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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
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.
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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 \textit{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 \textit{Rb} gene) and the D13S118 locus (distal telomERICally to \textit{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 \textit{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 \textit{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 \textit{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.
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>NHS</td>
<td>Normal human skin</td>
</tr>
<tr>
<td>NMSC</td>
<td>Nonmelanoma skin cancer</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen activated protein</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RCSi</td>
<td>Royal College of Surgeons in Ireland</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>RTR</td>
<td>Renal transplant recipient</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SSCP</td>
<td>Single strand conformational polymorphism</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris borate EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>NNN’N’ Tetramethylethlenediamine</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris(hydroxymethyl)aminomethane hydrochloride</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UVA</td>
<td>Ultraviolet A radiation</td>
</tr>
<tr>
<td>UVB</td>
<td>Ultraviolet B radiation</td>
</tr>
<tr>
<td>XP</td>
<td>Xeroderma pigmentosum</td>
</tr>
<tr>
<td>α-P&lt;sup&gt;32&lt;/sup&gt;dATP</td>
<td>P&lt;sup&gt;32&lt;/sup&gt;-labelled dATP</td>
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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 bum tend to develop less skin cancers than those that burn but never tan (Evans et al. 1988). However, the incidence in individuals who never bum 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

<table>
<thead>
<tr>
<th>Low-risk HPV types</th>
<th>High-risk HPV types</th>
</tr>
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<tbody>
<tr>
<td>HPV type</td>
<td>Associated with</td>
</tr>
<tr>
<td>6</td>
<td>Genital mucosa</td>
</tr>
<tr>
<td>11</td>
<td>Genital mucosa</td>
</tr>
<tr>
<td>13</td>
<td>Oral mucosa (Heck’s disease)</td>
</tr>
<tr>
<td>32</td>
<td>Oral mucosa (Heck’s disease)</td>
</tr>
<tr>
<td>34</td>
<td>Anogenital (intrepithelial neoplasia, BP)</td>
</tr>
<tr>
<td>40</td>
<td>Anogenital (intrepithelial neoplasia)</td>
</tr>
<tr>
<td>42</td>
<td>Anogenital (intrepithelial neoplasia, vulvar papilloma)</td>
</tr>
<tr>
<td>43</td>
<td>Anogenital (intrepithelial neoplasia, vulvar hyperplasia)</td>
</tr>
<tr>
<td>44</td>
<td>Anogenital (condylomas)</td>
</tr>
<tr>
<td>53</td>
<td>Anogenital (normal mucosa)</td>
</tr>
<tr>
<td>54</td>
<td>Genital (condylomata acuminata)</td>
</tr>
<tr>
<td>55</td>
<td>Genital (BP)</td>
</tr>
<tr>
<td>57</td>
<td>Anogenital (intrepithelial neoplasia), also found in cutaneous lesions</td>
</tr>
<tr>
<td>59</td>
<td>Anogenital (intrepithelial neoplasia)</td>
</tr>
<tr>
<td>61</td>
<td>Anogenital (intrepithelial neoplasia)</td>
</tr>
<tr>
<td>62</td>
<td>Anogenital (intrepithelial neoplasia)</td>
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<td>64</td>
<td>Anogenital (intrepithelial neoplasia)</td>
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<td>67</td>
<td>Anogenital (intrepithelial neoplasia)</td>
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<td>68</td>
<td>Anogenital (intrepithelial neoplasia)</td>
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<tr>
<td>69</td>
<td>Anogenital (intrepithelial neoplasia)</td>
</tr>
<tr>
<td>70</td>
<td>Anogenital (vulvar papilloma)</td>
</tr>
<tr>
<td>71</td>
<td>AIN</td>
</tr>
<tr>
<td>72</td>
<td>Oral (papillomas in immunosuppressed individuals)</td>
</tr>
<tr>
<td>73</td>
<td>Oral (papillomas in immunosuppressed individuals)</td>
</tr>
<tr>
<td>74</td>
<td>AIN</td>
</tr>
</tbody>
</table>

AIN anal intraepithelial neoplasia, BP Bowenoid papulosis.
<table>
<thead>
<tr>
<th>Cutaneous HPV types</th>
<th>EV-specific HPV types</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV type</td>
<td>Associated with</td>
</tr>
<tr>
<td>1</td>
<td>Myrmecia wart (v. plantaris)</td>
</tr>
<tr>
<td>2</td>
<td>Common wart (v. vulgaris)</td>
</tr>
<tr>
<td>3</td>
<td>Plane wart (v. plana)</td>
</tr>
<tr>
<td>4</td>
<td>Common wart (frequently palmar)</td>
</tr>
<tr>
<td>7</td>
<td>Butcher’s wart (proliferative hand warts)</td>
</tr>
<tr>
<td>10</td>
<td>Plane or intermediate wart (EV- associated)</td>
</tr>
<tr>
<td>26</td>
<td>Plane or intermediate wart (mostly in immunosuppressed patients)</td>
</tr>
<tr>
<td>27</td>
<td>Plane or intermediate wart (mostly in immunosuppressed patients)</td>
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<tr>
<td>28</td>
<td>Plane or intermediate wart</td>
</tr>
<tr>
<td>29</td>
<td>Intermediate or common wart</td>
</tr>
<tr>
<td>34</td>
<td>Bowen’s disease (mainly genital)</td>
</tr>
<tr>
<td>36</td>
<td>Actinic keratosis (mainly EV-HPV)</td>
</tr>
<tr>
<td>37</td>
<td>Keratoacanthoma (single case)</td>
</tr>
<tr>
<td>38</td>
<td>Malignant melanoma (single case) (EV-associated)</td>
</tr>
<tr>
<td>41</td>
<td>Warts, SCC</td>
</tr>
<tr>
<td>48</td>
<td>SCC (in immunosuppressed patients)</td>
</tr>
<tr>
<td>49</td>
<td>Warts, premalignant lesions (in immunosuppressed patients) (EV-associated)</td>
</tr>
<tr>
<td>57</td>
<td>Inverted maxillary papilloma (mainly genital)</td>
</tr>
<tr>
<td>60</td>
<td>Epidermoid plantar cyst</td>
</tr>
<tr>
<td>63</td>
<td>Myrmecia cystic wart</td>
</tr>
<tr>
<td>65</td>
<td>Pigmented wart</td>
</tr>
<tr>
<td>75</td>
<td>Common wart (in immunosuppressed patients)</td>
</tr>
<tr>
<td>76</td>
<td>Common wart (in immunosuppressed patients)</td>
</tr>
<tr>
<td>77</td>
<td>Common wart (in immunosuppressed patients)</td>
</tr>
</tbody>
</table>
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 \textit{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 \textit{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 \textit{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, Sureutherford 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 be 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 G0/G1 phase when E6 is removed, suggesting a role for E6 in regulating the cell-cycle. Subpopulations of E6/EJras transformed cell lines that have acquired spontaneous mutations in p53 do not arrest in G0/G1 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. gadd45, p21 and mdm2 (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^Waf/Cip1 and p27^Kip1 (Funk et al. 1997, Jones et al. 1997). In p21^Waf/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^Waf/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 bcl2 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

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, mdm2. 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 mdm2 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 D1-cdk4-Rb pathway plays a key role in the regulation of the G1-S phase transition. One of these four genes is mutated or altered in nearly all known cancers (Hall and Peters 1996, Sherr 1996). p16\textsuperscript{INK4} is a negative regulator of cyclin D1-cdk4 and is inactivated in many forms of cancer (Levine 1997). Cyclin D1 amplification and overexpression and cdk4 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 D1-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 D1-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\textsuperscript{Waf1/Cip1} (El-Deiry et al. 1993). The net result of p21\textsuperscript{Waf1/Cip1} expression is the hypophosphorylation of Rb, thus preventing the release of the E2F transcription factor complex and cell cycle arrest at the G1 boundary (Agarwal et al. 1998). p21\textsuperscript{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 GADD45, 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 death (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\(^{\text{Waf1/Cip1}}\) 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 also play a role in establishing basal levels of p53. Some of the cellula functions of p53 can be deregulated or compromised by the expression of Myc, Bcl2, or E1B. Control of p53 activity includes the Mdm autoregulatory loop. The intact set of p53-dependent pathways preserve genomic integrity by arresting cells via p21 induction, or by promoting apoptosis via Bax. p53 also helps to regulate entry into mitosis, spindle formation and cell cycle checkpoints that are likely to involve DNA 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 G₁-S boundary. Its gene product is negatively regulated by cell cycle-dependent phosphorylation catalysed by cyclin-dependent kinases (CDKs) in the late G₁ 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 G₁ 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 G₁ 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 G₁ (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\textsubscript{i} progression to cyclin D. This is supported by the fact that p21\textsuperscript{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\textsubscript{i} 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\textsuperscript{INK4a}-induced G\textsubscript{i} 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

Rb, p107 and p130 complex formation with the E2F transcription factors varies according to the phase of the cell cycle. During quiescence (G0) the major E2F species is p130-E2F4, which is thought to repress the activity of the E2F1 and cdc2 genes and maintain a state of non-cycling (Vairo et al. 1995, Johnson 1995, Tommasi and Pfeifer 1995). Rb-E2F complexes are found in mid- to late G1 phase and are maintained into the S and G2 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 (Sellars 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 G₁ arrest.

The interaction of tumour suppressor genes, oncogenes and the response to DNA damage in relation to G₁ arrest or induction of apoptosis suggests an important role for Rb/p107/p130 in p53-mediated regulation of the G₁-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 G₁-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 D1-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$_2$O 10ml

