altered band migration in the SCC F25AT compare with normal band migration in FT4 and FT5. F25AT was shown in a previous study to have a mutation in codon 248 of exon 7. **E** PCR-SSCP analysis of p53 exon 8 depicting altered band migration in the colorectal carcinoma cell line SW680 compared with normal migration in FT6 and B2. SW680 has a characterised mutation in exon 8.



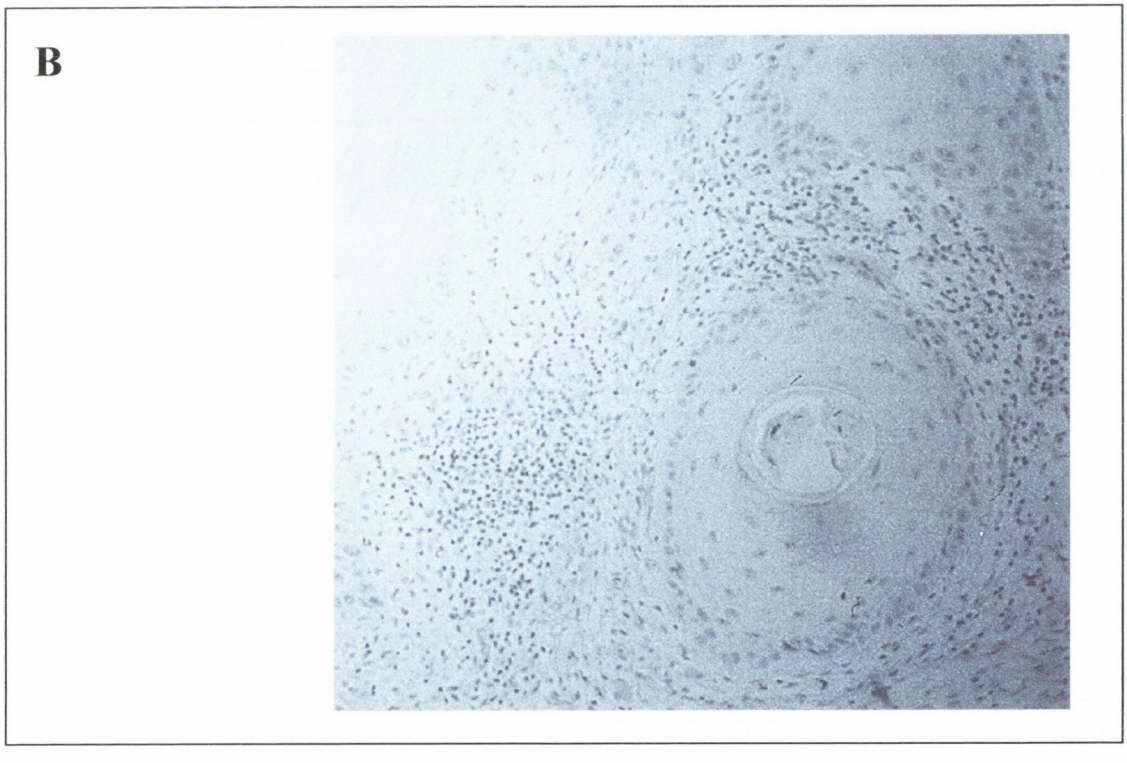


Figure 3.11 A p53 immunostaining with the antibody DO-7 depicting basal layer expression of p53 in the SCC F2ET. **B** Mdm2 immunostaining with the antibody SM7 in the same lesion. Note that there is no Mdm2 expression in the cells that express p53.

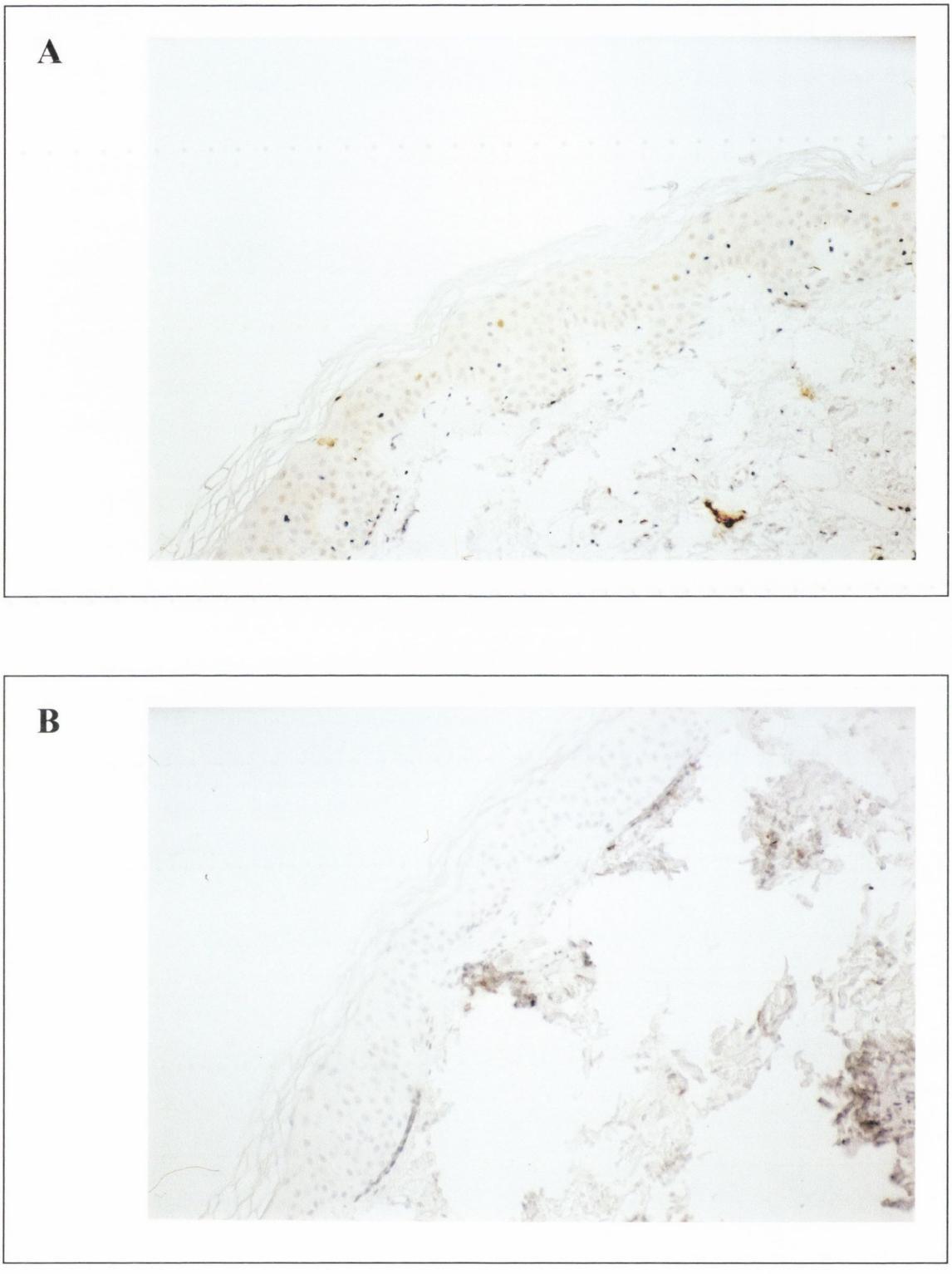
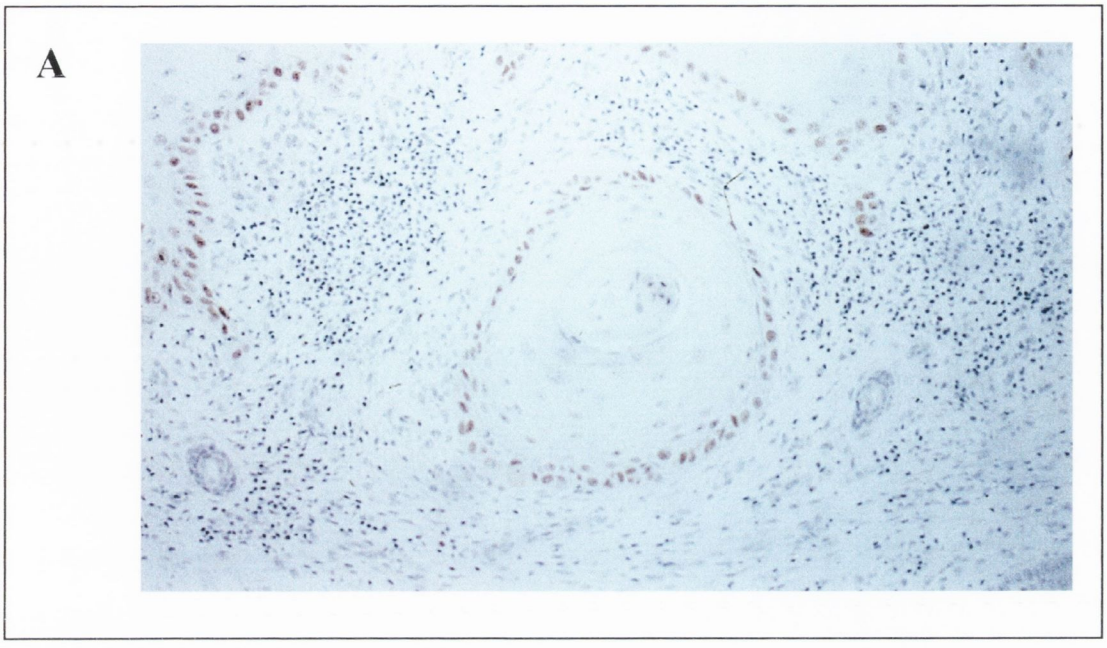


Figure 3.12 **A** Mdm2 immunostaining with the SM7 antibody in a normal human skin biopsy taken 24h post UV-irradiation depicting Mdm2 expression in isolated cells of the upper epithelium. **B** Negative control of the above.



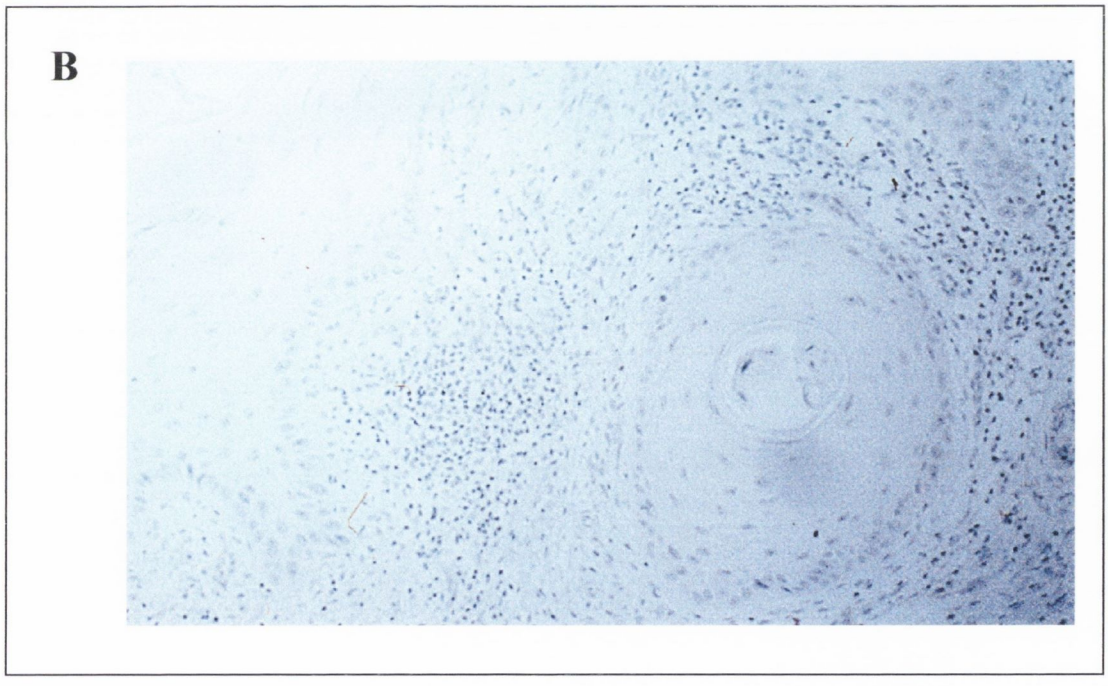


Figure 3.13 A p53 immunostaining with the DO-7 antibody depicting basal layer p53 expression in the SCC F2ET. **B** p21 immunostaining with the antibody p21(187) in the same lesion. Note that there is no expression of p21 in the cells that express p53.

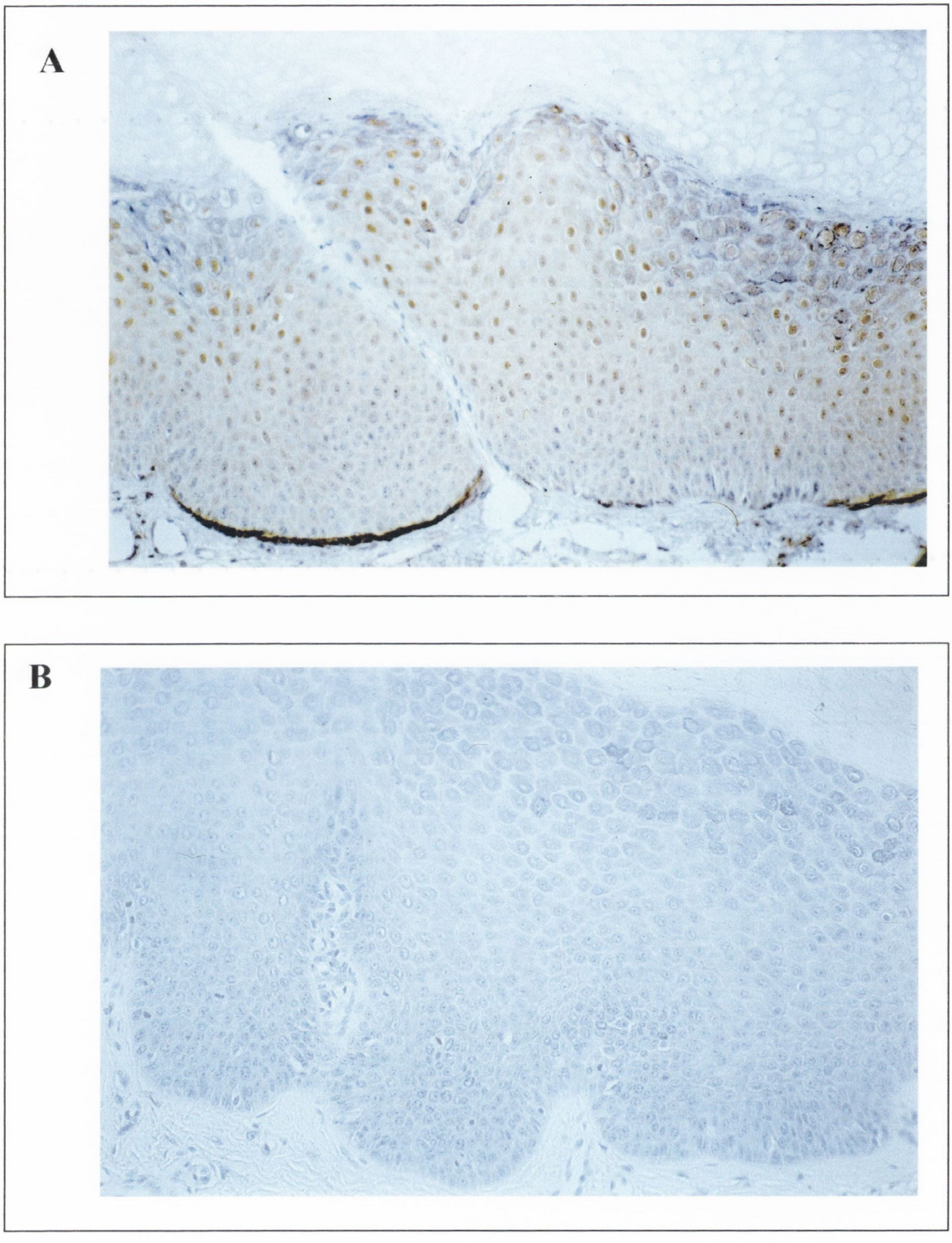


Figure 3.14 **A** p21 immunostaining with the p21(187) antibody in the wart W37A depicting intense p21 expression in the upper layers of the lesion. **B** Negative control of the above.

3.4 Discussion.

The *p53* tumour suppressor gene negatively regulates cellular proliferation in response to genotoxic stresses by inhibiting cells from passing the G_1 cell cycle boundary. Under normal conditions this allows the cell time to correct any DNA damage accrued in interphase before progressing to mitosis and passing the damaged DNA to daughter cells.

In this chapter, the expression of p53 in benign and malignant lesions from renal transplant recipients and immunocompetent skin cancer patients was examined. The results show that p53 is frequently detected by immunohistochemistry in these lesions and that the expression of p53 differs markedly in benign and malignant tissue. In the viral warts analysed in this study, 60% (9/15) showed no detectable levels of p53 and the remaining 40% (6/15) exhibited only low levels of focal, basal layer p53 expression in a small number of cells. This pattern of p53 expression has been previously described (Khorshid *et al.* 1996). Previous studies on p53 expression in warts have produced varying results. Some researchers have found no p53 expression in warts or normal skin (Mc Gregor *et al.* 1992). Others have suggested that there is p53 expression in the warts of EV patients, but no expression in the viral warts of non-EV patients (Piazarro *et al.* 1995). Numerous studies have linked the overexpression of p53 with an increase in proliferative activity or tumour progression (Kerschmann *et al.* 1994, Shimizu *et al.* 1997, Saito *et al.* 1999). It also appears that p53 overexpression correlates with increased proliferation associated with HPV replication. The levels of p53, PCNA and Ki-67 have all been shown to be increased in epidermal layers just below layers where HPV DNA is abundant (Lu *et al.* 1999). It has been suggested that p53 induction of p21

expression could allow p21 to trigger keratinocyte differentiation and thus initiate HPV DNA replication (Lu *et al.* 1999).

The expression patterns for p53 observed in the cutaneous SCCs analysed in this study were strikingly different from those in benign viral warts. No expression of p53 was detected in 33% (5/15) of the SCCs examined. Negative p53 staining in tumours has been attributed to a number of reasons. Firstly, technical considerations such as the choice of antibody and the choice of antigen retrieval method can greatly influence the outcome of any immunohistochemical procedure. These considerations need to be taken into account when evaluating immunohistochemical data. The antibody used in this study (DO-7) was ranked as the most sensitive and specific in an assessment of six antibodies for the immunohistochemical detection of p53 (Baas *et al.* 1994).

There may also be biological reasons for the lack of p53 expression in these tumours. Gross chromosomal deletion can result in the abolition of p53 expression (Sakatani *et al.* 1998), however in the case of the SCCs not expressing p53 in this study, PCR bands for each of exons 5-8 were readily amplified thus this seems unlikely. Point mutations that result in the generation of stop codons can abrogate p53 production so that it is not detected. Point mutations that do not result in the stabilisation of the protein such that its level in the cell is detectable immunohistochemically can also generate false negatives (Wynford-Thomas 1992). However, when exons 5-8 of *p53* in the SCCs that demonstrated no detectable p53 expression were analysed by PCR-SSCP analysis, none appeared to have any mutations.

In 60% (9/15) of the SCC analysed p53 overexpression was detected either diffusely expressed throughout the invasive component of the tumour or localised to the basal layer. Detection of p53 in a variety of cutaneous tumours (e.g. cutaneous basal cell carcinomas, soft tissue sarcomas arising in burn scars, SCC in psoriasis patients and

advanced cutaneous lymphomas) has been associated with stabilising UV-induced mutations in the *p53* gene (Rosenstein *et al.* 1999, Nakanishi *et al.* 1999, Nataraj *et al.* 1997, McGregor *et al.* 1999). In cutaneous SCCs of renal transplant recipients, p53 overexpression is commonly observed (Gibson *et al.* 1997) and again this accumulation of p53 has been attributed to stabilising UV-induced mutations (Brash *et al.* 1991; Bennett *et al.* 1996; McGregor *et al.* 1997). Mutation of *p53* is believed to be an early event in cutaneous carcinogenesis (Campbell *et al.* 1993, Ferrandiz *et al.* 1999) and that the lack of UV-induced, p53-mediated apoptosis in these cells places them at a selective advantage over other cells in the skin (Ziegler *et al.* 1994).

Of the p53-positive SCCs analysed in this study, only one (F25AT) was shown to harbour any mutation of the *p53* gene. This sample was also analysed in a previous study in our lab (Bennett *et al.* 1997). In fact the *p53* gene in F25AT has two point mutations, one in exon 5 resulting in an amino acid substitution from an alanine to a threonine at codon 161 and another in exon 7 resulting in an amino acid substitution from and arginine to a tryptophan at codon 248 (Bennett *et al.* 1997). The mutation at codon 161 is novel and has not been described in cutaneous tumours before, however codon 248 is recognised as a mutation hot-spot (Ziegler *et al.* 1993). Though a band shift in exon 5 was observed in the sample F27AT, subsequent sequencing revealed no mutation. This may be a function of a normal *p53* allele diluting the mutated allele when amplifying the exon 5 PCR product. It is possible that the SCCs that exhibited diffuse expression of p53 in the invasive tumour could have mutations in exons outside exons 5-8 or in non-coding/regulatory regions. However, as 80% (4/5) of these SCCs were deemed wild-type by PCR-SSCP analysis and exons 5-8 are the mutational hotspots for UV-induced p53 mutations in non-melanoma skin cancers (Ziegler *et al.* 1993) this would seem unlikely.

It has been suggested that the E6 protein of low-risk HPV types can complex with p53, rendering it inactive rather than promoting its degradation, thus inactivating p53 but allowing detection by immunohistochemistry (Lassus & Ranki 1996). In oral squamous cell carcinomas, SCCs of the head and neck and certain anogenital lesions, HPV DNA is found in cells both expressing p53 and in those that do not (Koh *et al.* 1998, Aggelopoulou *et al.* 1998, Adams *et al.* 1999, Caruso *et al.* 1998). In anal canal carcinomas, p53 expression was found to correlate with HPV status, however, HPV+p53+ patients showed no difference in tumour aggressiveness compared with HPV-p53- patients (Indinnimeo *et al.* 1999).The accumulation of wild-type p53 in the cutaneous SCCs analysed in this study does not appear to be related to the HPV status of the tumours. HPV-negative SCCs showed diffuse expression of p53, basal layer expression of p53 and no expression of p53.

Previous studies have detected a much higher incidence of *p53* mutations in cutaneous SCCs (Brash *et al.* 1991, McGregor *et al.* 1997). The fact that most of the tumours analysed in this study have wild-type p53 suggests another mechanism of p53 inactivation. Numerous studies have noted the accumulation of p53 without mutation in other cancers and pre-malignant lesions (Lang *et al.* 1994, Castren *et al.* 1998, Haapajarvi *et al.* 1999). The accumulation of wild-type p53 could be due to a defect in the degradation pathway that normally regulates its level in the cell. To examine if this was the case, Mdm2 expression in the p53-positive SCCs was examined. Mdm2 is induced by p53 and promotes the export of p53 to the cytoplasm where it is then degraded via the ubiquitin protein degradation pathway (Kubbutat *et al.* 1997). The E6 oncoprotein of high-risk mucosal HPV 16 and 18 can functionally substitute for Mdm2 in this process (Vousden 1993). Overexpression of Mdm2 itself can result in the abrogation of p53-mediated cell-cycle arrest and repair mechanisms (Wang *et al.*

1999a) however, this can be overcome by activation of p53 through phosphorylation at Ser-15 (Gao *et al.* 1999). In the p53-positive tumours analysed in this study, no expression of Mdm2 was detected however, suggesting that the accumulation of wild-type p53 is not due to a defect in Mdm2-regulated p53 degradation.

p53-mediated Mdm2 expression requires not just stabilisation but also activation of p53 (Reviewed in Oren 1999). It is possible that defective post-translational modification of p53 could render it stable, allowing it to accumulate in the cell, but transcriptionally inactive, thus effecting the expression of downstream genes. To examine whether this could to be the case in the wild-type p53-positive cutaneous SCCs, the expression of another p53 target gene, the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$, was examined. The induction of $p21^{Waf1/Cip1}$ expression by p53 results in cell-cycle arrest at the G_1 boundary thus allowing the cell to repair any DNA damage before the cell-cycle resumes (El-Deiry *et al.* 1993, Deng *et al.* 1995). In sun-exposed normal epidermis, $p21^{Waf1/Cip1}$ is co-expressed with wild type p53 (Inohara *et al.* 1996). In the wild-type p53-expressing SCCs analysed in this study, no expression of $p21^{Waf1/Cip1}$ was detected either in the tumour or in adjacent normal tissue. The lack of $p21^{Waf1/Cip1}$ expression in response to accumulated wild-type p53 may suggest that the p53 does not function effectively as a transcriptional activator. Other researchers have described a melanoma cell line that expresses wild-type p53 that lacks DNA-binding activity (Haapajarvi *et al.* 1999). This hypothesis is supported by the fact that other genes downstream of p53 (*gadd*45 and *bax*) were also found not to be expressed in the p53-expressing SCCs in this study (Data not shown). However limited amounts of tissue prevented the study of the expression of more genes involved in the p53 response to genotoxic stress.

Several researchers have shown that in addition to its p53-dependent induction, $p21^{Waf1/Cip1}$ can also be induced by p53-independent means (Jiang *et al.* 1995, Vidal *et al.* 1995, Ng *et al.* 1999, Haapajarvi *et al.* 1999). TGF-β has been shown to strongly increase the expression of $p21^{Waf1/Cip1}$ in squamous cell carcinoma cell lines with p53 mutations, and weak induction by γ-radiation has been observed in the same cell lines (Yoneda *et al.* 1999). Therefore, other pathways of $p21^{Waf1/Cip1}$ induction may also be defective or over-ridden in the SCCs analysed.

In summary, the results of this study show that the expression of p53 in malignant lesions of RTR and ICP is altered compared with benign lesions. The p53 expressed in cutaneous SCCs is predominantly wild-type, though it does not appear to evoke a normal response to genotoxic stress.

Chapter 4

Molecular genetic analysis of the *Rb* tumour suppressor gene and immunohistochemical analysis of Rb protein expression in renal transplant recipients and immunocompetent skin cancer patients

4.1 Introduction.

The rare childhood cancer retinoblastoma is partially caused by the inactivation of the *Rb* tumour suppressor gene, located on the long arm of chromosome 13 at 13q14.2. Its gene product is a nuclear phosphoprotein that plays a critical role in the regulation of cell proliferation, differentiation and signal transduction (reviewed in Ewen 1998). Loss of Rb function leads to the development of a wide range of inherited and sporadic forms of cancer (Hall & Peters 1996, Sherr 1996). The inactivation of Rb can occur through mutation of the gene or by interaction with the oncoproteins of DNA tumour viruses.

Loss of heterozygosity (LOH) is regarded as an important genetic mechanism in the development of malignant neoplasia and is responsible for inherited retinoblastoma. LOH at 3p and 17p has been previously described in cutaneous SCCs (Quinn *et al.* 1994, Ahmadian *et al.* 1998). In SCCs of the head and neck, LOH is frequently detected at 13q14 (Gupta *et al.* 1999). Deletions of portions of chromosome arm 13q or mutations of genes located on this arm are commonly detected in a large number of different human cancers. Most notably the *Rb* gene and *BRCA2* (a tumour suppressor gene associated with a predisposition to breast cancer) located at 13q12.3 (van den Berg *et al.* 1996). In cervical carcinomas, the E7 oncoproteins of HPV16 and 18 bind competitively to Rb thus inhibiting the binding of Rb to its normal physiological partners (Dyson *et al.* 1989).

There is no evidence to suggest that the E7 protein of the EV-HPV types prevalent in cutaneous SCCs acts in a similar manner to the E7 oncoprotein of HPV16 and 18. Therefore, another mechanism of Rb inactivation may occur. The following describes a study to examine the integrity of the *Rb* gene in cutaneous SCCs. Allelic loss at the D13S153 locus (located in exon 2 of the *Rb* gene) and at the D13S118 locus

(distal telomerically to *Rb* at 13q14.3) was examined in 13 histologically confirmed cutaneous SCCs. The effect of LOH on Rb expression in these tumours was also examined immunohistochemically. A comparison of Rb expression in cutaneous SCCs and benign viral warts was also carried out.

4.2 Methods.

4.2.1 Buffers and solutions.

Tris Buffer 0.05M, pH 7.6

6.1g Tris

40ml 1M HCl

H_2O to 1000ml

Tris Buffered Saline

500ml Tris Buffer

38.25g NaCl

125µl Tween 20

H_2O to 5000ml

Hydrogen Peroxide Solution

9ml Hydrogen peroxide (30% w/v)

300ml H_2O

Citrate Solution pH 6.0

2.94g Trisodium citrate

1000ml H_2O

4.2.2 Samples for LOH analysis.

Thirteen histologically proven SCCs and matched peripheral blood as normal controls were chosen for LOH analysis.

4.2.3 Tissue Section cutting of cutaneous SCCs for LOH analysis.

Paraffin embedded cutaneous squamous cell carcinomas were sectioned on a microtome as outlined in section 3.2.3. Two 8µm sections were cut in each case. The slides were incubated at 55°C overnight to ensure adequate adhesion. The sections were dewaxed in two changes of xylene, cleared in two changes of alcohol brought to water. The sections

were then stained with haematoxylin and eosin and dehydrated through two changes of alcohol. The slides were then allowed to air dry.

4.2.4 Stereoscopic microdissection.

Tumour samples were microdissected to remove any normal tissue prior to LOH analysis. Reference slides were used in each case to determine regions of invasive squamous cell carcinoma and thus determine the exact locations of the lesions to be microdissected. Tissue for microdissection was moistened with a drop of sterile 70% (v/v) ethanol. Using direct stereomicroscope visualisation (Zoom stereomicroscope (0.8x-3.5x zoom with 10x eyepiece and 1.5x objective)) normal tissue was dissected away from invasive regions using a scalpel blade. Tumour cells were then picked up on the scalpel blade and transferred to a 0.5ml eppendorf containing a drop of sterile 70% (v/v) ethanol. Gloves were worn throughout the microdissection procedure and a new sterile blade was used for each microdissection. The surface of the stereomicroscope was cleaned with sterile 70% (v/v) ethanol in between each case.

4.2.5 Isolation of total genomic DNA from microdissected SCCs and peripheral blood.

Total genomic DNA was isolated from microdissected tumour tissue by proteinase K digestion (0.5mg/ml in 100µl of lysis buffer (see section 2.2.1) adapted from Wright and Manos 1990, Harris and Jones 1997) at 55°C for 72h. Following incubation, the proteinase K was inactivated by heating to 95°C for 10 min. The tubes were then centrifuged at 12,000g for 30 sec. The remaining supernatant was used for the LOH analysis. Total genomic DNA was extracted from peripheral blood as described in section 5.2.3.

4.2.6 PCR detection of microsatellite markers D13S153 and D13S118.

Microsatellite markers were amplified using the primers described in table 4.1 and under the conditions described in table 4.2. Approximately 100ng of total genomic DNA extracted from microdissected tumours and peripheral blood was used as template for all PCR protocols. All PCR protocols were carried out using *Taq* Polymerase (1.5U per 50µl reaction). Positive, negative and contamination controls were included in every PCR protocol.

4.2.7 Analysis of loss of heterozygosity.

Analysis of LOH at the D13S153 and D13S118 loci was performed on an ALFexpress automated DNA sequencer using the Allele links software package. CY5-labelled PCR products were run on 6%(w/v) polyacrylamide gels and measured by laser excitation of the CY5 label. PCR product size was determined by comparison with CY5-labelled size markers (50-500bp). The intensity of CY5-labelled PCR bands representing each allele was calculated as a peak area for each band. Using the peak areas determined, allele ratios for each tumour sample and its corresponding normal sample were calculated and used to determine LOH the D13S153 and D13S118 loci (see Figure 4.1 for sample calculation).

CY5-labelled PCR products were diluted between 1:2 and 1:10 in ALF loading dye before loading, depending on the concentration of each product. Prior to loading, the samples and the CY5-labelled size marker were heated to 95°C for 5 min and immediately quenched on ice. Two sets of PCR products from each case were analysed on separate ALF gels and a mean peak ratio calculated and used to determine allelic loss. The cut-off point for determining LOH on the ALFexpress automated DNA sequencer used in this study was previously calculated as 0.74 (*ie* 99.5% of paired normal cases would give an allele ratio of 0.74 or greater) (Butler 1999). Therefore, any tumour samples with an allele ratio of 0.74 or less when compared with the normal allele ratio were deemed to show allelic loss.

4.2.8 Samples for immunohistochemical analysis of Rb expression.

The 13 cutaneous SCCs analysed for LOH at the D13S153 and D13S118 loci were analysed immunohistochemically for Rb expression. In addition, the expression of Rb in a further 9 cutaneous SCCs and 14 viral warts from renal transplant recipients and immunocompetent skin cancer patients was also analysed immunohistochemically in order to determine if there was any difference in the level of expression between benign and malignant skin lesions. All samples were histologically proven. A positive control of a breast tumour with known Rb expression was included.

4.2.9 Tissue section cutting of samples for immunohistochemical analysis of Rb expression in benign and malignant skin lesions of RTR and ICP.

Sectioning of paraffin-embedded SCCs for the immunohistochemical detection of Rb expression was carried out as outlined in section 3.2.3. The sections were dewaxed in two changes of xylene, cleared in two changes of alcohol and brought to water. Endogenous peroxidase activity was blocked by incubating the sections in hydrogen peroxide solution for 15 min. For the detection of Rb the sections were subjected to microwave pre-treatment in 0.01M sodium citrate solution (pH 6.0) for 22 min at full power (850W).

4.2.10 Immunohistochemical detection of Rb expression.

Following blocking of endogenous peroxidase and antigen retrieval, the slides containing the tissue sections were placed under running water for 5 min. The slides were then placed in incubation trays and the sections were covered in TBS pH 7.2 and incubated for 10 min. Excess TBS was then drained away and the area around the sections was dried with a clean tissue. The sections were then covered with normal goat serum (NGS) diluted 1/10 in TBS and incubated for 10 min. The NGS was then drained off and any excess wiped away. The upper section on each slide was then covered with Rb1 mouse monoclonal antibody (Dako) diluted 1/25 in TBS and incubated for 40 min. Rb1 detects both phosphorylated and non-phosphorylated Rb protein. The lower (negative control) section on each slide was covered with TBS alone. The sections were then washed twice with TBS (4 min each wash). The TBS was then drained off, any excess wiped away and the sections were covered with goat antimouse secondary antibody (10µl reagent C from the Dako Duet kit mixed with 10µl NGS made up to 1ml with TBS) and incubated for 30 min. The sections were then washed twice with TBS (4 min each wash). The TBS was drained off and any excess was wiped away. The sections were then covered with streptavidin/biotinylated horseradish peroxidase (10µl reagent A and 10µl reagent B from the Dako duet kit made up to 1ml with TBS) and incubated for 30 min. The sections were then washed twice with TBS (4 min each wash). The TBS was drained off and any excess wiped away. The sections were then covered in approximately 300µl of DAB reagent, which was prepared according to the manufacturer's instructions. The sections were incubated with DAB reagent until brown nuclear staining was visible and then they were washed in running water. The sections

were counterstained with Harris haematoxylin, dehydrated in two changes of alcohol, cleared and coverslipped using a Tissue-Tek coverslipping machine.

