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Polymorphism of cutaneous human papillomaviruses

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Polymorphism of cutaneous human papillomaviruses

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ABSTRACT

Human papillomaviruses (HPV) are etiologic agents of many epithelial tumors in humans. The broad spectrum of HPV-induced pathologies ranges from common warts to neoplastic lesions of the cervix uteri. Numerous recent studies reported the presence of HPV sequences in precancerous and malignant skin tumors. A particularly high prevalence of HPV DNA could be demonstrated in skin tumors of immunosuppressed transplant recipients. Surprisingly, a substantial proportion of HPV types involved turned out to belong to the larger group of HPV types originally believed to be exclusively associated with tumors in patients with epidermodysplasia verruciformis (EV). Of total 81 of cutaneous samples were collected from renal transplant recipients (RTR), skin squamous cell carcinoma (SCC), actinic keratosis (AK) and participants without skin lesion. Samples were analyzed using two published primer pairs (FAP59/64 and HVP2/B5) to detect HPV DNA in cutaneous samples. We were able to detect HPV DNA in 91% (68/75) from RTR, SCC, AK and participants without skin lesion. Direct sequencing and sequencing of cloned amplicons were performed and compared. By comparing FAP59/64 versus HVP2/B5 primer pairs we were able to detect HPV DNA in 100% (8/8) of RTR with FAP59/64 versus 13% (1/8) with HVP2/B5, in 88% (7/8) versus 25% (2/8) of participants with SCC, in 100% (8/8) versus 17% (2/12) of participants with AK and in 87% (41/47) versus 9% (4/47) of participants without skin lesion. Three novel types were described (LIO1, LIO2, LIO3). Multiples types were found in 75% (6/8) of RTR samples, 83% (10/12) of AK samples, 71% (5/7) of SCC samples, 50% (6/11) of samples from normal participants older than 50 years, 50% (15/30) of samples from normal participants younger than 50 years old.

Keywords: HPV, SCC, RTR, AK, PCR.

RÉSUMÉ

Les papillomaviruse humains (HPV) sont les agents étiologiques du cancer du col de l'utérus. Le spectre des pathologies induites par HPV s'étend des verrues communes aux lésions néoplasiques du col de l'utérus. Des études récentes ont rapporté la présence de séquences de HPV dans les lésion précancéreuses et malignes de la peau. Un taux élevé de détection d' ADN de HPV est démontré dans les tumeurs de la peau de patients ayant recu une greffe de rein. Etonnamment, une proportion substantielle des types de HPV impliqués est révéléé appartenir au plus grand groupe de types de HPV associés aux tumeurs des patients avec epidermodysplasia verruciformis (EV). 81 échantillons cutanés recueillis chez des patients receveurs d'une greffe rénale (RTR), avec carcinome squameu de la peau (SCC), de kératose actinique (AK) et sans lésion de la peau, ont été analysé par PCR avec les amorces dégénérés FAP59/64 et HVP2/B5. Ces paires d'amorces ont été utilisée pour détecter l'ADN de HPV dans de échantillons cutanés. Nous pouvions détecter de l'ADN de HPV dans 91% (68/75) des spécimens. Le typage par séquençage direct, après clonage des amplicons a été exécuté et les resultats comparés. En comparant FAP59/64 et HVP2/B5, nous pouvions détecter de l'ADN de HPV pour 100% (8/8) des patients avec RTR avec FAP59/64 contre 13% (1/8) avec HVP2/B5, pour 88% (7/8) contre 25% (2/8) des participants avec SCC, pour 100% (8/8) contre 17% (2/12) des participants avec AK et pour 87% (41/47) contre 9% (4/47) des participants sans lésion de la peau. Trois types originaux ont été décrit (LIO1, LIO2, LIO3). Plusieurs types de HPV ont été trouvé dans 75% (6/8) des échantillons de RTR, 83% (10/12) des échantillons de AK, 71% (5/7) des échantillons de SCC, 50% (6/11) des échantillons des participants normaux âgés de 50 ans et plus, 50% (15/30) des échantillons des participants normaux plus jeunes que 50 ans.

Les mots clés : HPV, SCC, RTR, AK, PCR.

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LIST OF ABBREVIATIONS

AK :	Actinic keratosis
BPV:	Bovine papillomavirus
BCC:	Basal cell carcinoma
Bp:	Base pair
CIN:	cervical intraepithelial neoplasia
CSF-1R:	Colony-stimulating factor-1 receptor
DNA:	Deoxy-riboneuclic acid
EGF:	Epidermal growth factor
EGFR:	Epidermal growth factor receptor
EV:	Epidermodysplasia verruciformis
FISH:	Fluorescence in situ hypridization
HPV:	Human papillomavirus
Kd:	Kilodalton
LCR:	Long control region
MED:	Minimal erythema dose
NMSC:	Non-melanoma skin cancer
NCR:	Non-coding region
Nt:	Nucleotide
NIKS cells:	previously named Bcl-Ep/SL cells
ORF:	Open reading frame
PCR:	Polymerase chain reaction
PV:	Papillomavirus
PDGFR:	Platelets-derived growth receptor B
PUVA:	Psoralen and UVA light treatment
RTR:	Renal transplant recipients
RNA:	Riboneuclic acid

SV40:	Simian vacuolating Virus 40
SCC:	Squameous cell carcinoma
UVR:	Ultraviolet rays
UV:	Ultraviolet
UVA:	Ultraviolet A
UVB:	Ultraviolet B
UVC:	Ultraviolet C
VW:	Viral warts
WT:	Wild type

DEDICATE

I dedicate this memory to my parents for their encouragement and support, to my husband and my kids.

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My thanks go to my research director Dr. Francois Coutlee, for his availability all during the course of my master. My thanks go also to the attending research Simon Gagnon and Pierre Forest.

LITERATURE REVIEW

Introduction

Human papillomaviruses (HPV) are small double-stranded DNA viruses found in a wide variety of proliferative lesions of epithelial origin. In recent years there has been a considerable increase in the number of identified human papillomavirus types. There are currently more than 100 distinct types based on DNA sequence homology, but several groups and partially characterized novel sequences have now been described, predicting the existence of many more. Accumulating epidemiological and experimental data strongly support that there is a relation between HPV infection and benign or malignant neoplasia. Although the nature of the association is not definitive, certain cutaneous HPV types are found in a high percentage of non-melanoma skin cancers (NMSC) but no HPV types have been associated specifically with NMSC in the general population. Some cutaneous HPV types (HPV-1, 2, 4, 7, 57) are associated with benign planter/palmer and common skin warts [1]. On the other hand, evidence suggesting an etiologic role for HPV in NMSC comes from studies on the rare disorder "epidermodysplasia verruciformis" (EV). About 30% of patients with this disease develop squamous cell carcinomas (SCC) of the skin, especially on sun-exposed sites [2].

In recent years, several consensus primer-mediated PCR techniques that allow for the detection of a wide range of HPV genotypes have been designed. Many of these methods have been used for detection of HPV types in healthy skin as well as in NMSC. These methods have included single round PCR using one pair of degenerate primers [3], combinations of degenerate primers [4,5,6] or nested PCRs using two pairs of degenerate primers [7]. In our study we compared two primer pairs (FAP59/64 and HVP2/B5) optimized to detect cutaneous HPV infection [3,4,8,9,10,11].

1 HUMAN PAPILLOMAVIRUS:

1.1 History

Human papillomaviruses (HPV) are small, non-enveloped double-stranded DNA epitheliotropic viruses [1]. They belong to the papillomaviridae family, members of which infect squamous epithelia and cause proliferative diseases (papilloma) in a number of vertebrates, including man, non-human primates, cattle, rabbits and dogs, in a highly species-specific manner.

