Cutaneous Human Papillomaviruses

Hazard, Kristina

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Medical Microbiology, Institution of Laboratory Medicine
Lund University, Malmö University Hospital, Malmö, Sweden.

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KRISTINA HAZARD

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Faculty opponent

Professor Herbert Pfister,
Institute of Virology,
University of Cologne, Cologne, Germany

Medical Microbiology, Institution of Laboratory Medicine
Lund University, Malmö University Hospital, Malmö, Sweden.

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KRISTINA HAZARD

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Till mamma och pappa

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ABSTRACT

Human papillomaviruses (HPVs) exist as more than 100 distinct types. Variants of HPVs appear to be common findings while HPV subtypes have been considered rare. New information about subtypes has recently been reported by us. Our characterisation of subtype HPV38b[FA125] and the identification of several HPV isolates representing putative subtypes have considerably extended the knowledge about this taxon.

Cutaneous HPVs are frequently found in healthy skin and some types have also been implicated in non-melanoma skin cancer in immunocompetent as well as in immunosuppressed individuals. However, data on whether these infections persist over time is limited. We recently reported that in a cohort of renal transplant recipients and matched healthy controls, 43% (19/44) of the cutaneous HPV infections persisted after 6.3 years. However, we did not detect any significant association between persistent infections and age, sex, immunosuppressive treatment, history of warts, or genus of HPV.

The heterogeneity of cutaneous HPVs, especially in the genus *Beta*-papillomavirus, has been extended through our characterisation of three new types, HPV93, 96, and 107.
The prevalence of these three types as well as HPV38 and its subtype HPV38b[FA125] and the recently described HPV92, was analysed in skin lesions and paired healthy skin. All types were only detected in low amounts and in low viral loads. However, the binding ability of the E7 protein of HPV92, 93 and 96 to the tumour suppressor protein Rb suggests a possible role for these types in the development of skin cancer.

LIST OF PAPERS

This thesis is based on the following papers.

I. **Hazard, K**., Karlsson, A.., Andersson, K., Ekberg, H., Dillner, J., and Forslund, O.
Cutaneous Human Papillomaviruses persist on healthy skin.
Journal of Investigative Dermatology 2007 Jan;127(1):116-9.

II. **Hazard, K**., Eliasson, L., Dillner, J., and Forslund, O.
Subtype HPV38b[FA125] demonstrates heterogeneity of Human Papillomavirus type 38.
International Journal of Cancer 2006 Sep 1;119(5):1073-7

III. **Hazard, K**., Andersson, K., Dillner, J., and Forslund, O.
Human Papillomavirus subtypes are not uncommon.
Virology, in press.

IV. Vasiljević, N., **Hazard K**., Eliasson, L., Ly, H., Hunziker, A., de Villiers, E-M., Norrild, B., Dillner, J., and Forslund, O.
Characterisation of two novel cutaneous Human Papillomaviruses, HPV93 and HPV96.
Journal of General Virology, 2007 May 1; 88 (5): 1479-1483

V. **Hazard, K**., Vasiljević, N., Dillner, J., and Forslund, O.
Isolation of a novel *Beta*-2 Human Papillomavirus from actinic keratosis.
Manuscript.

ABBREVIATIONS

Ab	Antibody
AK	Actinic keratosis
Arg	Arginine
Asp	Asparagine
BCC	Basal cell carcinoma
bp	Base pair
BPV	Bovine papillomavirus
CRPV	Cottontail rabbit papillomavirus
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
E	Early (region)
E6-AP	E6-associated protein
EV	Epidermodysplasia verruciformis
FDA	US Food and Drug Administration
GAG	Glycose-amino glycans
Gly	Glycine
His	Histidine
HPV	Human papillomavirus
HR	High-risk
HSPG	Heparin-sulphate proteoglycans
hTERT	Human telomerase reverse transcriptase
Ig	Immunoglobulin
L	Late (region)
LCR	Long control region
LR	Low-risk
MEGA	Molecular Evolutionary Genetic Analysis
NCR	Non-coding region
NMSC	Non-melanoma skin cancer
nt	Nucleotide
ORF	Open reading frame
ORI	Origin of replication
PCR	Polymerase chain reaction
pRb	Retinoblastoma protein
Pro	Proline
PV	Papillomavirus
RNA	Ribonucleic acid
RTR	Renal transplant recipient
SK	Seborrhoeic keratosis
SCC	Squamous cell carcinoma
URR	Upstream regulatory region
UV	Ultraviolet radiation
VLP	Virus-like particle

INTRODUCTION

HISTORY

In the early 1900's, papillomavirus (PV) research began with the first report of cell-free transmission of human warts.[1] In 1933, Richard Shope identified the first animal PV, cottontail rabbit papillomavirus (CRPV),[2, 3] and he recognised it as the etiological agent of cutaneous warts (papillomas) in the cottontail rabbit. In 1935, Rous described that warts induced by CRPV had the potential to transform into malignant processes,[4] and this discovery initiated the field of tumour virology. The first DNA tumour virus was identified.

In the 1950's, the carcinogenic potential of human papillomavirus (HPV) in patients with the rare hereditary disease epidermodysplasia verruciformis (EV) was discovered.[5] However, due to the absence of conventional tissue culture systems for *in vitro* viral propagation of PVs, it was not until the introduction of molecular biology methods that the first papillomavirus genomes, HPV1a and bovine papillomavirus (BPV) type 1, were successfully cloned in bacteria.[6, 7] A few years later, *in vitro* transforming assays were developed which permitted analyses of viral functions involved in the induction of cellular proliferation.[8-10]

In 1976, zur Hausen proposed that HPV infection might be the putative sexually transmitted agent for cervical cancer.[11] In the early 1980s, HPV16 and 18[12, 13] were identified, which provided the field with HPV types that were present in most cervical cancers. In 1987, the first epidemiological study on HPV and cervical cancer was published,[14] and since then studies from all over the world have established the etiological link between HPV and cervical cancer.[15, 16]

HPV also causes genital warts[17-19] (condyloma accuminata), cutaneous warts,[20, 21] and some types of HPV have also been associated with the development of non-melanoma skin cancer.[22-25]

In 2006, the US Food and Drug Administration (FDA) approved a quadrivalent[26, 27] prophylactic HPV vaccine, that protects against initial infection with HPV16 and 18, which together cause 70% of cervical cancers worldwide.[28] The vaccine showed a very high efficacy against persistent infections, as well as protection against HPV6 and 11, which cause approximately 90% of genital warts.[29]

CHARACTERISTICS AND CLASSIFICATION

The papillomaviruses belong to the family *Papillomaviridae*, and are small non-enveloped, DNA viruses. The papillomavirus particle is about 55 nm in diameter, and contains a single molecule of double-stranded closed circular DNA about 8,000 base pairs in size, contained in a protein capsid with icosahedral symmetry (see back cover).

The papillomaviruses infect a large number of different species e. g. humans, cattle, rabbits, and nonhuman primates.[30, 31] In general, the papillomaviruses are highly species specific, and there are no examples of a papillomavirus from one species causing a productive infection in a second species.

Papillomaviruses have a tissue tropism for epithelial cells, either cutaneous or mucosal, and can cause infections of the skin, or in the mucosal epithelium of the genital tract, the oral pharynx, and the oesophagus.[32] Papillomaviruses replicate in the nucleus of squamous epithelial cells.

GENOMIC ORGANISATION

The viral DNA is associated with cellular histones to form a chromatin-like complex.[33, 34] Most papillomaviruses have six well-described non-structural viral genes: E1, E2, E4, E5, E6, and E7, and two structural genes: the major capsid protein L1 and the minor capsid protein L2. All potential protein coding sequences occur in homologous positions on one strand of the PV genome. Transcriptional studies of the RNAs encoded by the papillomaviruses indicate that only one strand serves as template for transcription.[35]

The papillomavirus genome is divided into three domains based on their location and putative functional properties (Fig. 1). The early (E) region encodes viral regulatory proteins involved in DNA replication, transcription, and transformation of infected cells.[36] The late (L) region encodes structural proteins that make up the viral capsid. There is a region in the genome in which there are no open reading frames (ORFs). This region has been referred to by several terms, including the long control region (LCR), the upstream regulatory region (URR), and the non-coding region (NCR). It is defined as the part of the genome between the stop codon of the L1 ORF and the start codon of the E6 gene. This region contains the origin of replication (ORI) and numerous control signals for DNA replication and transcription.

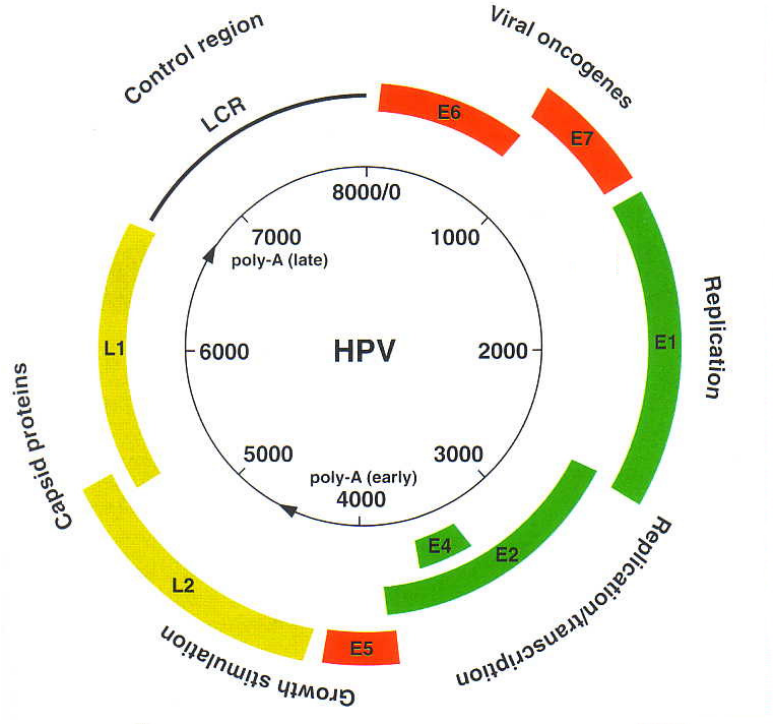

Figure 1. Schematic figure of the HPV genome with positions of the open reading frames of the early (E) and late (L) genes as well as the long control region (LCR).