4.3 Results.

4.3.1 Loss of heterozygosity at the D13S153 and D13S118 loci in cutaneous SCCs of renal transplant recipients.

The rate of informativity was 77% (10/13) at the D13S153 locus and 85% (11/13) at the D13S118 locus. The allele ratio for each tumour and its corresponding normal control at the D13S153 locus is shown in tables 4.3 and 4.4. The determination of LOH at the D13S153 locus applying a previously established LOH cut-off point of 0.74 is shown in table 4.5. The allele ratio for each tumour and its corresponding normal control at the D13S118 locus is shown in tables 4.6 and 4.7. LOH at D13S118 was also determined by applying the established LOH cut-off point of 0.74 (Table 4.8).

At the D13S153 locus, 40% (4/10) of informative cases showed allelic loss (see figure 4.2 for example). In all cases the degree of loss was approximately 50% or greater. At the D13S118 locus, 45% (5/11) informative cases demonstrated allelic loss (see figure 4.3 for example). However, the degree of loss was less than 50% in 4 of the 5 cases. Only one SCC (F3AT) showed a large degree of allelic loss (89%) at D13S118. Overall 64% (7/11) of informative cases demonstrated allelic loss at either D13S153 or D13S118 (Figure 4.4). Only two SCCs showed loss of both markers.

4.3.2 Immunohistochemical detection of Rb expression in cutaneous SCCs analysed for LOH.

The relationship between allelic loss and Rb expression is shown in table 4.9. Of the SCCs analysed for LOH, 92% (12/13) showed Rb expression in the invasive tumour. Of the samples that demonstrated LOH at the D13S153 locus, 75% (3/4) retained the ability to express Rb. Overall, 86% (6/7) of the SCCs that demonstrated LOH at either locus were found to express Rb.

4.3.3 Expression of Rb in benign and malignant skin lesions of renal transplant recipients and immunocompetent skin cancer patients.

The expression of Rb in cutaneous SCCs and viral warts of RTR and ICP is shown in table 4.10. In the case of the SCCs analysed, 82% (18/22) were found to be Rb-positive. Only 18% (4/22) of the SCCs analysed showed no detectable expression of Rb. In the case of the viral warts analysed, 86% (12/14) demonstrated Rb expression and only 14% (2/14) showed no expression of Rb.

Table 4.1 PCR primer used for detection of LOH at D13S153 and D13S118.

Primer	Sequence	Source
1312R	5' GAA ATA GTA TTT GGA CCT GGG 3'	Research Genetics Inc
1312TG	5' CCA CAG ACA TCA GAG TCC TT 3'	Research Genetics Inc
AFM058xd6a	5' AGC ATT GTT TCA TGT TGG TG 3'	Research Genetics Inc
AFM058xd6m	5' CAG CAG TGA AGG TCT AAG CC 3'	Research Genetics Inc

Table 4.2 Optimised PCR conditions for the detection of LOH at D13S153 and D13S118.

Primer pair	Cycling conditions	Product Size	[$MgCl_2$]	[dNTP]	[Primer]
1312R/TG	94°C 1min, 55°C 1 min, 72°C 1 min, 30 cycles.	187bp-201bp	1.5mM	200μM	1μM
AFM058xd6a/m	94°C 1 min, 55°C 1 min, 72°C 1 min, 30 cycles.	212bp-236bp	1.5mM	200μM	0.25μM

Table 4.3 Allele ratios for cutaneous SCCs used to calculate LOH at the D13S153 locus.

Samples	Allele ratio 1st run	Allele ratio 2nd run	Average allele ratio
F2AT	6.66	1.78	4.22
F2CT	2.56	1.61	2.09
F2DT	5.88	4.16	5.02
F2FT	1.26	1.31	1.28
F4AT	1.03	1.23	1.13
F17BT	1.49	1.88	1.69
F22AT	1.31	1.03	1.17
F23AT	4.54	9.09	6.81
F24AT	NI	NI	N/A
F28AT	1.33	1.75	1.54
F1AT	NI	NI	N/A
F10AT	0.63	1.36	1.47
F3AT	NI	NI	N/A

Table 4.4 Allele ratios for matched normal samples used to calculate LOH at the D13S153 locus.

Sample	Allele ratio 1st run	Allele ratio 2nd run	Average allele ratio
B2	1.08	1.08	1.08
B4	1.11	1.09	1.10
B17	1.25	1.39	1.32
B22	1.20	1.56	1.38
B23	1.07	1.11	1.09
B24	NI	NI	N/A
B28	1.18	1.66	1.45
B1	NI	NI	N/A
B10	1.12	1.08	1.10
B3	NI	NI	N/A

Table 4.5 Calculation of LOH at the D13S153 locus.

Samples	Average tumour allele ratio	Average normal Allele ratio	Tumour Vs Normal allele ratio	LOH
F2AT/B2	4.22	1.08	0.25	YES
F2CT/B2	2.09	1.08	0.51	YES
F2DT/B2	5.02	1.08	0.21	YES
F2FT/B2	1.28	1.08	0.84	NO
F4AT/B4	1.13	1.10	0.97	NO
F17BT/B17	1.68	1.32	0.78	NO
F22AT/B22	1.17	1.38	0.85	NO
F23AT/B23	6.81	1.09	0.16	YES
F24AT/B24	N/A	N/A	N/A	N/A
F28AT/B28	1.54	1.45	0.94	NO
F1AT/B1	N/A	N/A	N/A	N/A
F10AT/B10	1.47	1.10	0.75	NO
F3AT/B3	N/A	N/A	N/A	N/A

Table 4.6 Allele ratios for cutaneous SCCs used to calculate LOH at the D13S118 locus.

Samples	Allele ratio 1st run	Allele ratio 2nd run	Average allele ratio
F2AT	1.64	1.07	1.05
F2CT	2.12	1.92	2.02
F2DT	1.59	1.49	1.54
F2FT	1.79	1.64	1.72
F4AT	3.03	3.33	3.18
F17BT	1.42	1.42	1.42
F22AT	1.3	1.75	1.53
F23AT	3.03	2.78	2.91
F24AT	NI	NI	N/A
F28AT	1.22	1.33	1.27
F1AT	NI	NI	N/A
F10AT	1.04	1.11	1.08
F3AT	9.09	9.09	9.09

Table 4.7 Allele ratios for matched normal samples used to calculate LOH at the D13S118 locus.

Sample	Allele ratio 1st run	Allele ratio 2nd run	Average allele ratio
B2	1.36	1.36	1.36
B4	1.49	1.63	1.56
B17	1.00	1.00	1.00
B22	1.42	1.38	1.40
B23	1.49	1.61	1.55
B24	NI	NI	N/A
B28	1.25	1.25	1.25
B1	NI	NI	N/A
B10	1.15	1.10	1.13
B3	1.02	1.02	1.02

Table 4.8 Calculation of LOH at the D13S118 locus.

Samples	Average tumour allele ratio	Average normal Allele ratio	Tumour Vs Normal allele ratio	LOH
F2AT/B2	1.05	1.36	0.76	NO
F2CT/B2	2.02	1.36	0.67	YES
F2DT/B2	1.54	1.36	0.88	NO
F2FT/B2	1.72	1.36	0.79	NO
F4AT/B4	3.18	1.56	0.49	YES
F17BT/B17	1.42	1.00	0.70	YES
F22AT/B22	1.53	1.40	0.91	NO
F23AT/B23	2.91	1.55	0.53	YES
F24AT/B24	N/A	N/A	N/A	N/A
F28AT/B28	1.27	1.25	0.98	NO
F1AT/B1	N/A	N/A	N/A	N/A
F10AT/B10	1.08	1.13	0.96	NO
F3AT/B3	9.09	1.02	0.11	YES

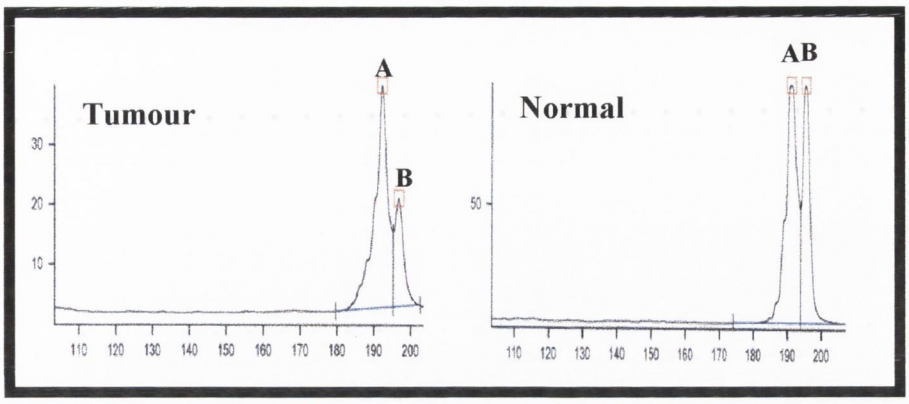
Table 4.9 LOH in cutaneous SCCs at the D13S153 and D13S118 loci: correlation with Rb immunohistochemistry.

SCC	LOH at D13S153	LOH at D13S118	Rb Expression
F2AT	YES	NO	+ve
F2CT	YES	YES	+ve
F2DT	YES	NO	-ve
F2FT	NO	NO	+ve
F4AT	NO	YES	+ve
F17BT	NO	YES	+ve
F22AT	NO	NO	+ve
F23AT	YES	YES	+ve
F24AT	NI	NI	+ve
F28AT	NO	NO	+ve
F1AT	NI	NI	+ve
F10AT	NO	NO	nd
F3AT	NI	YES	+ve

nd= not determined
NI= non-informative

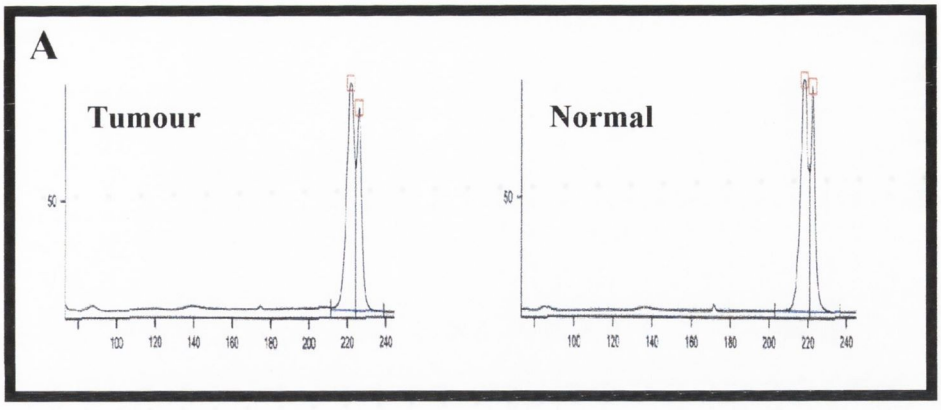
Table 4.10 Expression of Rb in benign and malignant skin lesions of renal transplant recipients and immunocompetent skin cancer patients.

Lesion	Rb +ve	Rb -ve
SCCs	82% (18/22)	18% (4/22)
Viral warts	86% (12/14)	14% (2/14)



Name	Peak Area
Tumour	**A**- 1.00
	B- 0.43
Normal	**A**- 1.00
	B- 0.92

Figure 4.1 Example of the calculation of allele ratios and determination of LOH at the D13S153 locus using the Allele links™ software package on the ALFexpress™ automated DNA sequencer. The peak areas for both the tumour sample and its matched normal are determined. The peak of greatest intensity in the normal sample is designated as "normal A". The corresponding peak in the tumour sample is designated as "tumour A". Using the formula Normal A/Normal B divided by Tumour A/Tumour B it is possible to express the difference in allele ratios as a percentage loss in the tumour sample. For example in this case, Normal A/Normal B is equal to 1.00/0.92 and Tumour A/Tumour B is equal to 1.00/0.43. This gives an allele ratio of 1.09 for the Normal sample and 2.33 for the Tumour sample. The percentage loss is therefore the allele ratio of the normal sample divided by the allele ratio of the tumour sample, which in this case is 1.09/2.33 which equals 0.47 representing a 53% loss of one of the alleles in the tumour at the D13S153 locus.



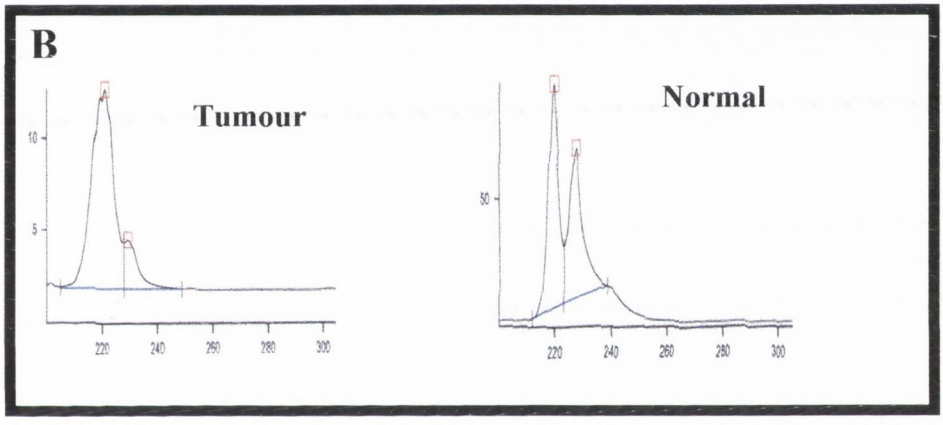


Figure 4.2 A Sample fluorogram generated on the ALFexpress ™ genetic analyser using the Allele links ™ software package depicting the alleles present at the D13S153 locus in the SCC F4AT and its matched normal B4. The retention of both alleles in the tumour sample can be clearly seen. **B** Sample fluorogram generated on the ALFexpress ™ genetic analyser using the Allele links ™ software package depicting the alleles present at the D13S153 locus in the SCC F2AT and its matched normal B2. The loss of one of the alleles in the tumour sample can clearly be seen.

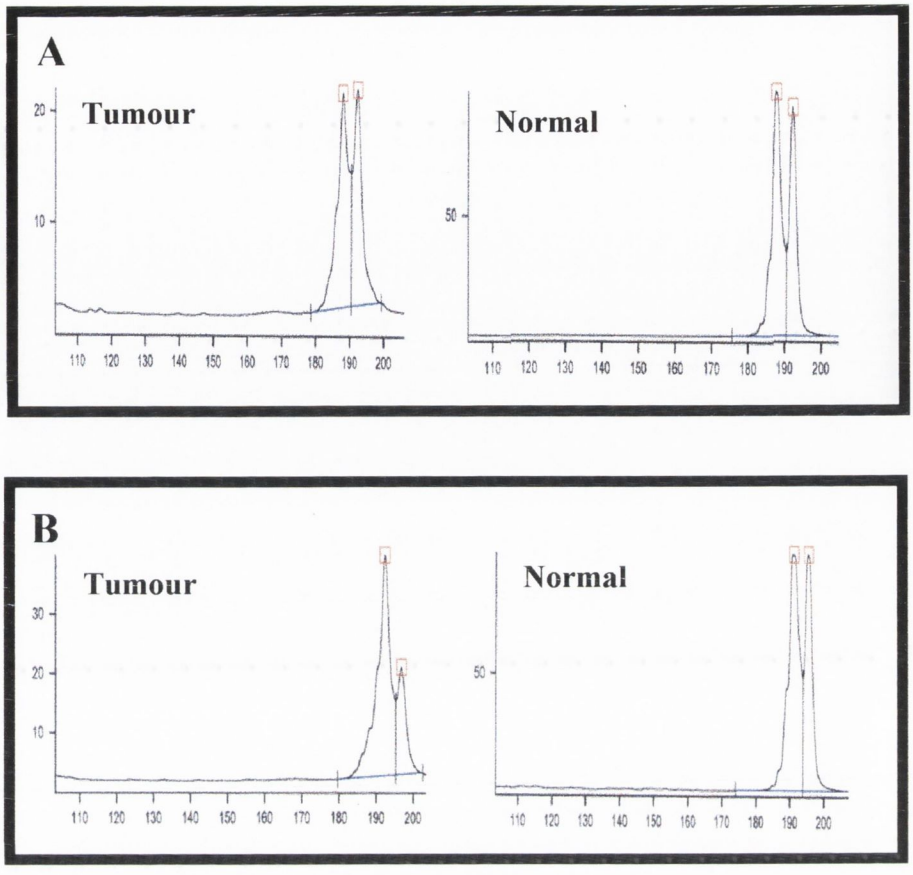


Figure 4.3 A Sample fluorogram generated on the ALFexpress ™ genetic analyser using the Allele links ™ software depicting the alleles present at the D13S118 locus in the SCC F10AT and its matched normal B10. The retention of both alleles in the tumour sample can clearly be seen. **B** Sample fluorogram generated on the ALFexpress ™ genetic analyser using the Allele links ™ software depicting the detection of the alleles present at the D13S118 locus in the SCC F4AT and its matched normal B4. The loss of the second allele in the tumour can clearly be seen.

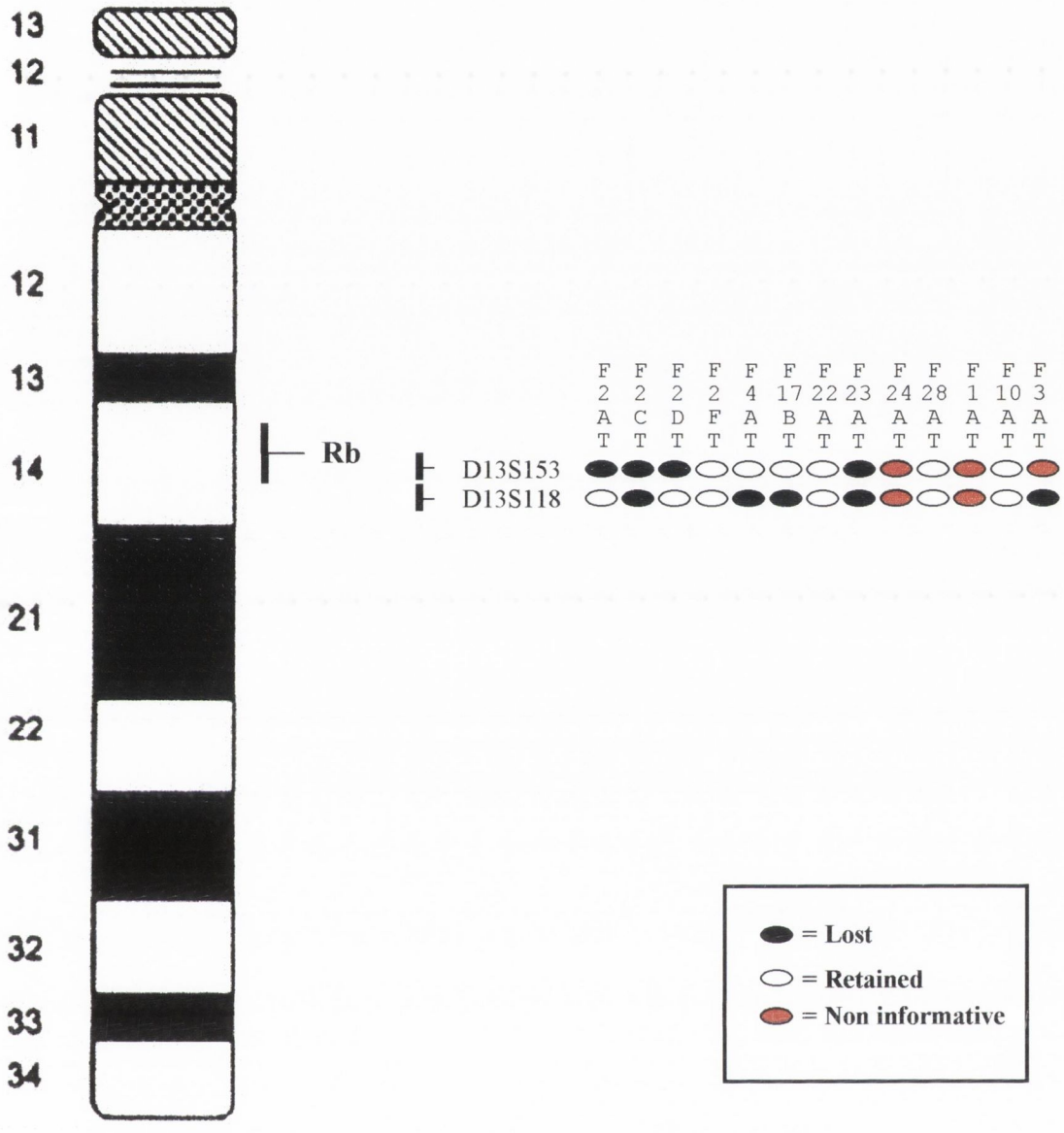


Figure 4.4 Diagram of loss of heterozygosity on the long arm of chromosome 13 in cutaneous squamous cell carcinomas from renal transplant recipients. The location of the *Rb* tumour suppressor gene is shown, as are the locations of the D13S153 and D13S118 microsatellite markers.

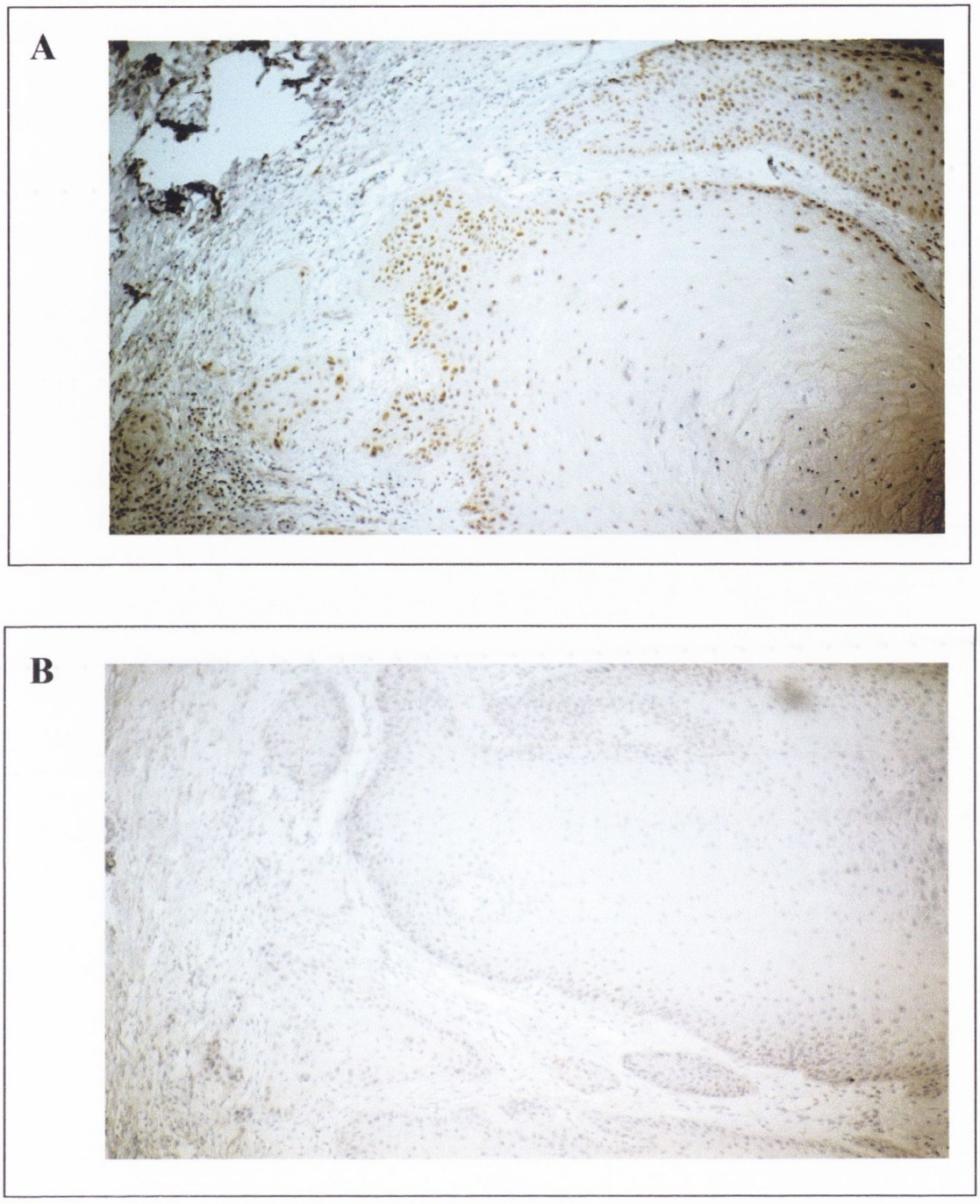


Figure 4.5 **A** Rb immunostaining with the RB1 antibody in the SCC F2AT depicting Rb expression in a tumour that demonstrated LOH at D13S153 (*ie* loss within the *Rb* gene). **B** Negative control of the above.

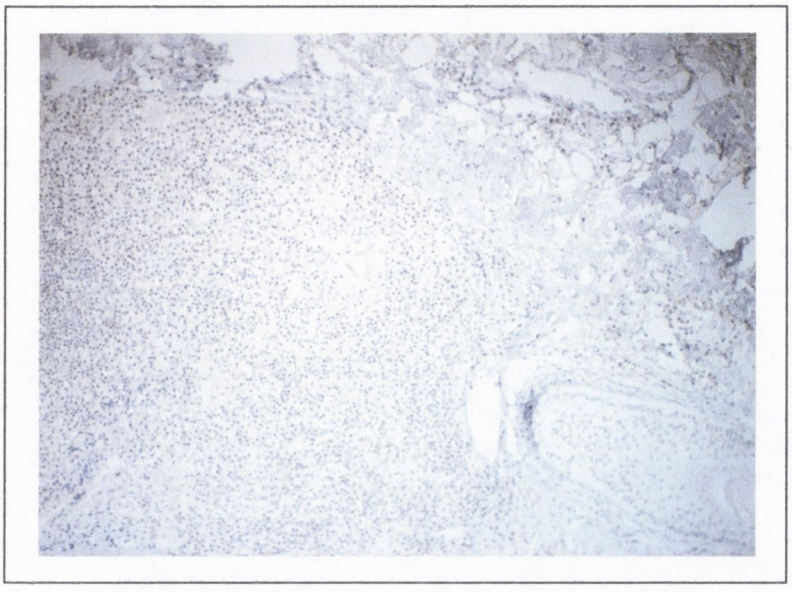


Figure 4.6 Rb immunostaining with the RB1 antibody in the SCC F2DT depicting negative Rb expression in a tumour that demonstrated LOH at D13S153.

4.4 Discussion.

The mechanism of LOH has been shown to play a causal role in the development of basal cell carcinoma, malignant melanoma and various other types of skin cancer (Holmberg *et al.* 1996, Funk *et al.* 1998). LOH in cutaneous squamous cell carcinomas has primarily been previously detected on chromosome 9, though a minimal amount of LOH has been observed at 3p, 13q and 17p (Kushida *et al.* 1999). Non-random allelic loss at 3p, 11p and 13q has been associated with HPV-mediated immortalisation of keratinocytes and the subsequent loss of terminal differentiation (Steenbergen *et al.* 1998). Actinic keratosis, a UV-related precancerous lesion of the skin has also been shown to exhibit a high degree of LOH at these same chromosomal regions (Kushida *et al.* 1999).

The cut-off point for determining LOH varies widely in other studies. Some researchers have used a cut-off point of 0.50 (*ie* a 50% reduction or greater)(Cawkwell *et al.* 1993, Huettner *et al.* 1998). However, this cut-off point was established on non-microdissected tissue and assumes that tumours with no contaminating normal tissue will give an allele ratio of 0.00. However, when the clonality of tumours is questionable and accumulated genetic damage is accrued as the tumour progresses this may not always be the case. Other researchers have applied a cut-off point of 0.70 (Baffa *et al.* 1996) or 0.80 (Man *et al.* 1996), however no explanation for choosing either was offered. Previous studies have also determined LOH by densitometric analysis of PCR bands and this technique is not as sensitive as fluorescent based quantitation. For the purposes of LOH determination, a cut-off point of 0.74 was used which was previously determined on the same ALFexpress DNA sequencer used in this study (Butler 1999). This cut-off point was determined by analysing the variation among paired normal

samples of microdissected paraffin embedded tissue, similar to those used in this study and it was determined that 99.5% of normal cases would give an allele ratio of 0.74 or greater. The wide variation in allele ratios observed in some of the tumour cases analysed in this study probably reflects the nature of the DNA extracted from paraffin-embedded tissues. The normal samples showed much less variation in allele ratios as DNA extracted from peripheral blood is less degraded than that extracted from archival material. However, even when both upper and lower allele ratios are used to calculate LOH separately, the determination of chromosomal loss does not alter.

The results of this study show that LOH at 13q14.2-13q14.3 is frequent in cutaneous SCCs of renal transplant recipients. Overall 64% (7/11) of informative cases showed loss of one or other microsatellite marker. This level of allelic loss is far greater than that reported in any other study of LOH in cutaneous SCCs. Previous studies have estimated the degree of LOH in cutaneous SCCs to range from 7% to 28%, predominantly on chromosome 9 (Quinn *et al.* 1994, Kushida *et al.* 1999).

Despite the high level of allelic loss in and around the *Rb* tumour suppressor gene demonstrated in these tumours, loss of Rb expression was only seen in one of the 13 SCCs examined for LOH. This would suggest that another tumour suppressor gene in the region 13q14.2-13q14.3 could be inactivated by the allelic loss shown in these tumours. In contrast, laryngeal tumours, oesophageal cancer and Merkel cell carcinomas with allelic loss at 13q14.2 generally show abnormal staining patterns for Rb when examined immunohistochemically (Takes *et al.* 1997, Xing *et al.* 1999, Leonard & Hayard 1997).

Frequent allelic loss at 13q14.2-13q14.3 has been demonstrated in a wide variety of other cancers, in particular SCC of the head and neck, lung cancer, breast cancer and oral SCC (Gupta *et al.* 1999, Tamura *et al.* 1997, Hamann *et al.* 1996,

Ogawara *et al.* 1998). In many cases, Rb does not appear to be inactivated suggesting that another tumour suppressor gene near the D13S153 and D13S118 loci may play a role in the development of these tumours (if Rb is not mutated or the loss of one allele doesn't affect the cell through haplo-insufficiency)(Yoo *et al.* 1994, Ogawara *et al.* 1998, Gupta *et al.* 1999). In B cell chronic lymphocytic leukaemia (B-CLL), allelic loss at 13q14.3 is frequent, though normal Rb expression is retained (Liu *et al.* 1993). Two putative tumour suppressor genes *leu*1 and *leu*2 were thought to be critically lost in all cases of B-CLL, however it has since been shown that this is not the case and therefore *leu*1 and *leu*2 may not be tumour suppressor genes after all (Rondeau *et al.* 1999). A further 46 expressed sequence tags (ESTs) have been assigned to the region 13q14.3 and any one of these could be a candidate tumour suppressor gene whose loss might contribute to a wide variety of human cancers (Bezieau *et al.* 1998).

The level of Rb expression in all of the cutaneous SCCs examined, both from RTR and ICP, was similar to that in benign viral warts from the same patients. This observation questions the role that the *Rb* gene plays in cutaneous SCCs. Amplification of 11q13 in SCC of the head and neck is well known and may target the cyclin D1 gene (Yoo *et al.* 1994). In oesophageal cancer it has been suggested that the Rb pathway could be circumvented by either Rb inactivation or cyclin D1 overexpression (Jiang *et al.*. 1993). LOH at chromosome 9p21, where the $p16^{INK4}$ locus is located, has been detected in 71% of sporadic melanomas (Kumar *et al.* 1999). The increase in proliferative potential associated with Rb inactivation can be seen in cells with $p16^{INK4}$ inactivation through mutation or altered methylation and in cells with overexpression of cdk4. Thus Rb itself may be expressed normally but its function in regulating the cell cycle may be diminished by other means (Reddel 1998).

In summary, the results of this study show that a high degree of chromosomal instability at 13q14.2-13q14.3 exists in cutaneous SCCs of renal transplant recipients. The expression Rb in the tumours exhibiting allelic loss in and around the *Rb* locus appears to be normal, suggesting that another as yet unknown tumour suppressor gene could be inactivated by the allelic loss observed.

Chapter 5

Analysis of the p53 codon 72 polymorphism in skin cancer patients and an ethnically matched control group

5.1 Introduction.

Nonmelanoma skin cancers commonly harbour multiple human papillomaviruses (HPV), though a causal role for the virus in cutaneous tumourigenesis is yet to be confirmed. A recent report (Storey *et al.* 1998) suggests that a common polymorphism at codon 72 of the p53 tumour suppressor gene (Matlashewski *et al.* 1987) might be a risk factor in the development of HPV-associated cancers. The replacement of a proline residue with an arginine residue at position 72 of the p53 gene product was found to marginally increase the susceptibility of the protein to degradation by the E6 oncoprotein of high-risk HPV types 16 and 18. The presence of an arginine residue was also found to markedly increase the susceptibility of p53 to degradation by the E6 protein of low-risk HPV type 11.

A number of subsequent reports (Rosenthal *et al.* 1998, Sonoda *et al.* 1999, Giannoudis *et al.* 1999) demonstrated that individuals that were homozygous for the arginine variant of the protein were at no greater risk of developing cervical cancer. However, one report suggested that in Swedish and Italian women with HPV 16-positive cervical disease, the incidence of cancer was higher in those patients homozygous for the arginine isoform (Zehbe *et al.* 1999).

As cutaneous malignancies more frequently harbour multiple low-risk HPV types (Surentheran *et al.* 1998, De Villiers *et al.* 1997, Shamanin *et al.* 1996) and the arginine isoform of p53 is susceptible to low-risk HPV E6-mediated degradation, there may therefore be an increased risk of developing skin cancer associated with possessing the arginine variant of p53. The following study describes the examination of the distribution of p53 codon 72 genotypes in skin cancer patients and an ethnically matched control group a portion of whom previously had their HPV status determined.

5.2 Methods.

5.2.1 Buffers.

Blood buffer A

0.32 M Sucrose,

10 mM Tris-HCl (pH 7.5),

5 mM $MgCl_2$, 1% Triton X-100.