**Luria Bertani (LB) Broth**

- 10g Tryptone
- 5g Yeast extract
- 10g NaCl
- H$_2$O 1000ml

**LB agar**

- 10g Tryptone
- 5g Yeast extract
- 10g NaCl
- 15g Agar
- H$_2$O 1000ml

**Long Ranger gel**

- 6ml 10X TBE
- 6ml Long Ranger gel solution
- 27g Urea
- H$_2$O 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™ 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 dH2O, 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 dH₂O 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 manufacturer'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 70°C 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 analysed 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 faint 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.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>7PA</td>
<td>5’ TCTCCCCAAGGCACGTG 3’</td>
<td>Bennett et al. 1996</td>
</tr>
<tr>
<td>7PS</td>
<td>5’ AAGAGGTGGGCCAGGGG 3’</td>
<td>Bennett et al. 1996</td>
</tr>
<tr>
<td>MY09</td>
<td>5’ CGTCCMARRGGAWACTGATC 3’</td>
<td>Manos et al. 1989</td>
</tr>
<tr>
<td>MY11</td>
<td>5’ GCMCAGGGWCATAAAYAATGGG 3’</td>
<td>Manos et al. 1989</td>
</tr>
<tr>
<td>CP65</td>
<td>5’ CARGGTCAYAAAYATGGYAT 3’</td>
<td>Berkhout et al. 1995</td>
</tr>
<tr>
<td>CP66</td>
<td>5’ AATCARMGTTTACWGT 3’</td>
<td>Berkhout et al. 1995</td>
</tr>
<tr>
<td>CP69</td>
<td>5’ GWTAGATCWACATYCCARAA 3’</td>
<td>Berkhout et al. 1995</td>
</tr>
<tr>
<td>CP70</td>
<td>5’ AAYTTTCGTCCYARAGRAWATTGRTC 3’</td>
<td>Berkhout et al. 1995</td>
</tr>
<tr>
<td>HPV20E6UP</td>
<td>5’ ACTAAGATACCCAGCCACCC 3’</td>
<td>This work</td>
</tr>
<tr>
<td>HPV20E6LOW</td>
<td>5’ TTGGCAGCTTACATTTCTC 3’</td>
<td>This work</td>
</tr>
<tr>
<td>HPV17E6UP</td>
<td>5’ ACCTTGTGATTCCATTAGT 3’</td>
<td>This work</td>
</tr>
<tr>
<td>HPV17E6LOW</td>
<td>5’ CTGTTGTGCTATTTTCTCTA 3’</td>
<td>This work</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Cycling conditions</th>
<th>Product Size</th>
<th>[MgCl₂]</th>
<th>[dNTP]</th>
<th>[Primer]</th>
</tr>
</thead>
<tbody>
<tr>
<td>7PS/7PA</td>
<td>95°C for 2 min, 61°C for 2 min, and 72°C for 2 min, 35 cycles</td>
<td>229bp</td>
<td>1.5 mM</td>
<td>0.25mM</td>
<td>30 pmol/50μl rxn</td>
</tr>
<tr>
<td>MY09/11</td>
<td>95°C for 30s, 55°C for 30s, and 72°C for 1 min, 30 cycles</td>
<td>450bp</td>
<td>1.5mM</td>
<td>0.25mM</td>
<td>30 pmol/50μl rxn</td>
</tr>
<tr>
<td>CP65/70</td>
<td>95°C for 1 min, 50°C for 1.5 min, and 72°C for 2 min, 5 cycles</td>
<td>Approx. 400bp</td>
<td>3.4mM</td>
<td>0.25mM</td>
<td>30 pmol/50μl rxn</td>
</tr>
<tr>
<td></td>
<td>95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, 35 cycles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP66/69</td>
<td>95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, 30 cycles</td>
<td>Approx. 400bp</td>
<td>3.4mM</td>
<td>0.25mM</td>
<td>30 pmol/50μl rxn</td>
</tr>
<tr>
<td>HPV20E6UP/LOW</td>
<td>95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, 30 cycles</td>
<td>183bp</td>
<td>1.5mM</td>
<td>0.25mM</td>
<td>30 pmol/50μl rxn</td>
</tr>
<tr>
<td>HPV17E6UP/LOW</td>
<td>95°C for 1 min, 48°C for 1 min, and 72°C for 2 min, 30 cycles</td>
<td>216bp</td>
<td>1.5mM</td>
<td>0.25mM</td>
<td>30 pmol/50μl rxn</td>
</tr>
</tbody>
</table>
Table 2.3 HPV types detected in RTR and ICP viral warts.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample</th>
<th>Immune Status</th>
<th>MY09/MY11</th>
<th>CP65-70</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>W2A</td>
<td>RTR</td>
<td>HPV2a/HPV57</td>
<td>HPV23-rel</td>
</tr>
<tr>
<td>2</td>
<td>W14A</td>
<td>RTR</td>
<td>HPV57</td>
<td>HPV38-rel</td>
</tr>
<tr>
<td></td>
<td>W14B</td>
<td></td>
<td>HPV10</td>
<td>HPVX14b</td>
</tr>
<tr>
<td>3</td>
<td>W37A</td>
<td>RTR</td>
<td>HPV10</td>
<td>RTRX5/RTRX6</td>
</tr>
<tr>
<td>4</td>
<td>W46A</td>
<td>RTR</td>
<td>HPV17</td>
<td>HPV23-rel</td>
</tr>
<tr>
<td>5</td>
<td>W50A</td>
<td>RTR</td>
<td>HPV10</td>
<td>HPV2a/HPV23-rel/RTRX10</td>
</tr>
<tr>
<td>6</td>
<td>W55A</td>
<td>RTR</td>
<td>nd</td>
<td>HPV23-rel</td>
</tr>
<tr>
<td></td>
<td>W55B</td>
<td></td>
<td>nd</td>
<td>RTRX10</td>
</tr>
<tr>
<td>7</td>
<td>W56A</td>
<td>RTR</td>
<td>-ve</td>
<td>RTRTX10-rel</td>
</tr>
<tr>
<td>8</td>
<td>W57A</td>
<td>RTR</td>
<td>HPV57</td>
<td>HPV23-rel</td>
</tr>
<tr>
<td>9</td>
<td>W58A</td>
<td>RTR</td>
<td>-ve</td>
<td>HPV23-rel</td>
</tr>
<tr>
<td>17</td>
<td>WN1A</td>
<td>ICP</td>
<td>-ve</td>
<td>HPV2a/HPV10-rel</td>
</tr>
<tr>
<td>18</td>
<td>WN2A</td>
<td>ICP</td>
<td>-ve</td>
<td>HPV20</td>
</tr>
<tr>
<td></td>
<td>WN2B</td>
<td></td>
<td>-ve</td>
<td>HPV5-rel/HPV15-rel</td>
</tr>
<tr>
<td>19</td>
<td>WN5A</td>
<td>ICP</td>
<td>-ve</td>
<td>HPV23-rel</td>
</tr>
<tr>
<td></td>
<td>WN5B</td>
<td></td>
<td>-ve</td>
<td>HPV23-rel</td>
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<td></td>
<td>WN5C</td>
<td></td>
<td>-ve</td>
<td>HPV20/HPV38-rel</td>
</tr>
<tr>
<td>20</td>
<td>WN6A</td>
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<td>HPVx2b</td>
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<tr>
<td>21</td>
<td>WN7A</td>
<td>ICP</td>
<td>-ve</td>
<td>HPV23-rel</td>
</tr>
</tbody>
</table>

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.

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

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.

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

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.

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>MY09/MY11</th>
<th>CP65-70</th>
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<tbody>
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<td>-ve</td>
<td>-ve</td>
</tr>
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<td>3</td>
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<td>7</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>8</td>
<td>-ve</td>
<td>HPV38-rel/ HPV23-rel</td>
</tr>
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<td>9</td>
<td>-ve</td>
<td>HPV23</td>
</tr>
<tr>
<td>10</td>
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<td>-ve</td>
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<td>-ve</td>
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<td>-ve</td>
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<td>-ve</td>
</tr>
<tr>
<td>15</td>
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</tr>
<tr>
<td>20</td>
<td>-ve</td>
<td>-ve</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Clinical Diagnosis</th>
<th>Histological Diagnosis</th>
<th>Percentage Positive</th>
<th>Predominant HPV types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinically &quot;common warts and plane warts&quot; (n=19)</td>
<td>Non-invasive squamous lesions</td>
<td>100% (19/19)</td>
<td>EV-associated types and common cutaneous types.</td>
</tr>
<tr>
<td>Normal Human Skin from skin cancer patients (n=9)</td>
<td>Normal Human Skin</td>
<td>33% (3/9)</td>
<td>EV-associated types</td>
</tr>
<tr>
<td>Normal human skin from the general population (n=20)</td>
<td>Normal Human Skin</td>
<td>15% (3/20)</td>
<td>EV-associated types</td>
</tr>
<tr>
<td>Clinically SCC (n=21)</td>
<td>Invasive SCC</td>
<td>86% (18/21)</td>
<td>EV-associated types</td>
</tr>
</tbody>
</table>
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 W2, 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 W2, 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 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. All gels were stained with ethidium bromide and visualised under UV light.
>gb:HPV15 Human papillomavirus type 15 genomic DNA.
Length = 7412

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

F2BT : 2 AATCACT0T0T0TACTAGCAGATAACCAAGGAAATCAAAATTACTATACTATT61
\[\ldots\]
HPV15 : 6711 AACAGAT0T0T0TATCT0TTTGCAGATAACCAAGGAAATCAAAATTACTATTASTATT6770

F2BT : 62 ACCTCTGATGGCAA0CCATAAATT0ATATAATCCACAAGATATCAAAATTATTAAAAGA121
\[\ldots\]
HPV15 : 6771 ACCTCTGATGGCAA0CCATAAATT0ATATAATCCACAAGATATCAAAATTATTAAAAGA6830

F2BT : 122 CATG0GAAAGATATCAATCTCTATCTATGCAATG0T0T0TAAAATACCTTTAAAAAGCT191
\[\ldots\]
HPV15 : 6831 CATG0GAAAGATATCAATCTCTATCTATGCAATG0T0T0TAAAATACCTTTAAAAAGCT6990

F2BT : 182 GAGGATTAAACACAAATTTGCAAATTTGAAATTTGCTATTGTTTCAATTTGAGCTATTGTTTCAATTTGAGCTATTGTTTCAATTTG421
\[\ldots\]
HPV15 : 6991 GAGGATTAAACACAAATTTGCAAATTTGAAATTTGCTATTGTTTCAATTTGAGCTATTGTTTCAATTTG4950

F2BT : 242 TTGTTGC246
\[\ldots\]
HPV15 : 6951 TTGTTGC6957

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\textsubscript{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.

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SSCP sample loading buffer</strong></td>
<td>95% formamide, 20mM EDTA (pH 8.5), 0.1M NaOH, 0.025% bromophenol blue, 0.025% xylene cyanol</td>
</tr>
<tr>
<td><strong>Wash Buffer A</strong></td>
<td>100ml 20x SSC, 10ml 10% SDS, 890ml H₂O</td>
</tr>
<tr>
<td><strong>SSCP gel fixing solution</strong></td>
<td>H₂O /ethanol/acetic acid (89.5/10/0.5 v/v)</td>
</tr>
<tr>
<td><strong>Wash Buffer B</strong></td>
<td>25ml 20X SSC, 10ml 10% SDS, 965ml H₂O</td>
</tr>
<tr>
<td><strong>SSCP gel silver stain</strong></td>
<td>0.2g AgNO₃, 200ml H₂O</td>
</tr>
<tr>
<td><strong>Wash Buffer C</strong></td>
<td>5ml 20X SSC, 10ml SDS, 985ml H₂O</td>
</tr>
<tr>
<td><strong>SSCP gel developer solution</strong></td>
<td>4.5g NaOH, 30mg sodium borohydride, 1.2ml formaldehyde (37%), 300ml H₂O</td>
</tr>
</tbody>
</table>
Tris Buffer 0.05M, pH 7.6

6.1g Tris
40ml 1M HCl
H₂O to 1000ml

Tris Buffered Saline

500ml Tris Buffer
38.25g NaCl
125μl Tween 20
H₂O to 5000ml

Hydrogen Peroxide Solution

9ml Hydrogen peroxide (30% w/v)
300ml H₂O

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 cutaneous squamous cell carcinoma with a known p53 mutation. A normal human skin biopsy taken 24h post-UV irradiation was used as 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) pH 7.2) for 6 min at room temperature. For the detection of Mdm2 the sections were subjected to microwave pre-treatment in 0.01 M 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 was 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 dH2O 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-32dATP, dH2O 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 electrophoresed 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 \( p21^{\text{Waf1/Cip1}} \) 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 \( p21^{\text{Waf1/Cip1}} \) was included and clearly demonstrated the expression of \( p21^{\text{Waf1/Cip1}} \), 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.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target</th>
<th>Working dilution</th>
<th>Antigen retrieval</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO-7</td>
<td>p53</td>
<td>1/160</td>
<td>None</td>
<td>Dako</td>
</tr>
<tr>
<td>P21 (187)</td>
<td>p21</td>
<td>1/25</td>
<td>Pronase</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Mdm2 (SMP7)</td>
<td>Mdm2</td>
<td>1/4000</td>
<td>Microwave</td>
<td>Santa Cruz</td>
</tr>
</tbody>
</table>
Table 3.2 PCR primers used for the amplification of p53 exons 5-8 for SSCP analysis.

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

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

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Cycling conditions</th>
<th>Product Size</th>
<th>[MgCl2]</th>
<th>[dNTP]</th>
<th>[Primer]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5PS/PA</td>
<td>95°C 2 min, 45°C 2 min, 72°C 2 min, 32 cycles.</td>
<td>192bp</td>
<td>1.5mM</td>
<td>200μM</td>
<td>0.6μM</td>
</tr>
<tr>
<td>5*PS/PA</td>
<td>95°C 2 min, 58°C 2 min, 72°C 2 min, 32 cycles.</td>
<td>229bp</td>
<td>1.5mM</td>
<td>200μM</td>
<td>0.6μM</td>
</tr>
<tr>
<td>6PS/PA</td>
<td>95°C 2 min, 47°C 2 min, 72°C 2 min, 32 cycles.</td>
<td>221bp</td>
<td>1mM</td>
<td>200μM</td>
<td>0.6μM</td>
</tr>
<tr>
<td>7PS/PA</td>
<td>95°C 2 min, 61°C 2 min, 72°C 2 min, 32 cycles.</td>
<td>221bp</td>
<td>1.5mM</td>
<td>200μM</td>
<td>0.6μM</td>
</tr>
<tr>
<td>8PS/PA</td>
<td>95°C 2 min, 50°C 2 min, 72°C 2 min, 32 cycles.</td>
<td>254bp</td>
<td>1.5mM</td>
<td>200μM</td>
<td>0.6μM</td>
</tr>
</tbody>
</table>
Table 3.4 p53 expression patterns in viral warts.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lesion</th>
<th>Adjacent</th>
<th>Number of +ve cells</th>
<th>Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>W46A</td>
<td>++</td>
<td>++</td>
<td>&lt;10%</td>
<td>Basal lesion/basal adjacent</td>
</tr>
<tr>
<td>WN1A</td>
<td>-ve</td>
<td>++</td>
<td>nd</td>
<td>Basal adjacent</td>
</tr>
<tr>
<td>W56A</td>
<td>+</td>
<td>+</td>
<td>&lt;10%</td>
<td>Basal lesion/basal adjacent</td>
</tr>
<tr>
<td>W14B</td>
<td>+</td>
<td>+</td>
<td>&lt;10%</td>
<td>Suprabasal lesion/suprabasal adjacent</td>
</tr>
<tr>
<td>W58A</td>
<td>+</td>
<td>+</td>
<td>&lt;10%</td>
<td>Basal lesion/basal adjacent</td>
</tr>
<tr>
<td>W55A</td>
<td>+</td>
<td>+</td>
<td>&lt;10%</td>
<td>Basal lesion/basal adjacent</td>
</tr>
<tr>
<td>WN2A</td>
<td>-ve</td>
<td>-ve</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>WN2B</td>
<td>-ve</td>
<td>-ve</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>WN4A</td>
<td>-ve</td>
<td>-ve</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>W50A</td>
<td>-ve</td>
<td>-ve</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>W37A</td>
<td>-ve</td>
<td>-ve</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>W14A</td>
<td>-ve</td>
<td>-ve</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>W57A</td>
<td>-ve</td>
<td>-ve</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>W55B</td>
<td>-ve</td>
<td>-ve</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>W2A</td>
<td>-ve</td>
<td>-ve</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

+= Weak expression
++= Moderate expression
nd = not determined
Table 3.5 p53 expression patterns in cutaneous squamous cell carcinomas.