Viral proteins

E1

The E1 ORF is the largest one in the papillomavirus genome and it is relatively well conserved among all of the PVs. The size of E1 differs between different phylogenetic genera, with the largest ORFs found among the *Alpha*-papillomaviruses.[37] The E1 protein is the only papillomavirus protein with enzymatic activity, and a hexameric form of the protein is associated with the DNA-dependent ATPase and DNA helicase activities fundamental to its initiator function in DNA replication.[38, 39]

E1 is the only viral factor that is directly involved in plasmid replication, and the protein is required for both the initiation and elongation of viral DNA synthesis.[40] E1 binds specifically to the ORI, and this binding is stabilised through interactions with the E2 protein.[41, 42] Although not essential for origin-dependent DNA replication *in vitro*, E2 greatly stimulates the ability of E1 to initiate DNA replication. E2 may also stimulate viral DNA replication by recruiting host replication factors to the origin. The E1:E2 complex is a precursor to a larger multimeric E1 complex, which after the removal of E2 can alter the replication origin and ultimately unwind the DNA.[43]

E2

The E2 protein is relatively well conserved among the papillomaviruses, and is an important regulator of viral transcription and replication. The E2 protein consists of three functional domains: a sequence-specific DNA-binding and dimerisation domain located in the carboxyl-terminal region of the protein, a transactivating domain located within the amino-terminal half of the protein, and a central hinge domain.[44] The DNA-binding and the transactivating domains are relatively well conserved among different papillomaviruses, while the hinge region is not, either in size or in amino acid composition.

The E2 protein binds to the consensus sequence $ACCN_6GGT$[45] that is found repeatedly in the URR of all papillomaviruses[46] with the exception of HPV41, which lacks these consensus sites.[47] E2 can regulate transcription from promoters containing these E2-binding sites.

E2 is a multifunctional protein, and its functions as a transcriptional activator and repressor are likely mediated by interactions with specific cellular factors. In addition to its role as a transcriptional regulator, E2 has critical roles in viral DNA replication and in plasmid maintenance. E2 has also been shown to induce apoptosis.[48, 49]

E2 utilizes a cellular protein known as Bromodomain-4 to tether the viral genome to cellular chromosomes.[50] This tethering to the cell's nuclear matrix ensures acurate distribution of viral genomes to each daughter cell after cell division.

E4

The E4 ORF of the papillomaviruses is located in the early region, yet is expressed as a late gene with a role in productive infection. It overlaps the E2 ORF but in a different reading frame and therefore encodes a protein with an entirely different amino acid sequence.

In general, the E4 protein is not well conserved among different PVs.

A viral transcript formed by splicing a few codons from the beginning of E1 to E4 appears to be the major RNA in HPV-induced lesions for the viruses that have been studied.[51-54]

The E4 protein is expressed in cells in which vegetative viral DNA replication is ongoing. The expression of E4 is not coincident with the expression of the capsid proteins,[55] and E4 expression precedes the expression of L1.[56]

The precise role of E4 in the viral life cycle is unclear. However, the HPV16 E4 protein has been shown to interact with intermediate filament of the host cell, leading to collapse of the cytokeratin network and thereby release of mature virus particles.[57-60]

E5

Among the mucosal HPVs, the high risk (HR) types codes for an E5 protein, while the low-risk (LR) types either lack a definable E5 ORF and/or translation start codon for E5.[61] However, it should be noted that the E5 gene is not expressed in most HPV-positive cancers, suggesting that if the E5 gene does stimulate cell proliferation *in vivo*, it presumably functions in benign papillomas and not in the cancers. It might also participate in the initiation of the carcinogenic process or in some other aspects of the viral-host cell interaction relevant to the pathogenesis of the HPV infection.

With the exception of HPV5[62] the cutaneous HPV types do not encode E5, while for BPV1 E5 is the major transformation protein.[63, 64]

E6

The primary function of the E6 protein is to inactivate the tumor suppressor protein p53.

E6 contains two zinc finger motifs[65] ($CxxC(x)_{29}CxxC$) which determine the protein conformation or protein-protein interactions for DNA binding that are essential for transformation.[66]

E6 and E7 together can extend the life span of human keratinocytes and lead to the outgrowth of immortalised clones that are resistant to terminal differentiation. This property is dependent on the full-length E6 protein. Mutational analysis has shown that the putative HPV16 E6* protein, encoding a truncated form of E6, cannot provide this function.[67] It appears that the E6*, if it is actually made *in vivo*, might function in a dominant negative manner to inhibit some of the activities of the full-length E6 oncoprotein of the cancer associated HPV types.[68]

The E6 and E7 proteins are structurally related and are conserved, at least in part, among all of the PVs.

E7

The E7 protein was the first oncogene of HR HPVs to be identified, and it is predominantly found in the nucleus.[69, 70] The major antigenic region of E7 for both low-risk and high-risk HPVs is found in the N-terminus part of the protein.

In most papillomavirus types, the primary function of the E7 protein is to inactivate members of the retinoblastoma tumour suppressor protein (pRb) family. The LxCxE motif necessary for pRb binding is in the C-terminal part of E7, whereas the residues important for pRb degradation are localised in the N-terminal region.[71-73] pRb act as a negative

regulator of the cell cycle and is regulated by phosphorylation.[74] E7 contains a casein kinase II phosphorylation site that leads to phosphorylation of E7 during the G_1 and S phases of the cell cycle.[75]

The E7 proteins of low-risk HPV types bind pRb with about a 10-fold lower efficiency than the E7 proteins of high-risk HPV types. Sequence comparison of the pRb-binding sites revealed a single consistent amino acid sequence difference between the HR and the LR E7 proteins: an aspartic acid residue (Asp21 in HPV16 E7) corresponding to a glycine residue in the LR E7 sequence (Gly22 in HPV6 E7). Substitution of this residue in the respective E7 genes revealed that this single amino acid residue was the principal determinant responsible for the difference in pRb-binding affinity and in the transforming capacity of the LR and the HR E7 proteins.[76, 77]

L1

The virus capsid consists of two virally encoded structural proteins, the major capsid protein L1, and the minor capsid protein L2.[78]

The L1 ORF is a highly conserved region among papillomaviruses. Five L1 monomers form a pentameric capsomer, and 72 such capsomers make up the viral capsid.[79] L1 represents about 80% of the total viral protein in the capsid.

L1 can self-assemble into virus-like particles (VLPs) that approximate the structure of native virions.[80-84] The ability to self-assemble is dependent on the amino acid at position 202. Efficient self-assembly is seen with Asp202, while histidine (His) at this position requires L2 for assembly.[81, 84, 85]

X-ray crystallographic analyses of these VLPs showed that surface loops (Fig. 2) contain the sites of largest sequence variation among HPV types. Also, these loops are likely locations of neutralising epitopes[86-89] against which virus-neutralising anti-L1 antibodies are generated. These antibodies are essentially type specific.[90-93]

VLPs assembled *in vitro* are the basis of prophylactic vaccines against several HPV types.

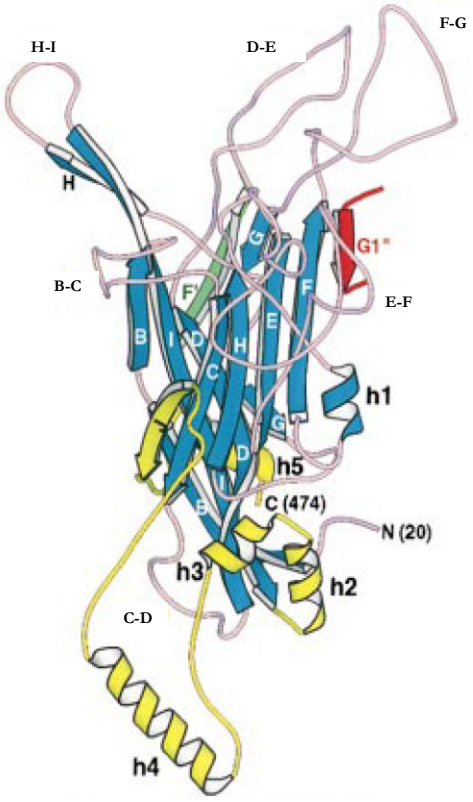

Figure 2. HPV16 L1 monomer with the surface exposed hyper-variable loops B-C, D-E, E-F, F-G, and H-I. (Adapted from Chen, 2000).[94]

L2

The minor capsid protein L2 is required for encapsidation of the viral genome.[93, 95]

L2, situated more internally of the capsid than L1, can also induce neutralising antibodies through a small segment that is exposed at the surface.[96, 97 98-100] These neutralising antibodies are less potent than anti-L1 antibodies, [98, 100, 101] but they appear to show some cross-reactivity to heterologous HPV types.[102, 103]

L1 proteins of cutaneous HPV types have a shorter amino acid sequence than the L2 proteins, while the opposite is seen in mucosal types.[104]

L2 cannot self-assemble into VLPs, however, it has been shown that the viral capsid assembly of L1 is enhanced 50-fold together with L2.[80]

Upstream regulatory region (URR)

This region comprises the part of the PV genome between the stop codon of the L1 ORF and the start codon of E6, and it is in general not well conserved between the papillomaviruses. The URR contains enhancer elements that are responsive to cellular factors as well as to virally encoded transcriptional regulatory factors. It is thought that these constitutive enhancer elements are essential for the initial expression of the viral gene

after virus infection and they may also be important in the maintenance of viral latency. In addition, the URR contains promoter regions, the origin of replication and the TATA-box for the early transcripts. Transcription is regulated by cellular factors and virally-encoded transcription factors that interact with promoters and enhancers. Several transcription-binding sites have been identified in the URR.