Blood buffer B

0.075 M NaCl,

0.02 M EDTA.

5.2.2 Patients and Volunteers.

Peripheral blood was taken from 55 skin cancer patients with cutaneous SCC attending the Dermatology Clinic at Beaumont Hospital and 115 ethnic Irish medical students and staff from the Royal College of Surgeons in Ireland and volunteers taking part in a study of pneumonia.

5.2.3 Total genomic DNA extraction.

Total genomic DNA was extracted from medical student and RCSI staff blood samples using the Isolate II kit according to the manufacturer's instructions. Total genomic DNA was extracted from skin cancer patients and control volunteers taking part in a study of pneumonia by the following method: blood was collected in 10 ml Li/Heparin tubes, transferred to centrifuge tubes and mixed with 0.2ml of 0.5M EDTA (pH 8.0). Eighty millilitres of Blood buffer A was added and the tubes were centrifuged at 7000rpm (IEC Centra-8 centrifuge) for 15 min. Following centrifugation, the supernatant was

discarded and the pellet resuspended in 4.5 ml of Blood buffer B. The resuspended pellets were transferred to sterile polypropylene tubes and 200ml of Proteinase K (20mg/ml) and 250ml 10% SDS were added and the tubes were incubated overnight at 37°C. Following overnight incubation, 5ml of phenol was added and mixed vigorously. Following mixing, the tubes were centrifuged at 1500 rpm (IEC Centra-8 centrifuge) for 5 min. The upper aqueous phase was removed and transferred to a sterile polypropylene tube and 5ml of chloroform: isoamyl alcohol (24:1 v/v) was added. The tubes were shaken vigorously and centrifuged at 1500 rpm (IEC Centra-8 centrifuge) for 5min. The upper aqueous phase was removed and 500 µl of 3M sodium acetate (pH 5.2) and 5ml ice-cold ethanol were added. The tubes were inverted until precipitated DNA was visible. The tubes were then centrifuged at 3500 rpm (IEC Centra-8 centrifuge) for 15 min. The ethanol was removed and the pellet washed in 70% (v/v) ethanol. The tubes were then centrifuged at 3500 rpm (IEC Centra-8 centrifuge) for 15 min, the 70% ethanol was discarded and the pellets allowed to dry at 37°C overnight. The DNA was resuspended in 500µl dH_2O.

5.2.4 Amplification of p53 codon 72 proline and arginine sequences.

The amplification of p53 proline and arginine sequences was performed as previously described by Storey *et al.* using approximately 100ng of total genomic DNA as template (see Table 5.1 and 5.2 for primer sequences and cycling conditions) (see Figure 5.1 for schematic of PCR strategy). The products of each of the two PCR amplifications were combined and analysed by 12% (w/v) acrylamide gel electrophoresis. Acrylamide gels were stained with ethidium bromide and visualised under UV light (sample gel shown in figure 5.2).

5.2.5 Statistical analysis.

χ^2 analysis was used to examine the differences in proportions of the p53 codon 72 genotypes between the skin cancer patients and the control group. The odds ratio for possessing the arginine variant and the development of skin cancer was also calculated. Exact binomial 95% confidence intervals were calculated for the proportions of the genotypes in each group.

5.3 Results.

The proportions of the p53 codon 72 genotypes found were 78% (43/55) arginine homozygous, 2% (1/55) proline homozygous and 20% (11/55) heterozygous among skin cancer patients and 79% (91/115) arginine homozygous, 3.5% (4/115) proline homozygous and 17.5% (20/115) heterozygous among the control population (figure 5.3). Statistical analysis showed no significant differences in the distribution of the various alleles between the control group and the skin cancer group (χ^2= 0.49, df=2, p=0.78). The odds ratio for the association of the arginine variant with the development of skin cancer was calculated at 1.06 (95% confidence interval 0.49 to 2.3).

Table 5.1 PCR primers used for the amplification of p53PRO/p53ARG sequences from skin cancer patients and control volunteers.

Primer	Sequence	Reference
p53Pro+	5' GCC AGA GGC TGC TCC CCC 3'	Storey *et al.* 1998
p53-	5' CGT GCA AGT CAC AGA CTT 3'	Storey *et al.* 1998
p53+	5' TCC CCC TTG CCG TCC CAA 3'	Storey *et al.* 1998
p53Arg-	5' CTG GTG CAG GGG CCA CGC 3'	Storey *et al.* 1998

Table 5.2 Optimised PCR conditions for the amplification of p53PRO/p53ARG sequences from skin cancer patients and control volunteers from the general population.

Primer pair	Cycling conditions	Product Size	[$MgCl_2$]	[dNTP]	[Primer]
p53Pro+/p53-	94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, 32 cycles	177bp	1.5mM	200 nM	40ng per 50µl rxn.
p53+/p53Arg-	94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, 32 cycles	141bp	1.5mM	200nM	40ng per 50µl rxn.

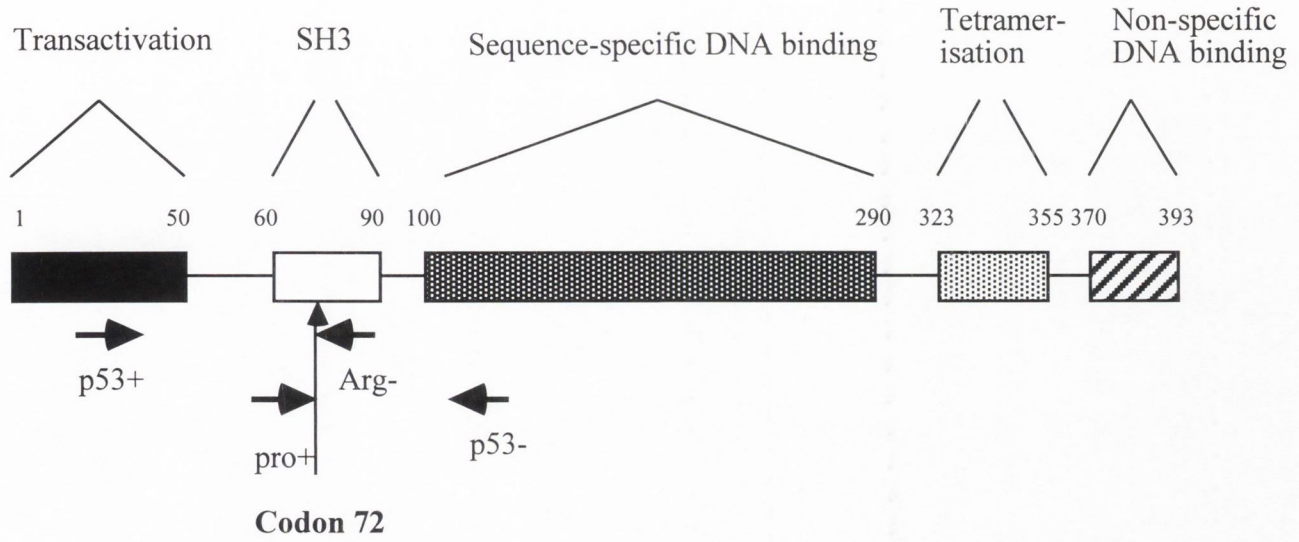


Figure 5.1 Schematic diagram of the PCR strategy used to amplify proline and arginine sequences at codon 72 of the p53 tumour suppressor gene. The p53+/Arg- primer pair amplify a 144bp fragment if the p53 sequence has an arginine codon at position 72, and the pro+/p53- primer pair amplify a 171bp fragment if the p53 sequence has a proline codon at position 72. Adapted from Storey *et al.* 1998.

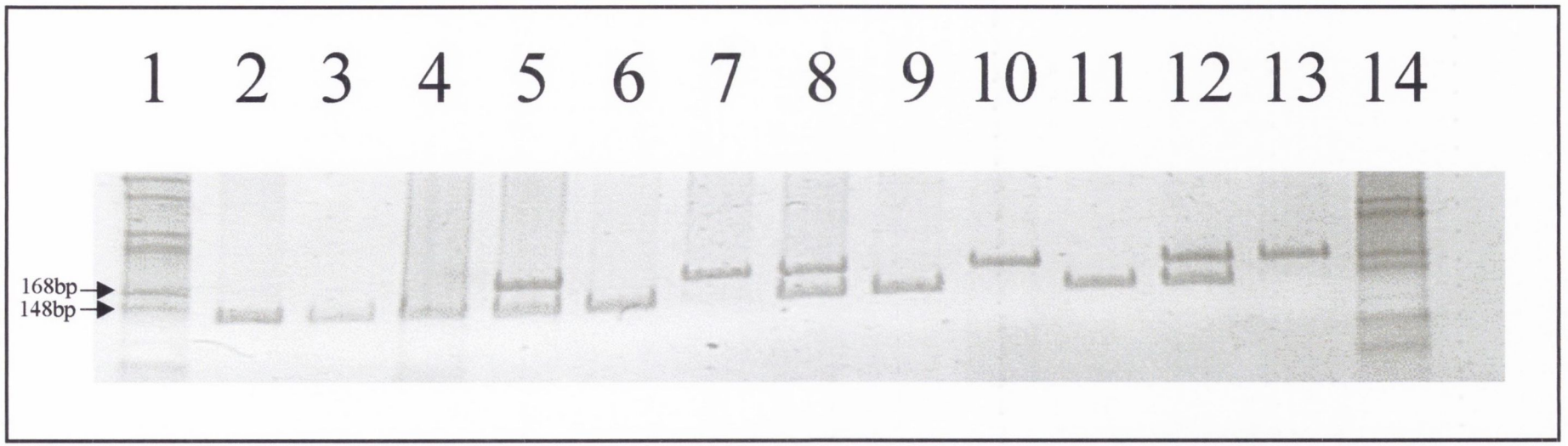


Figure 5.2 Sample 12% (w/v) acrylamide gel depicting the three p53 codon 72 genotypes detected in the skin cancer patients and the control population. Lanes 1 and 14 contain molecular weight marker (HaeIII-digested pBluescript), lanes 2, 3, 4, 6, 9 and 11 show the single 144bp band amplified from individuals who are arginine homozygotes for the codon 72 polymorphism. Lanes 7, 10 and 13 show the single 171bp band amplified from the p53 gene of individuals who are proline homozygotes for the codon 72 polymorphism. Lanes 5, 8 and 12 show both the 144bp arginine band and the 171bp proline band amplified from the p53 gene of heterozygous individuals. Gel was stained with ethidium bromide and visualised under UV light.

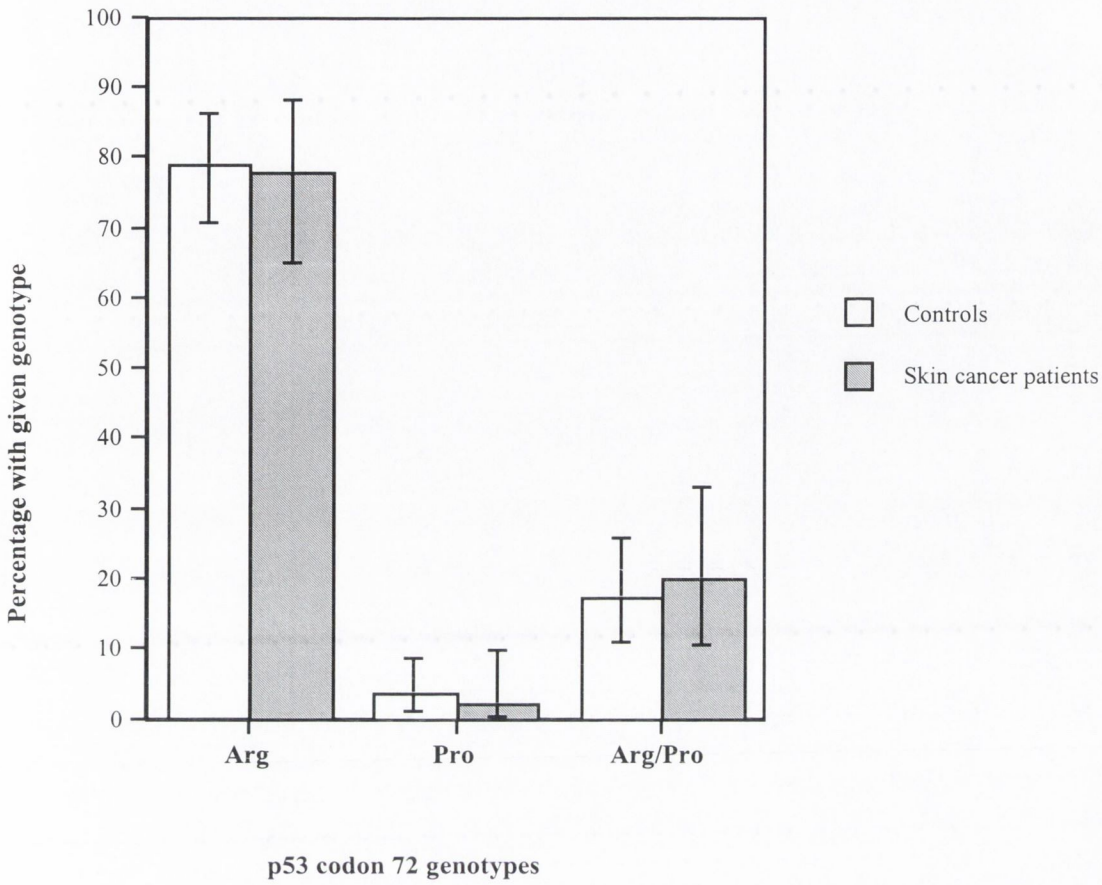


Figure 5.3 Bar chart depicting the distribution of p53 codon 72 genotypes in skin cancer patients and controls. Error bars represent exact binomial 95% confidence intervals. Arg = arginine homozygotes, Pro = proline homozygotes, Arg/Pro = heterozygotes.

5.4 Discussion.

The p53 codon 72 polymorphism has been implicated as a risk factor in a number of human cancers (Sorensen *et al.* 1997, Buller *et al.* 1997, Sjalander *et al.* 1996) and proline homozygotes have been demonstrated to exhibit a greater risk of developing lung cancer (Wang *et al.* 1999b, Jin *et al.* 1995). A recent study (Storey *et al.* 1998) suggests that the arginine isoform of p53 is susceptible to degradation by the E6 protein of low-risk HPV type 11. As cutaneous carcinomas frequently harbour multiple low-risk HPV types and not high-risk HPV16/18 it would seem that the presence of an arginine instead of a proline could be a significant risk factor in the development of these tumours. All of the skin cancer patients analysed in the study by Storey *et al.* were either arginine homozygotes (75%) or heterozygotes (25%) and were positive for common cutaneous and EV-associated HPV types. They suggest that the arginine isoform of p53 confers a susceptibility to tumourigenesis in HPV-associated skin cancers.

The HPV status of 34 of the 55 skin cancer patients analysed in this study was previously described in chapter 2, along with the HPV status of 20 of the 115 control volunteers. The prevailing HPV types in both groups were similar (cutaneous and EV-associated HPV types), though the detection rate in control subjects was somewhat lower. However, as described in chapter 2, latent or subclinical infections with cutaneous and EV-associated HPV types appear to be common in the general population.

The results of this study do not confirm the hypothesis proposed by Storey *et al.*. They show that in the Celtic population there is no apparent link between the presence of HPV, the p53 codon 72 genotype and the development of cutaneous carcinomas. It is

possible that in populations less prone to skin cancer where the arginine isoform of p53 is less prevalent (Beckman *et al.* 1994), it could still confer increased susceptibility to malignancy. However it has been shown that in some populations, where the proline isoform predominates, that individuals homozygous for the arginine isoform are at no greater risk of developing cervical carcinomas (Minaguchi *et al.* 1998, Ngan *et al.* 1999, Yamashita *et al.* 1999). Recently, intratype variation of the E6 gene in HPV16 has been shown to determine whether the p53 codon 72 arginine variant confers an increased risk of developing cervical cancer (van Duin *et al.* 2000). HPV16 types with a G instead of a T at position 350 of the E6 gene were found to be significantly over-represented in arginine homozygous women with cervical cancer. Whether or not intratype variation or the specific HPV type present in cutaneous lesions confers an increased risk of developing cutaneous cancers in conjunction with codon 72 arginine homozygosity is yet to be established.

Chapter 6

General discussion

6.1 Summary of results.

Human papillomaviruses (HPV) are the most common virus to infect human skin and are known to cause infectious warts. Links between a number of genital HPV types and cervical carcinomas have been demonstrated. Though HPV are ubiquitous in the general population they have until recently been overlooked as aetiological agents of non-melanoma skin cancer. This study was designed to determine the prevalence of HPV in viral warts, squamous cell carcinomas and normal skin from both renal transplant patients and immunocompetent patients and in normal skin from the general population. Forty-nine benign and malignant lesions (viral warts (19), squamous cell carcinomas (21) and normal human skin (9)) from 34 skin cancer patients and 20 normal human skin samples from the control population were examined for HPV. Two degenerate PCR strategies were used to amplify a region of the viral L1 gene, which was subsequently cloned and sequenced.

Of the skin cancer patient lesions analysed, 100% of the viral warts (19/19), 86% of the squamous cell carcinomas (18/21) and 22% of normal human skin samples (2/9) were found to be HPV-positive. Following sequencing, viral warts were shown to harbour either common cutaneous HPV types or EV-associated HPV types and all of the HPV-positive SCCs and normal human skin were shown to harbour EV-associated HPV types. Fifteen per cent of the normal human skin samples (3/20) from the control population were found to be HPV-positive and again following sequencing were shown to contain EV-associated HPV types.

Renal transplant recipients frequently develop numerous benign and malignant skin lesions. In chapter 2 the viral aetiology of these skin lesions was determined suggesting that EV-HPV may be involved in the evolution of cutaneous malignancies in

both RTR and immunocompetent skin cancer patients. Studies on anogenital cancers have highlighted the potential role of HPV in human carcinogenesis however, clinical and experimental data imply that HPV alone is not sufficient to induce cancer. The p53 tumour suppressor gene is the most frequently mutated gene in a wide range of human cancers. Under normal circumstances, the p53 gene product regulates the response of the cell to genotoxic stresses such as UV-radiation. A decrease in p53 function (be it through mutation or otherwise) may lead to uncontrolled cellular proliferation, the accumulation of DNA damage and ultimately cancer. Chapter 3 describes an immunohistochemical study to evaluate the expression of p53 in benign and malignant skin lesions from renal transplant recipients and immunocompetent skin cancer patients. The effect of p53 mutations on the expression patterns observed was examined by PCR-SSCP analysis and direct cycle-sequencing. The expression of the p53-regulated cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ and Mdm2, the p53-induced oncoprotein involved in targeting p53 for ubiquitin-mediated degradation, was also examined in p53-positive cells. The expression of p53 in benign and malignant lesions was found to be markedly different. p53 was only expressed in 40% (6/15) of viral warts analysed. The expression was confined to the basal layer both in the lesion and in adjacent normal skin and the level of expression was low and only in a small number of cells (<10%). Of the cutaneous squamous cell carcinomas (SCC) analysed, 60% (9/15) demonstrated p53 expression. Two different patterns of expression were observed. Basal layer expression in both the invasive tumour and adjacent normal skin was observed in 50% of the p53-positive SCCs and in the remaining 50%, p53 was expressed diffusely throughout the invasive tumour and in the basal layer of adjacent normal skin. The level of expression was high and in a large number of cells (>30% to >80%). PCR-SSCP analysis revealed that only one of the SCCs expressing p53 harboured a *p53* mutation and that the

accumulated p53 in the remaining tumours was wild-type. No Mdm2 or $p21^{Waf1/Cip1}$ expression was detected in the p53-positive SCCs indicating that although the accumulated p53 is stable, it does not function effectively as a transcriptional activator.

Loss of function of the *Rb* tumour suppressor gene, located on chromosome 13, is common in many inherited and sporadic forms of cancer. Inactivation of its gene product by oncogenic HPV plays a key role in the genesis of cervical cancer. Chapter 2 demonstrates that non-melanoma skin cancers of renal transplant recipients and immunocompetent skin cancer patients also frequently harbour potentially oncogenic HPV types. However, there is no evidence to suggest that the HPV types associated with cutaneous malignancies act in a manner similar to oncogenic mucosal types, therefore another mechanism of Rb deregulation may occur. Chapter 4 describes a study to examine the integrity of the *Rb* gene in HPV-associated SCCs from RTR and ICP. Loss of heterozygosity at the *Rb* locus was examined in 13 histologically confirmed SCCs using the D13S153 microsatellite marker, which is located in exon 2 of the *Rb* gene. Loss of a second marker, D13S118, distal telomerically to the *Rb* gene at 13q14.3 was also analysed. Of the 13 SCCs examined 11 were informative (*ie* 2 SCCs were homozygous for both microsatellite markers). LOH at the D13S153 locus was found in 40% (4/10) of informative SCCs and LOH at the D13S118 locus was found in 45% (5/11) of informative cases. Overall, 64% (7/11) of informative cases showed LOH at one or other locus. This represents a high degree of chromosomal instability in these tumours. The expression of the *Rb* gene product in the 11 informative cases was analysed immunohistochemically. Expression of Rb was detected in 91% (10/11) of the SCCs examined. No correlation between the HPV status of the tumours and the expression of Rb was found. Though the only SCC not to demonstrate Rb expression also demonstrated LOH at the D13S153 locus, the remaining SCCs that had also

demonstrated LOH at 13q14 expressed Rb. Therefore another tumour suppressor gene located at 13q14 may be responsible for the genesis of these tumours. The expression of Rb was also examined in a further 9 SCCs and in 15 benign viral warts from the same patients. The levels of expression in both viral warts and SCCs was comparable, therefore the Rb pathway may be deregulated by some other means in cutaneous SCCs.

Non-melanoma skin cancers frequently harbour multiple human papilloma virus types. A recent report suggests that a polymorphism of the p53 tumour suppressor gene that results in the substitution of a proline residue with an arginine residue at position 72 of the p53 protein might act as a risk factor in human papillomavirus-associated malignancies. Chapter 5 examines the role this polymorphism might play in the development of cutaneous carcinomas.

Blood samples were taken from 55 skin cancer patients and 115 ethnically matched volunteers. A polymerase chain reaction-based assay was used to determine the p53 codon 72 genotype of the skin cancer patients and control individuals.

The proportions of p53 codon 72 genotypes were 78% arginine homozygous, 2% proline homozygous and 20% heterozygous among skin cancer patients and 79% arginine homozygous, 3.5% proline homozygous and 17.5% heterozygous among the control population. Statistical analysis showed no significant differences in the distribution of the two p53 isoforms between the skin cancer patients and the control population.

These results suggest that there is no correlation between the presence of HPV, the p53 codon 72 arginine polymorphism and the development of skin cancer.

6.2 General discussion.

The key to facilitating molecular studies on the role of HPV in cutaneous malignancies lies in the identification of the high-risk HPV types involved. In the case of cervical carcinomas, the fact that HPV16 and HPV18 account for approximately 70% of the HPV types detected in these tumours has allowed researchers to decipher the precise role the virus plays in anogenital tumourigenesis. To date, this has not been possible in the case of HPV-associated skin cancers.

The wide range of HPV types detected in skin lesions reflects both the variation in detection techniques used and an apparent difference in the manner in which cutaneous HPV types infect human skin compared with the infection of mucosal tissues by genital HPVs. Anogenital lesions generally only harbour a single HPV type, whereas cutaneous lesions frequently demonstrate infection with multiple types. Whether all of the HPV types isolated from a single lesion contribute to the tumourigenic process is unknown. Some may act as co-factors, others merely as "innocent by-standers".

The concurrence of many other researchers that EV-associated HPV are the most prevalent types found in cutaneous SCCs suggests that should HPV play a role in the development of skin cancer, these are the most likely group to be involved. However, the prevalence at which EV-HPV types are detected in skin lesions may reflect a bias in the PCR-based detection techniques used. The nested-PCR assay used to detect EV types is much more sensitive than the single step degenerate PCR used to detect other HPV types. Vladimir Shamanin at the HPV reference laboratory in Heidelberg uses several rounds of PCR to detect mucosal types in cutaneous lesions. PCR products are separated on an agarose gel, a portion of the gel where a band should be is excised, invisible DNA is purified from the band and used as template for the

same PCR reaction again. This process is repeated up to six times before the presence of mucosal HPV DNA can be detected in cutaneous lesions (V. Shamanin, personal communication). This would suggest that either co-infection with mucosal types occurs in cutaneous lesions but that the mucosal types are present at a far lower level than cutaneous types, or that after six rounds of PCR, contamination with DNA from mucosal types in the lab are being detected.

Another interesting phenomenon is highlighted by the results demonstrated in this thesis. The number of new HPV types is expanding rapidly and many of the new types are being isolated from cutaneous malignancies. Few new HPV types are being described in viral warts. Why this should be is unknown. The mutation frequency in high risk mucosal types is believed to be low. However, it is possible that as viral DNA is maintained episomally in cutaneous cancers and is replicated in tandem with the host cell DNA a measure of genetic drift occurs. The environment in which viral DNA is replicated is prone to mutation by UV-radiation and tumour cells often have defective excision repair mechanisms. Given the long duration of HPV infection, it is not unreasonable to suggest that such an environment would promote the accumulation of many mutations in viral DNA and account for the emergence of the large number of new viral types being described. Whatever the role HPV play in the development of cutaneous cancers, other factors must also be involved.

Deregulation of the p53 pathway occurs through UV-induced mutation in approximately 50% of cutaneous SCCs. The rate of mutation in the tumours analysed in this study is significantly lower than previous published studies. However, as all of the control mutations included in the SSCP assay were detected in this study, the results would appear to be valid. No known sampling bias could account for this discrepancy and there appeared to be no difference between the renal transplant recipients and the

immunocompetent skin cancer patients with respect to mutation rate. However, while the accumulation of wild type p53 has been described previously in other cancers, the downstream effects of this accumulation have not been examined and as such the p53 phenotype described in the cutaneous SCCs analysed in this study represents the first such examination. Cell-lines with p53 deficient in DNA-binding activity have been described, but tumours *in vivo* with the same phenotype have not. The cell culture-based studies that show inhibition of p53-dependent transactivation at high p53 levels are misleading in that the levels of p53 attained through overexpression of an exogenous gene are far greater than would be seen physiologically, though it has been suggested that at particularly high physiological concentrations, p53 may induce apoptosis rather than cell cycle arrest (K. Vousden, personal communication). Thus the "squelching" of basal transcription machinery by high concentrations of wild type p53, while an attractive model, may not actually explain the reason for repression of transcriptional activation. It is possible that a much simpler explanation exists. Defective tetramerisation would result in the accumulation of transcriptionally inactive, wild type p53 that presumably doesn't induce the Mdm2 autoregulatory loop.

The results of the LOH analysis of chromosome 13 in this study suggest that a tumour suppressor gene located at 13q14.3 may be lost in cutaneous SCCs. The fact that this region is also lost in a wide range of other tumours lends credence to this hypothesis and the frequency at which this region is lost in these and other tumours suggests that this putative tumour suppressor plays a significant role in the prevention of cancer. To date, none of the candidate genes located in this region appear to act as tumour suppressors and further analysis of the remaining ESTs located at 13q14.3 is required in order to identify other possible candidates.

The patterns of expression observed in the cutaneous SCCs and viral warts analysed for Rb expression in this study suggest that there is no aberrant expression of Rb in these tumours. Rather, the Rb pathway is deregulated elsewhere. The fact that the p53 pathway appears to be frequently inactivated in similar cutaneous SCCs may offer one such source of deregulation. Alterations in $p16^{INK4}$ and cyclin D1 expression may also have a deregulatory effect on the Rb pathway and should be examined in these tumours to ascertain if this is the case.

Finally, the results of this study show no apparent link between arginine homozygosity at codon 72 of p53 and the development of HPV-associated skin cancer. The recent suggestion that intratype variation in the HPV16 E6 gene could determine whether or not possessing the arginine p53 isoform is a risk factor for HPV-associated cervical cancer does not apply to HPV-associated skin cancers as HPV16 is rarely found in cutaneous malignancies. Infection with specific cutaneous or EV-associated HPV types and arginine homozygosity may however play a role and skin cancers should be assessed for both p53 codon 72 status and HPV status in order to determine if such a scenario exists.

6.3 Future work.

The results described in this thesis have raised a number of questions regarding the biology of cutaneous squamous cell carcinomas. Firstly, the high prevalence of EV-associated HPV types detected in the SCCs examined here suggests a possible role for these viruses in the genesis of skin cancer. However, the simultaneous detection of these same HPV types in normal skin from both skin cancer patients and the general population implies that should HPV play a role in cutaneous carcinogenesis, differential

behaviour of the virus and other factors must contribute to the tumourigenic process. To this end, the expression of putative viral oncogenes in benign and malignant tissue should be examined further to determine if there is a true difference in the behaviour of the viruses in different tissues. A keratinocyte cell culture-based system would allow the examination of differential EV-HPV promoter activity in benign and malignant cells.

The p53 expression patterns observed in benign and malignant skin lesions described in this study show that p53 expression is altered in malignant cells. The particular p53 phenotype described here warrants further investigation. Simple immunohistochemical analysis to differentiate monomeric p53 from tetrameric p53 should be carried out to examine whether the repression of transcriptional activation observed here is due to inefficient assembly of active p53 molecules. Alterations in the expression of a wide range of other genes both downstream and upstream of p53 should be examined in these tumours using gene profiling technology to compare the SCCs with a benign state.

The level of chromosomal instability observed at 13q14.2-13q14.3 in the SCCs analysed in this study also warrants further investigation. More samples should be examined to determine if the degree of LOH observed here is true. More microsatellite markers should be examined along the long arm of chromosome 13 to determine the minimum area of loss in these tumours and the expression of candidate ESTs located in the minimum area of loss should be examined in these tumours using RT-PCR analysis.

Finally, the p53 codon 72 genotypes of skin cancer patients should be correlated with infection with specific HPV types.

6.4 Conclusions.

The results of this study show that EV-associated HPV types are the most prevalent viral types detected in cutaneous squamous cell carcinomas from renal transplant recipients and immunocompetent skin cancer patients. Should HPV play a role in the genesis of skin cancer these viral types would be the most likely candidates. Whether HPV play an active or passive role in skin carcinogenesis remains to be clarified.

Key pathways of cellular control and maintenance of genomic stability appear to be altered in the tumours examined in this study. The expression of p53 is markedly different in benign and malignant tissue and the p53 expressed in malignant cells appears to be transcriptionally silent. Mutations in the *p53* gene do not account for this repression of transcriptional activity, however the precise mechanism of inactivation remains to be discovered.

A high degree of chromosomal instability on the long arm of chromosome 13 is found in cutaneous SCCs. However, the expression of Rb seems unaffected by this instability. In addition, the expression of Rb appears to be unaltered in malignant skin lesions compared with benign skin lesions. This would suggest that the Rb pathway is deregulated at a point other than Rb and also that loss of an unknown tumour suppressor located at13q14.2-13q14.3 may be involved in the genesis of these tumours. However, further investigation into the minimum area of loss on chromosome 13 is required to accurately define where the putative novel tumour suppressor is located.

Finally, the results of this study suggest that there is no correlation between the presence of HPV, the p53 codon 72 arginine polymorphism and the development of skin cancer.

References

Adachi A, Kiyono T, Hayashi Y Ohashi M and Ishibashi M (1996) Detection of human papillomavirus type 47 DNA in malignant lesions from epidermodysplasia verruciformis by protocols for precise typing of related HPV DNAs. *J Clin Microbiol* **34**, 369-375.

Adams V, Schmid S, Zariwala M, Schmid M, Kleihues P, Briner J and Schafer R (1999) Prevalence of human papillomavirus DNA in head and neck cancer carrying wild-type or mutant p53 tumour suppressor genes. *Anticancer Res* **19**(1A), 1-6.

Agarwal ML, Taylor WR, Chernov MV, Chernova MB and Stark GR (1998) The p53 network. *J Biol Chem* **273**, 1-4.

Aggelopoulou E, Troiungos C, Goutas N, Skarlos D, Papadimitriou C and Kittas C (1998) Immunohistochemical detection of p53 protein in HPV positive oral lesions. *Anticancer Res* **18**(6A), 4511-4515.

Ahmadian A, Ren ZP, Williams C, Ponten F, Odeberg J and Ponten J (1998) Genetic instability in the 9q22.3 region is a late event in the development of squamous cell carcinoma. *Oncogene* **17**, 1837-1843.

Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215**, 403-410.

Amundson SA, Myers TG and Fornace Jr AJ (1998) Roles for p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress. *Oncogene* **17**(25), 3287-3299.

Androphy EJ, Dvoretzky J and Lowry DR (1985) X-linked inheritance of epidermodysplasia verruciformis: genetic and virologic studies of a kindred. *Arch Dermatol* **121**, 864-868.

Androphy EJ (1994) Molecular biology of human papillomavirus infection and oncogenesis. *J Invest Dermatol* **103**, 248-256.

Artuc M, Nurnberg W, Platzer M, Czarnetzki BM and Schadendorf D (1995) Differential promoter activity in benign and malignant human cells of skin origin. *Exp Dermatol* **4**(5):317-321.

Astori G, Lavergne D, Benton C, Hockmayr B, Egawa K, Garbe C and de Villiers EM (1998) Human papillomaviruses are commonly found in normal skin of immunocompetent hosts. *J Invest Dermatol* **110**(5), 752-755.

Baas IO, Mulder JWM, Offerhaus GJA, Vogelstein B and Hamilton S (1994) An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. *J Pathol* **172,** 3-12.

Baffa R, Negrini M, Mandes B, Rugge M, Ranzani GN, Hirohashi S and Croce CM (1996) Loss of heterozygosity for chromosome 11 in adenocarcinoma of the stomach. *Cancer Res* **56**, 268-272.

Banks L and Matlashewski G (1996) Biochemical and biological activities of the HPV E5 proteins. In Lacey C (ed) Papillomavirus reviews: current research on papillomaviruses. Leeds University Press, Leeds, pp39-45.

Barak Y, Juven T, Haffner R and Oren M (1993) mdm2 expression is induced by wild type p53 activity. *EMBO J* **12**, 461-468.

Barr BBB, Benton EC, McLaren K, Bunney MH, Smith IW, Blessing K and Hunter JAA (1989) Human papilloma virus infection and skin cancer in renal allograft recipients. *Lancet* 124-128.

Bartek J, Bartkova J, Vojtesek B, Staskova Z, Rejthar A, Kovarik J and Lane DP (1990) Patterns of expression of the p53 tumour suppressor in breast tissues and tumours in situ and in vitro. *Int J Cancer* **46**(5), 839-844.

Beckman G, Birgander R, Sjalander A, Saha N, Holmberg PA, Kivela A and Beckman L (1994) Is p53 polymorphism maintained by natural selection? *Hum Hered* **44**(5), 266-270.