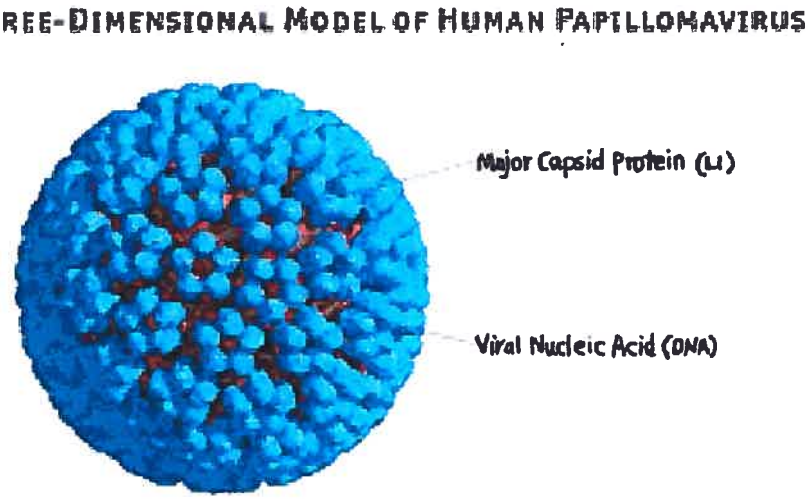

Figure (1): Human papillomavirus

HPV was the first tumor virus to be transmitted experimentally from one host to another. This was accomplished in 1894 by Licht [2] who transmitted warts from his brother to himself by inoculation of crude wart material. Ciuffo [3] in 1907 and Serra [4] one year later demonstrated that warts could be induced by cell- free filtrates of wart material. In 1919, Wile and Kingrey [5] successfully transmitted warts through a succession of human volunteers using sterile extracts of wart material. Electron

microscopic studies by Strauss et al [6], Melnick et al [7], Bunting [8] and Almeirta et al [9] confirmed a viral etiology for cutaneous warts.

Melinck in 1962 classified the papillomaviruses together with the polyomaviruses and SV40 (Simian Vacuolating Virus 40) in the papovaviridae family because they were small DNA viruses sharing ultra-structural features [10]. Reports of transmission of filtrates of warts, laryngeal papillomas, and genital tract condylomas to human volunteers who developed typical cutaneous warts at the sites of inoculation were interpreted as indicating that there was one type of human wart virus and that the site of infection and perhaps, the genetic makeup of the patient, determined the clinical appearance of cutaneous warts and mucosal papillomas [11]. However, recognition that there were different papillomavirus types and subtypes stimulated a new interest in the role of these viruses in hyperplasia and neoplasia arising in squamous epithelia of the anogenital and digestive tracts [12,13,14,15,16].

1.2 HPV Structure and Biology:

1.2.1 General biology of HPV

HPV virons are non-enveloped icosahedral capsids (50 to 55 nm in diameter) of 72 capsomeres [17]. They do not contain lipids and are inactivated by treatment with 0.4% formalin for 72 hours at 4°C [18]. Both complete and empty particles may be found in tissue samples [19,20].

Molecular analysis of PV DNA was first determined by Crawford [21] and Crawford [19,20]. Using stringent hybridization techniques without the aid of various restriction endonucleases, it was concluded that although PV from various species had similar structures and molecular weights, there was no polynucleotide sequence shared among the different genomes analyzed. The PV genome is found in virions and infects cells in three forms: a covalently closed, supercoiled molecule with a sedimentation coefficient of 23 S, uncoiled circular molecule with a sedimentation coefficient of 17 S,

and a linear molecule with a sedimentation coefficient of 16 S. [22,23]. The molecular size of the genome based on agarose gel electrophoresis and contour length measurement of DNA molecules by electron microscopy reveals a molecular weight of approximately $5x10^6$ Daltons, corresponding to 8000 base pairs, which is sufficient to code for proteins of 300,000 Daltons [12,13].

1.2.2 Molecular biology of papillomavirus oncogenes:

Papillomavirus genomes for each type contain 9-10 open reading frames. With variable splicing patterns, they have the potential to synthesize 12-15 gene products. The open reading frames labeled "E," for early, represent those genes in bovine papillomavirus (BPV) which were thought to be involved in episomal replication in cultured cells. The late, or "L," genes encode the viral capsid proteins. The number after E or L refers to the size of the peptide coded by the open reading frame, one being given to the largest peptide [24].

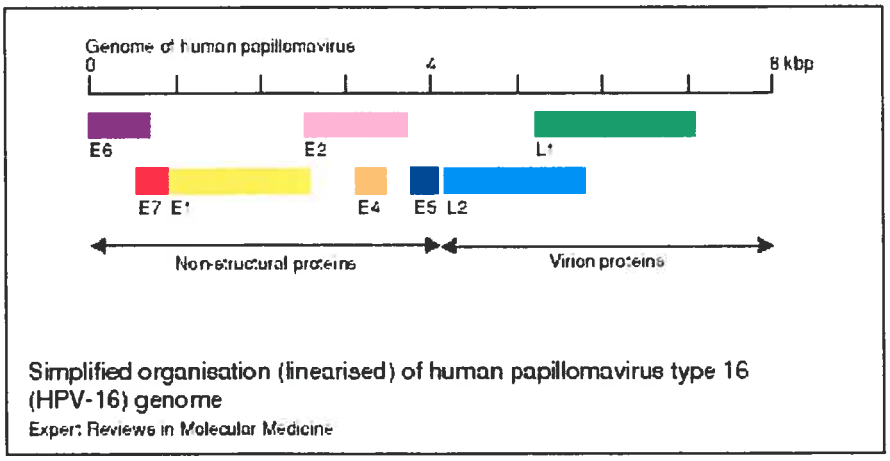

Figure (2): Simplified organization of the linearised genome of human papillomavirus type 16. The scale bar is in kilobase pairs. The rectangles represent the positions of various open reading frames (ORFs). The E genes encode proteins that are produced early in the infectious cycle (the non-structural proteins), whereas the L genes encode proteins that are produced late in infection (virion proteins that are necessary for virus assembly). The actual protein products of the genes are complex owing to the production of multiple messenger RNA (mRNA) transcripts.

Table (1): HPV gene products and their function.

Gene	Function
E1	Initiation of DNA replication
E2	Transcriptional regulation/DNA replication
E3	?
E4	Disrupts cytoskeleton?
E5	Transforming protein, interacts with growth factor receptors
E6	Transforming protein, binds to p53, leading to degradation of p53
E7	Transforming protein, binds to pRB
E8	?
L1	Major capsid protein
L2	Minor capsid protein

1.2.2.1 The E6 oncoprotein:

Papillomavirus E6 protein consists of about 150 amino acids believed to bind a zinc atom through two sets of cysteine repeats (cysteine X-X cysteine zinc fingers, where X is any amino acid) [25,26]. The human papillomavirus E6 proteins have moderate homology at the amino acid level, indicating that while they share functions, they may also differ. HPV-16 E6 has a half-life of 30-60 min and is present in transformed and cancer-derived cell lines at extremely low levels [27]. Several reports have demonstrated that high and low-risk E6 genes can stimulate transcription equally, suggesting that some E6 function may be relevant to viral replication rather than correlate with oncogenic potential [28,29,30].