The URR of all papillomaviruses contains repeated copies of the palindrome site $ACCGN_6CGGT$.[46] In HPV16 and 18, E2-binding to this site suppresses the expression of E6 and E7, since it is positioned close to the TATA-box of their promoter.[105, 106]

PHYLOGENY

Unlike most other virus groups, papillomaviruses are not referred to as serotypes. The classification of viral types is based on the species of origin and the extent and degree of relatedness of the viral genomes. The PVs are grouped into different genera, e. g. *Alpha*-, *Beta*-, and *Gamma*-papillomaviruses (Fig. 4).[107] Each genus is further divided into species containing one or several PV genotypes. In addition to genotypes, PVs are also grouped into subtypes and variants depending on their sequence similarity in the L1 gene.[107] Different genera share less than 60% nucleotide sequence similarity in the major capsid protein L1 ORF, while complete genome sequences of papillomaviruses of different genera share more than 23% but less than 43% sequence similarity. Different viral species within a genus share between 60% and 70% similarity, while different PV types have less than 90% but more than 70% sequence similarity to the closest known type in the L1 ORF. Subtypes and variants share between 90-98% and 98-99% sequence similarity, respectively, to any known PV type.

Papillomaviruses do not encode their own DNA polymerase but, instead, utilise the host cell enzymes for replication of the viral genome. Thus, they benefit from the high fidelity, proofreading capacity, and post-replication DNA repair mechanisms of the host DNA polymerase. This undoubtedly contributes to the relative stability of the PV genomes.

As recombination between different papillomaviruses has not been detected, it can be concluded that PVs have been, and are, evolving either by the slow accumulation of point mutations and/or by other non-stochastic mechanisms which result in establishment of specific genome types in a population.[108, 109]

Till date, more than 100 different HPVs have been completely characterised. In addition, there appears to exist about a hundred additional HPV types with sequence information so far available only from PCR amplimers.[31, 110-113]

HPV variants are quite commonly found and appear to associate with geographic regions and with biologic behaviour of the virus.[114, 115] By contrast, subtypes have been considered rare.[107, 116, 117] New insights into this matter is presented in paper III of this thesis. Needless to say, clinical and epidemiological studies that do not take the subtype diversity into account when designing and validating detection systems may give misleading results.

It is well known that HPV variants within the same type constitute the same serotype[118] and that different HPV types usually constitute different serotypes.[119] However, it is not well known whether HPV subtypes are serologically distinct or not, except for HPV5 (a member of the genus *Beta*) that has been reported to exist as serologically distinct subtypes.[120]

Except for HPV38b[FA125] previously reported by us,[121] the only completely characterised subtypes presented in the literature[107, 122-125] and available from GenBank belong to the genus *Alpha*-papillomavirus. Although the reasons for the infrequent reporting of HPV subtypes are not known, the issue of whether they are common or not has profound implications for design of virus detection systems and for understanding of the biology of HPV. It is also important to stress the fact that subtype is a taxon of its own, and should not be mistaken for different genotypes as is often seen in the literature.

Papillomavirus genera

All papillomaviruses known till date have been grouped into different genera based on their relatedness, as described above. Each genus is denoted by a Greek letter, and the human papillomaviruses are grouped into the genera *Alpha*-, *Beta*-, *Gamma*-, *Mu*-, and *Nu*-papillomaviruses. The other genera contain papillomaviruses isolated from various mammals and birds.

Alpha-papillomavirus

HPV types infecting genital epithelia are grouped into the genus *Alpha*-papillomavirus. However, some of the types classified into this genus also cause common skin warts.

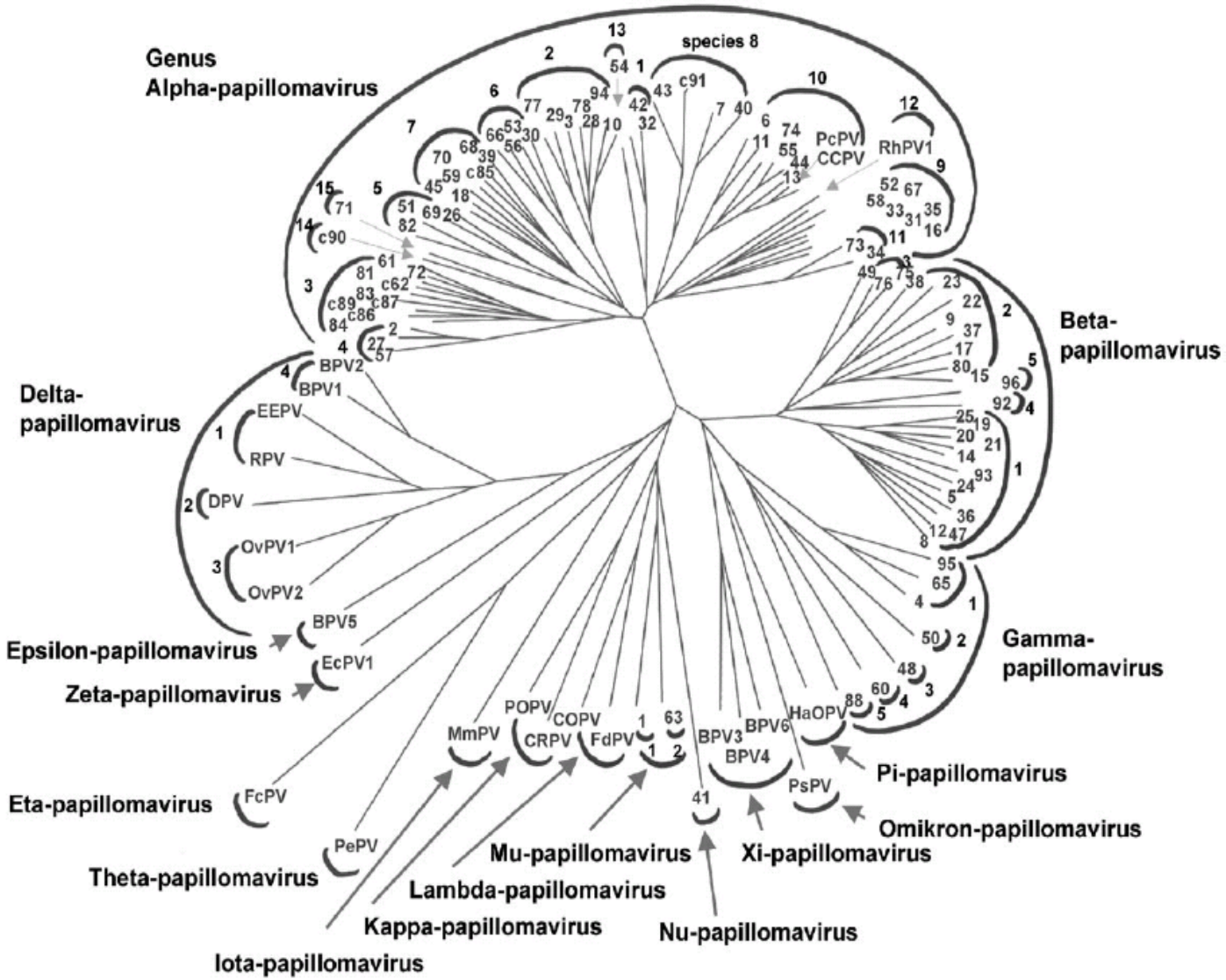

Figure 4. Phylogenetic tree presenting genus and species information for 118 papillomavirus types. (Adapted from de Villiers, 2004)[107]

The *Alpha*-papillomaviruses are divided into 15 different species, each containing closely related genotypes.
High-risk HPV types are predominantly found in species 5, 6, 7, 9, and 11, while low-risk types are grouped into species 3, 8, 10, and species 13-15.
The most common HPV types detected in cervical cancer, HPV16 and 18 which cause approximately 70% of all cancers, and their closely related types are found in species 7 and 9, while species 10 contains e. g. HPV6 and 11 responsible for 90% of genital warts.

Species 8 contains HPV7, HPV40, HPV43, and HPV91 which cause low-risk mucosal lesions. However, HPV7, also known as butcher's wart virus, frequently causes skin warts in butchers and meat handlers.
HPV types within species 4 deserve some extra attention. HPV2, 27, and 57 are classified as *Alpha*-papillomaviruses based on their relation to other HPVs in this genus. However, they cause common skin warts, and hence have a tropism for cutaneous epithelium. The same phenomenon is observed for the types in species 2 which more frequently cause cutaneous than mucosal lesions. Two of the types within this species, HPV10 and 3, usually cause skin warts.
Other quite peculiar findings among the *Alpha*-papillomaviruses are found in species 10 and 12. Clustering together with low-risk types such as HPV6 in species 10 is the Pygmy Chimpanzee PV (PcPV) and its subtype, the Common Chimpanzee PV (CCPV). In species 12 yet another non-human PV is found, Rhesus papillomavirus type 1 (RhPV1), which causes genital lesions in Rhesus monkeys.

Beta-papillomavirus

HPV types infecting the skin are divided into four different genera, *Beta*-, *Gamma*-, *Mu*-, and *Nu*-papillomaviruses, since they display larger sequence divergence than do the *Alpha*-papillomavirus types.
Cutaneous HPV types are frequently detected in healthy skin samples, demonstrating ubiquity of non-symptomatic infections with theses viruses.

The *Beta*-papillomaviruses are divided into 5 different species, but the majority of the types in this genus are found in species 1 and 2. These HPV types are commonly associated with skin lesions in EV patients, and predominantly HPV5 and 8 (species 1) are found in over 90% of theses tumours.

The latest human papillomavirus characterised, HPV107, was recently isolated by us and is reported in paper V of this thesis. HPV107 is classified into species 2 of the *Beta*-papillomaviruses, a group of viruses recently significantly associated with SCC[126] (Table 1). Furthermore, in the same study *Beta*-papillomaviruses within species 1 were significantly associated with seborrhoeic keratosis, a benign lesion of the skin.