Beijersbergen RL, Carlee L, Kerkhoven RM and Bernards R (1995) Regulation of the retinoblastoma protein-related p107 by G1 cyclin complexes. *Genes Dev* **9**, 1340-1353.

Bennett MA, O'Grady A, Kay EW, Leader M and Murphy GM (1997) p53 mutations in squamous cell carcinomas from renal transplant recipients. *Biochem Soc Trans* **25**, 342-345.

Benton EC, Shahidullah H, and Hunter JAA (1992) Human papillomavirus in the immunosuppressed. *Papillomavirus Rep* **3**, 23-26.

Berkhout RJM, Tieben LM, Smits HL, Bouwes Bavinck JN, Vermeer BJ and ter Schegget J (1995) Nested PCR approach for detection and typing of epidermodysplasia verruciformis-associated human papillomavirus types in cutaneous cancers from renal transplant recipients. *J Clin Microbiol* **33**, 690-695.

Bernard HU, Chan SY and Manos MM (1994) Identification and assessment of known and novel human papillomaviruses by polymerase chain reaction amplification, restriction fragment length polymorphisms, nucleotide sequence, and phylogenetic algorithms. *J Infect Dis* **170**, 1077-1085.

Berumen J, Casas L and Segura E **(**1994) Genome amplification of human papillomavirus type 16 and 18 in cervical carcinomas is related to the retention of E1/E2 genes. *Int J Cancer* **56**, 640-645.

Berumen J, Unger ER, Casas L and Figeuroa P (1995) Amplification of human papillomavirus types 16 and 18 in invasive cervical cancer. *Hum Pathol* **26**, 676-681.

Bezieau S, Devilder MC, Rondeau G, Cadoret E, Moisan JP and Moreau I (1998) Assignment of 48 ESTs to chromosome band 13q14.3 and expression pattern for ESTs located in the core region deleted in B-CLL. *Genomics* **52**(3), 369-373.

Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, Schiffman MH, Moreno V, Kurman R and Shah KV (1995) Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. *J Natl Cancer Inst* **87**, 796-802.

Boxman ILA, Berkhout RJM, Mulder LHC, Wolkers MC, Bouwes Bavinck JN, Vermeer BJ and ter Schegget J (1997) Detection of human papillomavirus DNA in plucked hairs from renal transplant recipients and healthy volunteers. *J Invest Dermatol* **108**, 712-715.

Boxman IL, Mulder LH, Russell A, Bouwes-Bavinck JN, Green A and ter Schegget J (1999) Human papillomavirus type 5 is commonly present in immunosuppressed and immunocompetent individuals. *Br J Dermatol* **141**(2), 246-249.

Brash DE, Seetharam S, Kraemer KH, Seidmann MM and Bredberg A (1987) Photoproduct frequency is not the major determinant of UV base substitution hot spots or cold spots in human cells. *Proc Natl Acad Sci USA* **84**(11), 3782-3786.

Brash DE, Rudolph JA and Simon JA (1991) A role of sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc Natl Acad Sci USA* **88**, 10124-10128.

Breuninger H, Black G and Rassner G (1990) Microstaging of squamous cell carcinomas. *Am J Clin Pathol* **94**, 624-627.

Buller RE, Sood A, Fullenkamp C, Sorosky J, Powills K and Anderson B (1997) The influence of the p53 codon 72 polymorphism on ovarian carcinogenesis and prognosis. *Cancer Gene Ther* **4**(4), 239-245.

Burns JE, Clark LJ, Yeudall WA, Mitchell R, Mackenzie K, Chang SE and Parkinson EK (1994) The p53 status of cultured human premalignant oral keratinocytes. *Br J Cancer* **70**, 591-595.

Butler D (1999) Investigation of molecular genetic and immunohistochemical markers in the progression of CIN I, CIN III and microinvasive carcinoma of the uterine cervix. M.Sc. Thesis, Trinity College Dublin.

Caelles C, Helmberg A and Karin M (1994) p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. *Nature* **370**, 220-223.

Campbell C, Quinn AG, Ro YS, Angus B and Rees JL (1993) p53 mutations are common early events that precede tumour invasion in squamous cell neoplasia of the skin. *J Invest Dermatol* **100**(6), 746-748.

Caruso ML and Valentini AM (1998) Immunohistochemical detection of p53 protein in anogenital lesions and its relationship with HPV status. *Anticancer Res* **18**(6A), 4097-4100.

Castren K, Vahakangas K, Heikkinen E and Ranki A (1998) Absence of p53 mutations in benign and pre-malignant male genital lesions with over-expressed p53 protein. *Int J Cancer* **77**(5), 674-678.

Cawkwell L, Bell S, Lewis F, Dixon M, Taylor G and Quirke P (1993) Rapid detection of allele loss in colorectal tumours using microsatellites and fluorescent DNA technology. *Br J Cancer* **67**, 1262-1267.

Chan S-Y, Delius H, Haipern AL and Bernard HU (1995) Analysis of genomic sequences of 95 papillomavirus types: uniting typing, phylogeny and taxonomy. *J Virol* **69**, 3074-3083.

Chow KNB and Dean DC (1996) Domains A and B in the Rb pocket interact to form a transcriptional repressor motif. *Mol Cell Biol* **16**, 4862-4868.

Chow LT and Broker TR (1994) Papillomavirus DNA replication. *Intervirology* **37**, 150-158.

Claudio PP, De Luca A, Howard CM, Baldi A, Firpo EJ, Paggi MG and Giordano A (1996) Functional analysis of pRb2/p130 interaction with cyclins. *Cancer Res* **56**, 2003-2008.

Cooper GM (1995) Tumour suppressor genes in human cancers. In: Oncogenes, Jones and Bartlett, Boston, pp145-161.

Crook T and Farthing A (1993) Human papillomavirus and cervical cancer. *Br J Hosp Med* **49**, 131-132.

Crook T and Vousden KH (1994) Interaction of HPV E6 with p53 and associated proteins. *Biochem Soc Trans* **22**, 52-55.

Crook T, Fisher C, Masterson PJ and Vousden KH (1994) Modulation of transcriptional regulatory properties of p53 by HPV E6. *Oncogene* **9**, 1225-1230.

Cullen AP, Reid R and Campion M (1991) Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. *J Virol* **65**, 606-612.

De Gregori J, Leone G, Ohtani K, Mirron A and Nevins JR (1995) E2F-1 accumulation bypasses a G1 arrest resulting from the inhibition of G1 cyclin-dependent kinase activity. *Genes Dev* **9**, 2873-2887.

de Jong-Tieben LM, Berkhout RJM, Smits HL, Bouwes Bavinck JN, Vermeer BJ, van der Woude FJ and ter Schegget J (1995) High frequency of detection of epidermodysplasia verruciformis-associated human papillomavirus DNA in biopsies from malignant and premalignant skin lesions from renal transplant recipients. *J Invest Dermatol* **105**, 367-371.

de Villiers E-M (1994) Human pathogenic papillomaviruses: An update. *Curr Top Microbiol Immunol* **86**, 1-12.

de Villiers EM, Lavergne D, McLaren K and Benton EC (1997) Prevailing papillomavirus types in non-melanoma carcinomas of the skin in renal allograft recipients. *Int J Cancer* **73**, 356-361.

Deau MC, Favre M and Orth G (1991) Genetic heterogeneity among human papillomaviruses (HPVs) associated with epidermodysplasia verruciformis: evidence for multiple allelic forms of HPV 5 and HPV8 E6 genes. *Virology* **184**, 492-503.

Debbas M and White E (1993) Wild type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes Dev* **7**, 546-554.

Deng C, Zhang P, Harper JW, Elledge SJ and Leder P (1995) Mice lacking p21/CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* **82**, 675-684.

Doorbar J (1996) The E4 proteins and their role in the viral life cycle. In Lacey C (ed) Papillomavirus reviews: current research on papillomaviruses. Leeds University Press, Leeds, pp31-38.

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

Dyson N, Dembski M, Fattaey A, Ngwu C, Ewen M and Helin K (1993) Analysis of p107-associated proteins: p107 associates with a form of E2F that differs from pRb-associated E2F. *J Virol***67**, 7641-7647.

El Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B (1993) WAF1, a potential mediator of p53 tumour suppression. *Cell* **75**, 817-825.

Eliyahu D, Michalovitcz D, Eliyahu S, Pinhasi-Kimhi O and Oren M (1989) Wild type p53 can inhibit oncogene-mediated focus formation. *Proc Natl Acad Sci USA* **86**, 8763-8767.

Euvrard S, Chardonnet Y, Pouteil-Noble C, Kanitakis J, Chignol MC, Thivolet J and Touraine JL (1993) Association of skin malignancies with various and multiple carcinogenic and noncarcinogenic human papillomaviruses in renal transplant recipients. *Cancer* **72**, 2198-2206.

Evans RD, Kopf AW, Lew RA, Rigel DS, Bart RS and Friedman RJ (1988) Risk factors for the development of malignant melanoma: review of case control studies. *J Dermatol Surg Oncol* **14**, 393-406.

Ewen ME (1998) Regulation of the cell cycle by the Rb tumour suppressor family. *Results Probl Cell Differ* **22**, 149-179.

Fan S, Chang JK, Smith ML, Duba D and Fornace AJ (1997) Cells lacking CIP1/WAF1 genes exhibit preferential sensitivity to cisplatin and nitrogen mustard. *Oncogene* **14**, 1875-1882.

Farthing AJ and Vousden KH (1994) Functions of human papillomavirus E6 and E7 oncoproteins. *Trends Microbiol* **2**, 170-174.

Favre M, Ramoz N and Orth G (1997) Human papillomaviruses: general features. *Clin Dermatol* **15**, 181-198.

Ferrandiz C, Fuente MJ, Fernandez-Figeuras MT, Bielsa I and Just M (1999) p53 immunohistochemical expression in early posttransplant-associated malignant and premalignant cutaneous lesions. *Dermatol Surg* **25**(2), 97-101.

Finlay CA, Hinds PW and Levine AJ (1989) The p53 proto-oncogene can function as a suppressor of transformation. *Cell* **57**, 1083-1093.

Fiscella M, Ullrich SJ, Zambrano N, Shields MT, Lin D, Lees-Miller SP, Anderson CW, Mercer WE and Apella E (1993) Mutation of the serine 15 phosphorylation site of human p53 reduces the ability of p53 to inhibit cell cycle progression. *Oncogene* **8**, 1519-1528.

Forslund O, Antonsson A, Nordin P, Stenquist B and Hansson BG (1999) A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. *J Gen Virol* **80**, 2437-2443.

Franco EL (1995) Cancer causes revisited: Human papillomavirus in cervical neoplasia. *J Natl Cancer Inst* **87**, 779-780.

Friedberg EC, Walker GC and Siede W (1995) *DNA repair and mutagenesis* (Am. Soc. For Microbiol., Washington, DC).

Fu L and Benchimol S (1997) Participation of the human p53 3' UTR in translational repression and activation following gamma-irradiation. *EMBO J* **16**, 4117-4125.

Fuchs PG and Pfister H (1996) Papillomaviruses in epidermodysplasia verruciformis. In Lacey C (ed) Papillomavirus reviews: current research on papillomaviruses. Leeds University Press, Leeds, pp253-261.

Funk JA, Waga S and Harry JB (1997) Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV16 E7 oncoprotein. *Genes Dev* **11**, 2090-2100.

Funk JA, Schiller PI, Barrett MT, Wong DJ, Kind P and Sander CA (1998) p16INK4a expression is frequently decreased and associated with 9p21 loss of heterozygosity in sporadic melanoma. *J Cutan Pathol* **25**, 291-296.

Gallagher RP, Ma B, McLean DI, Yang CP, Ho V, Carruthers JA and Warshawski LM (1990) Trends in basal cell carcinoma, squamous cell carcinoma and melanoma of the skin from 1973 through 1987. *J Am Acad Dermatol* **23**, 413-421.

Gao C, Nakajima T, Taya Y and Tsuchida N (1999) Activation of p53 in MDM2-overexpressing cells through phosphorylation. *Biochem Biophys Res Commun* **264**(3), 860-864.

Giaccia AJ and Kastan MB (1998) The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev* **12**(19), 2973-2983.

Giannoudis A, Graham DA, Southern SA and Herrington CS (1999) p53 codon 72 ARG/PRO polymorphism is not related to HPV type or lesion grade in low- and high-grade squamous intraepithelial lesions and invasive squamous carcinoma of the cervix. *Int J Cancer* **24**(83 pt 1), 66-69.

Gibson GE, O'Grady A, Kay EW, Leader M and Murphy GM (1997) p53 tumour suppressor gene protein expression in premalignant and malignant skin lesions of kidney transplant recipients. *Am J Dermatol* **36**(6 pt 1), 924-931.

Giles GG, Marks R and Foley P (1988) Incidence of non-melanocytic skin cancer treated in Australia. *Br Med J* **296**, 13-17.

Glass AG and Hoover RN (1989) The emerging epidemic of melanoma and squamous cell skin cancer. *JAMA* **262**, 2097-2100.

Gottlieb TM and Jackson SP (1993) The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. *Cell* **72**, 131-142.

Gravitt PE, Peyton CL, Alessi TQ, Wheeler CM, Coutlee F, Hildesheim A, Schiffman MH, Scott DR and Apple RJ (2000) Improved amplification of genital human papillomaviruses. *J Clin Microbiol* **38**(1), 357-361.

Green A, Battistuta D, Hart V, Leslie D and Weedon D (1996) Skin cancer in a sub-tropical Australian population: incidence and lack of association with occupation. *Am J Epidemiol* **144**, 1034-1040.

Gupta VK, Schmidt AP, Pashia ME, Sunwoo JB and Scholnick SB (1999) Multiple regions of deletion on chromosome arm 13q in head and neck squamous cell carcinoma. *Int J Cancer* **84**, 453-457.

Haapajarvi T, Pitkanen K and Laiho M (1999) Human melanoma cell line UV responses show independancy of p53 function. *Cell Growth Differ* **10**(3), 163-171.

Hall M and Peters G (1996) Genetic alterations of cyclins, cyclin-dependent kinases and cdk inhibitors in human cancer. *Adv Cancer Res* **68**, 67-108.

Haller K (1995) Differentiation-dependent transcription of the epidermodysplasia verruciformis-associated human papillomavirus type 5 in benign lesions. *Virology* **214**, 245-255.

Hamann U, Herbold C, Costa S, Solomayer EF, Kaufmann M, Bastert G, Ulmer HU, Frenzel H and Komitowski D (1996) Allelic imbalance on chromosome 13q: evidence for the involvement of *BRCA2* and *RB1* in sporadic breast cancer. *Cancer Res* **56**, 1988-1990.

Happle R (1999) Loss of heterozygosity in human skin. *J Am Acad Dermatol* **41**, 143-164.

Harris S and Jones DB (1997) Optimisation of the polymerase chain reaction. *Br J Biomed Sci* **54**, 166-173.

Harwood CA, Spink PJ, Surentheran T, Leigh IM, Hawke JL, Proby CM, Breuer J and McGregor JM (1998) Detection of human papillomavirus DNA in PUVA-associated no-melanoma skin cancers. *J Invest Dermatol* **111**(1), 123-127.

Harwood CA, McGregor JM, Proby CM and Breuer J (1999a) Human papillomaviruses and the development of non-melanoma skin cancer. *J Clin Pathol* **52**(4) 249-253.

Harwood CA, Spink PJ, Surentheran T, Leigh IM, de Villiers EM, McGregor JM, Proby CM and Breuer J (1999b) Degenerate and nested PCR: a highly sensitive and specific method for the detection of human papillomavirus infection in cutaneous warts. *J Clin Microbiol* **37**(11), 3545-3555.

Hatakeyama M and Weinberg RA (1995) The role of Rb in cell cycle control. *Prog Cell Cycle Res* **1**, 9-19.

Herrington CS (1995) Human papillomavirus and cervical neoplasia. II. Interaction of HPV with other factors. *J Clin Pathol* **48**, 1-6.

Hijmans EM, Voorhoeve PM, Beijersbergen RL, van't Veer LJ and Bernards R (1995) E2F-5, a new E2F family member that interacts with p130 in vivo. *Mol Cell Biol* **15**, 3082-3089.

Hinds PW, Mittnacht S, Dulic V, Arnold A, Reed SI and Weinberg SA (1992) Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* **70**, 993-1006.

Hofmann F and Livingston DM (1996) Differential effects of cdk2 and cdk3 on the control of pRb and E2F function during G1 exit. *Genes Dev* **10**, 851-861.

Hollstein M, Sidransky D, Vogelstein B and Harris CC (1991) p53 mutations in human cancers. *Science* **253**, 49-53.

Holmberg E, Rozell BL and Toftgard R (1996) Differential allele loss on chromosome 9q22.3 in human non-melanoma skin cancer. *Br J Cancer* **74**, 246-250.

Honda R and Yasuda (1999) Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumour suppressor p53. *EMBO J* **18**, 22-27.

Hopfl R, Bens G, Wieland U, Petter A, Zelger B, Fritsch P and Pfister H (1997) Human papillomavirus DNA in non-melanoma skin cancers of a renal transplant recipient: detection of a new sequence related to epidermodysplasia verruciformis associated types. *J Invest Dermatol* **108**, 53-56.

Howes KA, Ransom N, Papermaster DS, Lasudry JGH, Albert DM and Windle JJ (1994) Apoptosis or retinoblastoma: alternative fates of photoreceptors expressing the HPV-16 E7 gene in the presence or absence of p53. *Genes Dev* **8**, 1300-1310.

Howley PM (1996) Papillomaviridae: the viruses and their replication. In Fields BN, Kripke DM and Howley PM (eds.) Virology Vol 2. Lippincott-Raven, Philadelphia, pp2045-2076.

Huang LC, Clarkin KC and Wahl GM (1996) p53-dependent cell cycle arrests are preserved in DNA-activated protein kinase-deficient mouse fibroblasts. *Cancer Res* **56**, 2940-2944.

Huettner PC, Gerhard D, Li L, Gersell DJ, Dunnigan K, Kamarasova T and Rader JS (1998) Loss of heterozygosity in clinical stage 1B cervical carcinoma: relationship with clinical and histopathological features. *Human Pathol* **29**(4), 364-370.

Huibregste JM, Scheffner M and Howley PM (1993) Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. *Mol Cell Biol* **13**, 775-784.

Indinnimeo M, Cicchini C, Stazi A, Giarnieri E, French D, Limiti MR, Ghini C and Vecchione A (1999) Human papillomavirus infection and p53 nuclear overexpression in anal canal carcinomas. *J Exp Clin Cancer Res* **18**(1), 47-52.

Inohara S, Kitagawa K and Kitano Y (1996) Co-expression of $p21^{Waf1/Cip1}$ and p53 in sun-exposed epidermis, but not in neoplastic epidermis. *Br J Dermatol* **135**, 717-720.

Jablonska S and Orth G (1995) Cutaneous warts: clinical, histological and virological correlation. *Arch Dermatol Res* **287**, 616-617.

Jackson S and Storey A. Inhibition of UV-induced apoptosis by cutaneous HPV E6 proteins involved inactivation of Bak. *Unpublished results.*

Jiang W, Zhang YJ, Kahn SM, Hollstein MC, Santella RM, Lu SH, Harris CC, Montesano R and Weinstein IB (1993) Altered expression of cyclin D1 and retinoblastoma genes in human oesophageal cancer. *Proc Natl Acad Sci USA* **90**, 9026-9030.

Jiang H, Lin J, Su ZZ, Herlyn M, Kerbel RS, Weissmann BE, Welch DR and Fisher PB (1995) The melanoma differentiation-associated gene, mda-6, which encodes the cyclin-dependent kinase inhibitor p21, is differentially expressed during growth, differentiation, and progression in human melanoma cells. *Oncogene* **10**, 1855-1864.

Jin X, Wu X, Roth JA, Amos CI, King TM, Branch C, Honn SE and Spitz MR (1995) Higher lung cancer risk for younger African-Americans with the Pro/Pro p53 genotype. *Carcinogenesis* **16**(9), 2205-2208

Johnson DG, Schwarz JK, Cress WD and Nevins JR (1993) Expression of transcription factor E2F-1 induced quiescent cells to enter S phase. *Nature* **365**, 349-352.

Johnson DG (1995) Regulation of E2F-1 gene expression by p130 (Rb2) and D-type cyclin kinase activity. *Oncogene* **11**, 1685-1692.

Jones DL, Alani RM and Munger K (1997) The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2. *Genes Dev* **11**, 2101-2111.

Kato JY, Matsushime H, Hiebert SW, Ewen ME and Sherr CJ (1993) Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin-dependent kinase cdk4. *Genes Dev* **7**, 331-342.

Kelly DA, Young AR, McGregor JM, Seed PT, Potten CS and Walker SL (2000) Sensitivity to sunburn is associated with susceptibility to ultraviolet radiation-induced suppression of cutaneous cell-mediated immunity. *J Exp Med* **191**(3), 561-566.

Kerschmann RL, McCalmont TH and LeBoit PE (1994) p53 oncoprotein expression and proliferation index in keratoacanthoma and squamous cell carcinoma. *Arch Dermatol* **130**(2), 181-186.

Khorshid SM, Glover MT, Churchill L, McGregor JM and Proby CM (1996) p53 immunoreactivity in non-melanoma skin cancer from immunosuppressed and immunocompetent individuals: a comparative study of 246 tumours. *J Cutan Pathol* **23**(3), 229-233.

Koh JY, Cho NP, Kong G, Lee JD and Yoon K (1998) p53 mutations and human papillomavirus DNA in oral squamous cell carcinoma: correlation with apoptosis. *Br J Cancer* **78**(3), 354-359.

Kraemer KH, Lee MM, Andrews AD and Lambert WC (1984) DNA repair protects against cutaneous and internal neoplasia; evidence from Xeroderma pigmentosum. *Carcinogenesis* **5**, 511-514.

Kripke ML (1984) Immunological unresponsiveness induced by ultraviolet radiation. *Immunol Rev* **80**, 87-102.

Kripke ML (1990) Effects of UV radiation on tumour immunity. *J Natl Cancer Inst* **82**, 1392-1395.

Kubbutat MHG, Jones SN and Vousden KH (1997) Regulation of p53 stability by Mdm2. *Nature* **387**, 299-303.

Kubbutat MH, Ludwig RL, Levine AJ and Vousden KH (1999) Analysis of the degradation function of Mdm2. *Cell Growth Differ* **10**, 87-92.

Kumar R, Smeds J, Lundh Rozell B and Hemminki K (1999) Loss of heterozygosity at chromosome 9p21 (INK4-p14ARF locus): homozygous deletions and mutations in the p16 and p14ARF genes in sporadic primary melanomas. *Melanoma Res* **9**(2), 138-147.

Kushida Y, Miki H and Ohmori M (1999) Loss of heterozygosity in actinic keratoses, squamous cell carcinoma and sun-exposed normal appearing skin in Japanese: difference between Japanese and Caucasians. *Cancer Lett* **140**, 169-175.

Lane DP (1998) Awakening angels. *Nature* **394**, 616-617.

Lang FF, Miller DC, Pisharody S, Koslow M and Newcomb EW (1994) High frequency of p53 protein accumulation without p53 gene mutation in human juvenile pilocytic, low grade and anaplastic astrocytomas. *Oncogene* **9**(3), 949-954.

Lassus J and Ranki A (1996) Simultaneously detected aberrant p53 tumour suppressor protein and HPV-DNA localise mostly in separate keratinocytes in anogenital and common warts. *Exp Dermatol* **5**(2), 72-78.

Leigh IM and Glover MT (1995) Cutaneous warts and tumours in immunosuppressed patients. *J Royal Soc Med* **88**, 61-62.

Leonard JH and Hayard N (1997) Loss of heterozygosity of chromosome 13 in Merkel cell carcinoma. *Genes Chromosomes Cancer* **20**(1), 93-97.

Leveillard T, Andera L, Bisonette N, Schaeffer L, Bracco L, Egly JM and Waslyk B (1996) Functional interactions between p53 and the TFIIH complex are affected by tumour-associated mutations. *EMBO J* **15**, 1615-1624.

Levine AJ (1997) p53, the cellular gatekeeper for growth and cell division. *Cell* **88**, 323-331.

Lin SY, Black AR, Kostic D, Pajovic S, Hoover CN and Azizkhan JC (1996) Cell cycle-regulated association of E2F1 and Sp1 is related to their functional interaction. *Mol Cell Biol* **16**, 1667-1675.

Liu Y, Szekely L, Grander D, Soderhall S, Juliusson G, Gahrton G, Linder S and Einhorn S (1993) Chronic lymphocytic leukaemia cells with allelic deletions at 13q14 commonly have one intact *RB1* gene: evidence for role of an adjacent locus. *Proc Natl Acad Sci USA* **90**, 8697-8701.

London NJ, Farmery SM, Will EJ, Davison AM and Lodge JPA (1995) Risk of neoplasia in renal transplant patients. *Lancet* **346**, 403-406.

Lorincz AT, Reid R and Jenson B (1992) Human papillomavirus infection of the cervix: relative risk associations of 15 common anogenital types. *Obstet Gynecol* **79**, 329-337.

Lu X and Lane DP (1993) Differential induction of transcriptionally active p53 following UV or ionising radiation: defects in chromosome instability syndromes? *Cell* **75**, 765-778.

Lu H and Levine AJ (1995) Human TAFII31 protein is a transcriptional coactivator of the p53 protein. *Proc Natl Acad Sci USA* **92**, 5154-5158.

Lu S, Tiekso J, Hietanen S, Syrjanen K, Havu VK and Syrjanen S (1999) Expression of cell-cycle proteins p53, p21 (Waf 1), PCNA and Ki-67 in benign, premalignant and malignant skin lesions with implicated HPV involvement. *Acta Derm Venerol* **79**(4), 268-73.

Lutzner MA (1978) Epidermodysplasia verruciformis: an autosomal recessive disease characterised by viral warts and skin cancer. *Bull Cancer (Paris)* **65**, 169-182.

Maher VM, Dorney DJ, Mendrala AL, Konze-Thomas B and McCormick JJ (1979) DNA excision-repair processes in human cells can eliminate the cytotoxic and mutagenic consequences of ultraviolet irradiation. *Mutat Res* **62**(2), 311-323.

Majewski S, Skopinska-Rozewska E and Jablonska S (1986) Partial defects in cell-mediated immunity in patients with epidermodysplasia verruciformis. *J Am Acad Dermatol* **15**, 966-973.

Majewski S, Malejczyk J and Jablonska S (1990) Natural cell-mediated cytotoxicity against various target cells in patients with epidermodysplasia verruciformis. *J Am Acad Dermatol* **22**, 423-427.

Majewski S and Jablonska S (1995) Epidermodysplasia verruciformis as a model of human papillomavirus-induced genetic cancer of the skin. *Arch Dermatol* **131**, 1312-1318.

Majewski S, Jablonska S and Orth G (1997) Epidermodysplasia verruciformis. Immunological and non-immunological surveillance mechanisms: role in tumour progression. *Clin Dermatol* **15**, 321-334.

Man S, Ellis IO, Sibbering M, Blamey RW and Brook JD (1996) High levels of allele loss at the FHIT and ATM genes in non-comedo ductal carcinoma in-situ and grade 1 tubular invasive breast cancers. *Cancer Res* **56**, 5484-5489.

Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR and Wolinsky SM (1989) Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. *Cancer Cells* **7**, 209-214.

Mansat-Krzyzanowska E, Dantal J, Hourmant M, Litoux P, Soulillou JP and Dreno B (1997) Frequency of mucosal HPV DNA detection (types 6/11, 16/18, 31/35/51) in skin lesions of renal transplant patients. *Transpl Int* **10**(2), 137-140.

Marks R, Jolley D, Lecastas P and Foley P (1990) The role of childhood sunlight exposure in the development of solar keratoses and non-melanocytic skin cancer. *Med J Aust* **152**, 62-65.

Marks R, Staples M and Giles GG (1993) Trends in non-melanocytic skin cancer treated in Australia: the second national survey. *Int J Cancer* **53**, 585-590.

Matlashewski GJ, Tuck S, Pim D, Lamb P, Schneider J and Crawford LV (1987) Primary structure polymorphism at amino acid residue 72 of human p53. *Mol Cell Biol* **7**(2), 961-963.

Matlashewski G, Osborn K, Banks L, Stanley M and Crawford LV (1988) Transformation of primary human fibroblast cells with human papillomavirus type 16 and EJ-*ras*. *Int J Cancer* **42**, 232-238.

Mayo LD, Turchi JJ and Berberich SJ (1997) Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. *Cancer Res* **57**, 5013-5016.

McCann J (1999) Can skin cancers be minimised or prevented in organ transplant patients? *J Natl Cancer Inst* **91**(11), 911-913.

McGregor WG, Chen RH, Lukash L, Maher VM and McCormick JJ (1991) Cell cycle-dependent strand bias for UV-induced mutations in the transcribed strand of excision repair-proficient human fibroblasts but not in repair-deficient cells. *Mol Cell Biol* **11**(4), 1927-1934.

McGregor JM, Yu CC, Dublin EA, Levison DA and MacDonald DM (1992) Aberrant expression of p53 tumour-suppressor protein in non-melanoma skin cancer. *Br J Dermatol* **127**(5), 463-469.

McGregor JM, Berkhout RJM, Rozycka M, ter Schegget J, Bouwes Bavinck JN, Brooks L and Crook T (1997) p53 mutations implicate sunlight in post-transplant skin cancer irrespective of human papillomavirus status. *Oncogene* **15**, 1737-1740.

McGregor JM, Crook T, Fraser-Andrews EA, Rozycka M, Crossland S, Brooks L and Whittaker SJ (1999) Spectrum of p53 gene mutations suggests a possible role for ultraviolet radiation in the pathogenesis of advanced cutaneous lymphomas. *J Invest Dermatol* **112**(3), 317-321.

Medema RH, Herrera RE, Lam F and Weinberg RA (1995) Growth suppression by p16INK4 requires functional retinoblastoma protein. *Proc Natl Acad Sci USA* **92**, 6289-6293.

Minaguchi T, Kanamori Y, Matsushima M, Yoshikawa H, Taketani Y and Nakamura Y (1998) No evidence of correlation between polymorphism at codon 72 of p53 and risk of cervical cancer in Japanese patients with human papillomavirus 16/18 infection. *Cancer Res* **58**(20), 4585-4586

Moberg K, Starz MA and Lees JA (1996) E2F-4 switches from p130 to p107 and pRb in response to cell cycle entry. *Mol Cell Biol* **16**, 1436-1449.

Moller R, Reyman F and Hou-Jensen K (1979) Metastases in dermatological patients with squamous cell carcinoma. *Arch Dermatol* **115**, 703-705.

Monk BJ, Cook N, Ahn C, Vasilev SA, Berman ML and Wilczynski SP (1994) Comparison of the polymerase chain reaction and Southern blot analysis in detecting and typing human papillomavirus deoxyribonucleic acid in tumours of the lower female genital tract. *Diagn Mol Pathol* **3**, 283-291.

Moriwaki SI, Ray S, Tarone RE, Kraemer KH and Grossman L (1996) *Mutat Res DNA Repair* **364**, 117-123.

Morris JDH, Crook T, Bandara LR, Davies R, LaThangue NB and Vousden KH (1993) Human papillomavirus type 16 E7 regulates E2F and contributes to mitogenic signalling. *Oncogene* **8**, 893-898.

Myers G, Baker C and Munger K (1997) Human papillomaviruses 1997, a compilation and analysis of nucleic acid and amino acid sequences. Los Alamos, MN: Los Alamos National Laboratory.

Nakanishi H, Yoshikawa H, Sato N, Ochi T and Aozasa K (1999) Frequent p53 mutations in soft tissue sarcomas arising in burn scar. *Jpn J Cancer Res* **90**(3) 276-279.

Nataraj AJ, Wolf P, Cerroni L and Ananthaswamy HN (1997) p53 mutation in squamous cell carcinomas from psoriasis patients treated with psoralen + UVA (PUVA). *J Invest Dermatol* **109**(2), 238-243.

Nelson JH, Hawkins GA, Edlund K, Evander M, Kjellberg L, Wadell G, Dillner J, Gerasimova T, Coker AL, Pirisi L, Petereit D and Lambert PF (2000) A novel and rapid PCR-based method for genotyping human papillomaviruses in clinical samples. *J Clin Microbiol* **38**(2), 688-695.

Ng IO, Lam KY, Ng M and Regezi JA (1999) Expression of p21/waf1 in oral squamous cell carcinomas: correlation with p53 and mdm2 and cellular proliferation index. *Oral Oncol* **35**(1), 63-69.

Ngan HY, Liu VW and Liu SS (1999) Risk of cervical cancer is not increased in Chinese carrying homozygous arginine at codon 72 of p53. *Br J Cancer* **80**(11), 1828-1829

Nigro JM, Baker SJ, Priesinger AC, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S, Devilee P, Glover T, Collins FS, Weston A, Modali R, Harris CC and Vogelstein B (1989) Mutations in the p53 gene occur in diverse human tumour types. *Nature* **342**, 705-708.

Nishigori C, Yarosh DB and Donawho C (1996) The immune system in ultraviolet carcinogenesis. *J Invest Dermatol Symp Proc* **1**, 143-146.

Nixon RL, Dorevitch AP and Marks R (1986) Squamous cell carcinoma of the skin: accuracy of clinical diagnosis and outcome of follow-up in Australia. *Med J Aust* **144**, 235-239.

Ogawara K, Miyakawa A, Shiiba M, Uzawa K, Watanabe T, Wang XL, Sato T, Kubosawa Y, Kondo Y and Tanzawa H (1998) Allelic loss of chromosome 13q14.3 in human oral cancer: correlation with lymph node metastasis. *Int J Cancer* **79**, 312-317.

Oren M (1999) Regulation of the p53 tumour suppressor protein. *J Biol Chem* **274**, 36031-36034.

Orth G (1986) Epidermodysplasia verruciformis: a model for understanding the oncogenicity of human papillomaviruses. Ciba Foundation Symposium 120, Papillomaviruses, Wiley, Chichester, pp157-174.

Orth G (1987) Epidermodysplasia verruciformis. In: Salzman NP, Howley PM (eds.). *The papovaviridae. Vol.2: The papillomaviruses*. Plenum, New York, pp199-243.

Orth G (1994) Human papillomaviruses: general features. In: Webster RG & Granhoff A editors. *Encyclopaedia of virology; vol 2*, Academic Press, London, pp1013-1021.

Park JS, Boyer S, Mitchell K, Gilfor D, Birrer M, Darlington G, El Deiry W, Firestone GL, Munger K, Band V, Fisher PB and Dent P (2000) Expression of human papillomavirus E7 protein causes apoptosis and inhibits DNA synthesis in primary hepatocytes via increased expression of p21(Cip1/Waf1/MDA6). *J Biol Chem* **257**(1), 18-28.

Parker SL, Tong T, Bolden S and Wingo PA (1996) Cancer statistics 1996. *Ca Cancer J Clin* **46**, 5-27.