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

++ = Moderate expression  
+++ = Strong expression  
nd = not determined
Table 3.6 SSCP analysis of p53 exons 5-8

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lesion</th>
<th>Exon 5</th>
<th>Exon 6</th>
<th>Exon 7</th>
<th>Exon 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2DT</td>
<td>SCC</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>F24AT</td>
<td>SCC</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>F25AT</td>
<td>SCC</td>
<td>MUT</td>
<td>WT</td>
<td>MUT</td>
<td>WT</td>
</tr>
<tr>
<td>F27AT</td>
<td>SCC</td>
<td>WT</td>
<td>WT</td>
<td>MUT</td>
<td>WT</td>
</tr>
<tr>
<td>FT1</td>
<td>SCC</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>FT2</td>
<td>SCC</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>FT3</td>
<td>SCC</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>FT4</td>
<td>SCC</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>FT5</td>
<td>SCC</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>B2</td>
<td>NHS</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>B24</td>
<td>NHS</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>B25</td>
<td>NHS</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
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<td>B28</td>
<td>NHS</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>B30</td>
<td>NHS</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
</tbody>
</table>

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 G1 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 (gadd45 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^{\text{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^{\text{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^{\text{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 \textit{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 \textit{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 \textit{et al.} 1994, Ahmadian \textit{et al.} 1998). In SCCs of the head and neck, LOH is frequently detected at 13q14 (Gupta \textit{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 \textit{Rb} gene and \textit{BRCA2} (a tumour suppressor gene associated with a predisposition to breast cancer) located at 13q12.3 (van den Berg \textit{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 \textit{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 \textit{Rb} gene in cutaneous SCCs. Allelic loss at the D13S153 locus (located in exon 2 of the \textit{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
H₂O to 1000ml

Tris Buffered Saline

500ml Tris Buffer
38.25g NaCl
125µl Tween 20
H₂O to 5000ml

Hydrogen Peroxide Solution

9ml Hydrogen peroxide (30% w/v)
300ml H₂O

Citrate Solution pH 6.0

2.94g Trisodium citrate
1000ml H₂O

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.

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

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

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Cycling conditions</th>
<th>Product Size</th>
<th>[MgCl₂]</th>
<th>[dNTP]</th>
<th>[Primer]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1312R/TG</td>
<td>94°C 1 min, 55°C 1 min, 72°C 1 min, 30 cycles.</td>
<td>187bp-201bp</td>
<td>1.5mM</td>
<td>200μM</td>
<td>1μM</td>
</tr>
<tr>
<td>AFM058xd6a/m</td>
<td>94°C 1 min, 55°C 1 min, 72°C 1 min, 30 cycles.</td>
<td>212bp-236bp</td>
<td>1.5mM</td>
<td>200μM</td>
<td>0.25μM</td>
</tr>
</tbody>
</table>
Table 4.3 Allele ratios for cutaneous SCCs used to calculate LOH at the D13S153 locus.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Allele ratio 1st run</th>
<th>Allele ratio 2nd run</th>
<th>Average allele ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2AT</td>
<td>6.66</td>
<td>1.78</td>
<td>4.22</td>
</tr>
<tr>
<td>F2CT</td>
<td>2.56</td>
<td>1.61</td>
<td>2.09</td>
</tr>
<tr>
<td>F2DT</td>
<td>5.88</td>
<td>4.16</td>
<td>5.02</td>
</tr>
<tr>
<td>F2FT</td>
<td>1.26</td>
<td>1.31</td>
<td>1.28</td>
</tr>
<tr>
<td>F4AT</td>
<td>1.03</td>
<td>1.23</td>
<td>1.13</td>
</tr>
<tr>
<td>F17BT</td>
<td>1.49</td>
<td>1.88</td>
<td>1.69</td>
</tr>
<tr>
<td>F22AT</td>
<td>1.31</td>
<td>1.03</td>
<td>1.17</td>
</tr>
<tr>
<td>F23AT</td>
<td>4.54</td>
<td>9.09</td>
<td>6.81</td>
</tr>
<tr>
<td>F24AT</td>
<td>NI</td>
<td>NI</td>
<td>N/A</td>
</tr>
<tr>
<td>F28AT</td>
<td>1.33</td>
<td>1.75</td>
<td>1.54</td>
</tr>
<tr>
<td>F1AT</td>
<td>NI</td>
<td>NI</td>
<td>N/A</td>
</tr>
<tr>
<td>F10AT</td>
<td>0.63</td>
<td>1.36</td>
<td>1.47</td>
</tr>
<tr>
<td>F3AT</td>
<td>NI</td>
<td>NI</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Table 4.4 Allele ratios for matched normal samples used to calculate LOH at the D13S153 locus.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Allele ratio 1st run</th>
<th>Allele ratio 2nd run</th>
<th>Average allele ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2</td>
<td>1.08</td>
<td>1.08</td>
<td>1.08</td>
</tr>
<tr>
<td>B4</td>
<td>1.11</td>
<td>1.09</td>
<td>1.10</td>
</tr>
<tr>
<td>B17</td>
<td>1.25</td>
<td>1.39</td>
<td>1.32</td>
</tr>
<tr>
<td>B22</td>
<td>1.20</td>
<td>1.56</td>
<td>1.38</td>
</tr>
<tr>
<td>B23</td>
<td>1.07</td>
<td>1.11</td>
<td>1.09</td>
</tr>
<tr>
<td>B24</td>
<td>NI</td>
<td>NI</td>
<td>N/A</td>
</tr>
<tr>
<td>B28</td>
<td>1.18</td>
<td>1.66</td>
<td>1.45</td>
</tr>
<tr>
<td>B1</td>
<td>NI</td>
<td>NI</td>
<td>N/A</td>
</tr>
<tr>
<td>B10</td>
<td>1.12</td>
<td>1.08</td>
<td>1.10</td>
</tr>
<tr>
<td>B3</td>
<td>NI</td>
<td>NI</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Table 4.5 Calculation of LOH at the D13S153 locus.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Average tumour allele ratio</th>
<th>Average normal Allele ratio</th>
<th>Tumour Vs Normal allele ratio</th>
<th>LOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2AT/B2</td>
<td>4.22</td>
<td>1.08</td>
<td>0.25</td>
<td>YES</td>
</tr>
<tr>
<td>F2CT/B2</td>
<td>2.09</td>
<td>1.08</td>
<td>0.51</td>
<td>YES</td>
</tr>
<tr>
<td>F2DT/B2</td>
<td>5.02</td>
<td>1.08</td>
<td>0.21</td>
<td>YES</td>
</tr>
<tr>
<td>F2FT/B2</td>
<td>1.28</td>
<td>1.08</td>
<td>0.84</td>
<td>NO</td>
</tr>
<tr>
<td>F4AT/B4</td>
<td>1.13</td>
<td>1.10</td>
<td>0.97</td>
<td>NO</td>
</tr>
<tr>
<td>F17BT/B17</td>
<td>1.68</td>
<td>1.32</td>
<td>0.78</td>
<td>NO</td>
</tr>
<tr>
<td>F22AT/B22</td>
<td>1.17</td>
<td>1.38</td>
<td>0.85</td>
<td>NO</td>
</tr>
<tr>
<td>F23AT/B23</td>
<td>6.81</td>
<td>1.09</td>
<td>0.16</td>
<td>YES</td>
</tr>
<tr>
<td>F24AT/B24</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>F28AT/B28</td>
<td>1.54</td>
<td>1.45</td>
<td>0.94</td>
<td>NO</td>
</tr>
<tr>
<td>F1AT/B1</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>F10AT/B10</td>
<td>1.47</td>
<td>1.10</td>
<td>0.75</td>
<td>NO</td>
</tr>
<tr>
<td>F3AT/B3</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Table 4.6 Allele ratios for cutaneous SCCs used to calculate LOH at the D13S118 locus.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Allele ratio 1\textsuperscript{st} run</th>
<th>Allele ratio 2\textsuperscript{nd} run</th>
<th>Average allele ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2AT</td>
<td>1.64</td>
<td>1.07</td>
<td>1.05</td>
</tr>
<tr>
<td>F2CT</td>
<td>2.12</td>
<td>1.92</td>
<td>2.02</td>
</tr>
<tr>
<td>F2DT</td>
<td>1.59</td>
<td>1.49</td>
<td>1.54</td>
</tr>
<tr>
<td>F2FT</td>
<td>1.79</td>
<td>1.64</td>
<td>1.72</td>
</tr>
<tr>
<td>F4AT</td>
<td>3.03</td>
<td>3.33</td>
<td>3.18</td>
</tr>
<tr>
<td>F17BT</td>
<td>1.42</td>
<td>1.42</td>
<td>1.42</td>
</tr>
<tr>
<td>F22AT</td>
<td>1.3</td>
<td>1.75</td>
<td>1.53</td>
</tr>
<tr>
<td>F23AT</td>
<td>3.03</td>
<td>2.78</td>
<td>2.91</td>
</tr>
<tr>
<td>F24AT</td>
<td>NI</td>
<td>NI</td>
<td>N/A</td>
</tr>
<tr>
<td>F28AT</td>
<td>1.22</td>
<td>1.33</td>
<td>1.27</td>
</tr>
<tr>
<td>F1AT</td>
<td>NI</td>
<td>NI</td>
<td>N/A</td>
</tr>
<tr>
<td>F10AT</td>
<td>1.04</td>
<td>1.11</td>
<td>1.08</td>
</tr>
<tr>
<td>F3AT</td>
<td>9.09</td>
<td>9.09</td>
<td>9.09</td>
</tr>
</tbody>
</table>
Table 4.7 Allele ratios for matched normal samples used to calculate LOH at the D13S118 locus.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Allele ratio 1st run</th>
<th>Allele ratio 2nd run</th>
<th>Average allele ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2</td>
<td>1.36</td>
<td>1.36</td>
<td>1.36</td>
</tr>
<tr>
<td>B4</td>
<td>1.49</td>
<td>1.63</td>
<td>1.56</td>
</tr>
<tr>
<td>B17</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>B22</td>
<td>1.42</td>
<td>1.38</td>
<td>1.40</td>
</tr>
<tr>
<td>B23</td>
<td>1.49</td>
<td>1.61</td>
<td>1.55</td>
</tr>
<tr>
<td>B24</td>
<td>NI</td>
<td>NI</td>
<td>N/A</td>
</tr>
<tr>
<td>B28</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td>B1</td>
<td>NI</td>
<td>NI</td>
<td>N/A</td>
</tr>
<tr>
<td>B10</td>
<td>1.15</td>
<td>1.10</td>
<td>1.13</td>
</tr>
<tr>
<td>B3</td>
<td>1.02</td>
<td>1.02</td>
<td>1.02</td>
</tr>
</tbody>
</table>
Table 4.8 Calculation of LOH at the D13S118 locus.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Average tumour allele ratio</th>
<th>Average normal Allele ratio</th>
<th>Tumour Vs Normal allele ratio</th>
<th>LOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2AT/B2</td>
<td>1.05</td>
<td>1.36</td>
<td>0.76</td>
<td>NO</td>
</tr>
<tr>
<td>F2CT/B2</td>
<td>2.02</td>
<td>1.36</td>
<td>0.67</td>
<td>YES</td>
</tr>
<tr>
<td>F2DT/B2</td>
<td>1.54</td>
<td>1.36</td>
<td>0.88</td>
<td>NO</td>
</tr>
<tr>
<td>F2FT/B2</td>
<td>1.72</td>
<td>1.36</td>
<td>0.79</td>
<td>NO</td>
</tr>
<tr>
<td>F4AT/B4</td>
<td>3.18</td>
<td>1.56</td>
<td>0.49</td>
<td>YES</td>
</tr>
<tr>
<td>F17BT/B17</td>
<td>1.42</td>
<td>1.00</td>
<td>0.70</td>
<td>YES</td>
</tr>
<tr>
<td>F22AT/B22</td>
<td>1.53</td>
<td>1.40</td>
<td>0.91</td>
<td>NO</td>
</tr>
<tr>
<td>F23AT/B23</td>
<td>2.91</td>
<td>1.55</td>
<td>0.53</td>
<td>YES</td>
</tr>
<tr>
<td>F24AT/B24</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>F28AT/B28</td>
<td>1.27</td>
<td>1.25</td>
<td>0.98</td>
<td>NO</td>
</tr>
<tr>
<td>F1AT/B1</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>F10AT/B10</td>
<td>1.08</td>
<td>1.13</td>
<td>0.96</td>
<td>NO</td>
</tr>
<tr>
<td>F3AT/B3</td>
<td>9.09</td>
<td>1.02</td>
<td>0.11</td>
<td>YES</td>
</tr>
</tbody>
</table>
Table 4.9 LOH in cutaneous SCCs at the D13S153 and D13S118 loci: correlation with Rb immunohistochemistry.