The high-risk HPV-16 and 18 E6 proteins interact with p53, as do SV 40 large T antigen and adenovirus E1b [31]. HPV and other viruses presumably interfere with the ability of p53 to block cell division and DNA synthesis so that viral DNA can replicate to high levels. Indeed these mutations could permit genetic drift that can circumvent immune defenses. SV40 and adenovirus prevent p53 mediated cell cycle arrest by synthesizing large amounts of large T and E1b, which effectively hold p53 in inactive complexes. E6 protein eliminates p53 functions through a novel mechanism. It has been shown that formation of the E6-p53 complex in vitro induced p53 degradation through an ubiquitin-dependent mechanism [32]. High-risk E6 binds a 100-kDa protein, called E6-AP (for E6 Associated protein), which appears to be required for the binding of E6 to p53, and is necessary for degradation of p53 [33]. The gene for E6-AP is a member of the ubiquitin pathway for protein degradation [34]. It has been reported that although low-risk (HPV-6 and 11) E6 also binds p53 with reduced efficiency, it is not capable of inducing p53 degradation [35].

Despite these important findings, there are several lines of evidence that suggest that E6 possesses other functions. BPV E6 does not bind p53, yet fully transforms murine C127 cells [36]. BPV E6 and HPV6 E6, which do not induce p53 degradation in vitro or in vivo, can immortalize human mammary epithelial cells, although they are much less efficient than HPV-16 [37]. HPV-8, which is found in cutaneous SCC in patients suffering from epidermosysplasia verruciformis, does not bind or degrade p53 in vitro but, similarly to BPV E6, transforms mouse cells [38,39].

1.2.2.2 The E7 Oncoprotein:

The E7 oncoprotein is an acidic 98 amino acid phosphoprotein that has been localized in the nuclear matrix [40]. Two cysteine-X-X-cysteine motifs in the carboxy terminus of the protein mediate zinc binding and dimerization [41]. The recognition of the amino acid similarities between the HPV E7 protein and the DNA tumor virus transforming proteins SV40 Large T and adenovirus E1a facilitated elucidation of the biochemical properties of E7 [42,43]. These regions of similarity were shown to bind the

tumor suppressor gene product Rb and the related p107 proteins [44,45,46,47]. Both Rb and p107 regulate cell cycle division but act at different steps. The p107 binding region of E7 overlaps with but can be distinguished from the Rb binding domain [48,49].

It is though that when E7 binds Rb or p107, a transcription factor normally bound to Rb/p107 is released, because E7 and the factor bind the same pocket [50,51]. This factor, called E2F, is a sequence-specific DNA binding protein, and the DNA motif it recognizes is found in many genes essential for cell division [52]. There is evidence that the events induced by the Rb/ p107 association with E7 can lead to a complex yet coordinated cascade of positive and negative signals that allow a cell to replicate its DNA and divide. Other regions of E7 bind additional growth-related cellular factors. HPV-16 E7 has been identified in complexes with histone kinase, $p33^{cdk2}$ and cyclin A and casein kinase II, which phosophorylation may in part relate to the oncogenic potential of high-risk E7 [53,54,55,56].

Both HPV-16 and 18 E7 induced focus formation in murine cell line transformation assays [57,58]. Consistent with its interaction with Rb/p107, addition of E7 alone into primary human keratinocytes resulted in an increased rate of proliferation for an extended period of time, although cells eventually senesced [59]. Using very high-efficiency retroviral-mediated infection of primary human keratinocyes, it has been shown that low-risk HPV-6 E7 can cooperate with high-risk E6 to induce immortalization of primary human keratinocyes, and in the alternative mixing experiment, HPV-6 E6 cooperated with HPV-16 E7 to induce immortalization, although in both instances, the efficiency was less than with high-risk E6 and E7 [60]. As with E6, there remain unidentified properties of E7 that are required for its transforming activities. For example, mutations in E7 that do not affect Rb/p107 association interfered with its ability to transform and immortalize cells [61].

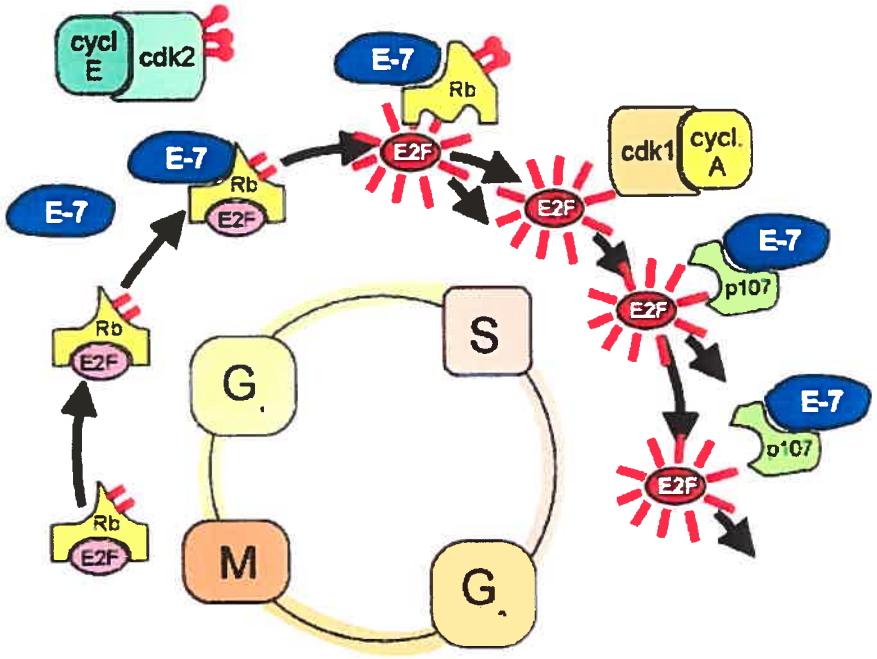

Figure (3): E7 Effects on Rb. E7 binding to Rb leads to the release of sequestered E2F, enabling the cell cycle to progress

1.2.2.3 The E5 oncoprotein:

The E5 protein represents another fascinating and ingenious means developed by papillomaviruses to prime cells for viral replication. An E5 gene has been identified in bovine, deer, elk, and some human papillomaviruses. These animal viruses cause dermal fibroblast proliferation (fibropapillomas) along with an epithelial component in their hosts. E5 protein is small: the BPV E5 gene product contains only 44 amino acids. The HPV E5 gene is often deleted or its gene not expressed in human cervical carcinomas, whereas E6 and E7 are always transcribed. This suggests that E5 plays a role at an early stage of viral carcinogenesis. BPV-1 and HPV-6c E5 have been reported to transform established murine fibroblast cell lines, whereas HPV-16 E5 did not [36,62,63].

Introduction of HPV-16 E5 into established murine keratinocyte lines induced their tumorigencity in nude mice, suggesting a potential role in human malignancy [64].

In contrast to the HPVs, the major transforming protein of bovine papillomavirus type 1 (BPV1) is the E5 protein, a 44-amino-acid highly hydrophobic protein that localizes predominantly to the Golgi and exists as homodimers [65,66,67]. The BPV1 E5 protein is able to transform both murine fibroblasts and keratinocytes in transformation assays in vitro [64,68]. The BPV1 E5 protein is able to bind to and activate the platelet-derived growth factor β receptor in the absence of exogenous ligand [69,70], and this has been shown to correlate with cellular transformation [71]. BPV1 E5 also binds to the 16-kDa pore-forming membrane component of the vacuolar proton ATPase (v-ATPase), a protein essential for the acidification of intracellular compartments such as lysosomes, endosomes, and the Golgi [72]. The binding of BPV1 E5 to the 16-kDa protein is able to cause alkalization of the Golgi, and this has been shown to correlate with cellular transformation [73]. Because of the structural similarities between the BPV1 E5 and HPV-16 E5 proteins, and because BPV-1 E5 has strong transforming potential, work was begun to determine if HPV-16 E5 was also an oncogene.