Pfister H (1992) Human papillomaviruses and skin cancer. *Seminars in Cancer Biology* **3**, 263-271.

Pfister H and Fuchs PG (1994) Anatomy, taxonomy and evolution of papillomaviruses. *Intervirology* **37**, 143-149.

Pim D, Storey A, Thomas M, Massimi P and Banks L (1994) Mutational analysis of HPV18 E6 identifies domains required for p53 degradation *in vitro*, abolition of p53 transactivation *in vivo* and immortalisation of primary BMK cells. *Oncogene* **9**, 1869-1876.

Pizzaro A, Gamallo C, Castresana JS, Gomez L, Palacios J, Benito N, Espada J, Fonseca E and Contreras F (1995) p53 protein expression in viral warts from patients with epidermodysplasia verruciformis. *Br J Dermatol* **132**(4), 513-519.

Polyak K, Waldman T, He TC, Kinzler KW and Vogelstein B (1996) Genetic determinants of p53-induced apoptosis and growth arrest. *Genes Dev* **10**, 1945-1952.

Proby CM and Harwood CA (1998) Role of human papillomaviruses in warts and cancer. *Hosp Med* **59**, 33-36.

Purdie KJ, Pennington J, Proby CM, Khalaf S, de Villeirs EM, Leigh IM and Storey A (1999) The promoter of a novel human papillomavirus (HPV77) associated with skin cancer displays UV responsiveness, which is mediated through a consensus p53 binding sequence. *EMBO J* **18**(19), 5359-5369.

Qin XQ, Livingston DM, Kaelin WG and Adams PD (1994) Deregulated transcription factor E2F-1 expression leads to S phase entry and p53-mediated apoptosis. *Proc Natl Acad Sci USA* **91**, 10918-10922.

Qin XQ, Livingston DM, Ewen M, Sellers WR, Arany Z and Kaelin WG (1995) The transcription factor E2F-1 is a downstream target of Rb action. *Mol Cell Biol* **15**, 742-755.

Quinn AG, Campbell C, Healy E and Rees JL (1994) Chromosome 9 allele loss occurs in both basal and squamous cell carcinomas of the skin. *J Invest Dermatol* **102**, 300-303.

Rajagopolan K, Bahry J and Tay CH (1972) Familial epidermodysplasia verruciformis of Lewandowsky and Lutz. *Arch Dermatol* **102**, 73.

Reddel R (1998) Genes involved in the control of cellular proliferative potential. *Ann NY Acad Sci* **854**, 8-19.

Reich NC and Levine AJ (1984) Growth regulation of a cellular tumour antigen, p53, in non-transformed cells. *Nature* **308**, 199-201.

Reznikoff CA, Yeager TR and Belair CD (1996) Elevated p16 at senescence and loss of p16 at immortalisation in human papillomavirus 16, E6, but not in E7, transformed human uroepithelial cells. *Cancer Res* **56**, 2886-2890.

Rodrigues NR, Rowan A, Smith ME, Kerr IB, Bodmer WF, Gannon JV and Lane DP (1990) p53 mutations in colorectal cancer. *Proc Natl Acad Sci USA* **87**(19), 7555-7559.

Rondeau G, Moreau I, Bezieau S, Cadoret E, Moisan JP and Devilder MC (1999) Exclusion of Leu1 and Leu2 as tumour suppressor genes in 13q14.3-deleted B-CLL. *Leukemia* **13**(10), 1630-1632.

Rose TTM, Schultz ER, Henckoff JG, Pietrovski S, McCallum CM and Henikoff S (1998) Consensus degenerate hybrid oligonucleotide primers for amplification of distantly-related sequences. *Nucl Acids Res* **26**(7), 1628-1635.

Rosenstein BS, Phelps RG, Weinstock MA, Bernstein JL, Gordon ML, Rudikokk D, Kantor I, Shelton R and Lebwohl MG (1999) p53 mutations in basal cell carcinomas arising in routine users of sunscreens. *Photochem Photobiol* B Biology **70**(5), 798-806.

Rosenthal AN, Ryan A, Al-Jehani RM, Storey A, Harwood C and Jacobs I (1998) p53 codon 72 polymorphism and risk of cervical cancer in UK. *Lancet* **352**, 871-75.

Rowe DE, Carroll RJ and Day DL (1992) Prognostic factors for local recurrence, metastasis and survival rates in squamous cell carcinoma of the skin, ear and lip. *J Am Acad Dermatol* **26**, 976-990.

Rudlinger R and Grob R (1989) Papillomavirus infection and skin cancer in renal allograft recipients. *Lancet* 1132-1133.

Saenz Robles MT, Symonds H, Chen J and van Dyke T (1994) Induction versus progression of brain tumour development: differential functions for the Rb- and p53-targeting domains of simian virus 40 T antigen. *Mol Cell Biol* **14**, 2686-2698.

Sailaja G, Watts RM and Bernard HU (1999) Many different papillomaviruses have low transcriptional activity in spite of strong epithelial specific enhancers. *J Gen Virol* **80**(7), 1715-1724.

Saito T, Nakajima T and Mogi K (1999) Immunohistochemical analysis of cell cycle-associated proteins p16, pRb, p53, p27 and Ki-67 in oral cancer and pre-cancer with special reference to verrucous carcinomas. *J Oral Pathol Med* **28**(5), 226-232.

Sakatani S, Kusakabe H, Kiyokane K and Suzuki K (1998) p53 gene mutations in squamous cell carcinoma occurring in scars: A comparison with p53 protein immunoreactivity. *Am J Dermatopathol* **20**(5), 463-467.

Salasche SJ (2000) Epidemiology of actinic keratoses and squamous cell carcinoma. *J Am Acad Dermatol* **42**(1 pt 2), 4-7.

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular cloning: A laboratory manual, Cold Spring Harbour Press, New York.

Sardet C, Vidal M, Cobrinik D, Geng Y, Onufryk C, Chen A and Weinberg RA (1995) E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. *Proc Natl Acad Sci USA*

Scheffner M, Werness BA, Huibregtse JM, Levine AJ and Howley PM (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63**, 1129-1136.

Scheffner M, Romanczuk H and Munger K (1994) Function of human papillomavirus proteins. *Curr Top Microbiol Immunol* **186**, 83-99.

Schiffman MH, Kiviat NB and Burk RD (1995) Accuracy and interlaboratory reliability of human papillomavirus DNA testing by hybrid capture. *J Clin Microbiol* **33**, 545-550.

Schneider-Manoury S, Croissant O and Orth G (1987) Integration of human papillomavirus type 18 : a possible early event in the progression of genital tumours. *J Virol* **61**, 3295-3298.

Sellers WR, Rodgers JW and Kaelin WG (1995) A potent transrepression domain in the retinoblastoma protein induces cell cycle arrest when bound to E2F sites. *Proc Natl Acad Sci USA* **92**, 11544-11548.

Sequeira LA and Cutler RE (1992) Occurrence of cancer in transplant recipients. *Dialysis Transplant* **21**, 143-151.

Seroz T, Hwang JR, Mocollin V and Egly JM (1995) TFIIH: a link between transcription, DNA repair and cell cycle regulation. *Curr Opin Genet Dev* **5**, 217-221.

Shah KV and Howley PM (1996) Papillomaviruses. In Fields BN, Kripke Dmand Howley PM (eds.) Virology Vol 2. Lippincott-Raven, Philadelphia, pp2077-2109.

Shamanin V, Delius H and de Villiers EM (1994a) Development of a broad spectrum PCR assay for papillomaviruses and its application in screening lung cancer biopsies. *J Gen Virol* **75**, 1149-1156.

Shamanin V, Glover M, Rausch C, Proby CM, Leigh IM, zur Hausen H and de Villiers EM (1994b) Specific types of human papillomavirus found in benign proliferations and carcinomas of the skin in immunosuppressed patients. *Cancer Res* **54**, 4610-4613.

Shamanin V, zur Hausen H, Lavergne D, Proby CM, Leigh IM, Neumann C, Hamm H, Goos M, Haustein U-F, Jung EG, Plewig G, Wolff H and de Villiers E-M (1996) Human papillomavirus infection in non-melanoma skin cancers from renal transplant recipients and non-immunosuppressed patients. *J Natl Cancer Inst* **88**, 802-811.

Sherr CJ (1996) Cancer cell cycles. *Science* **274**, 1672-1677.

Sherr CJ and Roberts JM (1995) Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev* **9**, 1149-1163.

Shieh SY, Ikeda M, Taya Y and Prives C (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* **91**, 325-334.

Shieh SY, Taya Y and Prives C (1999) DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, Ser20, requires tetramerisation. *EMBO J* **18**, 1815-1823.

Shiel A, Flavel S, Disney A and Matthew T (1985) Cancer development in patients progressing to dialysis and renal transplantation. *Transplant Progress* **17**, 1685-1688.

Shiffman NJ (1975) Squamous cell carcinomas of the skin of the pinna. *Can J Surg* **18**, 279-283.

Shimizu T, Muto M, Murakami T, Furumoto H, Mogami S and Asagami C (1997) Overexpression of p53 protein associated with proliferative activity as evaluated by Ki-67 immunostaining in well-differentiated squamous cell carcinoma of the skin. *Dermatology* **195**(3), 224-227.

Shirodkar S, Ewen M, De Caprio JA, Morgan J, Livingston DM and Chittenden T (1992) The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. *Cell* **68**, 157-166.

Sidransky D, von Eisenbach A, Tsai YC, Jones P, Summerhayes I, Marchall F, Paul M, Green P, Hamilton SR, Frost P and Vogelstein B (1991) Identification of p53 mutations in bladder cancers and urine samples. *Science* **252**, 706-709.

Sjalander A, Birgander R, Hallmans G, Cajander S, Lenner P, Athlin L, Beckman G and Beckman L (1996) p53 polymorphisms and haplotypes in breast cancer. *Carcinogenesis* **17**(4), 1313-1316.

Smith SE, Davis IC, Leshin B, Fleischer AB, White WL and Feldman S (1993) Absence of human papillomavirus in squamous cell carcinomas of nongenital skin from immunocompromised renal transplant patients. *Arch Dermatol* **129**, 1585-1588.

Smith ML and Fornace Jr AJ (1997) p53-mediated protective responses to UV radiation. *Proc Natl Acad Sci USA* **94**, 12255-12257.

Song S, Liem A, Miller JA and Lambert PF (2000) Human papillomavirus types 16 E6 and E7 contribute differently to carcinogenesis. *Virology* **267**(2), 141-150.

Sonoda Y, Saigo PE and Boyd J (1999) p53 codon 72 polymorphism and susceptibility to cervical cancer. *J Natl Cancer Inst* **91**, 557

Sorensen DM, Lewark TM, Haney JL, Meyers AD, Krause G and Franklin WA (1997) Absence of p53 mutations in squamous carcinomas of the tongue in nonsmoking and nondrinking patients younger than 40 years. *Arch Otolaryngol Head Neck Surg* **123**(5), 503-506.

Stark LA, Arends MJ, McLaren KM, Benton EC, Shahidullah H, Hunter JAA and Bird CC (1994a) Accumulation of p53 is associated with tumour progression in cutaneous lesions of renal allograft recipients. *Br J Cancer* **70**, 662-667.

Stark LA, Arends MJ, McLaren KM, Benton EC, Shahidullah H, Hunter JAA and Bird CC (1994b) Prevalence of human papillomavirus DNA in cutaneous neoplasms from renal allograft recipients supports a possible viral role in tumour promotion. *Br J Dermatol* **69**: 222-229.

Steenbergen RD, Hermsen MA, Walboomers JM, Meijer GA, Baak JP and Meijer CJ (1998) Non-random allelic losses at 3p, 11p and 13q during HPV-mediated immortalisation and concomitant loss of terminal differentiation of human keratinocytes. *Int J Cancer* **76**, 412-417.

Steger G and Pfister H (1992) In vitro expressed HPV8 E6 protein does not bind p53. *Arch Virol* **125**, 355-359.

Stern RS (1999) The mysteries of geographic variability in non-melanoma skin cancer incidence. *Arch Dermatol* **135**, 843-844.

Stern RS and Lange R (1988) Non-melanoma skin cancer occurring in patients treated with PUVA five to ten years after first treatment. *J Invest Dermaol* **91**, 120-124.

Stoler MH, Wolinsky SM, Whitbeck A, Broker TR and Chow LT (1989) Differentiation-linked human papillomavirus types 6 and 11 transcription in genital condylomata revealed by *in situ* hybridisation with message-specific RNA probes. *Virology* **172**, 331-340.

Storey A, Massimi P, Dawson K and Banks L (1995) Conditional immortalisation of primary cells by human papillomavirus type 18 E6 and EJ-*ras* defines an E6 activity in G_0/G_1 phase which can be substituted for mutations in p53. *Oncogene* **11**, 653-661.

Storey A, Thomas M, Kalita A, Harwood C, Gardiol D, Mantovani F, Breuer J, Liegh IM, Matlashewski G and Banks L (1998) Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. *Nature* **393**, 229-234.

Stubenrauch F, Hummel M, Iftner T and Laimins LA (2000) The E8E2C protein, a negative regulator of viral transcription, is required for extrachromosomal maintenance of human papillomavirus type 31 in keratinocytes. *J Virol* **74**(3), 1178-1186.

Surentheran T, Harwood CA, Spink PJ, Sinclair AL, Leigh IM, Proby CM, McGregor JM and Breuer J (1998) Detection and typing of human papillomaviruses in mucosal and cutaneous biopsies from immunosuppressed and immunocompetent patients and patients with epidermodysplasia verruciformis: a unified diagnostic approach. *J Clin Pathol* **51**, 606-610.

Takes RP, Baatenburg de Jong RJ, Schuuring E, Hermans J, Vis AA, Litvinov SV and van Krieken JH (1997) Markers for assessment of nodal metastasis in laryngeal carcinoma. *Arch Otolaryngol Head Neck Surg* **123**, 412-419.

Tamura K, Zhang X, Murakami Y, Hirohashi S, Xu HJ, Hu SX, Benedict WF and Sekiya T (1997) Deletion of three distinct regions on chromosome 13q in human small-cell lung cancer. *Int J Cancer* **74**, 45-49.

Tase T, Okagaki T and Clark BA (1988) Human papillomavirus types and localisation in adenocarcinoma and adenosquamous carcinoma of the uterine cervix: a study by *in situ* hybridisation. *Cancer Res* **48**, 993-998.

Thomas M and Banks L (1998) Inhibition of Bak-induced apoptosis by HPV-18 E6. *Oncogene* **17**(23), 2943-2954.

Thomas M and Banks L (1999) Human papillomavirus (HPV) E6 interactions with Bak are conserved amongst E6 proteins from high and low risk HPV types. *J Gen Virol* **80**(6), 1513-1517.

Thomas M, Massimi P, Jenkins J and Banks L (1995) HPV-18 E6 mediated inhibition of p53 DNA binding activity is independent of E6-induced degradation. *Oncogene* **10**, 261-268.

Thompson JD, Higgins DG and Gibson TJ (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting. *Nucl Acids Res* **22**, 4673-4680.

Thut CJ, Goodrich JA and Tjian R (1995) p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. *Genes Dev* **11**, 1974-1986.

Tieben LM, Berkhout RJM, Smits HL, Bouwes Bavink JN, Vermeer BJ, Briujn JA, van der Woude FJ and ter Schegget J (1994) Detection of epidermodysplasia verruciformis-like human papillomavirus types in malignant and pre malignant skin lesion of renal transplant recipients. *Br J Dermatol* **131**, 226-230.

Tommasi S and Pfiefer GP (1995) In vivo structure of the human cdc2 promoter: release of a p130-E2F-4 complex from sequences immediately upstream of the transcription initiation site coincides with induction of cdc2 expression. *Mol Cell Biol* **15**, 6901-6913.

Tommasino MJ, Adamczewski P, Carlotti F, Barth CF, Manetti R, Contorni M, Cavalieri F, Hunt T and Crawford L (1993) HPV 16 E7 protein associates with the protein kinase p33-cdk2 and cyclin A. *Oncogene* **8**, 195-202.

Tournier S, Leroy D, Goubin F, Ducommin B and Hyams JS (1996) Heterologous expression of the human cyclin-dependent kinase inhibitor p21Cip1 in the fission yeast, Schizosaccharomyces pombe reveals a role for PCNA in the chk1+ cell cycle checkpoint pathway. *Mol Cell Biol* **7**, 651-662.

Unger T, Juven-Gershon T, Moallem E, Berger M, Vogt Sionov R, Lozano G, Oren M and Haupt Y (1999) Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2. *EMBO J* **18**, 1805-1814.

Urbach F (1997) Ultraviolet radiation and skin cancer risk in humans. *J Photochem Photobiol B: Biology* **40**, 3-7.

Vairo G, Livingston DM and Ginsberg (1995) Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. *Genes Dev* **9**, 869-881.

van den Berg J, Johannsson O, Hakansson S, Olsson H and Borg A (1996) Allelic loss at chromosome 13q12-q13 is associated with poor prognosis in familial and sporadic breast cancer. *Br J Cancer* **74**, 1615-1619.

van der leest RJ, Zachow KR, Ostrow RS, Bender M, Pass F and Faras AJ (1987) Human papillomavirus heterogeneity in 36 renal transplant patients. *Arch Dermatol* **123**, 354-357.

van Duin M, Snijders PJ, Vossen MT, Klaassen E, Voorhorst F, Verhiejen RH, Helmerhorst TJ, Meijer CJ and Walboomers JM (2000) Analysis of human papillomavirus type 16 E6 variants in relation to p53 codon 72 polymorphism genotypes in cervical carcinogenesis. *J Gen Virol* **81**, 317-325.

van Ranst M, Kaplan JB and Burk RD (1992) Phylogenetic classification of papillomaviruses: correlation with clinical manifestations. *J Gen Virol* **73**, 2653-2660.

Vermeer BJ and Bouwes-Bavinck JN (1998) Relevance of photo-immunosuppression for viral infections (*ie* human papillomaviruses). *Eur J Dermatol* **8**, 205-206.

Vernon SD, Unger ER and Williams D (2000) Comparison of human papillomavirus detection and typing by cycle-sequencing, line blotting and hybrid capture. *J Clin Microbiol* **38**(2), 651-655.

Vidal MJ, Loganzo Jr F, de Oliveira AR, Hayward NK and Albino AP (1995) Mutations and defective expression of the Waf1 p21 tumour-suppressor gene in malignant melanomas. *Melanoma Res* **5**, 243-250.

Vousden KH (1993) Interactions of human papillomavirus transforming proteins with the products of tumour suppressor genes. *FASEB J* **7**, 872-879.

Wagner D, Ikenberg N, Boehm N and Gissmann L (1984) Identification of human papillomavirus in cervical swabs by deoxyribonucleic acid *in situ* hybridisation. *Obstet Gynecol* **64**, 767.

Wang H, Zeng X, Oliver P, Le LP, Chen J, Chen L, Zhou W, Agrawal S and Zhang R (1999a) MDM2 oncogene as a target for cancer therapy: an antisense approach. *Int J Oncol* **15**(4), 653-660.

Wang YC, Lee HS, Chen SK Chang YY and Chen CY (1999b) Prognostic significance of p53 codon 72 polymorphism in lung carcinomas. *Eur J Cancer* **35**(2), 226-230

Wei Q, Matanoski GM, Farmer ER, Hedayati MA and Grossman L (1996) DNA repair and ageing in basal cell carcinoma: a molecular epidemiology study. *Proc Natl Acad Sci USA* **90**, 1614-1618.

Weintraub SJ, Chow KN, Luo RX, Zhang SH, He S and Dean DC (1995) Mechanism of active transcriptional repression by the retinoblastoma protein. *Nature* **375**, 812-815.

Woo RA, McLure KG, Lees-Miller SP, Rancourt EE and Lee PWK (1998) DNA-dependent protein kinase acts upstream of p53 in response to DNA damage. *Nature* **394**, 700-704.

Wright DK and Manos MM (1990) Sample preparation from paraffin-embedded tissues. In (Innis MA, Gelfand DH, Sninsky JJ and White TJ eds) PCR protocols, Academic Press, Inc.

Wu L and Levine AJ (1997) Differential regulation of the p21/WAF-1 and mdm2 genes after high-dose UV irradiation: p53-dependent and p53-independent regulation of the mdm2 gene. *Mol Med* **3**, 441-451.

Wynford-Thomas D (1992) p53 in tumour pathology: can we trust immunocytochemistry? *J Pathol* **166**, 329-330.

Xing EP, Yang GY, Wang LD, Shi ST and Yang CS (1999) Loss of heterozygosity of the Rb gene correlates with pRb protein expression and associates with p53 alteration in human oesophageal cancer. *Clin Cancer Res* **5**(5), 1231-1240.

Yamashita T, Yaginuma Y, Saitoh Y, Kawai K, Kurakane T, Hayashi H and Ishikawa M (1999) Codon 72 polymorphism of p53 as a risk factor for patients with human papillomavirus-associated squamous intraepithelial lesions and invasive cancer of the uterine cervix. *Carcinogenesis* **20**(9), 1733-1736

Yan Y, Shay JW, Wright WE and Mumby MC (1997) Inhibition of protein phosphatase activity induces p53-dependent apoptosis in the absence of p53 transactivation. *J Biol Chem* **272**, 15220-15236.

Yang L, Mohr I, Fouts E, Lim DA, Nohaile M and Botchan M (1993) The E1 protein of bovine papillomavirus 1 is an ATP-dependent DNA helicase. *Proc Natl Acad Sci USA* **90**, 5086-5090.

Yoneda K, Yokoyama T, Yamamoto T, Hatebe T and Osaki T (1999) p53 gene mutations and p21 protein expression induced independently of p53, by TGF-beta and gamma-rays in squamous cell carcinoma cells. *Eur J Cancer* **35**(2), 278-283.

Yoo GH, Xu HJ, Brennan JA, Westra W, Hruban RH, Koch W, Benedict WF and Sidransky D (1994) Infrequent inactivation of the retinoblastoma gene despite frequent loss of chromosome 13q in head and neck squamous cell carcinoma. *Cancer Res* **54**, 4603-4606.

Yutsudo M, Tanigaki T, Kanda R, Sasagawa T, Inoue T, Jing P, Yong HH and Hakura A (1994) Involvement of human papillomavirus type 20 in epidermodysplasia verruciformis skin carcinogenesis. *J Clin Microbiol* **32**, 1076-1078.

Zehbe I, Voglino G, Wilander E, Genta F and Tomassino M (1999) Codon 72 polymorphism of p53 and its association with cervical cancer. *Lancet* **354**(9174), 218-219.

Zhang Y, Xiong Y and Yarbrough WG (1998) ARF promotes MDM2 degradation and stabilises p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumour suppression pathways. *Cell* **92**, 725-734.

Zhou J and Frazer IH (1995) Papovaviridae: capsid structure and capsid protein function. *Papillomavirus Rep* **6**, 59-64.

Ziegler A, Lefell DJ, Kanula S, Sharma HW, Gailani M, Simon JA, Halperin A, Baden HP, Shapiro BE, Bale AE (1993) Mutation hotspots due to sunlight in the p53 gene of non-melanoma skin cancers. *Proc Natl Acad Sci USA* **90**(9), 4216-4220.

Ziegler A, Jonason AS, Leffell DJ, Simon JA, Sharma HW, Kimmelman J, Remington L, Jacks T and Brash DE (1994) Sunburn and p53 in the onset of skin cancer. *Nature* **372**, 773-776.

zur Hausen H (1989) Host cell regulation of HPV transforming gene expression. *Princess Takamatsu Symp* **20**, 207-219.

zur Hausen H (1996) Papillomavirus infections: a major cause of human cancers. *Biochim Biophys Acta* **1288**, F55-F78.

zur Hausen H (1999) Papillomaviruses in human cancers. *Proc Assoc Am Phys* **111**, 581-587.

Materials

Materials

Chemicals, reagents and kits were obtained from the following suppliers:

Air Products, Ireland.

Liquid Nitrogen

American Type Culture Collection, USA.

SW680 colorectal carcinoma cell line.

Amersham International, Buckinghamshire, England.

Thermosequenase cycle sequencing kit, [α-P^{32}]dATP, Rapid-hyb hybridisation buffer.

BDH, Poole, Dorset, England.

Hydrochloric acid, Ethylenediaminetetra-acetic acid (EDTA), Sodium chloride, Sodium hydroxide, Sodium citrate, Bromophenol blue, Xylene cyanol, N,N-Methylenebisacrylamide, NNN'N'-Tetramethylethylenediamine (TEMED), Glycerol, Acetic acid, b-mercaptoethanol, Tween 20, Potassium phosphate, Hydrogen peroxide, Potassium dihydrogen phosphate, *di* potassium hydrogen orthophosphate, Haematoxylin, Eosin, Xylene, Proteinase K, Sodium borohydride, Silver nitrate.

Becton Dickinson, England.

3ml Luer-lok™ syringes.

Boeringer Mannheim GmbH Diagnostics, Germany.

10x TBE, 10x TAE, 20xSSC.

Dako, California, USA

Dako Duet kit, DO-7 anti-p53 mouse monoclonal antibody, Rb1 anti-Rb mouse monoclonal antibody.

Flowgen, Staffordshire, England.

Long Ranger™ gel solution.

Fuji Film Co., Japan.

Cronex 10s x-ray film.

Gibco LTD., Paisley, England.

Random hexanucleotide primers, superscript II reverse transcriptase, DNase I, DTT, TRIzol.

Lab-Scan, Analytical Sciences, Dublin, Ireland.

Absolute ethanol, Methanol, Chloroform, Isopropanol, Glacial acetic acid.

Millipore, Middlesex, England.

Millex-GV millipore filters.

Oligonucleotide Synthesis Unit, Queen's University, Belfast.

PCR primers, cy5-labelled sequencing primers, cy5-labelled LOH primers.

Oxoid, Basingstoke, Hampshire, England.

Tryptone, Yeast Extract, Bacteriological Agar.

Pharmacia, Uppsala, Sweden.

Sephadex G-50 Nick columns.

Polaroid Corp., MA, USA.

Polaroid 665 film.

Promega Corporation, Madison, WI, USA.

Taq DNA Polymerase, Deoxyadenosine 5'-triphosphate (dATP), Deoxycytidine 5'-triphosphate (dCTP), Deoxyguanosine 5' -triphosphate (dGTP), Deoxythymidine 5' -triphosphate (dTTP), 10x Thermo buffer (500mM KCL, 100mM Tris-HCL (pH 9.0 at 25^{o}C), 1% Triton X-100), RNasin, 25mM Magnesium chloride, 5-Bromo-4-chloro-3-indoyl-b-D-galactopyranoside (x-gal), Isopropyl-1-thio-b-D-galactopyranoside (IPTG), Promega Wizard^{TM} miniprep kit, T4 polynucleotide kinase, Wizard^{TM} PCR prep kit, 100 base pair DNA ladder, 6x loading dye, Total RNA isolation kit, prime-a-gene labelling kit.

Santa Cruz Biotechnology Inc., California, USA.

p21 (187) anti-p21 antibody, SMP7 anti-Mdm2 antibody.

Scotlab, Kirkshaws Road, Scotland.

Easigel [6% w/v acrylamide/0.3% w/v bis acrylamide, 7M urea, 1X TBE (ratio 19:1)].

Sigma Chemical Company Ltd., Poole, Dorset, England

Agarose, Ammonium persulphate, Bromophenol blue, Ethidium bromide, Mineral oil, Formaldehyde, Formamide, 3-[N-morpholino]propanesulfonic acid (MOPS), Ammonium acetate, Sodium acetate, Dithiothreitol, Bovine serum albumin (BSA), Phosphate buffered saline (PBS), agar, yeast extract, polyvinylpyrrolidone, salmon sperm DNA, Urea, Tris-HCl, Ampicillin, Ficoll, Poly A, sodium dodecyl sulphate, Diethyl pyrocarbonate (DEPC), Tris.

Stratagene Ltd., Cambridge, England.

PCR-Script™ Amp SK(+) cloning kit, *Pfu* polymerase, 10x *Pfu* polymerase reaction buffer,

Swann-Morton, Sheffield, England.

Scalpel blades.

Vector Labs, California, USA.

Vecta-bond reagent.

Whatman, Maidstone, England.

3MM Filter paper.

Zymed, USA.

Diaminobenzidine (DAB) reagent.

The following specialised equipment was used:

Pharmacia ALFexpress™ Automated DNA sequencer.

MJ Research DNA Engine™ Thermal Cycler.

Pharmacia Genequant™ Spectrophotometer.

Hybaid Hybridisation Oven.

Apple Power Macintosh.

Nikon BIOPHOT Microscope.

Nikon FX 35mm camera.

Nikon SMZ U Zoom stereomicroscope (0.8x-3.5x zoom with 10x eyepiece and 1.5x objective).

Reichert-Jung 2030 Microtome.

Appendices

APPENDIX I Alignments of HPV sequences amplified from RTR and ICP warts with known HPV types deposited in the Genbank database.

W2A (RTR)

```
emb|X55964|PAHPV2A Human papilloma virus type 2a complete DNA
           Length = 7860

 Score =  507 bits (256), Expect = e-142
 Identities = 289/300 (96%)
 Strand = Plus / Plus


Query: 1    ctttgggtgtggttgtgtctggtcaccaatattacaatagactggatgacactgaaaatg 60
            |||||||||||| ||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct: 6121 ctttgggtgtgggtgtgtctggtcacccatattacaatagactggatgacactgaaaatg 6180


Query: 61   cacacacacctgatacagctgatgatggcacggaaaacaattctatggattataaacaga 120
            |||||||||||||||||||||||||||| |||||||| ||||||||||||||||||||
Sbjct: 6181 cacacacacctgatacagctgatgatggcagggaaaacatttctatggattataaacaga 6240


Query: 121  cacagctgttcattcttggctgcaaacccccttattggtgagcactggtctaaaggtacca 180
            |||||||||||||||| |||||||||||| |||||||||||||||||||| |||||||
Sbjct: 6241 cacagctgttcattctgggctgcaaacccccctattggtgagcactggtctaagggtacca 6300


Query: 181  cctgtaatgggtcttctgctgcttgtgactgcccgcccttccaatttactaacacaacta 240
            ||||||||||||||||||||||| |||||||||||| ||||||||||||||||||||
Sbjct: 6301 cctgtaatgggtcttctgctgctggtgactgcccgcccctccaatttactaacacaacta 6360


Query: 241  ttgaggacgggaatatggttgaaacaggtttcggtgccttggattttgccactctgcagt 300
            |||||||||| |||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct: 6361 ttgaggacggggatatggttgaaacagggttcggtgccttggattttgccactctgcagt 6420


emb|X55965|PAHPV57 Human papillomavirus type 57 complete DNA
           Length = 7861

 Score =  650 bits (328), Expect = 0.0
 Identities = 352/360 (97%)
 Strand = Plus / Plus


Query: 1    ccctgcagtttacaaacaccactaatgaagatgggaatatggttgaaacccggttcgggg 60
            |||||||||||||||| ||||||| |||||||||| |||||||||||||| |||||||||
Sbjct: 6301 ccctgcagtttacaaataccactattgaagatggggatatggttgaaaccgggttcgggg 6360


Query: 61   cgctggattttgccgctctacagtccaacaaatcagatgtccccttgatatctgtacta 120
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct: 6361 cgctggattttgccgctctacagtccaacaaatcagatgtccccttggatatctgtacta 6420


Query: 121  acatatgtaaatatccagactatctgaagatggctgcagaaccttatggcgattctatgt 180
            |||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct: 6421 acatatgtaaatatccagactatctgaagatggctgcagacccttatggcgattctatgt 6480


Query: 181  tctttcccctgcgcaggaagcaaatgttcactcggcatttttttcaatcggggtgggtcga 240
            ||||| |||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6481 tcttttccctgcgcagggagcaaatgttcactcggcatttttttcaatcggggtgggtcga 6540
```

```
Query: 241  tgggtgacgccctcccggatgagctatatgtcaagagttctaccgtccagacccccggta 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6541 tgggtgacgccctcccggatgagctatatgtcaagagttctaccgtccagacccccggta 6600


Query: 301  gttatgtttatacctccactcccagtggctctatggtatcctctgaacagcagttattta 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6601 gttatgtttatacctccactcccagtggctctatggtatcctctgaacagcagttattta 6660


>gb:HPU31781 Human papillomavirus type 23, complete genome.
            Length = 7324

  Plus Strand HSPs:

 Score = 1315 (363.4 bits), Expect = 2.3e-101, P = 2.3e-101
 Identities = 315/380 (82%), Positives = 315/380 (82%), Strand = Plus / Plus

query:     1 caatcagctgtttgttactgtagcagataatacacgcaatactaattttagtatcagtgt 60
             ||| ||| ||||||| |||||||||||||||||||| ||||| || ||||||||||||||
sbjct:  6655 caaccagatgtttgtgactgtagcagataatacacgtaatacaaactttagtatcagtgt 6714

query:    61 taaatgtgaggatagcttagcaaattataatgctagtaatattagagaatatatgagaca 120
             ||    |||    || |||| ||| ||| |||| | ||| |||||||| | ||  |||||
sbjct:  6715 taccaatgacagcagtttagaaaagtatgatgccactaaaattagagagtttacaagaca 6774

query:   121 tgttgaagagtatcagttgtcttttatattacaattgtgcagaatacctttaaaggctga 180
             ||||||||| || ||  | ||||||||| |||| |||||||| ||||||||||||||| ||
sbjct:  6775 tgttgaagaataccaactttcttttatactacagttgtgcaggatacctttaaaggccga 6834

query:   181 agttttaacacgaatcaatgcaatgaactctgatattttagagaattggcaattgggctt 240
             || ||||||| ||| ||||| ||||| || |||||||||||||||||||| || || ||
sbjct:  6835 ggtcttaacacaaattaatgccatgaattcagatattttagagaattggcagttagggtt 6894

query:   241 tgtacctacaccaaataatgcagtacacgatatatataggtatttagcctcaaaggccgc 300
             ||| ||||||||| |||||||||| || || | ||| || ||||| || ||||||||| |
sbjct:  6895 tgttcctacaccagataatgcagttcatgacacatacagatatttggcttcaaaggccac 6954

query:   301 taagtgtcctgatgcagtacctgaaacccaaaaagaggatccttttggaaagtattcatt 360
             || ||||| |||||||||||||| || ||||||||||||||||||||||||||||||||
sbjct:  6955 aaaatgtccagatgcagtacctgacacgcaaaaagaggatccttttggaaagtattcatt 7014

query:   361 ctgggatgttgatctaacgg 380
             ||| |||||||| | || |
sbjct:  7015 ttggaatgttgatatgacag 7034
```