<table>
<thead>
<tr>
<th>SCC</th>
<th>LOH at D13S153</th>
<th>LOH at D13S118</th>
<th>Rb Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2AT</td>
<td>YES</td>
<td>NO</td>
<td>+ve</td>
</tr>
<tr>
<td>F2CT</td>
<td>YES</td>
<td>YES</td>
<td>+ve</td>
</tr>
<tr>
<td>F2DT</td>
<td>YES</td>
<td>NO</td>
<td>-ve</td>
</tr>
<tr>
<td>F2FT</td>
<td>NO</td>
<td>NO</td>
<td>+ve</td>
</tr>
<tr>
<td>F4AT</td>
<td>NO</td>
<td>YES</td>
<td>+ve</td>
</tr>
<tr>
<td>F17BT</td>
<td>NO</td>
<td>YES</td>
<td>+ve</td>
</tr>
<tr>
<td>F22AT</td>
<td>NO</td>
<td>NO</td>
<td>+ve</td>
</tr>
<tr>
<td>F23AT</td>
<td>YES</td>
<td>YES</td>
<td>+ve</td>
</tr>
<tr>
<td>F24AT</td>
<td>NI</td>
<td>NI</td>
<td>+ve</td>
</tr>
<tr>
<td>F28AT</td>
<td>NO</td>
<td>NO</td>
<td>+ve</td>
</tr>
<tr>
<td>F1AT</td>
<td>NI</td>
<td>NI</td>
<td>+ve</td>
</tr>
<tr>
<td>F10AT</td>
<td>NO</td>
<td>NO</td>
<td>nd</td>
</tr>
<tr>
<td>F3AT</td>
<td>NI</td>
<td>YES</td>
<td>+ve</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Rb +ve</th>
<th>Rb -ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCCs</td>
<td>82% (18/22)</td>
<td>18% (4/22)</td>
</tr>
<tr>
<td>Viral warts</td>
<td>86% (12/14)</td>
<td>14% (2/14)</td>
</tr>
</tbody>
</table>
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 & Hayward 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 leu1 and leu2 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 leu1 and leu2 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 p16INK4 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 p16INK4 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 (Surethiran 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.

<table>
<thead>
<tr>
<th>Blood buffer A</th>
<th>Blood buffer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.32 M Sucrose,</td>
<td>0.075 M NaCl,</td>
</tr>
<tr>
<td>10 mM Tris-HCl (pH 7.5),</td>
<td>0.02 M EDTA.</td>
</tr>
<tr>
<td>5 mM MgCl₂, 1% Triton X-100</td>
<td></td>
</tr>
</tbody>
</table>

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 dH2O.

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.

\( \chi^2 \) 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 ($\chi^2 = 0.49$, 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.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53Pro+</td>
<td>5' GCC AGA GGC TGC TCC CCC 3'</td>
<td>Storey et al. 1998</td>
</tr>
<tr>
<td>p53-</td>
<td>5' CGT GCA AGT CAC AGA CTT 3'</td>
<td>Storey et al. 1998</td>
</tr>
<tr>
<td>p53+</td>
<td>5' TCC CCC TTG CCG TCC CAA 3'</td>
<td>Storey et al. 1998</td>
</tr>
<tr>
<td>p53Arg-</td>
<td>5' CTG GTG CAG GGG CCA CGC 3'</td>
<td>Storey et al. 1998</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Cycling conditions</th>
<th>Product Size</th>
<th>[MgCl2]</th>
<th>[dNTP]</th>
<th>[Primer]</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53Pro+/p53-</td>
<td>94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, 32 cycles</td>
<td>177bp</td>
<td>1.5mM</td>
<td>200 nM</td>
<td>40ng per 50μl rxn.</td>
</tr>
<tr>
<td>p53+/p53Arg-</td>
<td>94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, 32 cycles</td>
<td>141bp</td>
<td>1.5mM</td>
<td>200nM</td>
<td>40ng per 50μl rxn.</td>
</tr>
</tbody>
</table>
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\(^{\text{Waf1/Cip1}}\) 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 p21Waf1/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


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.


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*


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.


Thermosequenase cycle sequencing kit, [α-32P]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.


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.


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.

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°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™ miniprep kit, T4 polynucleotide kinase, Wizard™ 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.

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


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.
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

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

```plaintext
Query: 1  ctttggggtgtggtgtgctgcaacaaatatcataaatagactggaactggagacactgaaatg 60
Sbjct: 6121 ctttggggtgtggtgtgctgcaacaaatatcataaatagactggaactggagacactgaaatg 6180

Query: 61  cacacacacctgatacagctgatgagccagaaaaaacttcatgtgattataaaacaca 120
Sbjct: 6181 cacacacacctgatacagctgatgagccagaaaaaacttcatgtgattataaaacaca 6240

Query: 121  cacagctgcttcctcctgctgcaaccccctattgtgagcactgtgctaaaggtacca 180
Sbjct: 6241 cacagctgcttcctcctgctgcaaccccctattgtgagcactgtgctaaaggtacca 6300

Query: 181  cctgtaatggtgcttcctgctgctagtgaccctggccctttgtaaaggtacca 240
Sbjct: 6301 cctgtaatggtgcttcctgctgctagtgaccctggccctttgtaaaggtacca 6360

Query: 241  ttagaggacgggaatagttgtaaaccaggttctctgctgctgactgtaaaggtacca 300
Sbjct: 6361 ttagaggacgggaatagttgtaaaccaggttctctgctgctgactgtaaaggtacca 6420

emb|X55965|PAHPV57 Human papillomavirus type 57 complete DNA

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

```plaintext
Query: 1  ccctgcagtttacaaacaccactaatgaagatgggaatatggttgaaaccgggttcgggg 60
Sbjct: 6301 ccctgcagtttacaaacaccactaatgaagatgggaatatggttgaaaccgggttcgggg 6360

Query: 61  cgctggatattttgccctctacagttcaacaaatcagatgtagtagtggtggttaacacttcgagt 120
Sbjct: 6361 cgctggatattttgccctctacagttcaacaaatcagatgtagtagtggtggttaacacttcgagt 6420

Query: 121  acatatgtaaatatccagactatctgaagatggctgcagaaccttatggcgattctatgt 180
Sbjct: 6421 acatatgtaaatatccagactatctgaagatggctgcagaaccttatggcgattctatgt 6480