Table 1. Prevalence of HPV DNA of *Beta*-papillomavirus species 2 in lesion and paired healthy skin biopsies from 349 patients. (Adapted from Forslund *et al.* 2007).[126]

Diagnosis	***Beta*-2 positive** (n=50), (%)	***Beta*-2 negative** (n=648), (%)	**Adjusted OR** (95% CI)[a]
Healthy skin	15 (30)	334 (52)	1.0 (referent)
Seborrhoeic keratosis (benign)	7 (14)	85 (13)	2.13 (0.81-5.58)
Actinic keratosis	5 (10)	44 (7)	1.75 (0.58-5.27)
Basal cell carcinoma	9 (18)	117 (18)	1.65 (0.68-3.97)
Squamous cell carcinoma	14 (28)	68 (11)	**4.40 (1.92-10.1)**

[a] Odds ratio adjusted for diagnosis, age, sex, skin type, self-reported previous sunburns, eye colour , and sun exposure of biopsy site.

Beta-papillomavirus, species 3 contains HPV49, 75, and 76 which are known to induce benign cutaneous lesions, while species 4 and 5 contain only one HPV type each, HPV92 and 96, respectively, causing pre- and malignant cutaneous lesions.

Gamma-papillomavirus

The *Gamma*-papillomaviruses contain seven types distributed over five different viral species.

Recently, two new HPV types, HPV101 and 103,[127] were isolated from cervicovaginal cells indicating possible relationship to the *Alpha*-papillomaviruses. However, phylogenetic analyses revealed that these two types are closely related to the *Gamma*-papillomaviruses and that they form a monophyletic group at the root of the *Gamma*- and *Pi*-papillomaviruses. The genus *Pi*-papillomavirus contains the Hamster oral papillomavirus (HaOPV).

Another interesting feature of HPV101 and 103 is the fact that both viruses lack the E6 open reading frame[127], a characteristic not reported in any other HPV types.

Mu-papillomavirus

The genus *Mu*-papillomavirus contains two HPV types, HPV1 and 63. They are quite distinct, but are still similar enough in their L1 ORF to be classified in the same genus. One interesting feature of these two types is the varying length of their upstream regulatory region, 982 bp in HPV1 compared to 558 bp in HPV63.

Nu-papillomavirus

The genus *Nu*-papillomavirus contains only one species with one type alone. HPV41 is quite interesting since it contains several large and uncharacterised open reading frames scattered throughout the genome. Further, it seems as if all E2 binding sites in the URR are modified.[47]

THE VIRAL LIFE CYCLE

The PV life cycle is closely linked to the differentiation program of the host cell. In order to induce a persistent infection, the virus must infect the basal cells in the epithelium since these are the only cells capable of dividing. The viral late functions, such as vegetative viral DNA synthesis, capsid protein synthesis, and virion assembly, occur exclusively in differentiated keratinocytes. The replicative phase of the papillomavirus life cycle has been difficult to study because of its link to the terminally differentiated epithelial cells. Papillomaviruses are thought to gain access to keratinocyte stem cells through small wounds, known as micro-traumas, in the skin or mucosal surface (Fig. 3).

The receptor that mediates HPV entry into host cells is not known. However, interactions between L1 and sulfated sugars on the cell surface is thought to promote initial attachment of the virus,[128, 129] and several receptors for attachment of PVs to host cells have been suggested. In 1997, α6 integrin was proposed as a putative receptor for PVs, since laminin, a substrate for α6 integrin receptors, was effective in preventing binding of HPV6 VLPs to the α6 integrin.[130, 131] Later on it was suggested that the α6β1 or the α6β4 integrins acted as receptors. α6β1 is expressed on a wide range of epithelial cells including platelets, lymphocytes and endothelial cells, while α6β4 is expressed in the basal cells and during wound healing, which correlates with viral entry. Papillomavirus virions can also bind to heparin and cell surface glycosaminoglycans (GAGs) on human keratinocytes, which may provide an initial binding event that could be followed by receptor binding and internalisation.[128] In 2001, Giroglou *et al.* [129] reported that heparin surface proteoglycans

(HSPGs) are essential for HPV infection. Heparan sulphate glycosaminoglycans (GAGs) have been proposed as the primary cell surface moieties mediating interaction with papillomavirus.[128, 129, 132]

After attachment, the virus is internalised from the cell surface and transported to membrane-enclosed vesicles called endosomes.[132, 133] The minor capsid protein L2 then disrupts the membrane of the endosomes, allowing the viral genome to escape and traffic, along with L2, to the cell nucleus.[134, 135]

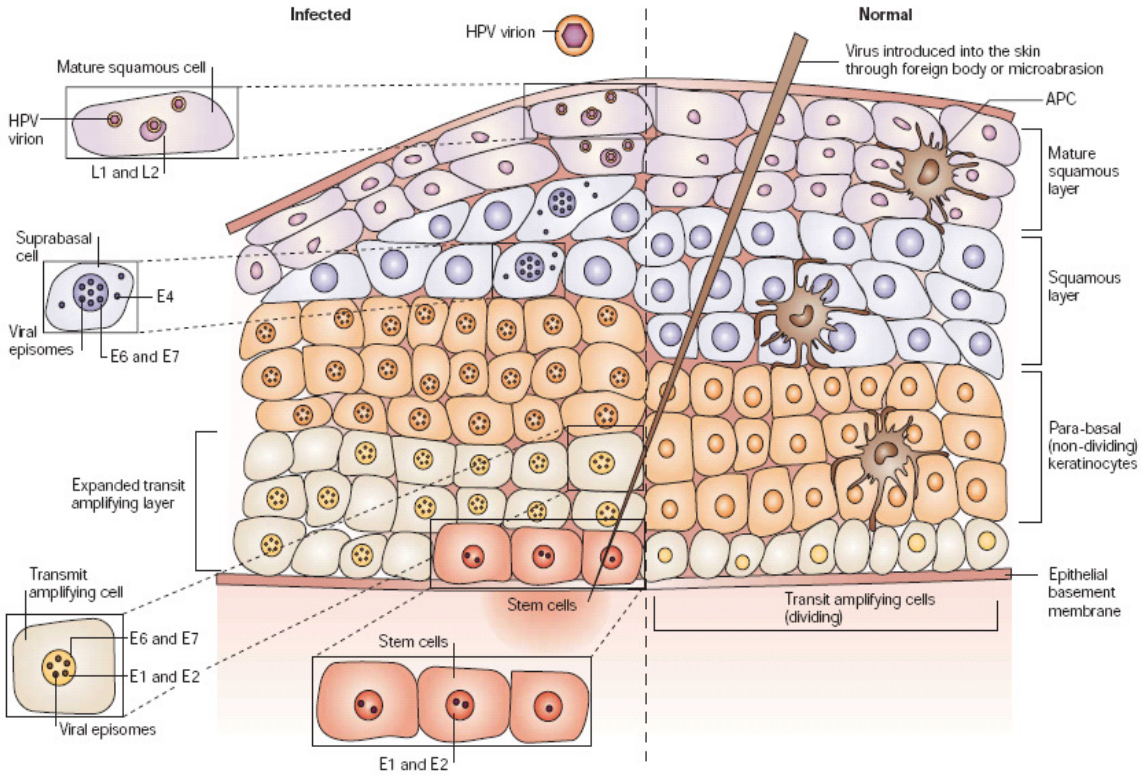

Figure 3. Stages of the papillomavirus life cycle, with their location in the squamous epithelium. Viral proteins are expressed sequentially with differentiation as shown and mature virions are produced only in the most superficial layers of the epithelium. (Adapted from Frazer, 2004).[136]

The papillomaviruses have three modes of viral DNA replication. The first occurs during the initial infection of basal keratinocytes by the virus, where there is an amplification of the viral genome to about 50 to 100 copies. After this stage, the viral genome is maintained as a stable episome.

Next phase is one of genomic maintenance, which occurs in dividing basal cells of the lower portion of the epidermis. In these cells, viral DNA is maintained as a stable multicopy plasmid. The viral genomes replicate an average of once per cell cycle during S

phase, in synchrony with the host chromosome[137] and are partitioned into the daughter cells. This type of DNA replication ensures a persistent and latent infection in the stem cells of the epidermis.

The third type of DNA replication is vegetative, which occurs in the more differentiated epithelial cells of the papilloma. In these cells, which no longer undergo cellular DNA synthesis, there is a burst of viral DNA synthesis, generating the genomes to be packaged into progeny virions. The mechanisms regulating the switch from plasmid maintenance to vegetative viral DNA replication are not known.

The fist viral genes to be expressed after infection are E1 and E2. They form a complex that binds to the ORI and thereby initiates DNA replication. After unwinding of the DNA the viral genome is ready for replication using cellular DNA replication factors.

At later stages in the HPV life cycle it is thought that E2 serves as a negative regulator of expression for the oncogenes E6 and E7 in latently HPV-infected basal layer keratinocytes.[138, 139] Genetic changes, such as integration of the viral DNA into a host cell chromosome, that inactivate E2 expression, tend to increase the expression of the E6 and E7 oncogenes which results in cellular transformation and possibly further genetic destabilization that in the end might result in cervical carcinoma.[140-142] The combination of E6 and E7 is required for the efficient immortalisation of primary human keratinocytes. E6 and E7 together can extend the life span of human keratinocytes and lead to the outgrowth of immortalised clones that are resistant to terminal differentiation.

E7 binds to the hypo-phosphorylated form of the retinoblastoma tumour suppressor protein (pRb), which leads to the functional inactivation of pRb and permits progression of the cell cycle into S phase.[74] This result in dissociation of the pRb-E2F complex and E2F can act as a transcriptional activator. The regulated conversion of the transcriptional activity of E2F between repressor and activator contributes to the regulation of G_1-to-S phase progression. The high-risk HPV E7 proteins contribute to carcinogenic progression, at least in part, by disrupting this regulatory network.[143]

When the cell cycle enters S phase there is an up-regulation in the expression of p53, a transcription factor regulating the cell cycle and hence functioning as a tumor suppressor.[144, 145] The function of p53 as a sequence-specific transcriptional activator[146, 147] is necessary for its activity in regulating cell growth and in tumour growth suppression. However, the HPV E6 protein can efficiently abrogate the transcriptional transactivation

activity of p53. Thus, E6 has anti-apoptotic activities and can interfere with the negative cell cycle regulatory functions of p53.