W14A (RTR)

```
emb|X55965|PAHPV57 Human papillomavirus type 57 complete DNA
           Length = 7861

 Score =  650 bits (328), Expect = 0.0
 Identities = 352/360 (97%)
 Strand = Plus / Plus


Query: 1    ccctgcagtttacaaacaccactaatgaagatgggaatatggttgaaacccggttcgggg 60
            |||||||||||||||| ||||||| |||||||||| |||||||||||||| |||||||||
Sbjct: 6301 ccctgcagtttacaaataccactattgaagatggggatatggttgaaaccgggttcgggg 6360


Query: 61   cgctggattttgccgctctacagtccaacaaatcagatgtccccttgatatctgtacta 120
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct: 6361 cgctggattttgccgctctacagtccaacaaatcagatgtcccctggatatctgtacta 6420


Query: 121  acatatgtaaatatccagactatctgaagatggctgcagaaccttatggcgattctatgt 180
            |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct: 6421 acatatgtaaatatccagactatctgaagatggctgcagacccttatggcgattctatgt 6480


Query: 181  tctttcccctgcgcaggaagcaaatgttcactcggcatttttcaatcggggtgggtcga 240
            ||||| |||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct: 6481 tcttttccctgcgcagggagcaaatgttcactcggcatttttcaatcggggtgggtcga 6540


Query: 241  tgggtgacgccctcccggatgagctatatgtcaagagttctaccgtccagacccccggta 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6541 tgggtgacgccctcccggatgagctatatgtcaagagttctaccgtccagacccccggta 6600


Query: 301  gttatgtttatacctccactcccagtggctctatggtatcctctgaacagcagttattta 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6601 gttatgtttatacctccactcccagtggctctatggtatcctctgaacagcagttattta 6660


>gb:HPU21875 Human papillomavirus type 38 L1 protein gene, partial cds.
           Length = 288

  Minus Strand HSPs:

 Score = 373 (104.1 bits), Expect = 2.4e-32, Sum P(2) = 2.4e-32
 Identities = 92/115 (80%), Positives = 93/115 (80%), Strand = Minus / Plus

query:   142 atgcagtgcatgrcacatatcgttaccttaattcaaaagctacaaaatgtccagatgcag 83
             || | || || |+ |||||||||||| | |  || ||||| || ||||||||||||||||
sbjct:   149 attctgtacacgatacatatcgttacataacatctaaagcaactaaatgtccagatgcag 208

query:    82 ttcaagaaacagaaaaggaagatccttttggtggatattcattttgggatgttga 28
             | |  |||||||||| |||||||| ||||||  |||| |||||||| |||| ||
sbjct:   209 tgcctgaaacagaaaaagaagatccctttggtcaatatacattttggaatgtgga 263
```

W14B (RTR)

```
emb|X74465|HPV10 Human papillomavirus type 10 genomic DNA
           Length = 7919

 Score =  793 bits (399), Expect = 0.0
 Identities = 416/420 (99%), Gaps = 2/420 (0%)
 Strand = Plus / Plus


Query: 71   aatggtatatgctgggctaac--attgtttgttactgtggtagacacgactcgcagtacc 128
            ||||||||||||||||||||  ||||||||||||||||||||||||||||||||||||
Sbjct: 6780 aatggtatatgctgggctaaccaattgtttgttactgtggtagacacgactcgcagtacc 6839


Query: 129  aatatgtgcttgtgtgttccttctgaggcctcccctgccactacgtatgacgccaccaaa 188
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6840 aatatgtgcttgtgtgttccttctgaggcctcccctgccactacgtatgacgccaccaaa 6899


Query: 189  tttaaagaatatttgaggcacggagaggaatatgatttgcagttcatttttcagttgtgt 248
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6900 tttaaagaatatttgaggcacggagaggaatatgatttgcagttcatttttcagttgtgt 6959


Query: 249  aaggtaacattgaccccggatattatggcctatttgcacaccatgaatagtagtttattg 308
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6960 aaggtaacattgaccccggatattatggcctatttgcacaccatgaatagtagtttattg 7019


Query: 309  gaggattggaactttgggttaactttgccaccgtccactagcttggaggacacatataga 368
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7020 gaggattggaactttgggttaactttgccaccgtccactagcttggaggacacatataga 7079


Query: 369  tctttgtcctcttcagccattacttgtcagaaagatacacccccaccgagaagcaggat 428
            | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7080 tttttgtcctcttcagccattacttgtcagaaagatacacccccaccgagaagcaggat 7139


Query: 429  ycctatgcaaaacttaacttttgggacgtagatcttaaggataggttttccctggacctg 488
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7140 ccctatgcaaaacttaacttttgggacgtagatcttaaggataggttttccctggacctg 7199
```

```
>gb:AF054875 Human papillomavirus isolate HPVX14b L1 protein gene, partial
cds.
            Length = 267

  Plus Strand HSPs:

 Score = 1164 (321.6 bits), Expect = 3.5e-91, P = 3.5e-91
 Identities = 248/267 (92%), Positives = 248/267 (92%), Strand = Plus / Plus

query:    53 ataagtgtaacaaatgatgcaggagctatagaagattatactgcacaaaatattagagaa 112
             |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
sbjct:     1 ataagtgtaacaaatgatgcaggagctatagaagactatactgcacaaaatattagagaa 60

query:   113 tatttgagacatgttgaagaatatcaatcatcagtaatactacaactatgtaaaatgcct 172
             || || |||||||||||||||||||||| |||||| ||||||||  |||||||||| |||
sbjct:    61 tacttaagacatgttgaagaatatcaattatcagtgatactacagttatgtaaaatacct 120

query:   173 ctaaatccaggagtactttctcagattaatgcaatgaattctggtattctagaagaatgg 232
             || ||||||| |||||||||||||||||||||||||||||| ||| ||||||||||||
sbjct:   121 ctgaatccagaagtactttctcagattaatgcaatgaattctggaattttagaagaatgg 180

query:   233 cagttaggctttgttcctactccagaaaatgctgtgcatgatacatatagatacatatat 292
             ||  |||||||||||||||| |||||||||||||| |||||||| ||||||||||||||
sbjct:   181 caactaggctttgttcctaccccagaaaatgctgtacatgatacgtatagatacatatat 240

query:   293 tctaaagctaccaaatgtccagatgaa 319
             |||||||||||| |||||||| ||||||
sbjct:   241 tctaaagctacaaaatgtcctgatgaa 267
```

W37A (RTR)

```
emb|X74465|HPV10 Human papillomavirus type 10 genomic DNA
            Length = 7919

 Score =  640 bits (323), Expect = 0.0
 Identities = 335/339 (98%)
 Strand = Plus / Plus


Query: 75   aatggtatatgctgggctaaccaattgtttgttactatggtagacacgactcgcagtacc 134
             ||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct: 6780 aatggtatatgctgggctaaccaattgtttgttactgtggtagacacgactcgcagtacc 6839


Query: 135  aatatgtgcttgtgtgttccttctgaggcctcccctgccactacgtacgacgccaccaaa 194
             |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct: 6840 aatatgtgcttgtgtgttccttctgaggcctcccctgccactacgtatgacgccaccaaa 6899


Query: 195  tttaaagaatatttggggcacggagaggaatatgatttgcagttcatttttcagttgtgt 254
             |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6900 tttaaagaatatttgaggcacggagaggaatatgatttgcagttcatttttcagttgtgt 6959


Query: 255  aaggtaacattgaccccggatattatggcctatttgcacaccatgaatagtagtttattg 314
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6960 aaggtaacattgaccccggatattatggcctatttgcacaccatgaatagtagtttattg 7019


Query: 315  gaggattggaactttgggttaactttgcctccgtccactagcttggaggacacatataga 374
             |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct: 7020 gaggattggaactttgggttaactttgccaccgtccactagcttggaggacacatataga 7079
```

```
Query: 375  tttttgtcctcttcagccattacttgtcagaaagataca 413
            |||||||||||||||||||||||||||||||||||||||
Sbjct: 7080 tttttgtcctcttcagccattacttgtcagaaagataca 7118


gb|L38922|PPHL1AN Human papillomavirus unidentified type (RTRX5) L1 gene,
partial
           cds.
           Length = 276

 Score =  511 bits (256), Expect = e-142
 Identities = 270/276 (97%)
 Strand = Plus / Plus


Query: 148 atagcagtttataatgattctggtgaaattaaagacattgcttcttatgattccactaag 207
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1   atagcagtttataatgattctggtgaaattaaagacattgcttcttatgattccactaaa 60


Query: 208 tttcgagaatttcaaagacatgtggaagaatatgagatttctttaattttacagttatgc 267
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 61  tttcgagagtttcaaagacatgtggaagaatatgagatttctttaattttacagttatgc 120


Query: 268 aaaattcctttaaaatcagaggtattagctcaaattaatgctatgaatcsyacaatactt 327
            |||||||||||||||||||||||||||||||||||||||||||||||||  |||||||||
Sbjct: 121 aaaattcctttaaaatcagaggtattagctcaaattaatgctatgaatcctacaatactt 180


Query: 328 gaggattggcaattaggttttgtgccaactcctgataatccaatacaggatgcttacaga 387
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 181 gaggattggcaattaggttttgtgccaactcctgataatccaatacaggatgcttacaga 240


Query: 388 tatttggattctctggctacacggtgaccagataaa 423
            |||||||||||| |||||||||||| |||||||||
Sbjct: 241 tatttggattctttggctacacggtgcccagataaa 276


>gb:PPHL1AO Human papillomavirus unidentified type (RTRX6) L1 gene, partial
            cds.
            Length = 267

  Plus Strand HSPs:

 Score = 1063 (293.7 bits), Expect = 1.7e-101, Sum P(2) = 1.7e-101
 Identities = 215/218 (98%), Positives = 215/218 (98%), Strand = Plus / Plus

query:   101 aaattagagaatttttaaggcacgtggaagagtatcaaatatctgtgatattacaactgt 160
             || ||||||||||||||||||||||||||||||||||||||||| ||||||||||||
sbjct:    50 aatttagagaatttttaaggcacgtggaagagtatcaaatatctgtaatattacaactgt 109

query:   161 gtaaagtatcactgcaacctgatgtgctagcccagatcgatgcaatgaattcaggtatat 220
             |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
sbjct:   110 gtaaagtatcactgcaacctgatgtgctagcccagatcaatgcaatgaattcaggtatat 169

query:   221 tagaagattggcagttaggatttgtaccaactcctgacaatgcagtacatgacacctata 280
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sbjct:   170 tagaagattggcagttaggatttgtaccaactcctgacaatgcagtacatgacacctata 229

query:   281 gatttataaattcctcagccactaaatgtccagataag 318
             |||||||||||||||||||||||||||||||||||||
sbjct:   230 gatttataaattcctcagccactaaatgtccagataag 267
```

W46A (RTR)

```
emb|X74469|HPV17 Human papillomavirus type 17 genomic DNA
           Length = 7427

 Score =  593 bits (299), Expect = e-167
 Identities = 323/331 (97%)
 Strand = Plus / Plus


Query: 1    acagtagctatcaaggtcgatctactgatgatagacaaaacacgtcacttgatcctaaac 60
            |||||||||||||||| |||||||||||||||||||||||||||| |||| |||||||
Sbjct: 6121 acagtagctatcaaggtggatctactgatgatagacaaaacacgtcatttgaccctaaac 6180


Query: 61   aagtgcagatgtttgttgtaggctgtgtaccttgtattggagaacatttggacagggctc 120
            |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct: 6181 aagtgcagatgtttgttgtaggctgtgtaccttgtattggagaacattgggacagggctc 6240


Query: 121  ctgtatgtggaaatgaacaaaacaatcaaacaggcctgtgtccaccattggaattaaata 180
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct: 6241 ctgtatgtgaaaatgaacaaaacaatcaaacaggcctgtgtccaccattggaattaaaaa 6300


Query: 181  acactgttatcgaagatggtgacatggttgacataggcttttgaaacattaataacaaag 240
            |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct: 6301 acactgttatcgaagatggtgacatggttgacataggctttggaaacattaataacaaag 6360


Query: 241  tgctttcatttaataaatcagatgtaagattagatatagttaatgaaacatgcaaatatc 300
            |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct: 6361 tgctttcatttaataaatcagatgtaagtttagatatagttaatgaaacatgcaaatatc 6420


Query: 301  ctgatttttaagcatggcaaatgatgttta 331
            ||||||||||||||||||||||||||||||
Sbjct: 6421 ctgatttttaagcatggcaaatgatgttta 6451


>gb:HPU31781 Human papillomavirus type 23, complete genome.
           Length = 7324

  Minus Strand HSPs:

 Score = 1337 (384.1 bits), Expect = 1.7e-105, P = 1.7e-105
 Identities = 316/378 (83%), Positives = 317/378 (83%), Strand = Minus / Plus

query:   379 caatcagctgtttgttacagtagcagataatacacgcaatactaattttagtatcagtgt 320
             ||| ||| ||||||| || |||||||||||||||| ||||| || ||||||||||||||
sbjct:  6655 caaccagatgtttgtgactgtagcagataatacacgtaatacaaactttagtatcagtgt 6714

query:   319 taaaagtgaggatagcttagcaaattataatgctagtaatattagagaatatatgagaca 260
             ||  | |||    || |||| ||| ||| |||| | ||| |||||||| | ||  |||||
sbjct:  6715 taccaatgacagcagtttagaaaagtatgatgccactaaaattagagagtttacaagaca 6774

query:   259 tgttgaagagtatcagttgtcttttatattacaattgtgcagaataccttaaaggctga 200
             ||||||||| || ||  | ||||||||| |||| |||||||| |||||||||||||| ||
sbjct:  6775 tgttgaagaataccaactttcttttatactacagttgtgcaggataccttaaaggccga 6834

query:   199 agttttaacacgaatcaatgcaatgaactctgatattttagagaattggcaattgggctt 140
              || ||||||| ||| ||||| ||||| || |||||||||||||||||||| || || ||
sbjct:  6835 ggtcttaacacaaattaatgccatgaattcagatattttagagaattggcagttagggtt 6894
```

```
query:   139 tgtacctacaccagataatgcagtacacgatatatataggtatttascctcaaaggccac 80
             ||| |||||||||||||||||| || || | ||| || ||||| +| ||||||||||
sbjct:  6895 tgttcctacaccagataatgcagttcatgacacatacagatatttggcttcaaaggccac 6954
query:    79 taagtgtcctgatgcagtacctgaaacccaaaaagaggatcttttggaaagtattcatt 20
              || ||||| |||||||||||||| || |||||||||||| ||||||||||||||||||
sbjct:  6955 aaaatgtccagatgcagtacctgacacgcaaaaagaggatcctttggaaagtattcatt 7014

query:    19 ttggaatgttgatctaac 2
             |||||||||||||| | ||
sbjct:  7015 ttggaatgttgatatgac 7032
```

W50A (RTR)

```
emb|X74465|HPV10 Human papillomavirus type 10 genomic DNA
           Length = 7919

 Score =  793 bits (399), Expect = 0.0
 Identities = 416/420 (99%), Gaps = 2/420 (0%)
 Strand = Plus / Plus


Query: 71   aatggtatatgctgggctaac--attgtttgttactgtggtagacacgactcgcagtacc 128
            ||||||||||||||||||||  ||||||||||||||||||||||||||||||||||||
Sbjct: 6780 aatggtatatgctgggctaaccaattgtttgttactgtggtagacacgactcgcagtacc 6839


Query: 129  aatatgtgcttgtgtgttccttctgaggcctcccctgccactacgtatgacgccaccaaa 188
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6840 aatatgtgcttgtgtgttccttctgaggcctcccctgccactacgtatgacgccaccaaa 6899


Query: 189  tttaaagaatatttgaggcacggagaggaatatgatttgcagttcatttttcagttgtgt 248
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6900 tttaaagaatatttgaggcacggagaggaatatgatttgcagttcatttttcagttgtgt 6959


Query: 249  aaggtaacattgaccccggatattatggcctatttgcacaccatgaatagtagtttattg 308
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6960 aaggtaacattgaccccggatattatggcctatttgcacaccatgaatagtagtttattg 7019


Query: 309  gaggattggaactttgggttaactttgccaccgtccactagcttggaggacacatataga 368
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7020 gaggattggaactttgggttaactttgccaccgtccactagcttggaggacacatataga 7079


Query: 369  tctttgtcctcttcagccattacttgtcagaaagatacacccccccaccgagaagcaggat 428
            | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7080 tttttgtcctcttcagccattacttgtcagaaagatacacccccccaccgagaagcaggat 7139


Query: 429  ycctatgcaaaacttaacttttgggacgtagatcttaaggataggttttccctggacctg 488
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7140 ccctatgcaaaacttaacttttgggacgtagatcttaaggataggttttccctggacctg 7199
```

```
emb|X55964|PAHPV2A Human papilloma virus type 2a complete DNA
           Length = 7860
 Score =  424 bits (214), Expect = e-117
 Identities = 214/214 (100%)
 Strand = Plus / Plus

Query: 1    ctttgggtgtgggtgtgtctggtcacccatattacaatagactggatgacactgaaaatg 60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6121 ctttgggtgtgggtgtgtctggtcacccatattacaatagactggatgacactgaaaatg 6180


Query: 61   cacacacacctgatacagctgatgatggcagggaaaacatttctatggattataaacaga 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6181 cacacacacctgatacagctgatgatggcagggaaaacatttctatggattataaacaga 6240


Query: 121  cacagctgttcattctgggctgcaaacccccctattggtgagcactggtctaagggtacca 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6241 cacagctgttcattctgggctgcaaacccccctattggtgagcactggtctaagggtacca 6300


Query: 181  cctgtaatgggtcttctgctgctggtgactgccc 214
            |||||||||||||||||||||||||||||||||
Sbjct: 6301 cctgtaatgggtcttctgctgctggtgactgccc 6334

gb|U31781|HPU31781 Human papillomavirus type 23, complete genome.
           Length = 7324
 Score =  216 bits (109), Expect = 6e-54
 Identities = 301/362 (83%), Gaps = 1/362 (0%)
 Strand = Plus / Minus

Query: 81   acatcccagaatgaatactttccaaaaggatcctctttttggggtttcaggtactgcatc 140
            |||| ||| |||||||||||||||||||||||||||||| | | ||||||||||||||
Sbjct: 7023 acattccaaaatgaatactttccaaaaggatcctcttttttgcg-tgtcaggtactgcatc 6965


Query: 141  aggacacttagtggcctttgaggctaaatacctatatatatcgtgtactgcattatctgg 200
             ||||| || |||||||||| || ||||| || ||| | || || ||||||||||||||
Sbjct: 6964 tggacattttgtggcctttgaagccaaatatctgtatgtgtcatgaactgcattatctgg 6905


Query: 201  tgtaggtacaaagcccaattgccaattctctaaaatatcagagttcattgcattgattcg 260
            |||||| ||||| || || |||||||||||||||||| || ||||| ||||| ||| |
Sbjct: 6904 tgtaggaacaaaccctaactgccaattctctaaaatatctgaattcatggcattaatttg 6845


Query: 261  tgctaaaacttcagcctttaaaggtatgctgcacaattataatataaaagacatctgata 320
            || ||| || || |||||||||||||| |||||||| | || ||||||||| |  || ||
Sbjct: 6844 tgttaagacctcggcctttaaaggtatcctgcacaactgtagtataaaagaaagttggta 6785


Query: 321  ctcatcaacatgtctcatatattctctaatattactagcattataatttactaagctatc 380
             || |||||||||||  || | |||||||| ||| | |||| ||| ||| |||| ||
Sbjct: 6784 ttcttcaacatgtcttgtaaactctctaattttagtggcatcatacttttctaaactgct 6725


Query: 381  ctcacttttaacactgatactaaaattagtattgcgtgtattatctgctacagtaacaaa 440
             ||| |  |||||||||||||||| || ||||| ||||||||||||||||||| |||||
Sbjct: 6724 gtcattggtaacactgatactaaagtttgtattacgtgtattatctgctacagtcacaaa 6665


Query: 441  ca 442
            ||
Sbjct: 6664 ca 6663
```

```
gb|U85663|HPU85663 Human papillomavirus strain RTRX10 major capsid protein L1
gene,
           partial cds
           Length = 337

 Score =  335 bits (169), Expect = 3e-90
 Identities = 187/193 (96%)
 Strand = Plus / Plus


Query: 1   ggctgataacacacgtaacactaaatttactattagtggtgccagtgatagtagcacagt 60
           ||||||||||||||||||||||||| |||||||||||| ||||||||||||||||||||
Sbjct: 1   ggctgataacacacgtaacactaattttactattagtgttgccagtgatagtagcacagt 60


Query: 61  gaattatgatgctggaaaattcagagaatacatgcgtcatgttgaagaatatcagctatc 120
           |||||||||||||||||| |||||||||||||||||||||||||||||||| ||||
Sbjct: 61  gaattatgatgctggaaaaatcagagaatacatgcgtcatgttgaagaatatcagttatc 120


Query: 121 atttatttttacaattatgtagaataccttagaggcagaagtgttaacacagcctaatgc 180
           |||||||||||||||||||||||||| |||||||||||||||||||||| ||||||
Sbjct: 121 atttatttttacaattatgtagaatacctttagaggcagaagtgttaacacagcttaatgc 180


Query: 181 tatgaatcatggg 193
           |||||||||||||
Sbjct: 181 tatgaatcatggg 193
```

W55A (RTR)

```
>gb:HPU31781 Human papillomavirus type 23, complete genome.
            Length = 7324

  Plus Strand HSPs:

 Score = 1315 (363.4 bits), Expect = 2.3e-101, P = 2.3e-101
 Identities = 315/380 (82%), Positives = 315/380 (82%), Strand = Plus / Plus

query:     1 caatcagctgtttgttactgtagcagataatacacgcaatactaattttagtatcagtgt 60
             ||| ||| ||||||| |||||||||||||||||||| ||||| || ||||||||||||||
sbjct:  6655 caaccagatgtttgtgactgtagcagataatacacgtaatacaaactttagtatcagtgt 6714

query:    61 taaatgtgaggatagcttagcaaattataatgctagtaatattagagaatatatgagaca 120
             ||    |||    || |||| ||| ||| |||| | ||| |||||||| | ||  |||||
sbjct:  6715 taccaatgacagcagtttagaaaagtatgatgccactaaaattagagagtttacaagaca 6774

query:   121 tgttgaagagtatcagttgtcttttatattacaattgtgcagaatacctttaaaggctga 180
             ||||||||| || ||  | ||||||||| |||| |||||||| |||||||||||||| ||
sbjct:  6775 tgttgaagaataccaactttcttttatactacagttgtgcaggatacctttaaaggccga 6834

query:   181 agttttaacacgaatcaatgcaatgaactctgatattttagagaattggcaattgggctt 240
              || ||||||| ||| ||||| ||||| || |||||||||||||||||| || || ||
sbjct:  6835 ggtcttaacacaaattaatgccatgaattcagatattttagagaattggcagttagggtt 6894

query:   241 tgtacctacaccaaataatgcagtacacgatatatataggtatttagcctcaaaggccgc 300
             ||| ||||||||| |||||||||| || || | ||| || ||||| || ||||||||| |
sbjct:  6895 tgttcctacaccagataatgcagttcatgacacatacagatatttggcttcaaaggccac 6954

query:   301 taagtgtcctgatgcagtacctgaaacccaaaaagaggatccttttggaaagtattcatt 360
              || ||||| |||||||||||||| || ||||||||||||||||||||||||||||||
sbjct:  6955 aaaatgtccagatgcagtacctgacacgcaaaaagaggatccttttggaaagtattcatt 7014
```

```
query:   361 ctgggatgttgatctaacgg 380
             ||| |||||||| | || |
sbjct:  7015 ttggaatgttgatatgacag 7034
```

W55B (RTR)

```
>gb:HPU85663 Human papillomavirus strain RTRX10 major capsid protein L1 gene,
            partial cds.
            Length = 337

  Plus Strand HSPs:

 Score = 618 (181.5 bits), Expect = 5.7e-47, P = 5.7e-47
 Identities = 181/274 (66%), Positives = 198/274 (72%), Strand = Plus / Plus

query:    24 gcagataatacacgcaatactaattttagtatcagtgttaaaagtgaggatagcttagca 83
             || ||||| ||||| || |||||||||| ||| ||||||   |||||   ||||  ||
sbjct:     2 gctgataacacacgtaacactaattttactattagtgttgccagtgatagtagcacagtg 61

query:    84 aattataatgctagtaatattagagaatatatgagacatgttgaagagtatcagttgtcy 143
             |||||| ||||| | || || |||||||| ||| | |||||||||||| |||||||| ||
sbjct:    62 aattatgatgctggaaaaatcagagaatacatgcgtcatgttgaagaatatcagttatca 121

query:   144 tttaaatammaatgtgcarrawaacctttaaaggstgragttttamamgaatcmatgcaa 203
             ||||  |  +|||     ++|+ ||||||| |||+ |+||| |||    |   +|
sbjct:   122 tttattttacaattatgtagaatacctttagaggcagaagtgttaacacagcttaatgct 181

query:   204 tgaactctgartawwttagagaaatggcaattgggctytgtamccacaccmgataawgca 263
                | ||   + |++|||||| || |||||||||| |+||||+| ||||| || ||+||
sbjct:   182 atgaatcatgggatattagaaaattggcaattggggtttgtacctacaccagacaatgct 241

query:   264 stacacgctatatataggkatwtagccyccmaag 297
             +| |||| ||  || |||+ |+|| | +|| |||
sbjct:   242 gtgcacgatacctacaggtgtatatcttccaaag 275
```

W56A (RTR)

```
>gb:HPU85663 Human papillomavirus strain RTRX10 major capsid protein L1 gene,
            partial cds.
            Length = 337

  Plus Strand HSPs:

 Score = 563 (156.0 bits), Expect = 8.2e-65, Sum P(2) = 8.2e-65
 Identities = 143/181 (79%), Positives = 143/181 (79%), Strand = Plus / Plus

query:    23 gcagataatacacgcaatactaattttagtatcagtgttaaaagtgaggatagcttagca 82
             || ||||| ||||| || |||||||||| ||| ||||||   |||||   ||||  ||
sbjct:     2 gctgataacacacgtaacactaattttactattagtgttgccagtgatagtagcacagtg 61

query:    83 aattataatgctagtaatattagagaatatatgagacatgttgaagagtatcagttgtct 142
             |||||| ||||| | || || |||||||| ||| | |||||||||||| |||||||| ||
sbjct:    62 aattatgatgctggaaaaatcagagaatacatgcgtcatgttgaagaatatcagttatca 121

query:   143 tttatattacaattgtgcagaataccttaaaggctgaagttttaacacgaatcaatgcv 202
             ||||| |||||||| || |||||||||||| |||| ||||| |||||||   | |||||
sbjct:   122 tttattttacaattatgtagaatacctttagaggcagaagtgttaacacagcttaatgct 181

query:   203 a 203
             |
sbjct:   182 a 182
```

W57A (RTR)

```
emb|X55965|PAHPV57 Human papillomavirus type 57 complete DNA
            Length = 7861

 Score =  571 bits (288), Expect = e-161
 Identities = 309/316 (97%)
 Strand = Plus / Plus


Query: 1    gaaaccgggttcggggcgctggaatttgccgctctacagtccaacaaaccagatgtcccc 60
            |||||||||||||||||||||| |||||||||||||||||||||| ||||||||||
Sbjct: 6345 gaaaccgggttcggggcgctggattttgccgctctacagtccaacaaatcagatgtcccc 6404


Query: 61   ttgaatatctgtactaacatatgtaaatatccagactatctgaagatggctgcagaccct 120
            ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6405 ttggatatctgtactaacatatgtaaatatccagactatctgaagatggctgcagaccct 6464


Query: 121  tattgcgattctatgttcttttccctgcgcaaggagcaaatgttcactcggcaatttttc 180
            ||| |||||||||||||||||||||||| |||||||||||||||||| ||||||
Sbjct: 6465 tatggcgattctatgttcttttccctgcgcagggagcaaatgttcactcggcatttttc 6524


Query: 181  aatcggggtgggtcgatgggtgacgccctcccggatgagctatatgtcaaaagttctacc 240
            |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct: 6525 aatcggggtgggtcgatgggtgacgccctcccggatgagctatatgtcaagagttctacc 6584


Query: 241  gtccagacccccggtagttatgtttatacctccactcccagtggctctatggtatcctct 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6585 gtccagacccccggtagttatgtttatacctccactcccagtggctctatggtatcctct 6644


Query: 301  gaacagcagttattta 316
            ||||||||||||||||
Sbjct: 6645 gaacagcagttattta 6660

gb|U31781|HPU31781 Human papillomavirus type 23, complete genome.
            Length = 7324

 Score =  177 bits (88), Expect = 1e-42
 Identities = 214/256 (83%), Positives = 214/256 (83%), Gaps = 2/256 (0%)


Query: 11   acatcccagaatgaatactttccaaaaggatcctctttttggggtttcaggtactgcatc 70
            |||| ||| |||||||||||||||||||||||||||||| | | ||||||||||||||
Sbjct: 7023 acattccaaaatgaatactttccaaaaggatcctctttttgcg-tgtcaggtactgcatc 6965


Query: 71   aggacacttagtggcytttgagstaaaatacctatatatatcgtgtactgcattatctgg 130
             ||||| || ||||| |||||    ||||| || ||| | || || ||||||||||||||
Sbjct: 6964 tggacattttgtggcctttgaagccaaatatctgtatgtgtcatgaactgcattatctgg 6905


Query: 131  tgtaggtacaaagcccaattgccaattctctmaaatatcagagttcattgcattgattcg 190
            |||||| ||||| || || |||||||||||| ||||||| || ||||| ||||| ||| |
Sbjct: 6904 tgtaggaacaaaccctaactgccaattctctaaaatatctgaattcatggcattaatttg 6845


Query: 191  tgttaaaacattcagcctttaaaggtattctgcacaattgtdatataaaagacaactgat 250
            |||||| ||  || |||||||||||||| |||||||| |||  ||||||||| |  || |
Sbjct: 6844 tgttaagac-ctcggcctttaaaggtatcctgcacaactgtagtataaaagaaagttggt 6786
```

```
Query: 251  actcttcaacatgtct 266
            | |||||||||||||
Sbjct: 6785 attcttcaacatgtct 6770
```

W58A (RTR)

```
>gb:HPU31781 Human papillomavirus type 23, complete genome.
            Length = 7324

  Plus Strand HSPs:

 Score = 612 (170.8 bits), Expect = 2.5e-43, P = 2.5e-43
 Identities = 149/186 (80%), Positives = 152/186 (81%), Strand = Plus / Plus

query:     1 caatcagctgtttgttactgtagcagataatacacgcaatactaattttagtatcagtgt 60
             ||| ||| ||||||| |||||||||||||||||||| ||||| || ||||||||||||||
sbjct:  6655 caaccagatgtttgtgactgtagcagataatacacgtaatacaaactttagtatcagtgt 6714

query:    61 taaaagtgaggatagcttagcaaattataatgctagtaatattagagratatatgagaca 120
             ||  | |||    || |||| ||| ||| |||| | ||| |||||||+ | ||  |||||
sbjct:  6715 taccaatgacagcagtttagaaaagtatgatgccactaaaattagagagtttacaagaca 6774

query:   121 tgttgaagartatcarttgtcttttatawtacaaatgtgcagaatacctttaaaggctga 180
             |||||||||+|| ||+ | |||||||| |||| ||||||| |||||||||||||| ||
sbjct:  6775 tgttgaagaataccaactttcttttatactacagttgtgcaggatacctttaaaggccga 6834

query:   181 agtttt 186
             || ||
sbjct:  6835 ggtctt 6840
```

WN1A (ICP)

```
emb|X55964|PAHPV2A Human papilloma virus type 2a complete DNA
            Length = 7860

 Score =  197 bits (97), Expect = 1e-48
 Identities = 112/114 (98%), Gaps = 1/114 (0%)
 Strand = Plus / Minus


Query: 53   agatctacatcccaaaaggtcagggaggcatagggatcggtaggggtcttaggaggtgta 112
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7123 agatccacatcccaaaaggtcagggaggcatagggatcggtaggggtcttaggaggtgta 7064


Query: 113  ggtttttgacatgtaatagcctgggactgcaaatatctattaggtatcctgtaa 166
            |||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct: 7063 ggtttttgacatgtaatagcctgggactgcaaatatcta-taggtatcctgtaa 7011
```

```
emb|X74465|HPV10 Human papillomavirus type 10 genomic DNA
           Length = 7919

 Score =  433 bits (217), Expect = e-119
 Identities = 246/257 (95%)
 Strand = Plus / Minus


Query: 162  gctagtggacggaggcaaagttaacccaaagtcccaatcctccaataggctactattcat 221
            ||||||||||| |||||||||||||||||| ||||||||||||  ||||||||||
Sbjct: 7061 gctagtggacggtggcaaagttaacccaaagttccaatcctccaataaactactattcat 7002


Query: 222  ggtgtgcaaataggccataatatccggggttaacgttaccttacacaactgaaaaatgaa 281
            |||||||||||||||||||||||||||| || ||||||||||||||||||||||||||
Sbjct: 7001 ggtgtgcaaataggccataatatccggggtcaatgttaccttacacaactgaaaaatgaa 6942


Query: 282  ctgcaaatcatattcctctccgtgcctcaaatattctttaaatttggtggcgtcatacgt 341
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6941 ctgcaaatcatattcctctccgtgcctcaaatattctttaaatttggtggcgtcatacgt 6882


Query: 342  agcggcasbggaggcctcagaaggaacacacaagcacatattggtactgcgggtcgtgtc 401
            || ||||  ||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct: 6881 agtggcaggggaggcctcagaaggaacacacaagcacatattggtactgcgagtcgtgtc 6822


Query: 402  taccactgtaacaaaca 418
            |||||| ||||||||||
Sbjct: 6821 taccacagtaacaaaca 6805
```

WN2A (ICP)

```
gb|U31778|HPU31778 Human papillomavirus type 20, complete genome.
           Length = 7757

 Score =  708 bits (356), Expect = 0.0
 Identities = 380/388 (97%)
 Strand = Plus / Plus


Query: 46   atcaactgtttgttactgtagtagatagtactcgaaatacaaattttagcatatcagttc 105
            ||||||| |||||||||||||||| |||||||||||||||||||||||||||||||||
Sbjct: 6908 atcaactatttgttactgtagtagataatactcgaaatacaaattttagcatatcagttc 6967


Query: 106  attcagaaaacactgatgtttctaaaattcaaaattatgattctcagaaatttcaagaat 165
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6968 attcagaaaacactgatgtttctaaaattcaaaattatgattctcagaaatttcaagaat 7027


Query: 166  atttaagacacgtagaagaatatgaaatttcattaattttacagctctgtaaagttcctt 225
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7028 atttaagacacgtagaagaatatgaaatttcattaattttacagctctgtaaagttcctt 7087


Query: 226  taacagctgaagttttagctcaaattaatgctatgaattcaaatatattagaggagtggc 285
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7088 taacagctgaagttttagctcaaattaatgctatgaattcaaatatattagaggagtggc 7147
```

```
Query: 286  agtgaggatttgtacctgcaccggataatcctatccacgatacatacagatatattaatt 345
             ||| |||||| || ||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7148 agttaggattcgttcctgcaccggataatcctatccacgatacatacagatatattaatt 7207

Query: 346  ctgcagctactagatgtcctgacaaaaatcctccaaaagaaagagaagatccttacaagg 405
             ||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct: 7208 ctgcagctactagatgtcctgataaaaatcctccaaaagaaagagaagatccttacaagg 7267


Query: 406  atctaaacttctggaatgttgatctatc 433
             |||||||||| |||||||||| |||||
Sbjct: 7268 atctaaacttttggaatgttgacctatc 7295
```