E5 seems to be important early in the course of infection. It stimulates cell growth by forming a complex with the epidermal growth-factor receptor, the platelet-derived growth-factor-B receptor and the colony-stimulating factor-1 receptor [74]. Recently, E5 has also been shown to prevent apoptosis following DNA damage [75]. However, as HPV-infected lesions progress to cervical cancer, the episomal viral DNA frequently becomes integrated into host-cell DNA. And a substantial part of the genome, commonly including the E5 coding sequence, is deleted [76]. So, E5 is not obligatory in late events of HPV-mediated carcinogenesis.

Unlike E6 and E7, the major viral oncoproteins, the E5 protein of HPV-16 is not commonly found in cervical carcinoma cells [77,78]. However, it is considered an oncogene given its ability to transform mouse fibroblasts and keratinocytes, cause mitogenic stimulation of human keratinocytes, and cooperate with E7 to stimulate

proliferation of human keratinocytes [64,79,80,81,82]. The E5 gene of HPV-16 is a 83-amino-acid hydrophobic membrane protein [83,84] that localizes to the Golgi apparatus, endoplasmic reticulum, and nuclear membrane [85].

Multiple studies have suggested that the HPV-16 E5 gene could interact with epidermal growth factor receptor (EGFR) signaling. Studies indicate that HPV-16 E5 causes an increased activation of the EGFR in the presence of ligand [79,80,81], and coimmunoprecipitation experiments indicate that HPV-16 E5 can form a complex with growth factor receptors [74]. The HPV-16 E5 protein also binds to the 16-kDa membrane component of the v-ATPase [85] and delays endosomal acidification in human keratinocytes [86]. It has been argued that in binding with the 16-kDa protein, E5 disrupts the 16-kDa protein-v-ATPase complex [87,88], which results in the inhibition of endosomal acidification.

E5 acts during the productive stage of the HPV-16 life cycle. A study showed that HPV-16 E5 mutants infecting basal cells results in a lower percentage of supra-parabasal cells undergoing DNA synthesis compared with cells harboring the (Wild Type) WT HPV-16 genomes [91]. Previously, it had been reported that E7 plays a critical role in the productive stage of the HPV-16 life cycle [89]. In that study, it was demonstrated that HPV-16 E7 mutants do not reprogram supra-parabasal cells to support DNA synthesis, and this defect correlates with the absence of viral DNA amplification. That study also established that cells harboring HPV-16 E7 mutants failed to modulate the differentiation program of raft cultures of NIKS cells and displayed reduced expression of L1, the major viral capsid protein expressed in the productive stage of the HPV-16 life cycle. In contrast, the HPV-16 E5 mutant genomes did modulate the differentiation program in raft cultures of NIKS cells, as was seen in NIKS harboring the WT HPV-16 genome. HPV-16 E5 mutants also expressed late viral proteins in the productive stage of the HPV-16 life cycle at the same levels as seen with HPV-16 WT. HPV-16 infected cells retained viral DNA amplification as analyzed by FISH. Thus, E5 plays a more subtle role during the productive stage of the viral life cycle than does E7.

A parallel study of E5 in the context of the HPV-31 life cycle by Fehrmann et al. [90] has also shown a subtle effect of E5 during the productive stage of the viral life cycle. In both studies, the disruption of E5 had no observable effect on the non-productive stage of the viral life cycle. Specifically, HPV-16 and HPV-31 with E5 mutations [90,91] could be maintained as nuclear plasmids. The E5 variation in HPV-16 was a deletion of an adenosine at position 30 of the E5 sequence. Cells harboring these E5 mutant genomes displayed similar growth kinetics in the absence or presence of EGF in monolayer cultures compared to cells harboring the WT HPV genome. In contrast, both studies noted defects during the productive stage of the viral life cycle.

Cells harboring HPV-16 E5 mutants displayed a significant reduction in the percent of supra-basal cells undergoing DNA synthesis in raft culture. They also found a lower induction of cyclins A and B and lower retention of proliferative potential in cells harboring HPV-31 E5 mutants, compared to those harboring WT genomes, upon suspension of those cell populations in semi-solid medium. In both studies, cells harboring E5 mutants retained the ability to amplify viral DNA upon induction of cellular differentiation. The investigators discerned a two-fold decrease in the degree of amplification in cells harboring E5 mutants compared to those harboring WT HPV-31 by Southern analysis. They also observed a twofold decrease in the frequency of cells supporting amplification of the E5 mutant genome, based on FISH analysis of organotypic raft cultures. However, this difference was not statistically significant. Southern analysis of DNA extracted from HPV-16 WT and HPV-16 E5 mutant rafts cultures was performed as described by Ozbun and Meyers [92] but they could not detect viral DNA amplification in the HPV-16 WT rafts or in rafts generated with a clone of cervical epithelial cells (W12E cells) that harbored episomal HPV-16 DNA. They believed that this absence of detectable amplification was due to the very low percentage of cells (0.09 to 3.48%) within a raft supporting viral DNA amplification as shown by their FISH analysis [91].

Studies have indicated that HPV-16 E5 is able to cooperate with E7 to induce proliferation, enhance immortalization, and promote anchorage-independent growth of

baby rat kidney cells [79,93]. In these studies, it was found that transfection of E5 alone into primary rodent cells had little effect on proliferation of these cells and that E7 alone was able to increase proliferation in comparison to the vector alone. However, cotransfection of E5 and E7 resulted in a significant increase in the amount of proliferating colonies over that of E7 alone. Considering that E5 contributes to the capacity of HPV-16 to reprogram differentiating cells to support DNA synthesis, a property also reliant on E7 [89,91], E5 could play a cooperative role with E7 in the productive stage of the viral life cycle [91].

The mechanism by which HPV-16 E5 is contributing to the productive stage of the viral life cycle is not yet clear. Many studies have suggested a link between HPV-16 E5 protein and the EGFR signaling pathway. These studies suggest that when treated with EGF, E5-expressing cells display anchorage-independent growth [81], increased mitogenic potential [62,82], and increased growth factor receptor signaling (with or without EGF) [67,94]. Whereas Laimins et al found little, if any, EGFR present in keratinocytes following suspension in semi-solid medium, other investigators clearly found that EGFR was present in the superficial layers of raft cultures of early-passage human foreskin keratinocytes or NIKS, albeit at lower levels than those observed in the basal layers [91,95]. A similar expression pattern of the EGFR in basal as well as supra-basal compartments of raft cultures has been reported in the context of an HPV-31-positive CIN 1 lesion-derived population, the CIN 612 9E cells [96]. In that study, the authors also monitored expression of HPV-31 E5 in the context of raft cultures and found that E5 protein levels were induced in a time-dependent manner, suggesting that its expression is tied to the differentiation and stratification of epithelial cells. Consistent with this observation, they detected E5-positive cells within the more superficial layers of the CIN 612 9E raft cultures. These data indicate that both E5 and one of its known targets, EGFR, are expressed within the terminally differentiating cell compartment in which we have observed an effect of E5 during the productive stage of the viral life cycle.

The binding of HPV-16 E5 to the 16-kDa component of the v-ATPase may also be important in E5's contribution during the productive stage of the viral life cycle [85]. It

has been shown that endosomal acidification of HPV-16 E5-expressing cells is inhibited and that this can lead to increased receptor recycling to the cell surface [86]. Another study has suggested that HPV-16 E5 affects trafficking from endocytic compartment rather than endosomal acidification [97]. Studies under way will allow us to determine which of these mechanisms are involved in E5's contribution during the productive stage of the viral life cycle.