Both HR and LR mucosal HPV E6 can bind to the C-terminus of p53. However, in the presence of a cellular ubiquitin ligase, the E6-associated protein (E6-AP), only the E6 proteins of HR HPVs can bind to the core of p53, which is essential for efficient degradation of this protein.[148, 149]

Another important function of the HR E6 protein is the activation of telomerase in infected cells. In normal cells, repeated rounds of DNA replication result in shortening of telomeres, eventually producing chromosomal instability and cellular senescence. HR HPV E6 proteins have been shown to increase telomeric length in epithelial cells by activating the catalytic subunit of the enzyme human telomerase reverse transcriptase (hTERT).[150-152]

Compared to HR HPV types, the E6 and E7 proteins of cutaneous HPVs in general have low binding affinity for p53 and pRb, and their ability to transform cells *in vitro* is low.[153, 154] However, in 2003, Caldeira *et al.*[22] showed that E6 and E7 of HPV38, a cutaneous HPV type, could deregulate the cell cycle control and thereby induce cellular transformation. They further showed that the HPV38 E7 protein efficiently inactivated pRb which resulted in deregulation of the G_1/S transition,[22] while HPV38 E6 was unable to promote p53 degradation.[155]

p53 is often mutated in skin cancers,[156] an event that is extremely rare in HPV16-induced cervical tumours.[157] UV-radiation has been shown to cause damage to p53,[158-160] and most likely this DNA damage is responsible for inactivation of p53, rendering E6-mediated p53 degradation unnecessary in skin tumourigenesis. An arginine (Arg) substitution of proline (Pro) at codon 72 of p53 is regarded as the most important UV-radiation induced p53 mutation, which gives rise to two distinct proteins with different ability to signal apoptosis following DNA damage.[161, 162]

A pro-apoptotic protein, Bak, is induced by UV-radiation.[163] Cutaneous HPV E6 has been shown to degrade this protein.[164] E6 of mucosal HPV types have also been shown to inhibit apoptosis by Bak.[162]

Little is known about the papillomavirus assembly or release. Virus particles are observed in the granular layer of the epithelium but not at lower levels. The virus is not believed to be cytolytic, and the release of the virion particles does not occur before the cornified layers of a keratinised epithelium.

HPV-ASSOCIATED DISEASES

Mucosal infections

Human papillomaviruses are considered to be one of the most common sexually transmitted disease agents in the world. They are grouped into mucosal and cutaneous types, depending on the epithelial cells they normally infect. The mucosal types may infect the genital skin, genital mucosa, or non-genital mucosa. These types are further subdivided into high-risk and low-risk types, depending on the risk for malignant progression of the lesions they induce in the cervix. The DNA of the high-risk HPVs can be distinguished from the DNA of the low-risk HPVs by their ability to immortalise primary cultures of human fibroblasts, human foreskin keratinocytes, and human cervical epithelial cells.[165-168] All of the genital tract HPV types (e. g. HPV6, 11, 16, and 18) are capable of transiently inducing cellular proliferation. However, only the HR HPVs are able to extend the life span and give rise to immortalised cell lines that are disobedient to differentiation signals.[166]

Cervical cancer

Persistent infection with one of the HR genital HPV types is a prerequisite for the development of cervical cancer,[169-172] which is the third most common cancer among women worldwide,[173-175] and the most common cancer of women in most developing countries.[176, 177]

About 85% of cervical cancers are squamous cell carcinomas, while most of the other cases are adenocarcinomas.

A number of human papillomaviruses have been implicated as the etiological agents for cervical cancer and other epithelial cancers.[178] For instance, the high-risk HPV types 16 and 18 together cause approximately 70% of cervical cancers worldwide.[28]

A persistent HPV infection is necessary but not sufficient for the development of cervical cancer. Co-factors associated with cervical cancer are multi-parity,[179] smoking habits,[180] use of oral contraceptives,[181] and other sexually transmitted infections, e. g. herpes simplex virus type 2,[182] and *Chlamydia trachomatis*.[183, 184]

HPV is also present in some cases of vulval and vaginal cancer, and penile cancer.

Genital warts

The low-risk types HPV6 and 11 cause approximately 90% of genital warts (condylomas).[29] Most condylomas are self-limiting, regressing spontaneously or after local treatment.

However, some lesions may persist for years, and in extreme cases even progress into so-called giant condylomas or Buschke-Löwenstein tumours of the vulva, penis and anus.[185]

Oral cancers

HPV infections of the oral cavity are frequently occurring,[186] and the two most prevalent HPV types in oral carcinomas are HPV16 and 18.[187, 188] Interestingly, the low-risk HPV types 6 and 11 can also be identified in some oral carcinomas.

Another condition caused by HPV is recurrent respiratory papillomatosis.[189] This is a rare condition of the airways and particularly affects young children. Most lesions are caused by HPV6 and 11, and in some cases these infections may be fatal.

Cutaneous infections

Healthy skin harbours a large spectrum of different HPV types,[31, 111] with more virus being detected in forehead samples than in samples from other body parts such as arms and thighs.[111] In addition, HPV is also often detected in plucked eyebrow samples.[190-192]

The persistence of cutaneous HPV infections has previously only been reported in renal transplant recipients, a group of patients commonly developing skin lesions with detectable HPV DNA. However, new results concerning persistence in both renal transplant recipients as well as in healthy individuals were recently presented by us (paper I of this thesis).

Skin warts

HPV1, 2, 3, 4, 10, 41, and 57 are associated with skin warts on the hands and feet, although they can arise in almost any location.[20, 21] Most of these benign lesions are present for several months and regress spontaneously within two years, although some may persist indefinitely. Butcher's warts which are predominantly found in meat-handlers and butchers are caused by HPV7.[193]

Patients with suppressed or impaired cellular immunity have a high prevalence of HPV-induced benign lesions and tumours. This is evident from renal transplant patients, of whom more than 90% develop skin warts and 40% develop skin cancer within 15 years of transplantation, representing a 50-to-100 fold increase compared to the general population.[194, 195]

Epidermodysplasia Verruciformis (EV)

The association between human papillomaviruses and cancer was first recognised in the 1950's in patients suffering from the rare hereditary cutaneous disorder epidermodysplasia verruciformis (EV).[5] Skin carcinomas normally develop in about one third of these patients and mainly arise at sun-exposed skin sites.[196-198] HPV5 and 8 are associated with skin cancer originating from benign lesions in these patients.[198, 199] EV-specific types, or antibody response to them, have also been found in patients with psoriasis and other conditions associated with rapid epidermal cell growth.[120, 200, 201] The relationship of HPV infection to these disease processes is unclear.

Non-melanoma skin cancer

Non-melanoma skin cancers (NMSC) are the most prevalent malignancies in the Caucasian population[202] and an increase in prevalence has been seen in European countries during the last few years.[203] NMSC comprises basal cell carcinoma (BCC) and squamous cell carcinoma (SCC),[204] and the standardised ratio of BCC to SCC is roughly 4:1.[205] The incidence of NMSC is 18-20 times greater than that of malignant melanoma. BCC arises *de novo*, which means there are no known precursor lesions, while precursor lesions for SCC include actinic keratosis (AK) and Bowen's disease (SCC *in situ*).[206] BCC has a relatively good prognosis, while SCC accounts for up to 20% of all deaths from skin cancer.[207]

NMSC are often found on sun-exposed sites in patients with the rare inherited genetic disease EV.[208] SCCs develop on sun exposed sites in 30-60% of affected patients by the fourth decade and specific HPV types, predominantly HPV5 and 8, are found in over 90% of theses tumours. HPV5 and 8 belong to the *Beta*-papillomaviruses, a phylogenetic subgroup of cutaneous HPVs,[107] which is also detectable in skin carcinomas, as well as in control specimens of healthy skin from the general population.[31, 154, 209-211]

Epidemiological studies have established a causal association between UV-radiation and NMSC.[207] Furthermore, fair skin and the immune status of the host are important risk factors. Immunosuppressed organ-transplant recipients have an up to 100-fold increased risk of SCC and a 10-fold increased risk of BCC, resulting in a reversal of the normal ratio of SCC to BCC.[212, 213]

UV-B radiation exposure of the skin leads to increased levels of the pro-apoptotic cellular Bak protein independent of p53 function. The E6 proteins of HPV5, 10, and 77 have been shown to target Bak for proteolytic degradation and to effectively inhibit UV-B-induced apoptosis.[163, 164]

Renal transplant recipients have a well documented 50- to 100-fold increased risk of cutaneous SCC.[194, 214] Several studies have reported HPV DNA in these cancers.[23, 24, 215]

A recent study[22] demonstrated that the early proteins E6 and E7 of HPV38, a *Beta*-2 papillomavirus, are sufficient to substantially increase the lifespan of primary human keratinocytes by deregulation of the cell cycle control. Furthermore, HPV38 E7 targets the retinoblastoma protein (pRb) with about the same efficiency as HPV16 E7.[22, 216] HPV38 has also been proposed to be a skin cancer-associated HPV type based on reported prevalences of 7–55% in BCC, and 13–46% in SCC.[22, 217, 218]

IMMUNOLOGY

The PVs infect primitive basal keratinocytes, most probably targeting stem cells. However, expression of high levels of viral proteins as well as viral assembly takes place in the upper layers of the squamous epithelia.[219-221] The time from infection to release of virus is approximately three weeks, the time required for the basal keratinocyte to undergo complete differentiation.

Since the papillomaviruses do not infect and replicate in antigen-presenting cells (APCs) located in the epithelium, and virus release does not cause lysis of keratinocytes, there is no opportunity for APCs to engulf virions and present virion-derived antigens to the immune system. Furthermore, there is no blood-borne phase of infection, so the immune system outside the epithelium has little opportunity to detect the virus. Thus, HPV infection is not accompanied by inflammation, and there is no obvious signal alerting the immune system to the virus's presence. This may result in persistent, chronic infection, as the host can remain ignorant of the pathogen for long periods.