WN2B (ICP)

```
gb|M22961|PPHDELCG Human papillomavirus type 5 DNA and naturally occurring
deletions in
            the late region of the virus.
            Length = 7746

 Score =  125 bits (63), Expect = 9e-27
 Identities = 171/207 (82%)
 Strand = Plus / Plus


Query: 181  cctttaaaagcagaagtcttggctcagataaatgccatgaacccctttattattggaggac 240
             |||||||| ||||| || ||||| ||||| ||||| |||||| | |  ||||||||||
Sbjct: 7108 cctttaaaggcagaggtattggcacagatcaatgcaatgaactcttcgttattggaggat 7167


Query: 241  tggcaattaggctttgtccttacacctgacaatccaattcatgatacctacagatttatt 300
             ||||| ||||| ||||| |  || ||||| |||||||||| || |||||||||| ||||
Sbjct: 7168 tggcagttaggatttgttcccactcctgataatccaattcaggacacctacagatatatt 7227


Query: 301  gactctttggctacccgatgccctgacaaaaatcctccaaaagaaaaacctgacccttat 360
             |||||||||||||| || || || || || |||||||| ||||||||    |||||||||
Sbjct: 7228 gactctttggctacacggtgtccagataagaatcctccgaaagaaaaggaagacccttat 7287


Query: 361  gagggcttaaacttttggaatgtagat 387
              |||||||| | |||||| ||||||||
Sbjct: 7288 aagggcttacatttttgggatgtagat 7314


emb|Y15176|HPVY15176 Human papillomavirus type 80 E6, E7, E1, E2, E4, L2, and
L1 genes
            Length = 7427

 Score =  685 bits (344), Expect = 0.0
 Identities = 371/380 (97%)
 Strand = Plus / Minus


Query: 46   gttagatctacattccagaatgtatattttccaaatgggtcttctttgtcctttggagca 105
             |||| |||||| ||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7105 gttaaatctacgttccaaaatgtatattttccaaatgggtcttctttgtcctttggagca 7046


Query: 106  actgcatcaggacatttagtagctttagagtcaatatatctgtaaatatcatgcacagca 165
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7045 actgcatcaggacatttagtagctttagagtcaatatatctgtaaatatcatgcacagca 6986
```

```
Query: 166  ttgtctggtgtgggaataaaccttagttgccactcttctaaaatacctgcattcattgca 225
            |||||||||||||||| ||||| ||||||||||||||||||||||||||||||||||||
Sbjct: 6985 ttgtctggtgtgggaacaaaccctagttgccactcttctaaaatacctgcattcattgca 6926


Query: 226  tttatctgtgttaacacctcagcctttaaaggcactttacatagttgtaatataatagct 285
            ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6925 tttatctgtgttaatacctcagcctttaaaggcactttacatagttgtaatataatagct 6866


Query: 286  aattgatattcttctacatgccttaaaaattctctaatattttgtgtattatattcagtt 345
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6865 aattgatattcttctacatgccttaaaaattctctaatattttgtgtattatattcagtt 6806


Query: 346  atagtactaccatcaggagtaacacttatggtgaaattagtgtttcttgtattatcagca 405
            |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6805 atagtactaccatcagaagtaacacttatggtgaaattagtgtttcttgtattatcagca 6746


Query: 406  acagtaacaaacatctgatt 425
            ||||||| |||||| |||||
Sbjct: 6745 acagtaataaacatttgatt 6726
```

WN5A (ICP)

```
gb|U31781|HPU31781 Human papillomavirus type 23, complete genome.
            Length = 7324

  Plus Strand HSPs:

 Score = 1324 (365.8 bits), Expect = 1.2e-99, P = 1.2e-99
 Identities = 316/380 (83%), Positives = 316/380 (83%), Strand = Plus / Plus

query:     1 caatcagctgtttgttacagtagcagataatacacgcaatactaattttagtatcagtgt 60
             ||| ||| ||||||| || |||||||||||||||| ||||| || ||||||||||||||
sbjct:  6655 caaccagatgtttgtgactgtagcagataatacacgtaatacaaactttagtatcagtgt 6714

query:    61 taaaagtgaggatagcttagcaaattataatgctagtaatattagagaatatatgagaca 120
             ||  | |||    || |||| ||| ||| |||| | ||| |||||||| | ||  |||||
sbjct:  6715 taccaatgacagcagtttagaaaagtatgatgccactaaaattagagagtttacaagaca 6774

query:   121 tgttgaagagtatcagttgtcttttatattacaattgtgcagaatacctttaaaggctga 180
             ||||||||| || ||  | ||||||||| |||| |||||||| ||||||||||||| ||
sbjct:  6775 tgttgaagaataccaactttcttttatactacagttgtgcaggatacctttaaaggccga 6834

query:   181 agttttaacacgaatcaatgcaatgaactctgatattttagagaattggcaattgggctt 240
             || ||||||| ||| ||||| ||||| || |||||||||||||||||| || || ||
sbjct:  6835 ggtcttaacacaaattaatgccatgaattcagatattttagagaattggcagttagggtt 6894

query:   241 tgtacctacaccagataatgcagtacacgatatatataggtatttagcctcaaaggccgc 300
             ||| |||||||||||||||||| || || | ||| || ||||| || ||||||||| |
sbjct:  6895 tgttcctacaccagataatgcagttcatgacacatacagatatttggcttcaaaggccac 6954

query:   301 taagtgtcctgatgcagtacctgaaacccaaaaagaggatccttttggaaagtattcatt 360
             || ||||| ||||||||||||| || ||||||||||||||||||||||||||||||||
sbjct:  6955 aaaatgtccagatgcagtacctgacacgcaaaaagaggatccttttggaaagtattcatt 7014

query:   361 ctgggatgttgatctaacgg 380
             ||| |||||||| | || |
sbjct:  7015 ttggaatgttgatatgacag 7034
```

WN5B (ICP)

```
gb|U31781|HPU31781 Human papillomavirus type 23, complete genome.
            Length = 7324

  Plus Strand HSPs:

 Score = 779 (216.6 bits), Expect = 3.4e-55, P = 3.4e-55
 Identities = 191/237 (80%), Positives = 191/237 (80%), Strand = Plus / Plus

query:     1 caatcagctgtttgttacagtagcagataatacacgcaatactaattttagtatcagtgt 60
             ||| ||| ||||||| || ||||||||||||||||| ||||| || ||||||||||||||
sbjct:  6655 caaccagatgtttgtgactgtagcagataatacacgtaatacaaactttagtatcagtgt 6714

query:    61 taaaagtgaggatagcttagcaaattataatgctagtaatattagagaatatatgagaca 120
             ||  | |||    || |||| ||| ||| |||| | ||| |||||||| | ||  |||||
sbjct:  6715 taccaatgacagcagtttagaaaagtatgatgccactaaaattagagagtttacaagaca 6774

query:   121 tgttgaagagtatcagttgtcktttatattacaattgtgcagaatgyctttaaaggctga 180
             ||||||||| || ||  | || |||||| |||| |||||||| ||  |||||||||| ||
sbjct:  6775 tgttgaagaataccaactttcttttatactacagttgtgcaggatacctttaaaggccga 6834

query:   181 agttttaacacgaatcaatgcaatgaactctgatattttagagaattggcaattggg 237
              || ||||||| ||| ||||| ||||| || ||||||||||||||||||| || ||
sbjct:  6835 ggtcttaacacaaattaatgccatgaattcagatattttagagaattggcagttagg 6891
```

WN5C (ICP)

```
gb|U31778|HPU31778 Human papillomavirus type 20, complete genome.
            Length = 7757

 Score =  525 bits (265), Expect = e-147
 Identities = 277/281 (98%)
 Strand = Plus / Plus


Query: 1    atatcagttcattcagaaaacactgatgtttctaaaattcaaaattatgattctcagaaa 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6958 atatcagttcattcagaaaacactgatgtttctaaaattcaaaattatgattctcagaaa 7017


Query: 61   tttcaagaatatttaagacacgtagaagaatatgaaatttcattaattttacagctctgt 120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7018 tttcaagaatatttaagacacgtagaagaatatgaaatttcattaattttacagctctgt 7077


Query: 121  aaagttcctttaacagctgaagttttagctcaaattaatgctatgaattcaaatatatta 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7078 aaagttcctttaacagctgaagttttagctcaaattaatgctatgaattcaaatatatta 7137


Query: 181  gaggagtggcagtgaggatttgtacctgcaccggataatcctatccacgatacatacaga 240
             |||||||||||||| |||||| || ||||||||||||||||||||||||||||||||||
Sbjct: 7138 gaggagtggcagttaggattcgttcctgcaccggataatcctatccacgatacatacaga 7197


Query: 241  tatattaattctgcagctactagatgtcctgacaaaaatcc 281
             |||||||||||||||||||||||||||||| ||||||||
Sbjct: 7198 tatattaattctgcagctactagatgtcctgataaaaatcc 7238
```

```
gb|U31787|HPU31787 Human papillomavirus type 38, complete genome.
           Length = 7400

 Score =  127 bits (64), Expect = 1e-27
 Identities = 161/194 (82%)
 Strand = Plus / Plus


Query: 13   gctgaggtgctaacccagattaatgctatgaattcaggtatattggaaaactggcaacta 72
            ||||| ||||| || ||||||||||||||||||| || ||||| ||||| |||||| ||
Sbjct: 6843 gctgaagtgctgacacagattaatgctatgaattctggaatattagaaaattggcaatta 6902


Query: 73   gggtttgttccaacaccagataatgcagtgcatgacacatatcgttaccttaattcaaaa 132
            || ||||| || || ||||| ||| | || || || ||||||||||| | |  || |||
Sbjct: 6903 ggctttgtacccaccccagacaattctgtacacgatacatatcgttacataacatctaaa 6962


Query: 133  gctacaaaatgtccagatgcagttcvtaaaacagaaaaggaagatccttttggtggatat 192
            || || |||||||||||||||| | | |||||||||| |||||||| ||||||  ||||
Sbjct: 6963 gcaactaaatgtccagatgcagtgcctgaaacagaaaaagaagatccctttggtcaatat 7022


Query: 193  tcattttggaatgt 206
             |||||||||||||
Sbjct: 7023 acattttggaatgt 7036
```

WN6A (ICP)

```
gb|AF097700|AF097700 Human papillomavirus isolate HPVX2b L1 protein gene,
partial cds
          Length = 276

 Score =  378 bits (190), Expect = e-103
 Identities = 190/190 (100%)
 Strand = Plus / Plus


Query: 4    agaatatgaagtatcacttattctacagctttgtaaaattccactaaaagctgaggtgct 63
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 87   agaatatgaagtatcacttattctacagctttgtaaaattccactaaaagctgaggtgct 146


Query: 64   agcacagattaatgcaatgaactctgacattttggaaagttggcagttaggttttgtacc 123
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 147  agcacagattaatgcaatgaactctgacattttggaaagttggcagttaggttttgtacc 206


Query: 124  tacaccagataatcctatccacgacacatacagatacttagattcattggctacccgctg 183
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 207  tacaccagataatcctatccacgacacatacagatacttagattcattggctacccgctg 266


Query: 184  cccagaaaaa 193
            ||||||||||
Sbjct: 267  cccagaaaaa 276
```

WN7A (ICP)

```
gb|U31781|HPU31781 Human papillomavirus type 23, complete genome.
           Length = 7324

  Plus Strand HSPs:

 Score = 1315 (363.4 bits), Expect = 4.1e-99, P = 4.1e-99
 Identities = 311/371 (83%), Positives = 311/371 (83%), Strand = Plus / Plus

query:     3 cagctgtttgttacagtagcagataatacacgcaatactaattttagtatcagtgttaaa 62
             ||| ||||||| || ||||||||||||||||| ||||| || ||||||||||||||||
sbjct:  6659 cagatgtttgtgactgtagcagataatacacgtaatacaaactttagtatcagtgttacc 6718

query:    63 agtgaggatagcttagcaaattataatgctagtaatattagagaatatatgagacatgtt 122
             | |||    || |||| ||| ||| |||| | ||| |||||||| | ||  |||||||||
sbjct:  6719 aatgacagcagtttagaaaagtatgatgccactaaaattagagagtttacaagacatgtt 6778

query:   123 gaagagtatcagttgtcttttatattacaattgtgcagaataccttaaaggctgaagtt 182
             ||||| || ||  | ||||||||| |||| |||||||| |||||||||||||| || ||
sbjct:  6779 gaagaataccaactttcttttatactacagttgtgcaggataccttaaaggccgaggtc 6838

query:   183 ttaacacgaatcaatgcaatgaactctgatattttagagaattggcaattgggctttgta 242
             ||||||| ||| ||||| ||||| || ||||||||||||||||||| || || |||||
sbjct:  6839 ttaacacaaattaatgccatgaattcagatattttagagaattggcagttagggtttgtt 6898

query:   243 cctacaccagataatgcagtacacgatatatataggtatttagcctcaaaggccactaag 302
             |||||||||||||||||| || || | ||| || ||||| || |||||||||| ||
sbjct:  6899 cctacaccagataatgcagttcatgacacatacagatatttggcttcaaaggccacaaaa 6958

query:   303 tgtcctgatgcagtacctgaaacccaaaaagaggatccttttggaaagtattcattctgg 362
             ||||| |||||||||||||| || ||||||||||||||||||||||||||||||| |||
sbjct:  6959 tgtccagatgcagtacctgacacgcaaaaagaggatccttttggaaagtattcattttgg 7018

query:   363 gatgttgatct 373
              |||||||| |
sbjct:  7019 aatgttgatat 7029
```

APPENDIX II Alignments of HPV sequences amplified from RTR and ICP SCCs with known HPV types deposited in the Genbank database.

F2BT (RTR)

```
>gb:HPV15 Human papillomavirus type 15 genomic DNA.
          Length = 7412

  Plus Strand HSPs:

 Score = 1199 (331.3 bits), Expect = 1.0e-91, P = 1.0e-91
 Identities = 243/247 (98%), Positives = 243/247 (98%), Strand = Plus / Plus

f2bt :     2 aatcagctgtttgttactgtagcagataacacaaggaatacaaattttactattagtgtt 61
             |||||| ||||| ||||||| |||||||||||||||||||||||||||||||||||||
hpv15:  6711 aatcagatgtttattactgttgcagataacacaaggaatacaaattttactattagtgtt 6770

f2bt :    62 acctctgatggtaatgccataaatgaatataattcacaagatatcagagaatttttaaga 121
             |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
hpv15:  6771 acctctgatggtaatgccataaatgaatataattcacaaaatatcagagaatttttaaga 6830

f2bt :   122 catgtggaagaatatcagttatctattattttgcaattgtgtaaaataccttaaaagct 181
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
hpv15:  6831 catgtggaagaatatcagttatctattattttgcaattgtgtaaaataccttaaaagct 6890

f2bt :   182 gaggtattaacacaaattaatgctatgaattcaggtattttagaagactggcaactaggg 241
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
hpv15:  6891 gaggtattaacacaaattaatgctatgaattcaggtattttagaagactggcaactaggg 6950

f2bt :   242 tttgttc 248
             |||||||
hpv15:  6951 tttgttc 6957
```

F2CT (RTR)

```
gb|AF003892|HPAF003892 Human papillomavirus strain P36-2 major capsid protein
L1 gene,
           partial cds
           Length = 419

 Score =  149 bits (75), Expect = 6e-34
 Identities = 99/104 (95%), Gaps = 1/104 (0%)
 Strand = Plus / Plus


Query: 1   ggagcgccaggacaacagactatgcccaacaattgaattggaaactacctatatagaaga 60
            |||||||||||||||||||||||||| |||||||||| ||||||| ||||||||||
Sbjct: 304 ggagcgccaggacaacagactatgcccaccaattgaattgaaaactacttatatagaaga 363


Query: 61  tggcgacatgccagatatagg-tttggaaatttaaacttcaaaa 103
            |||||||||| |||||||||| ||||||||||||||||||||
Sbjct: 364 tggcgacatggcagatataggttttggaaatttaaacttcaaaa 407
```

```
gb|AF003892|HPAF003892 Human papillomavirus strain P36-2 major capsid protein
L1 gene,
           partial cds
           Length = 419

 Score =  149 bits (75), Expect = 6e-34
 Identities = 99/104 (95%), Gaps = 1/104 (0%)
 Strand = Plus / Plus


Query: 1   ggagcgccaggacaacagactatgcccaacaattgaattggaaactacctatatagaaga 60
           ||||||||||||||||||||||||||||| |||||||||| ||||||| ||||||||||
Sbjct: 304 ggagcgccaggacaacagactatgcccaccaattgaattgaaaactacttatatagaaga 363


Query: 61  tggcgacatgccagatatagg-tttggaaatttaaacttcaaaa 103
           |||||||||| |||||||||| ||||||||||||||||||||
Sbjct: 364 tggcgacatggcagatataggttttggaaatttaaacttcaaaa 407
```

F2ET (RTR)

```
emb|X74469|HPV17 Human papillomavirus type 17 genomic DNA
           Length = 7427

 Score =  642 bits (324), Expect = 0.0
 Identities = 351/360 (97%)
 Strand = Plus / Plus


Query: 1    acagtagctatcaaggtcgatctactgatgatagacaaaacacgtcacttgatcctaaac 60
           ||||||||||||||| |||||||||||||||||||||||||| |||| |||||||
Sbjct: 6121 acagtagctatcaaggtggatctactgatgatagacaaaacacgtcatttgaccctaaac 6180


Query: 61   aagtgcagatgtttgttgtaggctgtgtaccttgtattggagaacatttggacagggctc 120
           |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct: 6181 aagtgcagatgtttgttgtaggctgtgtaccttgtattggagaacattgggacagggctc 6240


Query: 121  ctgtatgtggaaatgaacaaaacaatcaaacaggcctgtgtccaccattggaattaaata 180
           |||||||| |||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct: 6241 ctgtatgtgaaaatgaacaaaacaatcaaacaggcctgtgtccaccattggaattaaaaa 6300


Query: 181  acactgttatcgaagatggtgacatggttgacataggcttttgaaacattaataacaaag 240
           |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct: 6301 acactgttatcgaagatggtgacatggttgacataggctttggaaacattaataacaaag 6360


Query: 241  tgctttcatttaataaatcagatgtaagattagatatagttaatgaaacatgcaaatatc 300
           |||||||||||||||||||||||||| |||||||||||||||||||||||||||||||
Sbjct: 6361 tgctttcatttaataaatcagatgtaagtttagatatagttaatgaaacatgcaaatatc 6420


Query: 301  ctgatttttttaagcatggcaaatgatgtttatggtgatgcatgtttccttttcgccagac 360
           |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct: 6421 ctgatttttttaagcatggcaaatgatgtttatggtgatgcatgtttctttttcgccagac 6480
```

```
gb|U31787|HPU31787 Human papillomavirus type 38, complete genome.
           Length = 7400

 Score =  133 bits (67), Expect = 4e-29
 Identities = 214/263 (81%)
 Strand = Plus / Plus


Query: 109  gaatatatgagacatgtagaagaatatcaattatcatatatatttcagttatgtagcgta 168
            |||||| | |||||||| || ||||| ||||| |||| |||||| || || ||||  ||
Sbjct: 6774 gaatatttaagacatgttgaggaataccaattgtcatttatattgcaattgtgtaaggtt 6833


Query: 169  cccttagagactgaggtgctaacccagattaatgctatgaattcaggtatattagaaaac 228
            || ||| |  |||| ||||| || ||||||||||||||||||| || ||||||||||
Sbjct: 6834 cctttaaatgctgaagtgctgacacagattaatgctatgaattctggaatattagaaaat 6893


Query: 229  tggcaactagggtttgttccaacaccagataatgcagtgcatgacacatatcgttacctt 288
            |||||| |||| ||||| || || ||||| ||| | || || || ||||||||||| |
Sbjct: 6894 tggcaattaggctttgtacccaccccagacaattctgtacacgatacatatcgttacata 6953


Query: 289  aattcaaaagctacaaaatgtccagatgcagtccaagaaacagaaaaggaagatcctttt 348
            |  || ||||| || |||||||||||||||| |  |||||||||| |||||||| |||
Sbjct: 6954 acatctaaagcaactaaatgtccagatgcagtgcctgaaacagaaaaagaagatcccttt 7013


Query: 349  ggtggatattcattttggaatgt 371
            |||  |||| ||||||||||||
Sbjct: 7014 ggtcaatatacattttggaatgt 7036



>gb:HPU31785 Human papillomavirus type 36, complete genome.
           Length = 7722

  Plus Strand HSPs:

 Score = 1034 (287.0 bits), Expect = 1.1e-109, Sum P(2) = 1.1e-109
 Identities = 208/212 (98%), Positives = 210/212 (99%), Strand = Plus / Plus

query:    94 atgattacactgcagagcaatttagagaatatcaaaggcacgtggaggaatatgaaattt 153
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sbjct:  6996 atgattacactgcagagcaatttagagaatatcaaaggcacgtggaggaatatgaaattt 7055

query:   154 cattaatattacagctatgtaaggttcctctaaaggcagaagtattggctcagataaatg 213
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
sbjct:  7056 cattaatattacagctatgtaaggttcctctgaaggcagaagtattggctcagataaatg 7115

query:   214 ctatgaattcttctttattggragattggcagttaggttttgtacctactccagataacc 273
            ||||||||||||||||||||+|||||||||||||||||||||||||||||||||||||||
sbjct:  7116 ctatgaattcttctttattggaagattggcagttaggttttgtacctactccagataacc 7175

query:   274 ctattcwagacacctatcgatttattgattca 305
            ||||||+|||||||||||||| ||||||||||
sbjct:  7176 ctattcaagacacctatcgatatattgattca 7207
```

F2GT (RTR)

```
gb|U31780|HPU31780 Human papillomavirus type 22, complete genome.
           Length = 7368

 Score =  444 bits (224), Expect = e-123
 Identities = 285/300 (95%), Gaps = 3/300 (1%)
 Strand = Plus / Plus


Query: 1    actgaacgccaggaaggaacatcagatgatagaagaaatgtttcccttgatcctaaacag 60
            ||||||||||||||||||||||||||||||||||||||||||||  ||||||||||||||
Sbjct: 6061 actgaacgccaggaaggaacatcagatgatagaagaaatgtttcttttgatcctaaacag 6120


Query: 61   gttcaaatgtttatcattggatgtataccgtggggaggtgaatatttttataaagctcct 120
            |||||||||||||||||||||||||||||||   ||||||||||   ||||||||||||
Sbjct: 6121 gttcaaatgtttatcattggatgtataccgtgtttaggtgaatattgggataaagctcct 6180


Query: 121  gtttgtgaagatgcaggcagtca-gtaggattatgtcctccactagaatt--aaaatggt 177
            ||||||||||||||||||||||| |||||||||||||||||||||||||  |||||||
Sbjct: 6181 gtttgtgaagatgcaggcagtcaggtaggattatgtcctccactagaattaaaaaatggt 6240


Query: 178  gttatagaccatggagatatgtttgatataggatttccaaatataaataataaaacacta 237
            ||||||||  |||||||||||||||||||||||||  ||||||||||||||||||||||
Sbjct: 6241 gttatagaggatggagatatgtttgatataggatttggaaatataaataataaaacacta 6300


Query: 238  tcatttaatagatctgatgtaagcttagacattgtaaatgaaatctgtaaatatcctgat 297
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6301 tcatttaatagatctgatgtaagcttagacattgtaaatgaaatctgtaaatatcctgat 6360


>gb:PPHL1AJ Human papillomavirus unidentified type (RTRX1) L1 gene, partial
           cds.
           Length = 267

  Minus Strand HSPs:

 Score = 1290 (356.5 bits), Expect = 1.1e-101, P = 1.1e-101
 Identities = 262/267 (98%), Positives = 262/267 (98%), Strand = Minus / Plus

query:   330 atcagtgttactagtgaagacttaagtacagcaaaatatgatgctaaaaatatcagggaa 271
            |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
sbjct:     1 atcagtgttactagtgaagatttaagtacagcaaaatatgatgctaaaaatatcagggaa 60

query:   270 tatatgagacacgtagaagaatatcaattatcatttatattacagttatgtagggtaccc 211
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
sbjct:    61 tatatgagacatgtagaagaatatcaattatcatttatattacagttatgtagggtaccc 120

query:   210 tttgaggctgaggtgctaacccagattaatgctgtgaattcaggtatattagaaaactgg 151
            || |||||||||||||||||||||||||||| ||||||||||||||||||||||||||
sbjct:   121 ttagaggctgaggtgctaacccagattaatgctatgaattcaggtatattagaaaactgg 180

query:   150 caactagggtttgttccaacaccagataatgcagtgcatgacacatatcgttaccttaat 91
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
sbjct:   181 caactagggtttgttccaacaccagataatgcagtgcatgacacatatcgttaccttagt 240

query:    90 tcaaaagctacaaaatgtccagatgca 64
            ||||||||||||||||||||||||||
sbjct:   241 tcaaaagctacaaaatgtccagatgca 267
```

```
gb|U31787|HPU31787 Human papillomavirus type 38, complete genome.
           Length = 7400

 Score =  113 bits (57), Expect = 3e-23
 Identities = 159/193 (82%)
 Strand = Plus / Plus


Query: 181  ctgaggtgctaacccagattaatgctatgaattcagctatattagaaaactggcaactag 240
            |||| ||||| || |||||||||||||||||||| |  |||||||||| |||||| |||
Sbjct: 6844 ctgaagtgctgacacagattaatgctatgaattctggaatattagaaaattggcaattag 6903


Query: 241  gggttgttccaacaccagataatgcagtgcatgacacatatcgttaccttaattcaaaag 300
            |  |||| || || ||||| ||| | || || || |||||||||||| | |  || ||||
Sbjct: 6904 gctttgtacccaccccagacaattctgtacacgatacatatcgttacataacatctaaag 6963


Query: 301  ctacaaaatgtccagatgcagtccaagaaacagaaaaggaagatccttttggtggatatt 360
            | || |||||||||||||||| |  |||||||||| |||||||| ||||||  ||||
Sbjct: 6964 caactaaatgtccagatgcagtgcctgaaacagaaaaagaagatccctttggtcaatata 7023


Query: 361  cattttggaatgt 373
            |||||||||||||
Sbjct: 7024 cattttggaatgt 7036
```

F23AT (RTR)

```
gb|U31787|HPU31787 Human papillomavirus type 38, complete genome.
           Length = 7400

 Score =  155 bits (78), Expect = 1e-35
 Identities = 234/286 (81%)
 Strand = Plus / Plus


Query: 86   aatatgatgctaaaaatatcagggaatatatgagacatgtagaagaatatcaattatcat 145
            |||||||| ||  |||||| || |||||| | |||||||| || ||||| ||||| ||||
Sbjct: 6751 aatatgattctgcaaatattagagaatatttaagacatgttgaggaataccaattgtcat 6810


Query: 146  ttatatttcagttatgtagggtacccttagagactgaggtgctaacccagattaatgcta 205
            ||||||| || || |||| ||| || ||| |  |||| ||||| || ||||||||||||
Sbjct: 6811 ttatattgcaattgtgtaaggttcctttaaatgctgaagtgctgacacagattaatgcta 6870


Query: 206  tgaattcaggtatattagaaaactggcaactagggtttgttccaacaccagataatgcag 265
            ||||||| || |||||||||| |||||| |||| ||||| || || ||||| ||| | |
Sbjct: 6871 tgaattctggaatattagaaaattggcaattaggctttgtacccaccccagacaattctg 6930


Query: 266  tgcatgacacatatcgttaccttaattcaaaagctacaaaatgtccagatgcagtccaag 325
            | || || |||||||||||| | |  || ||||| || |||||||||||||||| |  |
Sbjct: 6931 tacacgatacatatcgttacataacatctaaagcaactaaatgtccagatgcagtgcctg 6990


Query: 326  aaacagaaaaggaagatccttttggtggatattcattttggaatgt 371
            |||||||||| |||||||| ||||||  |||| ||||||||||||
Sbjct: 6991 aaacagaaaaagaagatcccttttggtcaatatacattttggaatgt 7036
```

```
gb|L38918|PPHL1AJ Human papillomavirus unidentified type (RTRX1) L1 gene,
partial
           cds.
           Length = 267

 Score =  498 bits (251), Expect = e-139
 Identities = 263/267 (98%)
 Strand = Plus / Plus


Query: 103 atcagtgttactagtgaagacttaagtacagcaaaatatgatgctaaaaatatcagggaa 162
           |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct: 1   atcagtgttactagtgaagatttaagtacagcaaaatatgatgctaaaaatatcagggaa 60


Query: 163 tatatgagacatgtagaagaatatcaattatcatttatatttcagttatgtagggtaccc 222
           |||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct: 61  tatatgagacatgtagaagaatatcaattatcatttatattacagttatgtagggtaccc 120


Query: 223 ttagagactgaggtgctaacccagattaatgctatgaattcaggtatattagaaaactgg 282
           |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 121 ttagaggctgaggtgctaacccagattaatgctatgaattcaggtatattagaaaactgg 180


Query: 283 caactagggtttgttccaacaccagataatgcagtgcatgacacatatcgttaccttaat 342
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct: 181 caactagggtttgttccaacaccagataatgcagtgcatgacacatatcgttaccttagt 240


Query: 343 tcaaaagctacaaaatgtccagatgca 369
           |||||||||||||||||||||||||||
Sbjct: 241 tcaaaagctacaaaatgtccagatgca 267
```

F24AT (RTR)

```
gb|U31787|HPU31787 Human papillomavirus type 38, complete genome.
            Length = 7400

 Score =  127 bits (64), Expect = 1e-27
 Identities = 161/194 (82%)
 Strand = Plus / Plus


Query: 13   gctgaggtgctaacccagattaatgctatgaattcaggtatattggaaaactggcaacta 72
           ||||| ||||| || |||||||||||||||||| || ||||| ||||| |||||| ||
Sbjct: 6843 gctgaagtgctgacacagattaatgctatgaattctggaatattagaaaattggcaatta 6902


Query: 73   gggtttgttccaacaccagataatgcagtgcatgacacatatcgttaccttaattcaaaa 132
           || ||||| || || ||||| ||| | || || || |||||||||||| | |  || |||
Sbjct: 6903 ggctttgtacccaccccagacaattctgtacacgatacatatcgttacataacatctaaa 6962


Query: 133  gctacaaaatgtccagatgcagttcvtaaaacagaaaaggaagatccttttggtggatat 192
           || || |||||||||||||||| | | |||||||||| |||||||| ||||||  ||||
Sbjct: 6963 gcaactaaatgtccagatgcagtgcctgaaacagaaaaagaagatccctttggtcaatat 7022


Query: 193  tcattttggaatgt 206
            ||||||||||||
Sbjct: 7023 acattttggaatgt 7036
```

```
gb|L38918|PPHL1AJ Human papillomavirus unidentified type (RTRX1) L1 gene,
partial
           cds.
           Length = 267

 Score =  288 bits (145), Expect = 3e-76
 Identities = 151/153 (98%)
 Strand = Plus / Plus


Query: 1   gtacccttagaggctgaggtgctaacccagattaatgctatgaattcaggtatattggaa 60
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct: 115 gtacccttagaggctgaggtgctaacccagattaatgctatgaattcaggtatattagaa 174


Query: 61  aactggcaactagggtttgttccaacaccagataatgcagtgcatgacacatatcgttac 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 175 aactggcaactagggtttgttccaacaccagataatgcagtgcatgacacatatcgttac 234


Query: 121 cttaattcaaaagctacaaaatgtccagatgca 153
            |||| ||||||||||||||||||||||||||||
Sbjct: 235 cttagttcaaaagctacaaaatgtccagatgca 267
```

F26AT (RTR)

```
gb|AF054877|AF054877 Human papillomavirus isolate HPVX20 L1 protein gene,
partial cds
           Length = 267

 Score =  240 bits (121), Expect = 1e-61
 Identities = 130/133 (97%), Positives = 130/133 (97%)


Query: 1   cctcagcttctaaaggtactctgcgtaattgcaaaatataagatagctgatattcttcta 60
            |||||||||||||||||||||||| |||||||||||| ||||||||||||||||||||
Sbjct: 133 cctcagcttctaaaggtactctgcataattgcaaaataaaagatagctgatattcttcta 74


Query: 61  catgtctcatatactctctaatattttgagtattatattgtgcagtgcttacatcttcac 120
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct: 73  catgtctcatatactctctaatattttgagcattatattgtgcagtgcttacatcttcac 14


Query: 121 tggtaacactaat 133
            ||||||||||||
Sbjct: 13  tggtaacactaat 1
```

F27AT (RTR)

```
emb|X74468|HPV15 Human papillomavirus type 15 genomic DNA
           Length = 7412

 Score =  363 bits (183), Expect = 1e-98
 Identities = 186/187 (99%)
 Strand = Plus / Minus


Query: 51   gttagatctacattccaaaatgtatactttccaaatgggtcttctttgtcctttggttgt 110
            |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7090 gttaaatctacattccaaaatgtatactttccaaatgggtcttctttgtcctttggttgt 7031


Query: 111  acagcatcaggacatttagttgccttagagtcaatatatctataaatatcttgtacagcg 170
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7030 acagcatcaggacatttagttgccttagagtcaatatatctataaatatcttgtacagcg 6971


Query: 171  ttgtctggtgtaggaacaaaccctagttgccagtcttctaaaatacctgaattcatagca 230
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6970 ttgtctggtgtaggaacaaaccctagttgccagtcttctaaaatacctgaattcatagca 6911


Query: 231  ttaattt 237
            |||||||
Sbjct: 6910 ttaattt 6904
```