1.2.2.3.1 Interaction of E5 with cellular factors:

BPV-1 E5 forms complexes with different transmembrane proteins. Thus, E5 directly binds to the transmembrane domain to the platelet-derived growth receptor B (PDGFR) and functionally interacts with the epidermal growth factor receptor (EGFR) and the colony-stimulating factor-1 receptor (CSF-1R), [69,98,99]. Through these interactions, the receptors may be activated in a ligand-independent manner. Moreover, their signals, e.g. increased receptor phosphorylation, mitogen activated protein kinase activity and phospholipase C-γ-1 activity are enhanced even in the absence of a physiological ligand [67,100]. This may result from interference with receptor degradation and internalisation [101,102].

HPV-16 and HPV- 6 E5 also have been shown to co-operate with the EGFR and PDGFR [80,81,82]. Moreover, HPV-16 E5 enhances endothelin receptor signaling [103]. Whereas HPV-6 E5 also associates with the EGFR, the related erbB2 receptor and the PDGFR, HPV-16 E5 does not bind to cellular growth factor receptors [104]. Another common target of both BPV and HPV E5 proteins is a 16 kDa membrane pore protein representing a subunit of the H^+-dependent vacuolar ATPase [72,85]. For ATPase binding, the glutamine residue within the hydrophobic domain seems to play an important role [105]. As a result of this interaction, acidification of endosomes is inhibited. This has been suggested to be responsible for the prolonged retention and reduced degradation of the EGFR in the presence of the E5 protein [86]. However, the binding of HPV-16 E5 to the 16 kDa ATPase subunit could be dissociated from the E5-mediated EGFR overactivation [106].

1.3 Classification of HPV:

Until the late 1970s, papillomaviruses attracted little interest as they were only known as the causal agents of "warts", benign cutaneous lesions in some mammals and humans. Since warts are normally only a cosmetic problem but not a major threat to public health, they were mostly of academic interest. In the 1980s, newly developed powerful molecular biology techniques led to the detection of dozens of human papillomaviruses in benign and malignant mucosal lesions [14,107], such as cervical cancer and its precursor lesions, as well as in genital and laryngeal warts. Present data support the existence of more than 100 HPV types. The whole genomes of about 100 HPV types have been isolated and completely sequenced, while we have only indirect evidence for the other types, most often through the sequence of polymerase chain reaction (PCR) amplicons, which unambiguously identified the presence of HPV-related sequences. Description of HPV types has changed several times in parallel with technical progresses and phylogenetic analysis. An HPV type is now defined as an isolate whose L1 gene sequence is at least 10% dissimilar to that of any other known HPV types.

1.3.1 Classification according to sequences nucleotides:

In the mid-1950s to 1960s, papillomaviruses and polyomaviruses were analyzed by electron microscopy and basic nucleic acid analyses. These two groups of viruses were found to be the only one with double stranded circular DNA genomes, and non-enveloped particles consisting of icosahedral capsids. As a consequence, they were considered closely related and were placed into a common family, the papovaviruses (Papoviridae). Sequence and functional studies in the 1980s showed that these similarities were too superficial to establish relationship [108]. All polyomaviruses have a genome size around 5 kb, while the genome of papillomaviruses is close to 8 kb. Polyomaviruses transcribe mRNA from both strands, while papillomavirus transcription occurs only in one direction. And lastly, and most importantly, polyomaviruses and papillomaviruses do not share any substantial amount of nucleotide or amino acid sequence similarity, with the exception of a small homologous segment in the T-antigen and E1 genes, respectively

[109]. Since taxonomic classification should reflect natural relationships, it was concluded that these viruses form two separate families. Only fairly recently, the family "papillomaviruses" (Papillomaviridae) became officially recognized by the International Council on Taxonomy of Viruses (ICTV) [110].

1.3.1.1 Papillomavirus types:

Papillomaviruses are identified by the abbreviation PV and one or two letters indicating the host species. This can be derived from an English word, for example "HPV' for human papillomaviruses and "CRPV" for cottontail rabbit papillomaviruses, or the scientific name of the host, e.g. MnPV for Mastomys natalensis papillomavirus, which infects an African rat. HPV types are identified by numbers considering the historic sequence of their description, e.g. HPV-1, HPV-2, etc. [111]. Presently, and for the last 20 years, E.M. de Villiers at the Reference Center for Papillomaviruses at the German Cancer Research Center in Heidelberg has controlled this process [112]. New HPV types have to be registered by this center to confirm completeness of the genomic isolate. New types have to show 10% nucleotide sequence diversity in the L1 gene from all known HPV types. Thereafter, assignment of a new number and publication of this new HPV types is possible. HPV types, whose genome was generated by PCR rater than traditional cloning techniques, are identified by addition of the abbreviation "cand" (for candidate) before their number, e.g. candHPV-86 [110].

1.3.1.2 Subtypes:

The term "subtypes" was used in the 1980s to identify isolates of an HPV type with different restriction nuclease digestion patterns. Subsequently, this term became redefined as referring to an isolate whose L1 sequence is 2-10% different from that of any known type. A consequence of this redefinition was that several subtypes (e.g. HPV-6a, HPV-6b and HPV-6c) had to be eliminated, as they showed less than 2% sequence diversity. Surprisingly, today only three HPV isolates are known to fulfill this latter

subtype definition. HPV-46, HPV-55, and HPV-64 had been originally described as separate types, but their types status has now been cancelled, as they are subtypes of HPV-20, HPV-44, and HPV-34, respectively [110].

1.3.1.3 Variants:

"Variants" of HPV types differ by at most 2% in the ORF sequences or by at most 5% in the LCR of the original isolate characterized, also referred as "prototype" or "reference genome". Variants are identified by PCR-sequencing mainly. This strategy has been applied to numerous HPV types from isolates throughout the world [113,114,115,116,117,118]. The two principal observations from these studies are that there is apparently only a limited number (for example 20-100) of common variants for each HPV type, and that variants showed maximal divergence when they were sampled from different groups living in different countries. HPV types have these coevolved with the human species [119]. HPVs did not infect humans from an animal reservoir such as for some other viruses, like Ebola, the SARS coronavirus, or influenza. There are indications that variants of the same HPV type differ biologically and etiologically [120,121]. Such differences may contribute to the disparities in the incidence of cervical cancer throughout the world, although this question still requires substantial research before conclusions can be drawn [122].

1.3.2 Classification according the site of infections (Genital, cutaneous or mucosal):

It was found that different HPV types associated with similar lesions are sometimes only very distantly related to one another. For example, HPV-1, HPV-2, HPV-4 and HPV-41 are all found in wart-like cutaneous lesions, but are on remote branches of evolutionary trees. HPV-16 and HPV-18, the two HPV types that have become paradigms in the research on cervical carcinogenesis, are less related to one another than to some HPV types that are never found in cervical malignancies.

Table (2): Cutaneous types of HPV.

HPV Type	Non-genital Cutaneous Disease
1, 2, 4, 26, 27, 29, 41, 57, 65	Common warts (verrucae vulgaris)
1, 2, 4, 63	Plantar warts (myrmecias)
3, 10, 27, 28, 38, 41, 49	Flat warts (verrucae plana)
1, 2, 3, 4, 7, 10, 28	Butcher's warts (common warts of people who handle meat, poultry, and fish)
2, 27, 57	Mosaic warts
16	Ungual squamous cell carcinoma
2, 3, 10, 12, 15, 19, 36, 46, 47, 50	Epidermodysplasia verruciformis (benign)
5, 8, 9, 10, 14, 17, 20, 21, 22, 23, 24, 25, 37, 38	Epidermodysplasia verruciformis (malignant or benign)
37, 38	Non-warty skin lesions

Table (3): Mucosal types of HPV (non-genital).