Query: 181  tctttccccctgacaggttaaaatggttttctcattttctaatcggggttggtcga 240
Sbjct: 6481 tctttccccctgacaggttaaaatggttttctcattttctaatcggggttggtcga 6540
```
Query: 241  tgggtgacgccctcccggatgagctatatgtcaagagttctaccgtccagacccccggta 300

Sbjct: 6541 tgggtgacgccctcccggatgagctatatgtcaagagttctaccgtccagacccccggta 6600

Query: 301  gttatgttttatctccactcccagtggtctctatgtatccctctgaacacgagttttatta 360

Sbjct: 6601 gttatgttttatctccactcccagtggtctctatgtatccctctgaacacgagttttatta 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  caatcagctgtttgttatgtagctaatacactaataatgtcttttagatcagtg 60
sbjct: 6655 caaccaagatgttgctgtcagtaatacactaataatgtcttttagatcagtg 6714
query: 61  taaatgtgaggataagtttagaataatggtagatatatagagaaatatagaga 120
sbjct: 6715 taccactacagcattttgaaatattatgatgcatcaatataaattgagttttaaagcagaca 6774
query: 121  tgttgagagatctagttctttttattacatatgtcagacatcttttaaacgctga 180
sbjct: 6775 tgttgagagatctacagttctttttattacatatgtcagacatcttttaaacgctga 6834
query: 181  agtttttaacacgaatcaatgcaatctcttattttataagagttgacatgtttttgt 240
sbjct: 6835 gttttaataccaaattatgctcagtagatatatttagagtcagttttgt 6894
query: 241  tgtacctacaccaataatgcagtagtacagatatatagatagatatttagcctcaagccgc 300
sbjct: 6895 tgtttctacaccagataatgcagtagtacagatatatagatagatatttagcctcaagccgc 6954
query: 301  taagtgtcctgtagcagtagctactgaaccccaaaagaggttcctttttaaagatattcatt 360
sbjct: 6955 aaataattgcagcagtagctactgaaccccaaaagaggttcctttttaaagatattcatt 7014
query: 361  ctgggatgtgtatctcagc 380
sbjct: 7015 tttggaagatgtatctcagc 7034
**W14A (RTR)**

emb|X55965|PAHV57 Human papillomavirus type 57 complete DNA

Length = 7861

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

Query: 1
```
ccctgcagtttacaaacaccactaatgaagatgggaatatggttgaaacccggttcggg
```
Sbjct: 6301
```
ccctgcagtttacaaatccactattgaagatgggatatgtttgaaaccgggttcggg
```

Query: 61
```
cgctggatttttgcccgtctacagtccaaacaatcagatgctcccttttgatatctgtacta
```
Sbjct: 6361
```
cgctggatttttgcccgtctacagtccaaacaatcagatgtcccttttgatatctgtacta
```

Query: 121
```
acatatgtaaatattcagcactatctgtaagatggtgcagaaacaccttatgccgatctgatgt
```
Sbjct: 6421
```
acatatgtaaatattcagcactatctgtaagatggtgcagaaacaccttatgccgatctgatgt
```

Query: 181
```
tctttccccctgcagcaagaaacatatgttcactcgcctttttcaactccgggttggtctga
```
Sbjct: 6481
```
tctttccccctgcagcaagaaacatatgttcactcgcctttttcaactccgggttggtctga
```

Query: 241
```
tgggtgagcccctccccggatgagctatatgtcaagagttctacgcgtccagaccccggtta
```
Sbjct: 6541
```
tgggtgagcccctccccggatgagctatatgtcaagagttctacgcgtccagaccccggtta
```

Query: 301
```
gttatgttttatataccctacctcccgagtgtcttatgtatctccctgaacacagcagttatata
```
Sbjct: 6601
```
gttatgttttatataccctacctcccgagtgtcttatgtatctccctgaacacagcagttatata
```

>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
```
atgcagtgcagcccachacatatcgtttacttaattccaaagctaacaatatgtccagatgcag
```

sbjct: 149
```
atgcagtgcagcccachacatatcgtttacttaattccaaagctaacaatatgtccagatgcag
```

query: 82
```
tccagaaacagaaaggaagatctcccttttggtggtatctttttgggatgttgga
```

sbjct: 209
```
tccagaaacagaaaggaagatctcccttttggtggtatctttttgggatgttgga
```

147
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 aatggtatatgcctgggcctaa--atcttttacttgtgtagacacgactgcagttacc 128
Sbjct: 6780 aatggtatatgcctgggcctaaatattgtttgtttactgtgtagacacgactgcagttacc 6839

Query: 129 aatatgtgctttgttgtgcttccttgagcctcctgccccctgccccactacatagtagcgcaccaaaa 188
Sbjct: 6840 aatatgtgctttgttgtgcttccttgagcctcctgccccctgccccactacatagtagcgcaccaaaa 6899

Query: 189 tttaaagaatatttgagggacgagaggaatattgtgcattttttcagtttgtttgtg 248
Sbjct: 6900 tttaaagaatatttgagggacgagaggaatattgtgcattttttcagtttgtttgtg 6959

Query: 249 aaggtacattgaccccccgtgatatagttgaccacccatgaaatagataggattttttg 308
Sbjct: 6960 aaggtacattgaccccccgtgatatagttgaccacccatgaaatagataggattttttg 7019

Query: 309 gaggattggaaacttttgggtttaaccttggccacccgctagcttggagggacacatag 368
Sbjct: 7020 gaggattggaaacttttgggtttaaccttggccacccgctagcttggagggacacatag 7079

Query: 369 ttctttgtcttcctctctcagccatattctgtccagaaagataacacccccccacccgagagcaggtg 428
Sbjct: 7080 ttctttgtcttcctctctcagccatattctgtccagaaagataacacccccccacccgagagcaggtg 7139

Query: 429 ycctatgcacaaacctattttttggagcttagatccttaaaggatatggttttttccttgacctg 488
Sbjct: 7140 ccctatgcacaaacctattttttggagcttagatccttaaaggatatggttttttccttgacctg 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 ataa tgtgtacaa aatgtgcagagaactatagagagattactactgcaccacaattttagagaa 112
sbjct: 1 ataa tgtgtacaa aatgtgcagagaactatagagagattactactgcaccacaattttagagaa 60

query: 113 tatttgagacagtgttgagagaattatcactactagtgatagaaattttaggctt 172
sbjct: 61 tatttgagacagtgttgagagaattatcactactagtgatagaaattttaggctt 120

query: 173 cttaaatcgcagagtttcactttctcagattaaagtgaatattctgttatattcaagttaaagagatctt 232
sbjct: 121 cttaaatcgcagagtttcactttctcagattaaagtgaatattctgttatattcaagttaaagagatctt 180

query: 233 cagtttagggctttggctctactccagaaaattgcgtctgtgatagatacatacatatcatatatq 292
sbjct: 181 cagtttagggctttggctctactccagaaaattgcgtctgtgatagatacatacatatcatatatq 240

query: 293 tccaagaatccaaatgtcagatgaa 319
sbjct: 241 tccaagaatccaaatgtcagatgaa 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 aatggtatatgtcctgggctacaaatgtgttactataggtgagacacgactcgcagtgacc 134
Sbjct: 6780 aatggtatatgtcctgggctacaaatgtgttactataggtgagacacgactcgcagtgacc 6839

Query: 135 aatagtgtctgttgtgttctctctcgagcctcccctgccccactacgtcagcacgccaccaaaa 194
Sbjct: 6840 aatagtgtctgttgtgttctctctcgagcctcccctgccccactacgtcagcacgccaccaaaa 6999

Query: 195 tttaaggaatatgctgggcccagggagaggtaatatgtgtgttgctcctatttttcaggttgt 254
Sbjct: 6900 tttaaggaatatgctgggcccagggagaggtaatatgtgtgttgctcctatttttcaggttgt 6959

Query: 255 aaggtacatgtgaccacccggagatattatggtgcaacaccatgaataatagttatttattg 314
Sbjct: 6965 aaggtacatgtgaccacccggagatattatggtgcaacaccatgaataatagttatttattg 7019

Query: 315 gaggattggaaactttggtgattgtatactttgctcctggctcactagcctttgaggacacatatag 374
Sbjct: 7020 gaggattggaaactttggtgattgtatactttgctcctggctcactagcctttgaggacacatatag 7079
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  acagtagctatcaaqgtcagtctactgtatgatagagaaaaacagtctcttgtagtctaaac 60
Sbjct: 6121 acagtagctatcaaqgtgtagtctactgtatgatagagaaaaacagtctcttgaccctaaac 6180

Query: 61 aagtgcagatgtttgttgtaggctgtgtaccttgtattggagaacatttgacaggggctc 120
Sbjct: 6181 aagtgcagatgtttgttgtaggctgtgtaccttgtattggagaacatttgacaggggctc 6240

Query: 121 ctgtatgtgaaaatgaacaaaacaatcaaacaggcctgtgtccaccattggaattaaaata 180
Sbjct: 6241 ctgtatgtgaaaatgaacaaaacaatcaaacaggcctgtgtccaccattggaattaaaata 6300

Query: 181 acactgttatcgaagatggtgacatggttgacataggctttgaaacattaataacaaag 240
Sbjct: 6301 acactgttatcgaagatggtgacatggttgacataggctttggaaacattaataacaaag 6360

Query: 241 tgctttctattaataaatcagatgtaagtttagatatagttaatgaaacatgcaaatatc 300
Sbjct: 6361 tgctttctattaataaatcagatgtaagtttagatatagttaatgaaacatgcaaatatc 6420

Query: 301 ctgattttttaagcatggcaaatgatgttta 331
Sbjct: 6421 ctgattttttaagcatggcaaatgatgttta 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 caatcagctatcaaqgtcagtctactgtatgatagagaaaaacagtctcttgtagtctaaac 60
sbjct: 6655 caatcagctatcaaqgtcagtctactgtatgatagagaaaaacagtctcttgtagtctaaac 6714

query: 319 taaaagtgagatagtctactggaatttataattgatagagaaaattagagaaattagagaca 260
sbjct: 6715 taaaagtgagatagtctactggaatttataattgatagagaaaattagagaaattagagaca 6774

query: 259 tgttgaagatcttagttctttatatattcaaatgtgcagaaaaatactttagtctgaaagctga 200
sbjct: 6775 tgttgaagatcttagttctttatatattcaaatgtgcagaaaaatactttagtctgaaagctga 6834

query: 199 agttttaaacacagatactgaactctgtatatttgagaaatttgccaatgggctt 140
sbjct: 6835 agttttaaacacagatactgaactctgtatatttgagaaatttgccaatgggctt 6894
etnbIX55964#HPV2A 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  ctttggttggtgtgctttgtgctttgctagccttagcatattacaaatagctgtgagcactgaaatgt 60  
Sbjct: 6121  ctttggttggtgtgctttgtgctttgctagccttagcatattacaaatagctgtgagcactgaaatgt 6180

Query: 61 cacacacacctgtatacagctgtatgtagcaggaagggtaatttctatgattattaaacacag 120  
Sbjct: 6181 cacacacacctgtatacagctgtatgtagcaggaagggtaatttctatgattattaaacacag 6240

Query: 121 cacacacacctgtatacagctgtatgtagcaggaagggtaatttctatgattattaaacacag 180  
Sbjct: 6241 cacacacacctgtatacagctgtatgtagcaggaagggtaatttctatgattattaaacacag 6300

Query: 181 cctgtaatgggtctttgctgctggtgactgccc 214  
Sbjct: 6301 cctgtaatgggtctttgctgctggtgactgccc 6334

gbIU31781|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 acatccccagaatgaatactttccaaaaggatcctctttttggggtttcaggtactgcatc 140  
Sbjct: 7023 acatccccagaatgaatactttccaaaaggatcctctttttggggtttcaggtactgcatc 6965

Query: 141 aggacacatctatggcgcttttgaggctaaatacctatatatatctgtgactgtatatctgg 200  
Sbjct: 6964 tggacacatctatggcgcttttgaggctaaatacctatatatatctgtgactgtatatctgg 6905

Query: 201 tgtaggtacaagcccatactctctctctataatcagagttcagactgtatatctgg 260  
Sbjct: 6904 tgtaggtacaagcccatactctctctctataatcagagttcagactgtatatctgg 6845

Query: 261 tgtcaaaaacttcacgcttttaaaaggtatgctgcacaattataaatataaacaagcactgata 320  
Sbjct: 6844 tgtcaaaaacttcacgcttttaaaaggtatgctgcacaattataaatataaacaagcactgata 6785

Query: 321 ttcatacatatgctctctcatatatctctcaaatattactgcattataaatattactaagctatc 380  
Sbjct: 6784 ttcatacatatgctctctcatatatctctcaaatattactgcattataaatattactaagctatc 6725

Query: 381 ttcatacatatgctctctcatatatctctcaaatattactgcattataaatattactaagctatc 440  
Sbjct: 6724 ttcatacatatgctctctcatatatctctcaaatattactgcattataaatattactaagctatc 6665

Query: 441 ca 442  
Sbjct: 6664 ca 6663
Human papillomavirus strain RTRX10 major capsid protein L1 gene, partial cds

Score = 335 bits (169), Expect = 3e-90
Identities = 187/193 (96%) 
Strand = Plus / Plus

Query: 1 ggctgataacacagcttaacactaattttactattattagtggtgccagtgatagtagcacagt 60
Sbjct: 1 ggctgataacacagcttaacactaattttactattattagtggtgccagtgatagtagcacagt 60

Query: 61 gaattatgatgctggaaaatcagagaatacatgcgtcatgttgaagaatatcagctatc 120