On average, high-risk genital HPV infections persist for 12-18 months prior to being cleared by the immune system.[222, 223] This is considerably longer than the 5-6 months needed for the low-risk types.[224-226]

A fully functional immune system is necessary to clear HPV from the epidermis, as is illustrated by the critical importance of cell-mediated immune response in the resolution and control of HPV infections in immunosuppressed transplant patients, of whom approximately 77% develop warts at some time.[227]

T-cell immune response appears to be most important after the host has been infected and during wart regression.[228] The humoral immunity is of importance for the neutralisation and inactivation of virions, and thus for the prevention of the spread of HPVs to new sites

within the host and reducing the likelihood of re-infection. Protection appears to be largely type specific.

Natural HPV infection of the genital tract gives rise to a slow but modest measurable serum antibody response in most but not all infected individuals.[90, 229] The presence of HPV antibodies is long-lasting but does not contribute to clearance of established infections.[230]

Virus-neutralising anti-L1 antibodies are generated against epitopes at the surface of the viral capsid and are essentially type-specific.[90-93]

Antibodies elicited by natural infection with a specific HPV type do not confer protection, since seropositivity is not significantly associated with reduction in re-infection with homologous types.[231]

Patients who develop one HPV-associated tumour are at increased risk for a second one.[232-235] In renal transplant recipients, this association is almost certain to depend on their immunosuppression.

HPV VACCINE

The US Food and Drug Administration (FDA) recently approved a quadrivalent[26, 27] prophylactic HPV vaccine containing VLPs derived from the two most common oncogenic HPV types, HPV16 and 18, as well as VLPs derived from the two common genital wart types, HPV6 and 11. This vaccine has been tested in several Phase II trials, primarily in young women in their late teens or early twenties. These studies showed a substantial reduction in the incidence of infections with HPV16 and 18 which together cause 70% of cervical cancers worldwide.[28] Protection has been observed for at least 36 months following vaccination. The vaccine also showed protection against HPV6 and 11, which cause approximately 90% of genital warts.[29] In rare circumstances, HPV6 and 11 can cause serious diseases such as recurrent respiratory papillomatosis, a severe disease that may be fatal, and so-called giant condylomas or Buschke-Löwenstein tumours of the vulva, penis and anus.[185]

Phase II results have also been observed using a candidate vaccine[236]. This bivalent vaccine contains HPV16 and 18 VLPs, and very high levels of protection against HVP16 and 18 infection as well as HPV-associated cervical histologic abnormalities were observed in young women up to 27 months following vaccination with this vaccine.[237] Of interest, very

preliminary reports suggest that this bivalent vaccine might also induce some cross-protection against HPV31, 45, and 52.[237]

The quadrivalent and the bivalent vaccines are now under study in large Phase III trials[238] which should definitely demonstrate the ability of these vaccines to prevent acute and persistent HPV infections with the oncogenic or genital HPV types included in the vaccines, as well as to decrease the appearance of cervical intraepithelial neoplasia lesions, which are associated with, and are precursors of cervical cancer.

PRESENT STUDIES

AIMS

The purpose of the studies upon which this thesis is based was to:

- Investigate the persistence of cutaneous HPV infections over a period of several years, with special emphasis on expected differences between renal transplant recipients and healthy individuals.

- Characterise the complete genome of an HPV38 subtype, and screen for the presence of the prototype and the subtype in paired lesion and healthy skin biopsies from patients with non-melanoma skin cancer.

- Investigate whether sequence information of FA amplimers (~440 nt of the L1 ORF) is valid for taxonomic purposes, and if so identify putative subtypes in GenBank and in our in-house HPV database.

- Characterise new HPV types in the genus *Beta*-papillomavirus and investigate their prevalences in non-melanoma skin cancers.

- Examine the binding between the retinoblastoma tumour suppressor protein (pRb) and the E7 proteins of newly characterised HPV types.

MATERIALS AND METHODS

Sample collection

Swab samples

All swab samples analysed in the following studies were collected using cotton-tipped swabs pre-wetted with saline (0.9% NaCl solution). The swab was drawn back and forth approximately five times over the forehead skin in **paper I**, and over the lesions in **papers II, IV,** and **V**, and then suspended in 1 ml saline.

Stripped biopsies from non-melanoma skin patients

Before taking a biopsy, the skin was stripped with tape that was attached and removed five times, followed by a new piece of tape for another five times. Thereafter, a punch biopsy of approximately 2 mm in diameter was taken from the tumour.

The DNA from each biopsy was extracted and dissolved in 200 ml TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) using a simple phenol-free method.[110]

Paper I

Forehead swab samples from renal transplant recipients (RTRs) and sex- and age-matched healthy controls were collected from 1998-1999.[111] In 2005, new forehead swab samples were collected from 21 of the RTRs and 42 of the healthy individuals participating in the previously mentioned study. This resulted in a mean follow-up time of 6.3 years (range: 5.0-7.0 years).

At both sampling occasions, all subjects answered a questionnaire about presence/absence of warts, eczema, allergy, and history of any cancer.

All samples (from 1998/1999 and 2005) were tested *de novo* using the broad PCR primers FAP 59/64[110] followed by cloning and sequencing of three clones per sample.

Multivariate analysis was performed using questionnaire data from the baseline visit.

Paper II

In a previous study employing the above mentioned FA primers, a closely related HPV38 sequence, isolate FA125,[113] was detected in 16 samples from 978 stripped skin biopsies, while the prototype HPV38 was not detected at all.[126] In the present study, the complete genome of this isolate was completely characterised from a stripped punch biopsy taken from the forehead of an immunocompetent 78-year-old man with the diagnosis of actinic keratosis. The complete genome of the HPV38 subtype HPV38b8[FA125] was characterised through sequencing of overlapping PCR amplimers.

The presences and viral loads of this subtype as well as the prototype HPV38 were investigated in stripped skin lesions and paired healthy skin samples collected from patients with the diagnoses actinic keratosis (*n=52*), seborrhoeic keratosis (*n=47*), basal cell carcinoma (*n=118*), or squamous cell carcinoma (*n=52*) attending dermatology clinics in Sweden.

The samples were analysed with quantitative real-time PCR, and type specific primers and probes for the HPV38 and HPV38b[FA125] were designed using the Primer Express software program. No cross-hybridisation to any closely related HPV type was observed with any of the primer systems.

For all lesions positive for HPV38 or HPV38b[FA125], a swab sample taken from the top of the lesions before stripping was also analysed.

Paper III

Previous reports have stated that HPV subtypes are rare.[107, 116, 117] However, studies using the general FA primers have identified several putative type, subtype, and variant isolates (approximately 440 nt of the L1 ORF).[31, 113] Sequences of amplimers obtained with this PCR system have been compared to GenBank, and if not corresponding to any known HPV type, they have been given a provisional designation (FA-number). Currently our in-house HPV database contains approximately 220 such "FA" isolates.

A MedLine search identified seven HPV type-subtype pairs with a complete L1 ORF, and in order to investigate whether FA isolates can be used for taxonomic purposes, alignments of each of the seven genotype-subtype pairs as well as one type/subtype pair with complete L1 from our in-house database were performed using ClustalW, using sequence data from FA amplimers, from MY09/11 amplimers (approximately 409 nt of the L1 gene) and the complete L1 of each pair of genotype and subtype.

Paper IV

Two of the above mentioned FA fragments in our in-house HPV database are FAIMVS6 and FA47. Both of these isolates represent putative new HPV types, and the complete genomes of them are referred to as HPV93 and 96, respectively. HPV93 was isolated from a punch biopsy taken from the dorsum of the hand an immunocompetent 82-year-old man with the diagnosis of actinic keratosis, and HPV96 was isolated from a biopsy taken from an SCC *in situ* on the upper chest of an immunocompetent 75-year-old man.

The complete genomes of HPV93 and 96 were characterised through sequencing of overlapping PCR amplimers.

Prevalences and viral loads of HPV93 and 96, as well as the recently characterised HPV92,[239] were analysed in the same patient samples as used in paper II. The samples were analysed with quantitative real-time PCR, and type specific primers and probes for HPV92, 93, and 96 were designed using the Primer Express software program.

Furthermore, immunoprecipitation assays were performed to analyse the binding affinity between the retinoblastoma protein (pRb) and the E7 protein of HPV92, 93, and 96.

Paper V

In a recent study, a significant association between SCC and an infection with *Beta*-papillomaviruses in species 2 was detected.[126] Several putative new types within this species have been detected through previous studies, and we aimed at characterise the complete genome of at least one of these putative HPV types, isolate FA85, detected in 2.4% in of SCC samples in the study mentioned above.

HPV107, formerly known as FA85, was isolated and completely characterised from an immunocompetent 76-year-old male with the diagnosis of actinic keratosis.

The complete genome was characterised through sequencing of overlapping PCR amplimers covering the complete genome of HPV107.

The prevalences and viral loads of HPV107 as well as three other putative HPV types frequently detected in SCC[126] were examined in stripped skin lesions and paired healthy skin samples collected from patients with the diagnoses actinic keratosis (*n=50*), seborrhoeic keratosis (*n=46*), basal cell carcinoma (*n=118*), or squamous cell carcinoma (*n=50*) attending dermatology clinics in Sweden.

The FA fragments of HPV107, FA5, FA51, FA75, as well as closely related HPV types within species 2 of the *Beta*-papillomaviruses were aligned using ClustalW and a dendrogram was created using MEGA (Molecular Evolutionary Genetic Analysis) software, version 3.1.

RESULTS AND DISCUSSIONS

Paper I

Cutaneous Human Papillomaviruses persist on healthy skin

Overall, 71 different HPV types or putative types were detected in the collected samples. Among the healthy individuals, the prevalences of HPV were 69% (29/42) in the samples from 1998/1999 and 71% (30/42) in the follow-up samples from 2005. Of the 29 healthy individuals (17 females and 12 males) that were positive for HPV in their first sample, 48% (14/29) were positive for the same HPV type/putative type also in the second sample.