HPV Type	Non-genital Mucosal Disease
6, 11	Respiratory papillomatosis
6, 11, 16, 18	Squamous cell carcinoma of the lung
6, 11, 30	Laryngeal papilloma
16,18	Laryngeal carcinoma
57	Maxillary sinus papilloma
16,18	Squamous cell carcinoma of the sinuses
6,11	Conjunctival papillomas
16	Conjunctival carcinoma
13,32	Oral focal epithelial hyperplasia (Heck disease)
16,18	Oral carcinoma
16,18	Oral leukoplakia
16,18	Squamous cell carcinoma of the esophagus

Table (4): Mucosal types of HPV (Anogenital).

HPV Type	Ano-genital Disease
6, 11, 30, 42, 43, 44, 45, 54	Condylomata acuminate
16, 18, 34, 39, 42, 45	Bowenoid Papulosis
16, 18, 31, 34	Bowen disease
6,11	Giant condylomata (Buschke-Löwenstein tumors)
30, 34, 39, 40, 53, 57, 59, 61, 62, 64, 66, 67, 68, 69	Unspecified intraepithelial neoplasia
6, 11, 43	Low-grade intraepithelial neoplasia
31, 33, 35, 42, 44, 45, 51, 5216, 18, 56, 58, 59, 66	High-grade intraepithelial neoplasia
6, 11, 16, 18	Carcinoma of vulva Malignant Vulvar Lesions
16	Carcinoma of vagina
16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 66	Carcinoma of cervix
16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 66	Carcinoma of anus
16	Carcinoma in situ of penis (erythroplasia of Queyrat)
16,18	Carcinoma of penis

1.3.3 Classification according to the high risk or low risk

As a consequence of infection and viral persistence in the epithelium, a variety of proliferative lesions may arise in the skin or mucosa according to their preferential sites of infection. These include benign warts and condylomas but also dysplastic lesions, which may further de-differentiate and progress to cancer. Distinct clinical entities have been found to be associated with distinct HPV types. Based on the biological or clinical properties, HPV types have therefore been classified into "low-risk" types mainly

associated with benign disease only and "high-risk" types also associated with malignant disease (124).

In general, sometimes we are classifying types according to site of infection could led to confusing contradictions. Examples of these contradictions include:

- HPV-6 and HPV-11 are typically found in genital warts or condylomata acuminate in the genital tract, and they were therefore considered "genital" HPVs. However, they are also found in non-genital sites, for example in papillomas of the larynx [123].

- Genital warts can be of mucosal as well as cutaneous origin. HPV types involved include mucosal types as well as HPV-2, HPV-27, and HPV-57, which are causes of common warts, and HPV-7 [123].

1.4 Mechanism of human papillomavirus infection:

1.4.1 Entry:

Papillomaviruses gain access to the basal cells through physical breaks in the epithelial barrier. Basal cells are infected by one or two copies of the episomal viral DNA per cell. The extrachromosomal viral DNA in basal cells replicates in concert with normal cell division thus maintaining a constant number of episomal HPV genome per cell. Presumably, the viral DNA is maintained in the daughter cells in upper levels of the epithelium. It is in these strata of cells undergoing differentiation that viral RNAs are expressed at substantial levels [125]. The differentiation-specific cellular events promote viral transcription and DNA replication. Thus papillomavirus is shed to high numbers only in expendable, terminally differentiated cells sloughed off the epithelium. HPV are not lytic viruses [24].

1.4.2 Shedding:

After many copies of the circular viral DNA are synthesized in the upper strata, these genomes are incorporated into a particle, or capsid, which consists of the L1 and L2 proteins. The viral capsid protects its DNA as the keratinocytes terminally differentiate as well as after being shed into the environment. Importantly, papillomaviruses do not bud from the cell's plasma membrane and thus do not incorporate a membrane-derived lipid envelop. HPV particles are thus not sensitive to environmental stresses such as heat, soaps, or desiccation. After assembly, papillomaviruses are carried along with differentiated cells to the stratum corneum, where they are released with cell death due to apoptosis [24].

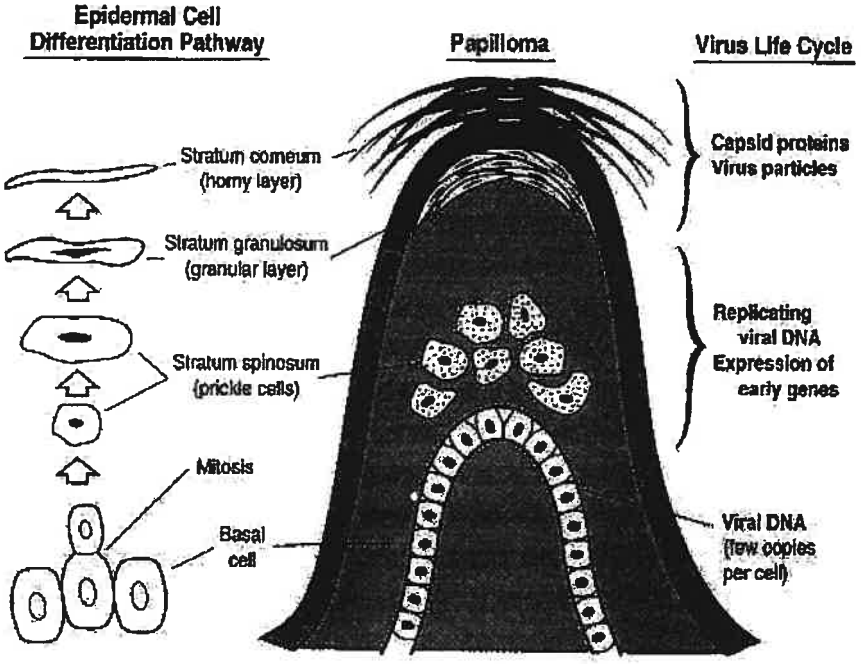

Figure (4): Schematic representation of a skin wart (papilloma). The papillomavirus life cycle is tied to epithelial cell differentiation. The terminal differentiation pathway of epidermal cells is shown on the left. Events in the virus life cycle are noted on the right. Late events in viral replication (capsid protein synthesis and virion morphogenesis) occur only in terminally differentiated cells.

1.4.3 Coordination of the viral replication cycle:

After entering the appropriate host cell, viral gene will be expressed in an ordered fashion. Within the non-coding region (NCR) of each virus are DNA sequences that are recognized by cellular transcription factors [126,127,128,129,130]. The papillomavirus genotypes vary in the type, array, and position of these sites, which could have important consequences for pathogenicity.

In addition to these binding sites for cellular factors, papillomaviruses encode a DNA binding protein designated E2. E2 binds the inverted palindrome 5'-ACCG NNNN CGGT-3' with very high affinity ($\sim 10^{-11}$M) [131], and multiple copies of this DNA palindrome are found in every papillomavirus genome [132]. Their position often varies among different genotype groups. E2 can stimulate, and under certain instances repress, viral transcription [133,134,135]. E2 is also required for viral DNA replication. When E2 binds its recognition site, it usually stimulates transcription from the nearby promoters in a classical "enhancer" mode.

The E2 protein averages about 400 amino acids with a monomeric molecular weight of approximately 50 kilo-Daltons (KD) and has distinct functional domains [136,137,138]. The carboxy terminal 100 amino acids of all papillomavirus E2 proteins constitute the DNA binding domain. This small region is sufficient for sequence-specific DNA binding and dimerization, which E2 must do to bind DNA [139]. The atomic structure of the BPV E2 protein bound to its cognate DNA site was found to fold as an unusual ß- barrel dimer with a DNA recognition helix crossing each monomer [140].

The amino terminal half of the E2 protein is necessary for activation of gene expression, so these amino acids must interface with the cell's transcription machinery. This region of E2 represents its transcription activation domain. When E2 binds a specific segment on the viral DNA through its DNA binding domain, its transcription activation domain recruits the cellular factors that lead to synthesis of the viral mRNA. One such

factor may be Sp1, although there is evidence that E2 activates transcription in concert with a variety of basal promoter factors [141,142].