WN3A (ICP)

```
gb|U31778|HPU31778 Human papillomavirus type 20, complete genome.
           Length = 7757

 Score =  696 bits (350), Expect = 0.0
 Identities = 383/391 (97%), Gaps = 1/391 (0%)
 Strand = Plus / Plus


Query: 45   caatcaactgtttgttacagtagtagataatactcgaaatacaaattttagcatatcagt 104
            ||||||||| |||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct: 6906 caatcaactatttgttactgtagtagataatactcgaaatacaaattttagcatatcagt 6965


Query: 105  tcattcagaaaacactgatgtttctaaaattcaaaattatgattctcagaaatttcaaga 164
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6966 tcattcagaaaacactgatgtttctaaaattcaaaattatgattctcagaaatttcaaga 7025


Query: 165  atatttaagacacgtagaagaatatgaaattccattaattttacagctctgtaaagttcc 224
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct: 7026 atatttaagacacgtagaagaatatgaaatttcattaattttacagctctgtaaagttcc 7085


Query: 225  tttaacagctgaagttttagctcaaattaaagctatgaattcaaatatattagaggagtg 284
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct: 7086 tttaacagctgaagttttagctcaaattaatgctatgaattcaaatatattagaggagtg 7145


Query: 285  gcagttaggatttgttcctgcaccggataatcctatccacgatacatacagatatattaa 344
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7146 gcagttaggattcgttcctgcaccggataatcctatccacgatacatacagatatattaa 7205
```

```
Query: 345  ttctgcagctactagatgtcctgataaaaatcctccaaaagaaagagaagatccttacaa 404
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7206 ttctgcagctactagatgtcctgataaaaatcctccaaaagaaagagaagatccttacaa 7265
```

FT3 (ICP)

```
gb|U31778|HPU31778 Human papillomavirus type 20, complete genome.
           Length = 7757

 Score =  638 bits (322), Expect = 0.0
 Identities = 337/342 (98%)
 Strand = Plus / Plus


Query: 10   tttgttacagtagtagataatactcgaaatacaaattttagcatatcagttcattcggaa 69
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct: 6916 tttgttactgtagtagataatactcgaaatacaaattttagcatatcagttcattcagaa 6975


Query: 70   aacactgatgtttctaaaattcaaaattatgattctcagaaatttcaagaatatttaaga 129
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6976 aacactgatgtttctaaaattcaaaattatgattctcagaaatttcaagaatatttaaga 7035


Query: 130  cacgtagaagaatatgaaatttcattaattttacagctctgcaaagttcctttaacagct 189
            |||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct: 7036 cacgtagaagaatatgaaatttcattaattttacagctctgtaaagttcctttaacagct 7095


Query: 190  gaagttctagctcaaattaatgctatgaattcaaatatattagaggagtggcagttagga 249
            |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7096 gaagttttagctcaaattaatgctatgaattcaaatatattagaggagtggcagttagga 7155


Query: 250  tttgttcctgcaccggataatcctatccacgatacatacagatatattaattctgcagct 309
            || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7156 ttcgttcctgcaccggataatcctatccacgatacatacagatatattaattctgcagct 7215


Query: 310  actagatgtcctgataaaaatcctccaaaagaaagagaagat 351
            |||||||||||||||||||||||||||||||||||||||||
Sbjct: 7216 actagatgtcctgataaaaatcctccaaaagaaagagaagat 7257
```

FT4 (ICP)

```
gb|U31779|HPU31779 Human papillomavirus type 21, complete genome.
           Length = 7779

 Score =  715 bits (360), Expect = 0.0
 Identities = 373/375 (99%), Gaps = 1/375 (0%)
 Strand = Plus / Minus


Query: 6    atctacatcccaaaatttcatatttttataaggatcttctcgttcttttggagggttttt 65
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7332 atctacatcccaaaatttcatatttttataaggatcttctcgttcttttggagggttttt 7273
```

```
Query: 66   atcaggacatctagkagctgcagagtcaatgtatctatatgtatcatgaataggattgtc 125
            ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7272 atcaggacatctagtagctgcagagtcaatgtatctatatgtatcatgaataggattgtc 7213


Query: 126  tggg-caggaacaaatcctaactgccattcttctaaaatatttgcattcattgcattaat 184
            |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7212 tggggcaggaacaaatcctaactgccattcttctaaaatatttgcattcattgcattaat 7153


Query: 185  ttgagctaagacttctgctgttaaaggaactttacataattgtaaaattaaagaaagttc 244
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7152 ttgagctaagacttctgctgttaaaggaactttacataattgtaaaattaaagaaagttc 7093


Query: 245  atattcttcaacgtgtcttaaatattcttgaaagctctcggctttataattttcaatttt 304
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7092 atattcttcaacgtgtcttaaatattcttgaaagctctcggctttataattttcaatttt 7033


Query: 305  agacacgtctgcattctcaggatttactgaaatactaaagtttgtgttacgagtgttgtc 364
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 7032 agacacgtctgcattctcaggatttactgaaatactaaagtttgtgttacgagtgttgtc 6973


Query: 365  tactactgtaacaaa 379
            |||||||||||||||
Sbjct: 6972 tactactgtaacaaa 6958
```

FT6 (ICP)

```
gb|AF097700|AF097700 Human papillomavirus isolate HPVX2b L1 protein gene,
partial cds
           Length = 276

 Score =  431 bits (207), Expect = e-119
 Identities = 263/281 (93%), Positives = 263/281 (93%), Gaps = 5/281 (1%)


Query: 54   attgctgtttatcaagaacagaaaaaggtgaaagagatacagagttacgattctaccaag 113
            |||||||||||||||||||||||||||| ||||| ||||||||||||||||||||||||
Sbjct: 1    attgctgtttatcaagaacagaaaaaggtaaaagaaatacagagttacgattctaccaag 60


Query: 114  tttaatgaattccamagacatgtgggragaatatgdaagtatcacttattctacagcttt 173
            || |||||||||| |||||||||  |||||||| ||||||||||||||||||||||||
Sbjct: 61   ttcaatgaattccaaagacatgtgg-aagaatatg-aagtatcacttattctacagcttt 118


Query: 174  gtaaaattccactaaaagctgaggtgctagcacagatttwatgcvaatgaacccygacat 233
            |||||||||||||||||||||||||||||||||||  |||| ||||||| | |||||
Sbjct: 119  gtaaaattccactaaaagctgaggtgctagcacagatt-aatgc-aatgaactctgacat 176


Query: 234  ttttkgaaagttggcagttakgttttgtaccyacaccagataatcctatccacgacacat 293
            |||  |||||||||||||| |||||||||| ||||||||||||||||||||||||||||
Sbjct: 177  ttt-ggaaagttggcagttaggttttgtacctacaccagataatcctatccacgacacat 235


Query: 294  acagatacttagattcattggckacccgcygcccagaaaaa 334
            |||||||||||||||||||| |||||| ||||||||||
Sbjct: 236  acagatacttagattcattggctacccgctgcccagaaaaa 276
```

FT7 (ICP)

```
gb|L38388|PPHL1FR Human papillomavirus L1 gene fragment.
          Length = 395

 Score =  565 bits (285), Expect = e-159
 Identities = 334/349 (95%), Gaps = 1/349 (0%)
 Strand = Plus / Plus


Query: 26  gataatacacgcaatactaattttagtatcagtgttaaaagtgaggatagcttagcaaat 85
           |||| |||||||| |||||||||||||||||||||||| ||||||||||||||||||
Sbjct: 1   gatactacacgcagtactaattttagtatcagtgttaaaaatgaggatagcttagcaaat 60


Query: 86  tataatgctagtaatattagagaatatatgagacatgttgaagagtatcagttgtctttt 145
           |||||||||||| |||||||||||||| |||||||||||||| ||||||||||||||||
Sbjct: 61  tataatgctagaaatattagagaatacatgagacatgttgaggagtatcagttgtctttt 120


Query: 146 atattacaattgtgcagaatacctttaagggctgaagttttaacacgaatcaatgcaatg 205
           |||||||||||||||||||||||||||| |||||| |||||||||| ||||||||||||
Sbjct: 121 atattacaattgtgcagaatacctttaaaggctgaggttttaacacaaatcaatgcaatg 180


Query: 206 aactctgatattttagagaattggcaattgggcgtttgtacctacaccagataatgcagt 265
           |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct: 181 aactctgatattttagagaattggcaattgggc-tttgtacctacaccagataatgcagt 239


Query: 266 acacgatatatataggtatttagcctcaaaggccactaagtgtcctgatgcagtgcctga 325
           |||||||| |||||||||||||||||||| |||||||||||||||||||||| |||||
Sbjct: 240 acacgatacatataggtatttagcctcaaaagccactaagtgtcctgatgcagtacctga 299


Query: 326 aacccaaaaagaggatccttttggaaagtattcattctgggatgttgat 374
           |||||||||||| |||||||||||||||||||||||| ||||||||
Sbjct: 300 aacccaaaaagaagatccttttggaaagtattcattctggaatgttgat 348
```

FT8 (ICP)

```
gb|L38918|PPHL1AJ Human papillomavirus unidentified type (RTRX1) L1 gene,
partial
          cds.
          Length = 267

 Score =  482 bits (243), Expect = e-134
 Identities = 261/267 (97%)
 Strand = Plus / Minus


Query: 129 tgcatctggacattttgtagcttttgaattaaggtaacgatatgcatcatgcactgcatt 188
           |||||||||||||||||||||||||||| |||||||||||||  ||||||||||||||
Sbjct: 267 tgcatctggacattttgtagcttttgaactaaggtaacgatatgtgtcatgcactgcatt 208


Query: 189 atctggtgttggaacaaaccctagttgccagttttctaatatacctgaattcatagcatt 248
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 207 atctggtgttggaacaaaccctagttgccagttttctaatatacctgaattcatagcatt 148


Query: 249 aatctgggttagcacctcagcctctaagggtaccctacataactgtaatataaatgataa 308
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 147 aatctgggttagcacctcagcctctaagggtaccctacataactgtaatataaatgataa 88
```

```
Query: 309 ttgagattcttctacatgtctcatatattccctgatatttttagcatcatattttgctgt 368
           |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 87  ttgatattcttctacatgtctcatatattccctgatatttttagcatcatattttgctgt 28


Query: 369 acttaagtcttcactagtatcactgat 395
           |||||| |||||||||||| |||||||
Sbjct: 27  acttaaatcttcactagtaacactgat 1
```

FT9 (ICP)

```
gb|U31781|HPU31781 Human papillomavirus type 23, complete genome.
           Length = 7324

 Score =  232 bits (117), Expect = 9e-59
 Identities = 300/361 (83%)
 Strand = Plus / Plus


Query: 69   tgtttgttacagtagcagataatacacgcaatactaattttagtatcagtgttaaaagtg 128
           ||||||| || |||||||||||||||| ||||| || |||||||||||||||  | ||
Sbjct: 6663 tgtttgtgactgtagcagataatacacgtaatacaaactttagtatcagtgttaccaatg 6722


Query: 129  aggatagcttagcaaattataatgctagtaatattagagaatatatgagacatgttgaag 188
           |    || |||| ||| ||| |||| | ||| |||||||| | ||  ||||||||||||
Sbjct: 6723 acagcagtttagaaaagtatgatgccactaaaattagagagtttacaagacatgttgaag 6782


Query: 189  agtatcagttgtcttttatattacaattgtgcagaatacctttaaaggctgtagttttaa 248
           | || ||  | ||||||||| |||| |||||||| |||||||||||||| |  || ||||
Sbjct: 6783 aataccaactttcttttatactacagttgtgcaggatacctttaaaggccgaggtcttaa 6842


Query: 249  cacgaatcaatgcaatgaactctgatattttagagaattggcaatggggctttgtaccta 308
           ||| ||| ||||| ||||| || |||||||||||||||||| |  || ||||| ||||
Sbjct: 6843 cacaaattaatgccatgaattcagatattttagagaattggcagttagggtttgttccta 6902


Query: 309  caccagataatgcagtacacgatatatataggtatttagcctcaaaggccactaagtgtc 368
           |||||||||||||| || || | ||| || ||||| || |||||||||| || ||||
Sbjct: 6903 caccagataatgcagttcatgacacatacagatatttggcttcaaaggccacaaaatgtc 6962


Query: 369  ctgatgcagtgcctgaaacccaaaaagaggatccttttggaaagtattcattctgggatg 428
           | |||||||| ||||| || |||||||||||||||||||||||||||||| ||| |||
Sbjct: 6963 cagatgcagtacctgacacgcaaaaagaggatccttttggaaagtattcattttggaatg 7022


Query: 429  t 429
           |
Sbjct: 7023 t 7023
```

FT10 (ICP)

```
gb|U31781|HPU31781 Human papillomavirus type 23, complete genome.
           Length = 7324

 Score =  216 bits (109), Expect = 6e-54
 Identities = 301/362 (83%), Gaps = 1/362 (0%)
 Strand = Plus / Minus


Query: 81   acatcccagaatgaatactttccaaaaggatcctctttttggggtttcaggtactgcatc 140
            |||| ||| |||||||||||||||||||||||||||||| | | ||||||||||||||
Sbjct: 7023 acattccaaaatgaatactttccaaaaggatcctctttttgcg-tgtcaggtactgcatc 6965


Query: 141  aggacacttagtggcctttgaggctaaatacctatatatatcgtgtactgcattatctgg 200
             ||||| || |||||||||| || ||||| || ||| | || || ||||||||||||||
Sbjct: 6964 tggacattttgtggcctttgaagccaaatatctgtatgtgtcatgaactgcattatctgg 6905


Query: 201  tgtaggtacaaagcccaattgccaattctctaaaatatcagagttcattgcattgattcg 260
            |||||| ||||| || || |||||||||||||||||||| || ||||| ||||| ||| |
Sbjct: 6904 tgtaggaacaaaccctaactgccaattctctaaaatatctgaattcatggcattaatttg 6845


Query: 261  tgctaaaacttcagcctttaaaggtatgctgcacaattataatataaaagacatctgata 320
            || ||| || || |||||||||||||| |||||||| | || ||||||||| |  || ||
Sbjct: 6844 tgttaagacctcggcctttaaaggtatcctgcacaactgtagtataaaagaaagttggta 6785


Query: 321  ctcatcaacatgtctcatatattctctaatattactagcattataatttactaagctatc 380
             || ||||||||||  || | |||||||| ||| | |||| ||| ||| |||| ||
Sbjct: 6784 ttcttcaacatgtcttgtaaactctctaattttagtggcatcatacttttctaaactgct 6725


Query: 381  ctcacttttaacactgatactaaaattagtattgcgtgtattatctgctacagtaacaaa 440
             ||| |  |||||||||||||||| || ||||| |||||||||||||||||||| |||||
Sbjct: 6724 gtcattggtaacactgatactaaagtttgtattacgtgtattatctgctacagtcacaaa 6665


Query: 441  ca 442
            ||
Sbjct: 6664 ca 6663
```

FT11 (ICP)

```
gb|U31781|HPU31781 Human papillomavirus type 23, complete genome.
           Length = 7324

 Score =  216 bits (109), Expect = 6e-54
 Identities = 301/362 (83%), Gaps = 1/362 (0%)
 Strand = Plus / Minus


Query: 81   acatcccagaatgaatactttccaaaaggatcctctttttggggtttcaggtactgcatc 140
            |||| ||| |||||||||||||||||||||||||||||| | | ||||||||||||||
Sbjct: 7023 acattccaaaatgaatactttccaaaaggatcctctttttgcg-tgtcaggtactgcatc 6965


Query: 141  aggacacttagtggcctttgaggctaaatacctatatatatcgtgtactgcattatctgg 200
             ||||| || |||||||||| || ||||| || ||| | || || ||||||||||||||
Sbjct: 6964 tggacattttgtggcctttgaagccaaatatctgtatgtgtcatgaactgcattatctgg 6905
```

```
Query: 201  tgtaggtacaaagcccaattgccaattctctaaaatatcagagttcattgcattgattcg 260
             |||||| ||||| || || |||||||||||||||||||| || ||||| ||||| ||| |
Sbjct: 6904 tgtaggaacaaaccctaactgccaattctctaaaatatctgaattcatggcattaatttg 6845


Query: 261  tgctaaaacttcagcctttaaaggtatgctgcacaattataatataaaagacatctgata 320
             || ||| || || ||||||||||||| |||||||| | || ||||||||| |  || ||
Sbjct: 6844 tgttaagacctcggcctttaaaggtatcctgcacaactgtagtataaaagaaagttggta 6785


Query: 321  ctcatcaacatgtctcatatattctctaatattactagcattataatttactaagctatc 380
              || |||||||||||  || | |||||||| ||| | |||| ||| ||| |||| ||
Sbjct: 6784 ttcttcaacatgtcttgtaaactctctaattttagtggcatcatacttttctaaactgct 6725


Query: 381  ctcacttttaacactgatactaaaattagtattgcgtgtattatctgctacagtaacaaa 440
              ||| |  |||||||||||||||| || ||||| |||||||||||||||||||| |||||
Sbjct: 6724 gtcattggtaacactgatactaaagtttgtattacgtgtattatctgctacagtcacaaa 6665


Query: 441  ca 442
             ||
Sbjct: 6664 ca 6663
```

FT13 (ICP)

```
gb|U31781|HPU31781 Human papillomavirus type 23, complete genome.
           Length = 7324

 Score =  214 bits (108), Expect = 2e-53
 Identities = 294/353 (83%), Gaps = 1/353 (0%)
 Strand = Plus / Minus


Query: 6    aatgaatactttccaaaaggatcctctttttggggtttcaggtactgcatcaggacactt 65
             |||||||||||||||||||||||||||||| | | |||||||||||||| ||||| ||
Sbjct: 7014 aatgaatactttccaaaaggatcctctttttgcg-tgtcaggtactgcatctggacattt 6956


Query: 66   agtggcctttgaggctaaatacctatatatatcgtgtactgcattatctggtgtaggtac 125
              |||||||||| || ||||| || ||| | || || |||||||||||||||||||| ||
Sbjct: 6955 tgtggcctttgaagccaaatatctgtatgtgtcatgaactgcattatctggtgtaggaac 6896


Query: 126  aaagcccaattgccaattctctaaaatatcagagttcattgcattgattcgtgctaaaac 185
             ||| || || |||||||||||||||||||| || ||||| ||||| ||| ||| ||| ||
Sbjct: 6895 aaaccctaactgccaattctctaaaatatctgaattcatggcattaatttgtgttaagac 6836


Query: 186  ttcagcctttaaaggtatgctgcacaattataatataaaagacatctgatactcatcaac 245
              || ||||||||||||| |||||||| | || ||||||||| |  || || || |||||
Sbjct: 6835 ctcggcctttaaaggtatcctgcacaactgtagtataaaagaaagttggtattcttcaac 6776


Query: 246  atgtctcatatattctctaatattactagcattataatttactaagctatcctcacttttt 305
             ||||||  || | |||||||| ||| | |||| ||| ||| |||| ||    ||| |  |
Sbjct: 6775 atgtcttgtaaactctctaattttagtggcatcatacttttctaaactgctgtcattggt 6716


Query: 306  aacactgatactaaaattagtattgcgtgtattatctgctacagtaacaaaca 358
             |||||||||||||| || ||||| |||||||||||||||||| |||||||
Sbjct: 6715 aacactgatactaaagtttgtattacgtgtattatctgctacagtcacaaaca 6663
```

APPENDIX III Alignments of HPV sequences amplified from NHS from RTR, ICP and the general population with known HPV types deposited in the Genbank database.

N17A (RTR)

```
gb|U85662|HPU85662 Human papillomavirus strain RTRX9 major capsid protein L1
gene,
           partial cds
           Length = 349

 Score =  416 bits (210), Expect = e-115
 Identities = 210/210 (100%)
 Strand = Plus / Plus


Query: 1   attagataatactagaaatacaaactttagtattgctgtacatcaagagcagaagcaggt 60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1   attagataatactagaaatacaaactttagtattgctgtacatcaagagcagaagcaggt 60


Query: 61  taaagaaatacaaaattatgattctgcaaagtttaatgaatttcaaagacatgttgagga 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 61  taaagaaatacaaaattatgattctgcaaagtttaatgaatttcaaagacatgttgagga 120


Query: 121 atatgaagtgtctctcattttacaattgtgtaaaattccattgaaagctgaggttcttgc 180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 121 atatgaagtgtctctcattttacaattgtgtaaaattccattgaaagctgaggttcttgc 180


Query: 181 acagattaatgcaatgaactctgatatttt 210
           |||||||||||||||||||||||||||||
Sbjct: 181 acagattaatgcaatgaactctgatatttt 210


 gb|U31786|HPU31786 Human papillomavirus type 37, complete genome.
           Length = 7421

 Score =  335 bits (169), Expect = 3e-90
 Identities = 224/240 (93%), Gaps = 2/240 (0%)
 Strand = Plus / Plus


Query: 1    tgttatgccagacattattttgtaagaggaaaaaatgtagttgatgctattcccgatgcc 60
           |||||||||||||||||||||||||||||   ||||||| |||||||||||||||| |
Sbjct: 6481 tgttatgccagacattattttgtaagaggggaaatgtaggtgatgctattcccgatggc 6540


Query: 61   actggtaatcagaaccacaaatattacttacctgccaaatcagacaagcagcagtatctg 120
           |||| ||||||| ||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct: 6541 actgttaatcaggaccacaaatattacttacctgccaaatcagaccagcagcagtatctg 6600


Query: 121  ttacgcaattctacctaaattcctactgttagtggatccttagtaacatctgatgctcag 180
           ||| ||||||||||||  |||| |||||||||||||| ||||||||||||||||||||||
Sbjct: 6601 ttaggcaattctacctattttcccactgttagtggatctttagtaacatctgatgctcag 6660


Query: 181  ctctctaacaggcc--tttggttacgcagagctcaaggtcacaacaatggcattttatgg 238
           |||| |||||||||  ||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6661 ctctttaacaggccttttttggttacgcagagctcaaggtcacaacaatggcattttatgg 6720
```

```
emb|X74468|HPV15 Human papillomavirus type 15 genomic DNA
           Length = 7412

 Score =  349 bits (176), Expect = 3e-94
 Identities = 329/380 (86%)
 Strand = Plus / Plus


Query: 1    aatcagatgtttgttactgttgctgataatacaagaaacactaatttcaccataagtgtt 60
            ||||||||||||| |||||||||| ||||| ||||| || || ||||| || || ||||||
Sbjct: 6711 aatcagatgtttattactgttgcagataacacaaggaatacaaattttactattagtgtt 6770


Query: 61   actcctgatggtagtactataactgaatataatacacaaaatattagagaatttttaagg 120
            ||  ||||||||| | | |||| |||||||||| |||||||||| ||||||||||||||
Sbjct: 6771 acctctgatggtaatgccataaatgaatataattcacaaaatatcagagaatttttaaga 6830


Query: 121  catgtagaagaatatcaattagctattatattacaactatgtaaagtgcctttaaaggct 180
            ||||| |||||||||||| ||| ||||||| || ||| | |||||| | |||||||| |||
Sbjct: 6831 catgtggaagaatatcagttatctattattttgcaattgtgtaaaataccttaaaagct 6890


Query: 181  gaggtgttaacacagataaatgcaatgaatgcaggtattttagaagagtggcaactaagg 240
            ||||| |||||||| || ||||| |||||| |||||||||||||||| ||||||||| ||
Sbjct: 6891 gaggtattaacacaaattaatgctatgaattcaggtattttagaagactggcaactaggg 6950


Query: 241  tttattcccacaccagacaatgctgtgcatgatatttacagatatattgactctaaagct 300
            ||| |||| |||||||||||| ||||| || |||||||| |||||||||||||||||| ||
Sbjct: 6951 tttgttcctacaccagacaacgctgtacaagatatttatagatatattgactctaaggca 7010


Query: 301  actaaatgtcctgatgcagttgctccaaaggacaaagaagacccatttggaaaatataca 360
            |||||||||||||||| ||    |||||||||||||||||||||||||||||| ||||||
Sbjct: 7011 actaaatgtcctgatgctgtacaaccaaaggacaaagaagacccatttggaaagtataca 7070


Query: 361  ttctggaatgtagatctaac 380
            || |||||||||||| ||||
Sbjct: 7071 ttttggaatgtagatttaac 7090
```

FN1 (ICP)

```
emb|AJ223858|HPVAJ3858 human papillomavirus type 24, L1 capsid gene strain
HPV24
           >gi|2894525|emb|AJ223859|HPVAJ3859 human papillomavirus
           type 24, L1 capsid gene strain HPV24
           Length = 496

 Score =  573 bits (289), Expect = e-161
 Identities = 325/337 (96%)
 Strand = Plus / Plus


Query: 1    tgctttagcagatatgtgcgtatataatccagaggaggaaagattggtatggggttgcag 60
            ||||||||||||||||| |||||||||||||| |||||||||||||||||||||||||||
Sbjct: 71   tgctttagcagatatgtccgtatataatccagagaaggaaagattggtatggggttgcag 130


Query: 61   aggagtagaaataggtagacgacaaccattaggtgtttttacaagtggacatccattatt 120
            |||||||||||||||||| ||||||||||||||||   ||||||||||||||||||||
Sbjct: 131  aggagtagaaataggtagaggacaaccattaggtgttgggacaagtggacatccattatt 190
```

```
Query: 121 taacaaagtgaatgacacagaaaaccctgtatcatatacgacacaagcatcgtccacaga 180
           |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct: 191 taacaaagtgaatgacacagaaaaccctgtatcatataggacacaagcatcgtccacaga 250


Query: 181 tgatagacaaattacctcatttgatcctaaacaaattcaaatgtttattataggttgtgc 240
           |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 251 tgatagacaaaatacctcatttgatcctaaacaaattcaaatgtttattataggttgtgc 310


Query: 241 accctgcataggagaacatggggaactagctgagaggtgtgctggtgataatattgatgc 300
           |||||||||||||||||| ||||| ||||||||||||||||||||||||| ||||||
Sbjct: 311 accctgcataggagaacattgggaagtagctgagaggtgtgctggtgataataatgatgc 370


Query: 301 tggtagatgtccacctattaatttggtaaattcagta 337
           |||||||||||||||||||| ||||||||||||||
Sbjct: 371 tggtagatgtccacctattaagttggtaaattcagta 407


emb|X74469|HPV17 Human papillomavirus type 17 genomic DNA
           Length = 7427

 Score =  472 bits (238), Expect = e-131
 Identities = 262/270 (97%)
 Strand = Plus / Plus


Query: 1    agtgcagatgtttgttgtaggctgtgtaccttgtattggagaacatttggacagggctcc 60
           |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct: 6182 agtgcagatgtttgttgtaggctgtgtaccttgtattggagaacattgggacagggctcc 6241


Query: 61   tgtatgtggaaatgaacaaaacaatcaaacaggcctgtgtccaccattggaattaaataa 120
           |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct: 6242 tgtatgtgaaaatgaacaaaacaatcaaacaggcctgtgtccaccattggaattaaaaaa 6301


Query: 121  cactgttatcgaagatggtgacatggttgacataggcttttgaaacattaataacaaagt 180
           |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct: 6302 cactgttatcgaagatggtgacatggttgacataggctttggaaacattaataacaaagt 6361


Query: 181  gctttcatttaataaatcagatgtaagattagatatagttaatgaaacatgcaaatatcc 240
           |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct: 6362 gctttcatttaataaatcagatgtaagtttagatatagttaatgaaacatgcaaatatcc 6421


Query: 241  tgaaattttaagcattgcaaatgatgttta 270
           |||  |||||||||| ||||||||||||||
Sbjct: 6422 tgatttttaagcatggcaaatgatgttta 6451
```

FN5 (ICP)

```
emb|X74463|HPV7 Human papillomavirus type 7 genomic DNA
           Length = 8027

 Score =  307 bits (155), Expect = 7e-82
 Identities = 220/240 (91%), Gaps = 3/240 (1%)
 Strand = Plus / Plus


Query: 1    atacccagattatttaggaatggctgcagaaccgtatggtaatagtttaaaattt---ct 57
            ||||||||||||||||||||||||||||||||||||||||||||||||||   |||   ||
Sbjct: 6481 atacccagattatttaggaatggctgcagaaccgtatggtaatagtttatttttttttct 6540


Query: 58   tagaggggaacaaatgtttgttaggcacccttttttaataggcagaaactactggagacag 117
            |||| | ||||||||||||||||||||||  |||| |   ||||| ||||||||||||||
Sbjct: 6541 tagaagagaacaaatgtttgttaggcacttttttaatagggcaggaactactggagacag 6600


Query: 118  tgttccaaatgatctatatataacaagttcatctaatcgcgcatctattgcaggcagtaa 177
            |||||||||||||| |||||||||| ||||||||||||||| ||||||||||||||||
Sbjct: 6601 tgttccaaatgatttatatataacaggttcatctaatcgcgcttctattgcaggcagtat 6660


Query: 178  ttattattccacaccaagtggctctctagttacctctgtttctcagatttttaataaacc 237
            |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct: 6661 ttattattccacaccaagtggctctctagttacctctgattctcagatttttaataaacc 6720
```

NHS8 (GENERAL POPULATION)

```
gb|U31787|HPU31787 Human papillomavirus type 38, complete genome.
           Length = 7400

 Score =  113 bits (57), Expect = 3e-23
 Identities = 159/193 (82%)
 Strand = Plus / Plus


Query: 181  ctgaggtgctaacccagattaatgctatgaattcagctatattagaaaactggcaactag 240
            |||| ||||| || ||||||||||||||||||| |  |||||||||| |||||| |||
Sbjct: 6844 ctgaagtgctgacacagattaatgctatgaattctggaatattagaaaattggcaattag 6903


Query: 241  gggttgttccaacaccagataatgcagtgcatgacacatatcgttaccttaattcaaaag 300
            |  |||| || || ||||| ||| | || || || |||||||||||| | |  || ||||
Sbjct: 6904 gctttgtacccaccccagacaattctgtacacgatacatatcgttacataacatctaaag 6963


Query: 301  ctacaaaatgtccagatgcagtccaagaaacagaaaaggaagatccttttggtggatatt 360
            | || |||||||||||||||| |  |||||||||| |||||||| ||||||  ||||
Sbjct: 6964 caactaaatgtccagatgcagtgcctgaaacagaaaaagaagatccctttggtcaatata 7023


Query: 361  cattttggaatgt 373
            |||||||||||||
Sbjct: 7024 cattttggaatgt 7036
```

```
gb|U31781|HPU31781 Human papillomavirus type 23, complete genome.
           Length = 7324

 Score =  161 bits (81), Expect = 3e-37
 Identities = 246/301 (81%)
 Strand = Plus / Minus


Query: 3    ggacacttagtggcctttgaggctaaatacctatatatatcgtgtactgcattatctggt 62
            ||||| || |||||||||||| || ||||| || ||| | || || ||||||||||||||
Sbjct: 6963 ggacattttgtggcctttgaagccaaatatctgtatgtgtcatgaactgcattatctggt 6904


Query: 63   gtaggtacaaagcccaattgccaattctctaaaatatcagagttcattgcattgattcgt 122
            ||||| ||||| || || ||||||||||||||||||| || ||||| ||||| ||| ||
Sbjct: 6903 gtaggaacaaaccctaactgccaattctctaaaatatctgaattcatggcattaatttgt 6844


Query: 123  gctaaaacttcagcctttaaaggtatgctgcacaattataatataaaagacatctgatac 182
            | ||| || || ||||||||||||| |||||||| | || ||||||||| |  || ||
Sbjct: 6843 gttaagacctcggcctttaaaggtatcctgcacaactgtagtataaaagaaagttggtat 6784


Query: 183  tcatcaacatgtctcatatattctctaatattactagcattataatttactaagctatcc 242
            || ||||||||||  || | |||||||| ||| | |||| ||| ||| |||| ||
Sbjct: 6783 tcttcaacatgtcttgtaaactctctaattttagtggcatcatacttttctaaactgctg 6724


Query: 243  tcacttttaacactgatactaaaattagtattgcgtgtattatctgctacagtaacaaac 302
            ||| |  |||||||||||||||| || ||||| |||||||||||||||||||| ||||||
Sbjct: 6723 tcattggtaacactgatactaaagtttgtattacgtgtattatctgctacagtcacaaac 6664
```

NHS9 (GENERAL POPULATION)

```
gb|U31781|HPU31781 Human papillomavirus type 23, complete genome.
           Length = 7324

 Score =  513 bits (259), Expect = e-144
 Identities = 292/300 (97%), Gaps = 1/300 (0%)
 Strand = Plus / Minus

Query: 1    ggacattttgtggcctttgaagccaaatatttgtatgtgtcaagaactgcattatgtggt 60
            ||||||||||||||||||||||||||||| |||||||||| |||||||||||| ||||
Sbjct: 6963 ggacattttgtggcctttgaagccaaatatctgtatgtgtcatgaactgcattatctggt 6904


Query: 61   gtaggaacaaaccctaactgtcaattctctaaattatctgaattcatggcattaatttgt 120
            |||||||||||||||||| |||||||||| ||||||||||||||||||||||||||||
Sbjct: 6903 gtaggaacaaaccctaactgccaattctctaaaatatctgaattcatggcattaatttgt 6844


Query: 121  gttaagacctcggcctttaaaagtatcctgcacaactgtagtataaaagaaagttggtat 180
            |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct: 6843 gttaagacctcggcctttaaaggtatcctgcacaactgtagtataaaagaaagttggtat 6784


Query: 181  tcttcaacatgtcttgtaaactctctaaatttagtggcatcatacttttctaaactgctg 240
            ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct: 6783 tcttcaacatgtcttgtaaactctctaattttagtggcatcatacttttctaaactgctg 6724


Query: 241  tcattggtaacactgatactaaagtttgtattaacgtgtattatctgctacagtcacaaa 300
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct: 6723 tcattggtaacactgatactaaagtttgtatt-acgtgtattatctgctacagtcacaaa 6665
```

NHS12 (GENERAL POPULATION)

```
gb|L38388|PPHL1FR Human papillomavirus L1 gene fragment.
          Length = 395

 Score =  351 bits (177), Expect = 1e-94
 Identities = 216/229 (94%)
 Strand = Plus / Minus


Query: 1   ctggtgtaggtacaaagcccaattgccaattctctaaaatatcagagttcattgcattga 60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 229 ctggtgtaggtacaaagcccaattgccaattctctaaaatatcagagttcattgcattga 170


Query: 61  ttcgtgctaaaacttcagcctttaaaggtatgctgcacaattataatataaaagacatct 120
           || ||| |||||| |||||||||||||||| |||||||||| |||||||||||||| ||
Sbjct: 169 tttgtgttaaaacctcagcctttaaaggtattctgcacaattgtaatataaaagacaact 110


Query: 121 gatactcatcaacatgtctcatatattctctaatattactagcattataatttactaagc 180
           ||||||| |||||||||||||| |||||||||||||| |||||||||||||||| ||||||
Sbjct: 109 gatactcctcaacatgtctcatgtattctctaatatttctagcattataatttgctaagc 50


Query: 181 tatcctcacttttaacactgatactaaaattagtattgcgtgtattatc 229
           |||||||| |||||||||||||||||||||||| |||||||| ||||
Sbjct: 49  tatcctcatttttaacactgatactaaaattagtactgcgtgtagtatc 1
```