Sbjct: 61 gaattatgatgctggaaaatcagagaatacatgcgtcatgttgaagaatatcagctatc 120

Query: 121 atttatatttacaattatgtagaatacctttagaggcagaagtgttaacacagcttaatgc 180
Sbjct: 121 atttatatttacaattatgtagaatacctttagaggcagaagtgttaacacagcttaatgc 180

Query: 181 tatgaatcatggg 193
Sbjct: 181 tatgaatcatggg 193

W55A (RTR)

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

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 caatcagctgtttgttactgtagcagataatacacgcaatactaattttagtatcagtgt 6714

query: 61 taaatgtgaggatagcttagcaaattataatgctagtaatattagagaatatatgagaca 120
sbjct: 6715 taaatgtgaggatagcttagcaaattataatgctagtaatattagagaatatatgagaca 6774

query: 121 tgttgaagagtatcagttgtcttttatattacaattatgctactatatagagagtttacaagaca 180
sbjct: 6775 tgttgaagagtatcagttgtcttttatattacaattatgctactatatagagagtttacaagaca 6834

query: 181 agttttaacacgaatcaatgcaatgaactctgatattttagagaattggcagttagggtt 240
sbjct: 6835 agttttaacacgaatcaatgcaatgaactctgatattttagagaattggcagttagggtt 6994

query: 241 tgtaccaatcaccacaaatatgctcagtacaccatatataaggctc'aagggctc 300
sbjct: 6995 tgtaccaatcaccacaaatatgctcagtacaccatatataaggctc'aagggctc 6954

query: 301 taaggctcctgatcgacatccttgaaacccaaaagagaggtcttttggagaagtttacatcatt 360
sbjct: 6955 taaggctcctgatcgacatccttgaaacccaaaagagaggtcttttggagaagtttacatcatt 7014
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: 361 ctgggatgttgatctaacgg 380

sbject: 7015 ttggaatgttgatatgacag 7034

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 gcagataataacgcaatacatttaaaaaatgtcaggttcgggtaaaggttttaatgtcaggtgcagca083

sbject: 2 gctgataacacgcaatacatttaaaaaatgtcaggttcgggtaaaggttttaatgtcaggtgcagca61

query: 84 aattataatgctagtaatattagagaatatatgagacatgttgaagagtatcagttgtct

sbject: 62 aattatagctgcagagatatacgtacgtatggttgaagatatacgtttatacgtatca121

query: 143 tttaatattacaattgtgcagagatatacctttaaaggctgaagttttaacacgaatcaatgcv

sbject: 122 tttaattttcaattatgtgtaatctttccagggcgagatattcagtttttatgtatcagttatc181

query: 204 tgaactctgtaawwtttagagaaattgcaattgggctytgtamccacaccmgataawgca

sbject: 182 atgaatcatggttttagagaaattgcaattggggtttgtacctacaccagacaatgtc241

query: 264 stacacgctatatagcatggggcttatctttaaaggcataatttcatcttccaaag

sbject: 242 gtgcacgatacctacaggtgtatatcttccaaag275

155
Query: 251 actcttcaacatgtct 266
Sbjct: 6785 actcttcaacatgtct 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 caatcagctttttagttcactgtagacgataaatcgcacacactaatatttagatcagttcgtg 60
Sbjct: 6655 caacccagatgttttgtgactctgacgagataatcgcacacacactaatatttagatcagttcgtg 6714

query: 61 taaaagttaggattatgtccttaataattataatgctgattgtaatatttagatgatcagttagagcaca 120
Sbjct: 6715 taccaatgacagagttttagaaagatagatgacgcccactaaaaattagagatgttataaagcaca 6774

query: 121 tgttgaagattatctgcttcctttttatatctacaatagtgacgacatctctttaaaggctg 180
Sbjct: 6775 tgttgaagattatcacaactctttttatatctacgttgcagcagattaccccttaaaggcaga 6834

query: 181 agttttt 186
Sbjct: 6835 ggtcctt 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 agatctacatccccaaaaggtcagggagcatagggatcgggttagggttaggtgtaa 112
Sbjct: 7123 agatccacatccccaaaaggtcagggagcatagggatacggttagggtttaggtgtaa 7064

Query: 113 ggttttttgacatgtaatagccccgtggagctcagttacatccatatctatgctctcttaa 166
Sbjct: 7063 ggttttttgacatgtaatagccccgtggagctcagttacatccatatctatgctctcttaa 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  gctagtggacggaggcaagttttaaccacaaagttccccatcctctcaaataggctactattcat 221
Sbjct: 7061 gctagtggacggaggcaagttttaaccacaaagttccccatcctctcaaataggctactattcat 7002

Query: 222  ggtgtgcaaatagggccataaatccgggttaacgttcctttacacactgaaatgaa 281
Sbjct: 7001 ggtgtgcaaatagggccataaatccgggttaacgttcctttacacactgaaatgaa 6942

Query: 282  ctgcaaatcataatctccctggtctgatcctcaaatatttttacctttctatggctgctcagttc 341
Sbjct: 6941 ctgcaaatcataatctccctggtctgatcctcaaatatttttacctttctatggctgctcagttc 6882

Query: 342  agtgccagggggccctcagagggadcaacacccaacataatagctgctgctgctgctc 401
Sbjct: 6881 agtgccagggggccctcagagggadcaacacccaacataatagctgctgctgctc 6822

Query: 402  taccactgtataacaca 418
Sbjct: 6821 taccactgtataacaca 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  atcaactgtttttacttagtagatagtactctgaatatctaatatttagctaatctccttc 105
Sbjct: 6908 atcaactgtttttacttagtagatagtactctgaatatctaatatttagctaatctccttc 6967

Query: 106  attcgaaacacactgtatggctttcataattctccaatagttattgtctcgaggatt 165
Sbjct: 6968 attcgaaacacactgtatggctttcataattctccaatagttattgtctcgaggatt 7027

Query: 166  atttagacacgctagaaggggctttacataattcctaaatggctctgccagttc 225
Sbjct: 7028 atttagacacgctagaaggggctttacataattcctaaatggctctgccagttc 7087

Query: 226  taaccagtgaaggttttagctcaaaaatattgtatgctgtaaatctaatattagaggtgagctgc 285
Sbjct: 7088 taaccagtgaaggttttagctcaaaaatattgtatgctgtaaatctaatattagaggtgagctgc 7147
null
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
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 caatcagctgttgctacacatgtacagataaatcagcaataactaattttagatacatcgtgt 60
Sbjct: 6655 caacccagatgttgtaacacctgtaatacagataaatcagcaataactaattttagatacatcgtgt 6714

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 atatcagttcattcagaaaacactgatgtttctaaatcagataatttagattctcagaaa 60
Sbjct: 6958 atatcagttcattcagaaaacactgatgtttctaaatcagataatttagattctcagaaa 7017

Query: 61 tttcagaaatatttagaacaagctgtaaaatgtctatcattattttacaggtctgt 120
Sbjct: 7018 tttcagaaatatttagaacaagctgtaaaatgtctatcattattttacaggtctgt 7077

Query: 121 aaagttcctttaacagctgaagttttagctcaaattaatgctatgaattcaaatatatta 180
Sbjct: 7078 aaagttcctttaacagctgtaaaatgtctatcattattttacaggtctgt 7137

Query: 181 gaggttgccagtaggtgagtttgtacctgcacccgataatcctcattcacgcgatacatacaga 240
Sbjct: 7138 gaggttgccagtaggtgagtttgtacctgcacccgataatcctcattcacgcgatacatacaga 7197

Query: 241 tatattaattctgcaacgtactagtgtctctgacaaaaatcc 281
Sbjct: 7198 tatattaattctgcaacgtactagtgtctctgacaaaaatcc 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 gctgaggtgctaatccagattaatgctatgaatcagtatatggaaaactggcaacta 72
Sbjct: 6843 gctgtaagtgtgctagacacagattaatgctatgaacttcgtgaaatättgaggaaattgcaatta 6902

Query: 73 gggttttgatttcacaccagataatgcatgctacatcacctcattttactcataattcaca 132
Sbjct: 6903 ggttttgattccacacccacagacatcctcgatccagatalcctcattacataaacatctaa 6962

Query: 133 gctacaatgattgcagttcacaattattcgcacatggcaaaactgcagtttccattgcattatat 192
Sbjct: 6963 gcaactaatttgccagatgtaaaatgctggaaacacgaaaaagaaagatcccttttggtaaat 7022

Query: 193 tcaattttggaatgt 206
Sbjct: 7023 acattttggaatgt 7036

WN6A (ICP)

gb|AP097700|AP097700 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 agaatatgaagtatcacttattctctacgtcatggtgctttaaatattctacataaaagctgaggtgct 63
Sbjct: 87 agaatatgaagtatcacttattctctacgtcatggtgctttaaatattctacataaaagctgaggtgct 146

Query: 64 agcacagattaatgcaactttcatgctacatggtgtgaatgggtagtttggtagtcttacc 123
Sbjct: 147 agcacagattaatgcaactttcatgctacatggtgtgaatgggtagtttggtagtcttacc 206

Query: 124 tacaccagataactccctactccagacacacataagatagatgctggtctccgctg 183
Sbjct: 207 tacaccagataactccctactccagacacacataagatagatgctggtctccgctg 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 cagctgtttgttacagtagcagataaatacgcagcaaatcaatctatttttagatcgtttaaa

sbjct: 6659 cagatgtttgttacagtagcagataaatacgcagcaaatcaatctatttttagatcgttacc

query: 63 agtgaggatagcttagcaaattataatgctagtaatattagagaatatatgagacatgtt

sbjct: 6719 aatgcacgcagtttagaaagtatgatgcacactaaattagaggtttacaagacatgtt

query: 123 gaagagttacagtgtgtcttttatattacaaattgtgcaagtaatccctttaaggctgaagtt

sbjct: 6779 gaagagttacagtgtgtcttttatattacaaattgtgcaagtaatccctttaaggctgaagtt

query: 183 ttaacacgaatcaatgcaatgaactctgatattttagagaattggcagttagggtttgtt

sbjct: 6839 ttaacacgaatcaatgcaatgaactctgatattttagagaattggcagttagggtttgtt

query: 243 cctacaccagataatgcaactacgcagataatatgtgattatctcaagttgcaactaag

sbjct: 6899 cctacaccagataatgcaactacgcagataatatgtgattatctcaagttgcaactaag

query: 303 tgtccctgatgcaatgctctggaaccccaaaaaagggactcttttggaaaaagatattctcgg

sbjct: 6959 tgtccctgatgcaatgctctggaaccccaaaaaagggactcttttggaaaaagatattctcgg

query: 363 gatggtgatat 373

sbjct: 7019 aatggtgatat 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

| Query | 62 aatcagctttgtactgtagcagataacacaaggaatacaaatatttactattagtt |
|       | 61 |
| Sbjct | 6711 aatcagatgtttattactgttgcaagataacacaaggaatacaaatatttactattagtt |

| Query | 122 acctctgtggttaaatgcccataatgaaatataatccacaagatattcaaggaatatttttaaga |
|       | 121 |
| Sbjct | 6771 acctctgtggttaaatgcccataatgaaatataatccacaagatattcaaggaatatttttaaga |

| Query | 182 catgtggaagaatatcagttatatattttgaatattgcataatcaggg |
|       | 181 |
| Sbjct | 6831 catgtggaagaatatcagttatatattttgaatattgcataatcaggg |

| Query | 242 tttgttc |
|       | 241 |
| Sbjct | 6951 tttgttc |

**F2CT (RTR)**

gb|AP003892|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 ggagcgccaggacacagactatggcccaacaatttggaatattgggaacactctatatatagaga |
|       | 60 |
| Sbjct | 304 ggagcgccaggacacagactatggcccaacaatttggaatattgggaacactctatatatagaga |

| Query | 61 tggcgacatgccagatataggtttggaaattttaaacttccaaa |
|       | 103 |
| Sbjct | 364 tggcgacatgccagatataggtttggaaattttaaacttccaaa |
gb|AP003892|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  ggagcgccaggacacagacactatgcgcccaacaattgaatgtgaaactacctatataagaaga 60
Sbjct: 304  ggagcgccaggacacagacactatgcgcccaacaattgaatgtgaaactacctatataagaaga 363

Query: 61  tggcgacatggccagatataagg-tttggaatattaactctcaaaaa 103
Sbjct: 364  tggcgacatggccagatataagg-tttggaatattaactctcaaaaa 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  acagtagctatcaaaggtcgatctactgtagatagacaaaacacgtcacttgatctaacaac 60
Sbjct: 6121  acagtagctatcaaaggtcgatctactgtagatagacaaaacacgtcacttgatctaacaac 6180

Query: 61  aagtgcagatgtttgttgtaggctgtgtaccttgtattggagaacatttggacagggctc 120
Sbjct: 6181  aagtgcagatgtttgttgtaggctgtgtaccttgtattggagaacatttggacagggctc 6240

Query: 121  ctgtatgtggaaatgaacaaaacaatcaaacaggcctgtgtccaccattgaatattaaaaa 180