The E2 DNA binding and transcription activation domains share considerable homology among the papillomaviruses. Between these two regions is a variable stretch of residues that are not conserved, and are generally thought to represent a "flexible hinge." The E4 reading frame overlaps this E2 hinge region and the papillomaviruses differ substantially in their E4 protein. E4 is not essential for viral transcription, replication, or transformation in vitro and is believed to serve a role in maturation of the viral capsid and escape of the viral particle from the dense intermediate filament network of the epithelial cell [143,144].

Papillomaviruses do not express high levels of their RNAs and proteins until the late stages of epithelial differentiation. To restrict expression of the viral genes, BPV and HPV generates truncated E2 proteins to act as transcriptional repressors [145,146,147,148]. These repressors lack the amino terminal transcription activation domain but retain a functional DNA binding domain [149]. Therefore they compete with full-length E2 proteins for binding the E2 recognition sites on the viral genome. In addition, the E2 protein normally is a dimer, and formation of a heterodimer between a full-length and truncated E2 protein represents another mechanism for the inactivation of E2-induced transcription [150]. Both full-length and truncated E2 can repress viral transcription factors to their recognition sites in the LCR. Dominance of the latter function may reduce viral expression in basal and parabasal kerationcytes.

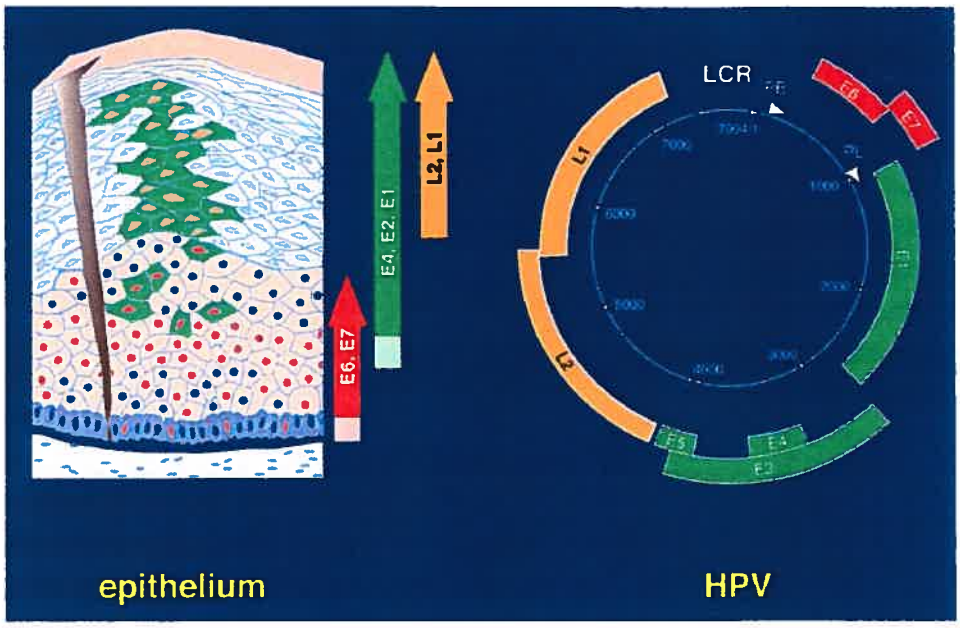

Figure (5): The 8000 base pair papillomavirus genome is shown diagrammatically on the right. Viral proteins are expressed in a highly organized pattern as an infected cell migrates towards the epithelial surface. The timing of viral protein expression in infected epithelium is shown on the left.

1.4.4 Replication of the viral genome:

The viral DNA must be selectively replicated in differentiated cells to produce a high level of infectious progeny in each cell. This would normally be very detrimental except that PV replication occurs in terminally differentiated cells. This highlights the central paradox of papillomavirus replication: it begins in a non-replicating cell layer in which the multitude of enzymes necessary for DNA synthesis is thought not to be present. Because papillomavirus do not encode a DNA polymerase or the associated factors necessary to duplicate DNA, they must induce the cell to replicate while differentiating. Consequently, the virus must mobilize these cellular factors to replicate the viral genome.

Papillomavirus utilizes two proteins, E2 and E1, to identify their genomes among the mass of host DNA. Mutations in the viral E1 gene interfere with autonomous replication of the viral DNA [151]. The E1 protein binds to E2 protein [152,153,154]. It is believed that E2 and E1 each bring a set of cellular factors to the viral DNA, and these factors replicate the viral DNA. E1 weakly binds a specific DNA sequence in the viral regulatory region, and this activity of E1 is greatly enhanced when it is complexed with E2 [155,156]. In BPV, the E1 binding site is adjacent to E2 sites, and this segment of viral DNA is sufficient for autonomous replication in murine cells when E1 and E2 proteins are expressed [157]. The E1 protein has helicase activity, which is necessary for separating the HPV DNA strands prior to their replication [158]. Both BPV and HPV E1 and E2 are necessary for viral DNA replication [159,160,161].

2 Human papillomavirus and skin cancer:

2.1 Normal skin:

The skin is considered the largest organ of the body and has many different functions. The skin functions in thermoregulation, protection, metabolic functions and sensation. The skin is divided into two main regions, the epidermis, and the dermis, each providing a distinct role in the overall function of the skin. The dermis is attached to an underlying hypodermis, also called subcutaneous connective tissue, which stores adipose tissue and is recognized as the superficial fascia of gross anatomy.

The epidermis is the most superficial layer of the skin and provides the first barrier of protection from the invasion of foreign substances into the body. The principal cell of the epidermis is called a keratinocyte. The epidermis is subdivided into five layers or strata. The outermost part of the epidermis is called the stratum corneum, or horny layer. It is composed of dead keratinocytes (the main type of cell of the epidermis) that are continually shed. Below the stratum corneum are layers of living keratinocytes, also called squamous cells. These cells form an important protein called keratin. Keratin contributes to the skin's ability to protect the rest of the body.

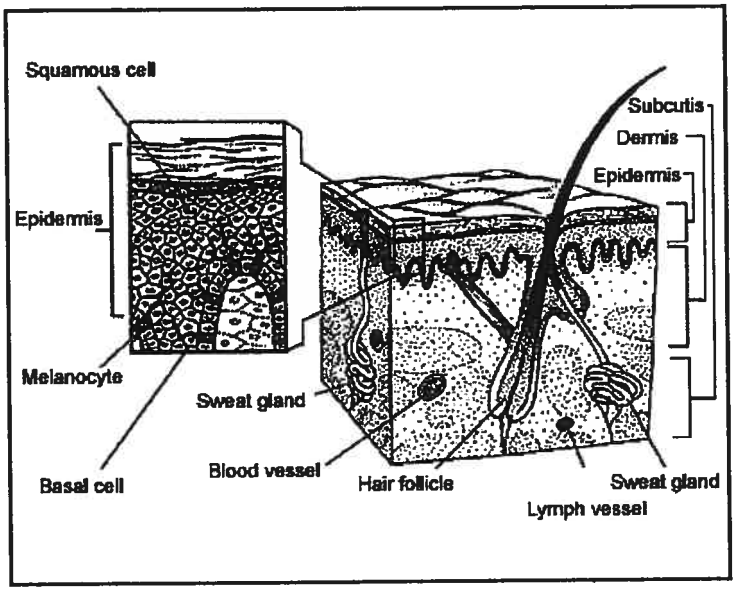

Figure (6): Section of skin showing epidermis and dermis layers.