Among the renal transplant recipients (RTRs), 71% (15/21) were positive for HPV in their first sample, and 90% (19/21) were positive at follow-up. Among the 15 RTRs (seven women and eight men) positive for HPV in the first sample, 33% (5/15) had a persistent infection.

Overall, 70% (44/63) of the subjects were positive for HPV DNA in their first sample and a persistent HPV infection was detected in 43% (19/44) of them.

Most persistent infections, 15/19 (79%), were *Beta*-papillomavirus infections, with HPV20 being the single most common type-specific persistent infection.

Eighteen subjects were positive for HPV in both samples, but with different types.

In this study, we report that cutaneous HPVs commonly persist for many years on healthy skin, with about half (48%) of the healthy individuals being positive for the same HPV type six years later. Considering the immunosuppression of the RTRs and the fact that these patients commonly develop skin lesions with detectable HPV DNA, it was surprising that persistent infections were not more common in this group than in healthy individuals. Further, persistence was not significantly associated with age, sex, immunosuppressive treatment, history of warts, or genus of HPV.

The knowledge that viral persistence is common in the natural history of cutaneous HPV infections is of interest for understanding the biology of these viruses and may be helpful in the continuing elucidation of their possible role in human skin disorders.

Paper II

Subtype HPV38b[FA125] demonstrates heterogeneity of Human Papillomavirus type 38.

The complete genome of the HPV38 subtype HPV38b[FA125] (GenBank accession no. DQ090005) was 7,400 bp which correlates with the size of HPV38. The E6 and E7

proteins showed 100% identity to HPV38, whereas the E4 protein only showed 94% identity.

Five of the 15 nucleotides at the 3'-end of the upstream regulatory region (URR) were mismatches between HPV38 and HPV38b[FA125]. However, this difference did not affect any of the known binding sites for transcriptional factors.

Eight amino acids of the L1 ORF differed between HPV38 and HPV38b[FA125]. Comparison with the three-dimensional structure of the L1 ORF of HPV16 showed that six of the altered amino acids were most likely positioned in the hyper-variable loops that extend toward the HPV capsid surface. Furthermore, the E4 protein, thought to be a major target for CD4+ responses to HPV, showed only 94% sequence similarity between prototype HPV38 and its subtype. These results suggest a possible immunological significance of the subtype heterogeneity.

HPV38 was detected in 7 (2.6%) and HPV38b[FA125] was detected in 5 (1.9%) of the 269 lesions. Two of 269 healthy skin biopsies (0.7%) were positive for HPV38b[FA125], while none was positive for HPV38. Only one patient was positive for the same virus type both in the lesion and in the healthy skin sample. None of the samples were positive for both HPV38 and HPV38b[FA125] (Table 2).

Even when combining the prevalences of HPV38 and HPV38b[FA125], we found considerably lower prevalences of these viruses in skin lesions than many other studies. This discrepancy is likely to be attributable to the removal of superficial cells with tape, which is known to reduce HPV DNA prevalences.[113]

Both prototype HPV38 and its subtype HPV38b[FA125] were more commonly found in skin lesions than in healthy skin. However, there were higher copy numbers on top of the skin lesions than in the lesions themselves. Hence, the role of HPV38 in the development of NMSC remains elusive.

Table 2. Presence and viral loads of HPV38, HPV38b[FA125], HPV92, HPV93, HPV96, HPV107, FA5, FA51, and FA75 in skin lesions. (CV calculated on three values).

Diagnosis	Number of patients	HPV type	Viral copies/cells		
			Lesion (CV %)	Healthy skin (CV%)	Top of lesion (CV%)
AK[1]	*Paper II*	HPV38	1/349 (76)	-	12/1 (58)
	(n=52)	HPV38b[FA125]	1/183 (41)[2]	-	9/1 (31)
		HPV38b[FA125]	1/600 (39)	-	-
		HPV38b[FA125]	1/1,849 (0.6)[2]	-	-
	Paper IV	HPV92	1/298 (2)	-	NA[1]
	(n=52)	HPV93[3]	1/380 (88)	-	NA[1]
		HPV96[3]	1/1,410 (84)	-	NA[1]
		HPV96	1/3,238 (162)	-	NA[1]
	Paper V	HPV107	1/156 (15)	-	2/1 (14)
	(n = 50)	HPV107	1/1,141 (41)	-	1/13 (24)
		FA5	1/438 (29)	-	1/13 (12)
		FA5	1/877 (2)[2]	-	-
		FA5	1/910 (13)	-	-
		FA5	1/2,147 (54)	-	2/1 (29)[2]
		FA5	1/3,042 (2)	-	-
		FA51	1/2,109 (88)	-	1/13 (50)
		FA75	1/256 (25)	-	1/5 (19)
		FA75	-	1/97 (13)[2]	NA[1]
SK[1]	*Paper II*	HPV38	1/167 (37)[2]	-	NA[1]
	(n=47)	HPV38	1/1,960 (75)	-	-
		HPV38b[FA125]	1/524 (37)	1/133 (63)	-
		HPV38b[FA125]	1/12,336 (41)[2]	-	-
	Paper V	HPV107	3/1 (18)	-	-
	(n = 46)	HPV107	1/48 (42)	-	-
		FA5	-	1/83 (24)[2]	NA[1]
BCC[1]	*Paper II*	HPV38	1/67,407 (1.5)[2]	-	1/29 (38)
	(n=118)	HPV38b[FA125]	-	1/254 (44)	1/20 (51)
	Paper IV	HPV92	1/1,948 (33)	-	NA[1]
	(n = 118)	HPV96	1/953 (25)	-	NA[1]
	Paper V	HPV107	1/8 (65)	-	137/1 (19)
	(n = 118)	HPV107	1/279 (33)	-	1/2 (14)
		HPV107	1/2,378 (36)	1/21 (59)	1/18 (19)
		HPV107	-	1/4,104 (51)[2]	NA[1]
		FA5	-	1/354 (9)[2]	NA[1]
		FA5	-	1/1,768 (19)[2]	1/2 (21)
		FA51	1/333 (5)	1/231 (65)	-
		FA51	1/6,383 (69)	-	1/211 (46)
		FA51	1/22,649 (8)[2]	-	-

Diagnosis	Number of patients	HPV type	Viral copies/cells		
			Lesion (CV %)	Healthy skin (CV%)	Top of lesion (CV%)
SCC[1]	*Paper II*	HPV38	1/142 (27)	-	-
	(n=52)	HPV38	1/1,107 (64)	-	1/2 (18)
		HPV38	1/12,875 (21)[2]	-	9/1 (3.6)
	Paper IV	HPV92	1/114 (39)	-	NA[1]
	(n = 52)	HPV92[4]	1/10,325 (20)	-	NA[1]
		HPV93[4]	1/45 (15)	1/2,987 (45)	NA[1]
	Paper V	FA5	1/2 (59)	-	4/1 (17)
	(n = 50)	FA5	1/35,279 (19)[2]	-	-
		FA75	1/4,390 (22)[2]	-	-
		FA75	-	1/1,669 (5)	NA[1]

[1] AK = actinic keratosis, SK = seborrhoeic keratosis, BCC = basal cell carcinoma, SCC = squamous cell carcinoma, NA = not analysed.
[2] CV calculated on two values.
[3, 4] Double infections in two patients.

Paper III

Human papillomavirus subtypes are not uncommon

The estimates of sequence similarity using the complete L1 ORF and the FA fragments were equivalent. Eight out of nine subtypes were classified as subtypes also when using the information of the FA fragments, while HPV27b was misclassified as a variant. Comparison with the sequences amplified by the degenerate primer pair MY09/11, another of the most frequently used general primer system that has about the same fragment length as the FA primer system, found very similar results, including misclassifying HPV27b as a variant.

We concluded that estimation of sequence similarity based on FA amplicons, representing about 30% of the L1 gene, produced only minor classifications errors and proceeded to search GenBank and our in-house HPV database for sequences corresponding to the FA amplicon. We found 103 HPV types/putative type isolates within the genus *Gamma*-papillomavirus, which substantially outnumbered the 54 and 58 types/putative type isolates that we found in the genera *Alpha*- and *Beta*-papillomaviruses, respectively. However, subtypes appeared to be more frequently detected in the genus *Beta*-papillomavirus where we identified 30 subtype/putative subtype isolates compared to 10 subtypes/putative subtypes in the genus *Gamma* and only seven subtype isolates among the *Alpha*-papillomaviruses. Compared to the number of type isolates, there were significantly more

subtypes detected within the genus *Beta* (30 subtypes versus 58 types) than in the genus *Gamma*-papillomaviruses (10 subtypes versus 103 types) ($p>0.001$).

The amino acid identities between the nine HPV type-subtype pairs for the entire L1 protein was on average 96.5% (range 94.5-98.4%). The amino acid sequence diversity between HPV15 and its subtype FA161, between HPV57 and HPV57b, and between HPV38 and HPV38b clustered within the hyper-variable loops of L1, while the amino acid differences were more evenly distributed over the complete L1 gene for the other genotype-subtype pairs. In addition, it is interesting to note that HPV15, 57, and 38 all have a cutaneous tropism; whereas the viruses with L1 amino acid differences spread out over the L1 gene mostly have a mucosal tropism.

If HPV subtypes confer partial immunity to each other or are even immunologically distinct, this may have implications for the population dynamics and natural history of HPV infections. Our findings that HPV subtypes are relatively common would therefore seem to motivate further studies on the immunological relatedness of HPV subtypes.

Paper IV

Characterisation of two novel cutaneous Human Papillomaviruses, HPV93 and HPV96

The complete genome of HPV93 (GenBank accession no. AY382778) was 7,450 bp and was closest related to HPV24 with 79% sequence similarity in the L1 ORF. The complete genome of HPV96 (GenBank accession no. AY382779) was 7,438 bp and was closest related to HPV92 with 71% similarity in the L1 gene.