Sbjct: 6241  ctgtatgtggaaatgaacaaaacaatcaaacaggcctgtgtccaccattgaatattaaaaa 6300

Query: 181  acactgttatcgagagatgtgtacgtgtacgtgtcaagtttgcacctgggcacgac 240
Sbjct: 6301  acactgttatcgagagatgtgtacgtgtacgtgtcaagtttgcacctgggcacgac 6360

Query: 241  tgcctttcatttaaataataactcagtagctaagcatctatctagatattttctaataataacaag 300
Sbjct: 6361  tgcctttcatttaaataataactcagtagctaagcatctatctagatattttctaataataacaag 6420

Query: 301  ctgatattttaagcatggcaatgtgttttcatgtgctgcatgtttttcccttgccagac 360
Sbjct: 6421  ctgatattttaagcatggcaatgtgttttcatgtgctgcatgtttttcccttgccagac 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 gaatatatgagacatgtagaaagatatatcatatatatattcagttatgtaggta 168

Sbjct: 6774 gaatatatatgagacatgtagaaagatatatcatatatatattcagttatgtaggta 6833

Query: 169 ccccttagagactggtgtgctaatcccaaccagataatgctatgtaatctgatatttagaaac 228

Sbjct: 6834 ccccttagagactggtgtgctaatcccaaccagataatgctatgtaatctgatatttagaaac 6893

Query: 229 tggcaactaggttttgttcccaacaccagataatgcagtcagctacatcgattacctt 288

Sbjct: 6894 tggcaactaggttttgttcccaacaccagataatgcagtcagctacatcgattacctt 6953

Query: 289 aattcacaacctaatagttccagatgcagctcacaagaccagaaaaagaagatctctttt 348

Sbjct: 6954 aattcacaacctaatagttccagatgcagctcacaagaccagaaaaagaagatctctttt 7013

Query: 349 ggtggatatctatttttgaatgt 371

Sbjct: 7014 ggtgatatctatttttgaatgt 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 atgattacactgcagagcaattagaataatcacaagggcaggtggaggaatagtaataatttt 153

sbjct: 6996 atgattacactgcagagcaattagaataatcacaagggcaggtggaggaatagtaataatttt 7055

query: 154 cattaaatattaacagctatgtaaggtttctctaatagggcagaagatattggttcagataatgt 213

sbjct: 7056 cattaaatattaacagctatgtaaggtttctctaatagggcagaagatattggttcagataatgt 7115

query: 214 ctattgaattctctttatattgttgragatgtgcagtttaggtttgtatacattctccagatataacc 273

sbjct: 7116 ctattgaattctctttatattgttgragatgtgcagtttaggtttgtatacattctccagatataacc 7175

query: 274 ctattcagacacacctatcgattattatgtgttca 305

sbjct: 7176 ctattcagacacacctatcgattattatgtgttca 7207

166
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

>gb:PPHLlAJ Human papillomavirus unidentified type (RTRXl) LI 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

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 gtttgtgaagatgcaggcagtcaggtaggattatgtcctccactagaattaaaaaatggt 177
Sbjct: 6181 gtttgtgaagatgcaggcagtcaggtaggattatgtcctccactagaattaaaaaatggt 6240
Query: 178 gttatagaggatggagatatgtttgatataggatttccaaatataaataataaaacacta 237
Sbjct: 6241 gttatagaggatggagatatgtttgatataggatttggaaatataaataataaaacacta 6300
Query: 238 tcatttaatagatctgatgtaagcttagacattgtaaatgaaatctgtaaatatcctgat 297
Sbjct: 6301 tcatttaatagatctgatgtaagcttagacattgtaaatgaaatctgtaaatatcctgat 6360

gb:PPHLlAJ Human papillomavirus unidentified type (RTRXl) LI 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 371
Sbjct: 1 atcagtgttactagtgaagacttaagtacagcaaaatatgatgctaaaaatatcagggaa 60
Query: 270 tatatgagacaacgtgtagaagaatatactcaattatcattttatattacagtttaggtaccc 311
Sbjct: 61 tatatgagacaacgtgtagaagaatatactcaattatcattttatattacagtttaggtaccc 120
Query: 210 tttgagggctgtagtcaacccagattaaatgtctgtgaatctcaggtatataggaaaaactgg 315
Sbjct: 121 ttaggggtcaggtgaacctatcagtttagtattataggaaaaactgg 180
Query: 150 caactagtgggtttgtcccccaacacacagataatgcagctcgcacactcgtacccatatccttaat 391
Sbjct: 181 caactagtgggtttgtcccccaacacacagataatgcagctcgcacactcgtacccatatccttaat 240
Query: 90 tcataagctcataaatgtttagcatca464
Sbjct: 241 tcataagctcataaatgtttagcatca464

167
gb|L38918|PPHL1AJ Human papillomavirus unidentified type (RTRX1) L1 gene, partial cds.
Score = 498 bits (251), Expect = e-139
Identities = 263/267 (98%)
Strand = Plus / Plus

Query: 103 atcagtgttactagtgaagacttaagtacagcaaaatatgatgctaaaaatatcagggaa 162
Sbjct: 1 atcagtgttactagtgaagatttaagtacagcaaaatatgatgctaaaaatatcagggaa 60

Query: 163 tatatgagacatgtagaagatttaagtacagcaaaatatgatgctaaaaatatcagggaa 222
Sbjct: 61 tatatgagacatgtagaagatttaagtacagcaaaatatgatgctaaaaatatcagggaa 120

Query: 223 ttagagacatgaggtgctaaccacagattaatgtcatgaattcaggtatatgtagataagaaactgg 282
Sbjct: 121 ttagagacatgaggtgctaaccacagattaatgtcatgaattcaggtatatgtagataagaaactgg 180

Query: 283 caacctagggttttgctcaacaccagattaatgtcagtcgtatgacagtatcttatctcttat 342
Sbjct: 181 caacctagggttttgctcaacaccagattaatgtcagtcgtatgacagtatcttatctcttat 240

Query: 343 tcaaaagctacaaaaatgtcagatgca 369
Sbjct: 241 tcaaaagctacaaaaatgtcagatgca 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 gctgaggtgctaaccacagattaatgtcagttatcaggtatatgtagaaatgtcagatgcaacta 72
Sbjct: 6843 gctgaggtgctaaccacagattaatgtcagttatcaggtatatgtagaaatgtcagatgcaacta 6902

Query: 73 gggtttttgcctcaacaccagattaatgctagtcgtatgcagttatatctttttacttagaaas 132
Sbjct: 6903 gggtttttgcctcaacaccagattaatgctagtcgtatgcagttatatctttttacttagaaas 6962

Query: 133 gctacaaaaatgtcagatgctagggctctgctacaaaaatgtcagatgctagggctctgctacaaaaatgtcagatgctagggctctgctacaaaaatgtcagatgctagggctctgctacaaaaatgtcagatgctagggctctgctacaaaaatgtcagatgctagggctctgctacaaaaatgtcagatgctaggg 192
Sbjct: 6963 gcaactaaatgtcagatgctagggctctgctacaaaaatgtcagatgctagggctctgctacaaaaatgtcagatgctagggctctgctacaaaaatgtcagatgctagggctctgctacaaaaatgtcagatgctagggctctgctacaaaaatgtcagatgctagggctctgctacaaaaatgtcagatgctaggg 7022

Query: 193 tcatttttgaatgt 206
Sbjct: 7023 acatttttgaatgt 7036
gb|L38918|PPHL1A9 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  gtaccctttagggctggtgctaaccacaagattatgtatcagatattcagggaa 60
Sbjct: 115 gtaccctttagggctggtgctaaccacaagattatgtatcagatattcagggaa 174

Query: 61  aactggcaactagggtttttgccaacaccagataatgcagcagtgcagcacatcgtac 120
Sbjct: 175 aactggcaactagggtttttgccaacaccagataatgcagcagtgcagcacatcgtac 234

Query: 121  ccttaattcaaaagctacaataatgtccagatca 153
Sbjct: 235 ccttagttcaaaagctacaataatgtccagatca 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  cctcagctttcctaaggtactctgtgtaattgcaaaatataagatagctgatattcttcta 60
Sbjct: 133 cctcagctttcctaaggtactctgtgtaattgcaaaatataagatagctgatattcttcta 74

Query: 61  cagtgctctctatataatattttgagttatattatgtgcagtgcgttacatcttc 120
Sbjct: 73  cagtgctctctatataatattttgagttatattatgtgcagtgcgttacatcttc 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 gttgatctacattccaaatgtatacttttccaaatgggcttttgggtt 110
Sbjct: 7090 gttaatcattccaaatgtatacttttccaaatgggcttttgggtt 7031

Query: 111 acagcatcaggacatttagttgcttagtgaactatatcctctcgcag 170
Sbjct: 7030 acagcatcaggacatttagttgcttagtgaactatatcctctcgcag 6971

Query: 171 ttgtcctgtttaggaacaaaccctagttgccagtcttctaaaatacctgaattcatagca 230
Sbjct: 6970 ttgtcctgtttaggaacaaaccctagttgccagtcttctaaaatacctgaattcatagca 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 caatcaactgtttttctacgtagtagataaactctgaaataaatttttgacctatcagt 104
Sbjct: 6906 caatcaactatattgtttactgtagtagataaactctgaaataaatttttgacctatcagt 6965

Query: 105 tcattcagaaaaacagtgttttatttcctaaatttagatttgtcagaaatttcaga 164
Sbjct: 6966 tcattcagaaaaacagtgttttatttcctaaatttagatttgtcagaaatttcaga 7025

Query: 165 atatattaagacacagtgaagatggactcatattttacagctggttaaatg 224
Sbjct: 7026 atatattaagacacagtgaagatggactcatattttacagctggttaaatg 7085

Query: 225 tttaacagctgaaagtttagctccaatattaaaggtatatgaatctctaaattttttgacctatcagt 284
Sbjct: 7086 tttaacagctgaaagtttagctccaatattaaaggtatatgaatctctaaattttttgacctatcagt 7145

Query: 285 gcagtttagaatgttttttcgacagggataactccctacccagataatcataattaa 344
Sbjct: 7146 gcagtttagaatgttttttcgacagggataactccctacccagataatcataattaa 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 tttgttacatgtagtagataatactcgaaatacaattttagcatatcagttcattcggaa 69

Sbjct: 6916 tttgttacatgtagtagataatactcgaaatacaattttagcatatcagttcattcggaa 6975

Query: 70 aacactgatgtttctaaaattcaaaattatgattctcagaaatttcaagaatatttaaga 129

Sbjct: 6976 aacactgatgtttctaaaattcaaaattatgattctcagaaatttcaagaatatttaaga 7035

Query: 130 cacgtagaagaatatgaaatttcattaattttacagctctgcaaagttcctttaacagct 189

Sbjct: 7036 cacgtagaagaatatgaaatttcattaattttacagctctgtaaagttcctttaacagct 7095

Query: 190 gaagttctagctcaaattaatgctatgaattcaaatatattagaggagtggcagttagga 249

Sbjct: 7096 gaagttctagctcaaattaatgctatgaattcaaatatattagaggagtggcagttagga 7155

Query: 250 tttgttcctgcaccggataatcctatccacgatacatacagatatattaattctgcagct 309

Sbjct: 7156 tttgttcctgcaccggataatcctatccacgatacatacagatatattaattctgcagct 7215

Query: 310 actagatgtctcgataaaatatctccaaaagaaagagaagat 351

Sbjct: 7216 actagatgtctcgataaaatatctccaaaagaaagagaagat 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 atctacatcccaaaatcttcatatattttatagggatcttctctcttttggagggtttttt 65

Sbjct: 7332 atctacatcccaaaatcttcatatattttatagggatcttctctcttttggagggtttttt 7273
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%)
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 gataatacagcagaataactaatttttagatatcagtttaaaagtgaggatagcttagcaaat 85
Sbjct: 1 gataactaacaagcagtactaatttttagatatcagtttaaaatgaggatagcttagcaaat 60

Query: 86 tataatgctagtaaatattagagaatatatgagacatgtgaaagagtacagtgtcttttt 145
Sbjct: 61 tataatgctgaaatatagagaatatactgagacagatgtgaggatagctgtcttttt 120

Query: 146 atattacattgtgcaagaaataccttaattttagatatcagtttaaaatgaggatagcttagcaaat 205
Sbjct: 121 atattacattgtgcaagaaataccttaattttagatatggtgtaggttaaaatgaggatagcttagcaaat 180

Query: 206 aacctctgatatattttgtagcttttgaattaaggtaacgatatgcatcatgcactgcatt 265
Sbjct: 181 aacctctgatatattttgtagcttttgaactaaggtaacgatatgtgtcatgcactgcatt 239

Query: 266 acacgatatatatagttgcaatatgggtttttgcattacatcagatataagcttg 325
Sbjct: 240 acacgatatatatagttgcaatatgggtttttgcattacatcagatataagcttg 299

Query: 326 aacccaaagaagagctttttggaagattattcattctggttagttg 374
Sbjct: 300 aacccaaagaagagctttttggaagattattcattctggttagttg 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 tgcatctgtagacctttttttgagctttttggaattaaggtacagatcgatattctgcaggtttt 188
Sbjct: 267 tgcatctgacatctttttttgagctttttggaactaaggtacagatcgatattctgcaggtttt 226

Query: 189 atctggtttggaaaacaaaccctagtgtgcagtttttctatatataactctgaaatctcagcatt 248