Phylogenetically, HPV93 was classified into species 1 of the *Beta*-papillomaviruses, whereas HPV96 represents the first HPV type within species 5 of this genus.

The upstream regulatory region (URR) of HPV93 and 96 was 397 bp and 399 bp, respectively, which is within the expected range for *Beta*-papillomaviruses.

The E6 protein of HPV93 and 96 both contained two conserved zinc-binding domains ($CxxC(x)_{29}CxxC$) identical to that of other HPV E6 proteins.

The E7 protein of HPV93 and 96 contained one zinc binding domain as well as the consensus pRb-binding motif (LxCxE).

HPV92 was detected in 3 (1.1%), HPV93 in 2 (0.7%), and HPV96 in 3 (1.1%) of 269 lesions. Double infections were observed in two samples, one from a patient diagnosed with AK and the other from a patient with SCC (Table 2).

pRb-binding assay

The immunoprecipitation assays showed that the E7 protein of HPV92, 93, and 96 possessed the ability to bind the tumour suppressor protein Rb. Similar results have previously been presented for HPV38 E7,[22] which support a possible role for cutaneous HPV types in the development of skin lesions.

Paper V

Isolation of a novel Beta-2 Human Papillomavirus from actinic keratosis

The complete genome of HPV107 (GenBank accession no. EF422221) was 7,562 bp, and was closest related to HPV80, with 74.3% sequence similarity in the L1 ORF.

HPV107, and the HPV isolates FA5 and FA75 were all closest related to HPV80 but showed only 71% (HPV107 and FA5), 79% (HPV107 and FA75), and 74% (FA5 and FA75) sequence similarity to each other, hence represents three separate HPV genotypes. Isolate FA51 was closest related to HPV38 with 76% sequence similarity in the FA fragment.

Phylogenetic analyses based on the FA fragments of HPV107, FA5, FA51, and FA75 classified them as *Beta*-papillomaviruses, in species 2, see figure 4.

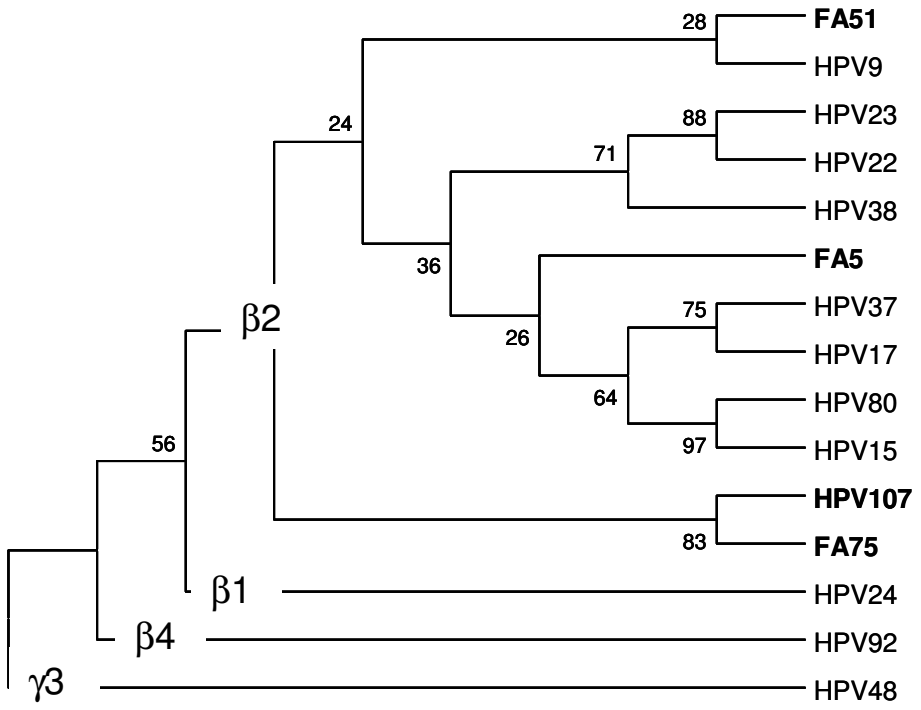

Figure 4. Dendrogram based on the alignment of FA fragments (~440 nt in the L1 open reading frame) of HPV107, FA5, FA51, FA75, and closely related HPV types. HPV24, 92, and 48 were added for orientation.

HPV107, FA5, FA51, and FA75 were more commonly found in skin lesions than in healthy skin, although there were higher copy numbers on top of the lesions, and only very low amounts in the skin lesions themselves (Table 2).
One patient was positive for HPV107, FA5, FA51, and FA75 with viral loads ranging from 1 viral copy/256 cells to 1 copy/2,109 cells. The top of lesion swab sample was also positive for all four types, while none of the viruses were detected in the corresponding healthy skin biopsy. This patient was also positive for HPV38, reported in paper II. These results confirm that multiple infections of HPV are common findings of the skin.

When considering the four viruses together, they were found most frequently in actinic keratosis lesions (9/50 lesions), which was significantly more often than in healthy skin (8/264 samples) (OR: 7.0, 95% CI: 2.2-22.0)

The characterisation of HPV107 expands the heterogeneity of HPV types in the genus *Beta*-papillomavirus, particularly in species 2. However, many additional putative types exist in this species and much more work will be required to fully elucidate its biological significance.

CONCLUDING REMARKS

Our results clearly show that not only infections with genital HPV types, but also cutaneous HPVs persist over time. With a mean time of follow-up of 6.3 years, nearly half of the healthy individuals had a persistent HPV infection. However, we found it surprising that persistent infections of HPVs were more common among healthy individuals than in renal transplant recipients, a patient group commonly developing skin lesion with detectable HPV DNA.

The knowledge that viral persistence is common in the natural history of cutaneous HPV infections is of interest for understanding the biology of these viruses and may be helpful in the continuing elucidation of their possible role in human skin disorders.

HPV persistence was particularly common among *Beta*-papillomaviruses, which is interesting since six new/putative new HPV types within this genus, and recently characterised by us, are presented in this thesis (HPV93, 96, 107, and the isolates FA5, FA51, and FA75). Another interesting fact is that HPV107, FA5, FA51, and FA75 are all classified into species 2, a group of viruses recently found to be significantly associated with SCC. However, much more work will be required to fully elucidate the biological significance if viruses in this species. However, *Beta*-papillomaviruses might be involved in early steps of development of skin cancer, and it is tempting to speculate that cutaneous HPV types may inhibit apoptosis in response to UV radiation and also to allow proliferation of genetically unstable cells. Among other things, this theory is supported by the ability of the E7 protein of HPV92, 93, and 96 to bind the retinoblastoma tumour suppressor protein (pRb).

HPV38 has been proposed to be a skin cancer-associated HPV type, and our characterisation of the subtype HPV38b[FA125] demonstrates heterogeneity of this type. Both the prototype and the subtype were more commonly found in skin lesions than in healthy skin. However, there were higher copy numbers on top of skin lesions than in the lesions themselves.

Furthermore, HPV subtypes seem to be more common than earlier anticipated, at least among HPVs with cutaneous tropism. It is interesting to note that the heterogeneity between cutaneous HPV subtypes and their genotypes predominantly clustered in the hyper-variable loops of L1, while mucosal HPV genotype/subtype heterogeneity was more evenly distributed over the protein.

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

En förutsättning för livmoderhalscancer är en bestående infektion med en av högrisk-typerna av genitala humana papillomvirus (HPV), till exempel HPV16 eller 18. Vi har nu visat att även infektioner med hud-HPV persisterar under en längre tid. Nästan hälften av de friska individerna i första studien testade positivt för samma HPV-typ efter 6.3 år, medan de njurtransplanterade patienterna i samma studie uppvisade betydligt lägre frekvenser av persistenta infektioner. Resultaten är intressanta eftersom dessa patienter har nedsatt immunförsvar med hänsyn till transplantatet, samt att de ofta utvecklar hudcancer, i vilka man i tidigare studier har detekterat DNA från HPV.

Våra resultat visade tydligt att det främst är HPV-typer inom klassificeringsgruppen *Beta*-papillomvirus (se figur 4 sid 20) som persisterar på huden. Detta är särskilt intressant med tanke på att samtliga sex nya/potentiellt nya HPV-typer som presenteras i den här avhandlingen (HPV93, 96, 107, FA5, FA51, och FA75) tillhör just denna grupp. Resultaten visar dock att en eventuell roll för HPV-typer i *Beta*-gruppen vid utvecklandet av icke-melanom hudcancer måste undersökas vidare, eftersom samtliga typer endast hittades i få prover samt i låga mängder. Virusen skulle dock kunna vara involverade i tidiga stadier av cancern, och det är frestande att spekulera kring möjligheterna för hud-HPV att förhindra den självprogrammerade celldöd som troligtvis slår till som svar på stark UV-bestrålning av huden, vilket i sin tur tillåter fortsatt proliferation av genetiskt skadade celler. Ett faktum som stödjer denna teori är att det virala proteinet E7 uttryckt av HPV92, 93 och 96 visade sig kunna binda till ett tumörreglerande kroppsligt protein.

I tidigare studier har HPV38 associerats med icke-melanom hudcancer. Vi visar nu på det faktum att HPV38 är ett heterogent virus, och att det utöver prototypen består av åtminstone en subtyp, HPV38b[FA125]. Både HPV38 och dess subtyp var vanligare i hudcancer än i frisk hud, men mängden virus var högre uppe på tumören än inne i densamma. Resultaten stödjer således inte hypotesen om att HPV38 skulle vara associerat med hudcancer.

Vad subtyper beträffar så verkar de i största allmänhet vara vanligare än vad som tidigare har rapporterats, framför allt hos hud-HPV. En detalj som var speciellt intressant är den att hos hud-HPV verkar olikheterna mellan prototypen och subtypen vara samlade i så kallade hypervariabla loopar i kapselproteinet L1 (se figur 2 sid 15), medan de hos genitala HPV-typer var mera jämnt spritt över hela proteinet.

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