Sbjct: 207 atctggtttggaaaacaaaccctagtgtgcagtttttctatatataactctgaaatctcagcatt 148

Query: 249 aacctctgatatattttgtagcttttgaattaaggtaacgatatgcatcatgcactgcatt 308
Sbjct: 147 aacctctgatatattttgtagcttttgaactaaggtaacgatatggtgtaggttaaaatgaggatagcttagcaaat 88
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: 309 ttgagattcttcacatgtctccatatatttcttgtc 368
Sbjct: 87 ttgatattcttcacatgtctccatatatttcttgtc 28

Query: 369 acttaagtccctcaagtaagtcctagat 395
Sbjct: 27 acttaatctccactgagactagat 1

Query: 69 tgttttgtaagtgacgataacgtaaatgagatagtttta 128
Sbjct: 6663 tgttttgtaagtgacgataacgtaaatgagatagtttta 6722

Query: 129 aggataagcttacatgatctatgtgatagttttaaagtc 188
Sbjct: 6723 acagcagtttagaagataatgatgactcactaataattagagagagttaatcagat 6782

Query: 189 agtatcagttgcttttatattgagctcttttatttgctttagtctcttgcatgcccaagagtttttctta 248
Sbjct: 6783 aatccaatctttttttctttcatttaaatgctgtggaatggcgattttttctta 6842

Query: 249 cacgaatcaatgcaatgaactctgatattttagagaattggcaatggggctttgtacct 308
Sbjct: 6843 cacaaatcattggatattcagttggtacatgatccgctttaatgcttttctctta 6902

Query: 309 caccagataatgcagatgatcaggtatatcagcttatattagctctaaagcgttaagtc 368
Sbjct: 6903 caccagataatgcagatgatcaggtatatcagcttatattagctctaaagcgttaagtc 6962

Query: 369 ctgatgcagttctgaaaccacaaaaagagctcttttggaagatt acctattccttgagatg 428
Sbjct: 6963 cagatgcagttctgacacacagcataaaagagctcttttggaagatt acctattccttgagatg 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 acatccccagaatgatacttttccaaaggtactctttttggggttttccagtgtactgcac 140

Sbjct: 7023 acatccccaaaatgataacttttccaaaggtactctttttgcg-tgtaaggtactgcac 6965

Query: 141 aggcacaccttagtggtccttttgaggttaatatatatatctgtgtacttgattatctgg 200

Sbjct: 6964 tggacatatttggccctttgaggcceaaatactgtatgtgtatgtcatgtacactgcatc 6905

Query: 201 tgtaggtacaaagccaaatgctgcaaatcctataatctgtgtacttgattaatttttcg 260

Sbjct: 6904 tgtaggaacaaaaaccttcaactgccaattctctaaaatatctgaattcatggcattaattttg 6845

Query: 261 tgtcaaaaaccttgagcttttaaaaaggtatgtgctgcacaaaatattataatataaaaaagcatctgata 320

Sbjct: 6844 ttgtagaagacctcggcgctttgaagccaaatatctgtatgtgtcatgaactgcattatctgg 6785

Query: 321 ctcattacaacatgtctctcatatatctctataattatattacgattataattacttaagctatc 380

Sbjct: 6784 tctttcaacatgtctcttgaaacctcattctctataattatattagttgcactcactcttc 6725

Query: 381 ctcacttttaacacgtgataattaataattttgtgtattatctgtctacgtaaaaa 440

Sbjct: 6724 gtcattggaacacgtgataattaataattttgtgtattatctgtctacgctcacaaga 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 acatccccagaatgatacttttccaaaggtactctttttggggttttccagtgtactgcac 140

Sbjct: 7023 acatccccaaaatgataacttttccaaaggtactctttttgcg-tgtaaggtactgcac 6965

Query: 141 aggcacaccttagtggtccttttgaggttaatatatatatctgtgtacttgattatctgg 200

Sbjct: 6964 tggacatatttggccctttgaggcceaaatactgtatgtgtatgtcatgtacactgcatc 6905
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
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,

<table>
<thead>
<tr>
<th>partial cds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length = 349</td>
</tr>
</tbody>
</table>

Score = 416 bits (210), Expect = e-115
Identities = 210/210 (100%)
Strand = Plus / Plus

Query: 1 attagataatcagaaatacaaaatctttagtatttgctgtacatcaagagcgagaaggt 60
Sbjct: 1 attagataatcagaaatacaaaatctttagtatttgctgtacatcaagagcgagaaggt 60

Query: 61 taagaagagatctcctctcattttacatgatccatctgagtcctctctcctgc 120
Sbjct: 61 taagaagagatctcctctcattttacatgatccatctgagtcctctctcctgc 120

Query: 121 atatgaagtgtctctcattttacaattgtgtaaaattccattgaaagctgaggttcttgc 180
Sbjct: 121 atatgaagtgtctctcattttacaattgtgtaaaattccattgaaagctgaggttcttgc 180

Query: 181 acagattaatcgaatgaactctgatatttt 210
Sbjct: 181 acagattaatcgaatgaactctgatatttt 210

gb|U31786|HPU31786 Human papillomavirus type 37, complete genome.

| Score = 335 bits (169), Expect = 3e-90 |
| Identities = 224/240 (93%), Gaps = 2/240 (0%) |
| Strand = Plus / Plus |

Query: 1 tgttatgccagacattatttttgtaagaggaaaaatgtagttgtgctatctccgatgcc 60
Sbjct: 6481 tgttatgccagacattatttttgtaagaggaaaaatgtagttgtgctatctccgatgcc 6540

Query: 61 actggtaatccagaaccacaatatattactcttcctgcaaattcagacaagcagcagtatctcg 120
Sbjct: 6541 actggtaatccagaaccacaatatattactcttcctgcaaattcagacaagcagcagtatctcg 6600

Query: 121 ttacgcaattctacccattcttctcttcctgctttcagatcttgatcctgatc 180
Sbjct: 6601 ttacgcaattctacccattcttctcttcctgctttcagatcttgatcctgatc 6660

Query: 181 ctctctaccaagccc--tttggattacgcagactcaagctcagactcagactcagactcag 238
Sbjct: 6661 ctctctaccaagccc--tttggattacgcagactcaagctcagactcagactcagactcag 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 aatcagatgtttggtggtctgtaaatatacaaggataacactaatatttcaccataagtgg 60
Sbjct: 6711 aatcagatgttttggtcagataacacaacaggaatcaaatattttactattagtggt 6770

Query: 61 acctctgtaggttagtactataactataactataatattacacaaattttgacaattttaagg 120
Sbjct: 6771 acctctgtaggttagttgcagataacacaacaggaatcaaatattttactattagtggt 6830

Query: 121 catgtggaagataattactgttgcagataacacaaggaatactaattttactattagtggt 180
Sbjct: 6831 catgtggaagataattactgttgcagataacacaaggaatactaattttactattagtggt 6950

Query: 241 tttattccacacacagacaatctgtgcatatttacagataatttacactctaaaggtc 300
Sbjct: 6951 tttggttcctacacacagacaacgctgtaacagataatttacagataattttactctaaaggtc 7010

Query: 301 actaatgtcctgtagttgcagtccttaccaaggataaagacacattttgaattattatacac 360
Sbjct: 7011 actaatgtcctgtagttgcagtccttaccaaggataaagacacattttgaattattatacac 7070

Query: 361 ttctggaatgtagatctaac 380
Sbjct: 7071 ttctggaatgtagatctaac 7090

FNI (ICP)

emb|AJ223858|HPVAJ3858 human papillomavirus type 24, L1 capsid gene strain HPV24

>gi|2894525|emb|AJ223858|HPV AJ3858 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 tgcctttagcagatatgtgtcgtatatataatcagagagagagatgttgttgttgttgtcag 60
Sbjct: 71 tgcctttagcagatatgtgtcgtatatataatcagagagagatgttgttgttgttgtcag 130

Query: 61 agagtagaataattaggtagagcagacaacatttagtggtttaaacaagttgacatccattatt 120
Sbjct: 131 agagtagaataattaggtagagcagacaacatttagtggtttaaacaagttgacatccattatt 190
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
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
ataccccagattatatttaggaattggcgacaaaccgtatgtgtatagttaatggtaataatgttaaaattt---ct 57
Sbjct: 6481 atacccagattatatttaggaattggcgacaaaccgtatgtgtatagttaatggtaataatgttaaaatttct 6540

Query: 58
tagaggggaacaatgtttgttaggcacccttttttaatagccagaaactacttggaagcag 117
Sbjct: 6541 tagaagagaacaaatgtttgttaggcaccttttttaatagccagaaactacttggaagcag 6600

Query: 118
tgttccaaatgtatctatatataacagttcatctaatcgcgccttttttaatagccagaaactacttggaagcag 177
Sbjct: 6601 tgttccaaatgtatctatatataacagttcatctaatcgcgccttttttaatagccagaaactacttggaagcag 6660

Query: 178
ttatattccccacaaacctaattggctctcttagttatcctgttactttttcagatatatatatataaaacc 237
Sbjct: 6661 ttatattccccacaaacctaattggctctcttagttatcctgttactttttcagatatatatatataaaacc 6720

NHS8 (GENERAL POPULATION)

gb|U31787|HPV31787 Human papillomavirus type 38, complete genome.
Length = 7400

Score = 113 bits (57), Expect = 3e-23
Identities = 159/193 (82%)
Strand = Plus / Plus

Query: 181
tctaggggtgctaatccccagattatgtatgtatagattagctataatgaaactggcaacctg 240
Sbjct: 6844 tctaggggtgctaatccccagattatgtatgtatagattagctataatgaaactggcaacctg 6903

Query: 241
ggtttgccacaccagataatcagtgcgtcaggacacatatcgttacatttataatttccaag 300
Sbjct: 6904 ggtttgccacaccagataatcagtgcgtcaggacacatatcgttacatttataatttccaag 6963

Query: 301
tctaaacatgtccccagatgccgctcggatgctaagggagatctctttttgggttattt 360
Sbjct: 6964 tcatctaaacatgtccccagatgccgctcggatgctaagggagatctctttttgggttattt 7023

Query: 361
cattttgggaatgt 373
Sbjct: 7024 cattttgggaatgt 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 ggacacttagtgccccctttggaggttaaatcttatatatctgtgtactgcattatctgtgtgtgt 62
Sbjct: 6963 ggacatttttggtgccctttgaagccaaatctctattctggtgtgtctcatgatcattatctgtgt 6904

Query: 63 gtaggtcacaagggcccaattttctctcaatattatcagcagttcatgcattggtcgtgc 122
Sbjct: 6903 gtaggaacaaacctcatactgcccaaatctcttatattatctggtatctgcatggtattaatat 6844

Query: 123 gctaaacactcagccttttaaaaaggtatgtgctgcaacatttagataatattatattattttacagctatcc 182
Sbjct: 6843 gttagacccctgccccctttaaaggtacattctgctgcaacactgtagatatattttataatttgat 6784

Query: 183 tccataacactgaattaaaaaattttagatgtactgctatgctacatgactaaccacaacc 242
Sbjct: 6783 tctctaaacactgataactaatttttagatgctactgctacatgactaaccacaacc 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 ggacatttttggtccctttggaggttaaatcttatatatctgtgtactgcattatctgtggt 60
Sbjct: 6903 ggacatttttggtccctttggaggttaaatcttatatatctgtgtactgcattatctgtggt 6904

Query: 61 gtaggaacaaaccctcataactgcataaatatttatctgatattctgctatgactaaccacaacc 120
Sbjct: 6843 gttagacccctgccccctttaaaggtacattctgctgcaacactgtagatatattttataatttgat 6784

Query: 121 gtttagacccctgccccctttaaaggtacattctgctgcaacactgtagatatattttataatttgat 180
Sbjct: 6843 gttagacccctgccccctttaaaggtacattctgctgcaacactgtagatatattttataatttgat 6784

Query: 181 tccataacactgcattttcattctatatttttagttggccatcactattttcctaatatctgtgct 240
Sbjct: 6783 tccataacactgcattttcattctatatttttagttggccatcactattttcctaatatctgtgct 6724

Query: 241 tccataacactgcataactaatttttagttggccatcactattttcctaatatctgtgct 300
Sbjct: 6723 tccataacactgcataactaatttttagttggccatcactattttcctaatatctgtgct 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  ctggtgtaggtacaaagcccaattgccaattctctaaatacagagttcattgcattga 60

Sbjct: 229 ctggtgtaggtacaaagcccaattgccaattctctaaatacagagttcattgcattga 170

Query: 61  ttggtgtataaaacttcagctcttttaaggtatgtgctgcaacaattataatatataaaagacatct 120

Sbjct: 169 ttggtgtataaaacttcagctcttttaaggtatgtgctgcaacaattataatatataaaagacatct 110

Query: 121  gatactcatcaacatgtctcatatattctctaatattactagcattataatttactaagc 180

Sbjct: 109 gatactcatcaacatgtctcatatattctctaatatttactagcattataatttactaagc 50

Query: 181  tattcctcaacttttaacactgatactaaatatagattgtgcgtgtattatc 229

Sbjct: 49 tattcctcaacttttaacactgatactaaatatagattgtgcgtgtattatc 1