The lowest part of the epidermis, the basal layer, is formed by basal cells. These cells continually divide to form new keratinocytes, which replace older keratinocytes that wear off of the skin surface. The basement membrane separates the epidermis from the deeper layers of skin. Melanocytes are also present in the epidermis. These skin cells produce the protective brown pigment called melanin. Melanin makes the skin tan or brown. It protects the deeper layers of the skin from the harmful effects of the sun.

The middle layer of the skin is called the dermis. The dermis is much thicker than the epidermis. It contains hair follicles, sweat glands, blood vessels, and nerves that are held in place by collagen. Collagen, which is made by skin fibroblasts, gives the skin its resilience and strength.

The last and deepest layer of the skin is called the subcutis. The subcutis and the lowest part of the dermis form a network of collagen and fat cells. The subcutis conserves heat and has a shock-absorbing effect that helps protect the body's organs from injury.

2.2 Types of nonmelanoma skin cancer (NMSC):

Skin cancers are classified into 2 general categories: nonmelanoma and melanoma. Nonmelanoma skin cancers (NMSC) are the most common cancers of the skin. Melanocytes can also form malignant melanoma and benign growths (moles). There are many types of NMSCs, but 2 types are most common--basal cell carcinoma and squamous cell carcinoma.

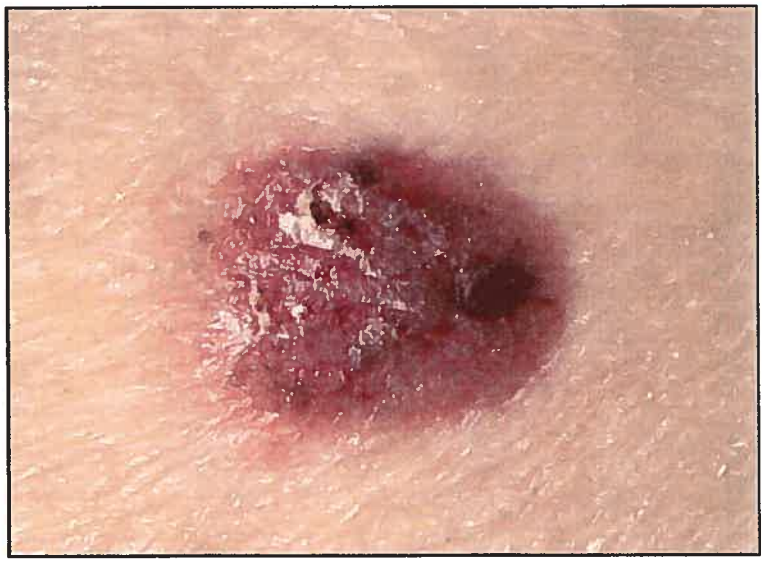

Figure (7): Squamous Cell Carcinoma

Squamous cell carcinomas develop in higher levels of the epidermis and account for about 20% of all skin cancers. They commonly appear on sun-exposed areas of the body such as the face, ear, neck, lip, and back of the hands. They can also develop within scars or skin ulcers elsewhere. Less often, they develop in the genital area. SCCs tend to be more aggressive than basal cell cancers. They are more likely to invade tissues beneath the skin, and slightly more likely to spread to lymph nodes and/or distant parts of the body.

Basal cell carcinoma begins in the lowest layer of the epidermis, called the basal cell layer. About 75% of all skin cancers are basal cell carcinomas. They usually develop on sun-exposed areas, especially the head and neck. Basal cell carcinoma was once found almost exclusively in middle-aged or older people. Now it is also being seen in younger people, probably because they are spending more time in the sun with their skin exposed. Basal cell carcinoma is slow growing. It is highly unusual for a basal cell cancer to spread to lymph nodes or to distant parts of the body. However, if a basal cell cancer is left untreated, it can grow into nearby areas and invade the bone or other tissues beneath the skin. After treatment, basal cell carcinoma can recur in the same place on the skin. Also, new basal cell cancers can start elsewhere on the skin. Thirty-five to fifty percent of people diagnosed with one basal cell cancer develop a new skin cancer within 5 years of the first diagnosis.

Less common types of NMSC include: Kaposi's sarcoma, cutaneous lymphoma, skin adnexal tumors, various types of sarcomas, and Merkel cell carcinoma. Together, these types account for less than 1% of NMSCs.

2.3 Precancerous and preinvasive skin conditions:

2.3.1 **Actinic keratosis:**

Actinic keratosis (AK) is thickened, scaly (keratotic) growth of the skin, also known as solar keratosis. Actinic keratosis are small, rough spots that may be pink-red or

flesh-colored. Usually they develop on the face, ears, back of the hands, and arms of middle-aged or older people with fair skin, although they can arise on other sun-exposed areas of the skin. Individuals with one actinic keratosis usually develop many more.

Actinic keratosis represents atypical keratinocytic proliferations confined to the epidermis. Histological changes of altered cell polarity, variation in cell size and basophilicity, enlarged nuclei, prominent nucleoi, and mitosis are present in AK, all of which are also seen in SCC [162,163,164]. The difference between AK and SCC is strictly architectural. AKs by definition are confined to foci in the epidermis, whereas SCC involves the full thickness of the epidermis with extension into adnexal epithelium and the dermis.

80% of AKs appear on sun-exposed skin of the head, neck, arms, and hands. They are more common in whites (17 per 1,000) than blacks (0.2 per 1,000). The clinical course of AK is unpredictable and ranges from spontaneous disappearance to progression to SCC with potential for metastasis. Estimates of progression from AK to SCC over 10 years have varied from 13% to 20% [165,166]. Although AKs have traditionally been described as premalignant, evidence now indicates that AKs and SCCs lie on a clinical, histological, cytological, and molecular continuum [162,167,168,169]. Thus, AKs are not premalignant but are truly malignant in that the cells comprising AK have already undergone neoplastic transformation, and the evolution from AK to SCC involving the dermis and deeper structures represents progression rather than transformation [170].

2.3.2 Squamous cell carcinoma in situ:

Squamous cell carcinoma in situ, also called Bowen's disease, is the earliest form of squamous cell skin cancer. The cells of these cancers are contained entirely within the epidermis and have not invaded the dermis. Bowen's disease appears as reddish patches. Compared with actinic keratosis, Bowen's disease patches tend to be larger, redder, more scaly, and crusted. Like invasive squamous cell skin cancers, the major risk factor is

overexposure to the sun. Bowen's disease of the anal and genital skin is often related to sexually transmitted infection with HPVtypes-16, 18, 31) the types that can also cause genital warts or preinvasive cervical disease.

3 Ultraviolet rays (UVR) and skin:

Most skin cancers are linked to sunburn or prolonged exposure to the sun. Skin cells are damaged by the electromagnetic radiation that makes up sunshine. The dangerous rays contain UV radiation and can penetrate deep into our cells and cause gene damage, the trigger for cancer. There are three types of UV: UVA, UVB and UVC.

3.1 UVA

UVA is the predominant type of UV radiation from the sun. It increases production of melanin in the skin, resulting in a temporary tan. UVA doesn't burn the skin. UVA rays cause aging, wrinkling, and loss of elasticity. UVA also increases the damaging effects of UVB, including skin cancer and cataracts. Long term exposure can lead to skin cancer.

3.2 UVB

UVB radiation makes up a very small proportion of the sun's UV radiation. But it can cause redness and burning. Prolonged exposure can result in blistering and second degree burns. Exposure to UVB rays is a risk factor for both NMSCs and malignant melanoma. UVB rays cause a much greater risk of skin cancer than UVA.

3.3 UVC

UVC radiation gets filtered out by the ozone layer and does not reach the earth. It can be artificially produced for example in arc welding lamps and is extremely damaging to the skin.

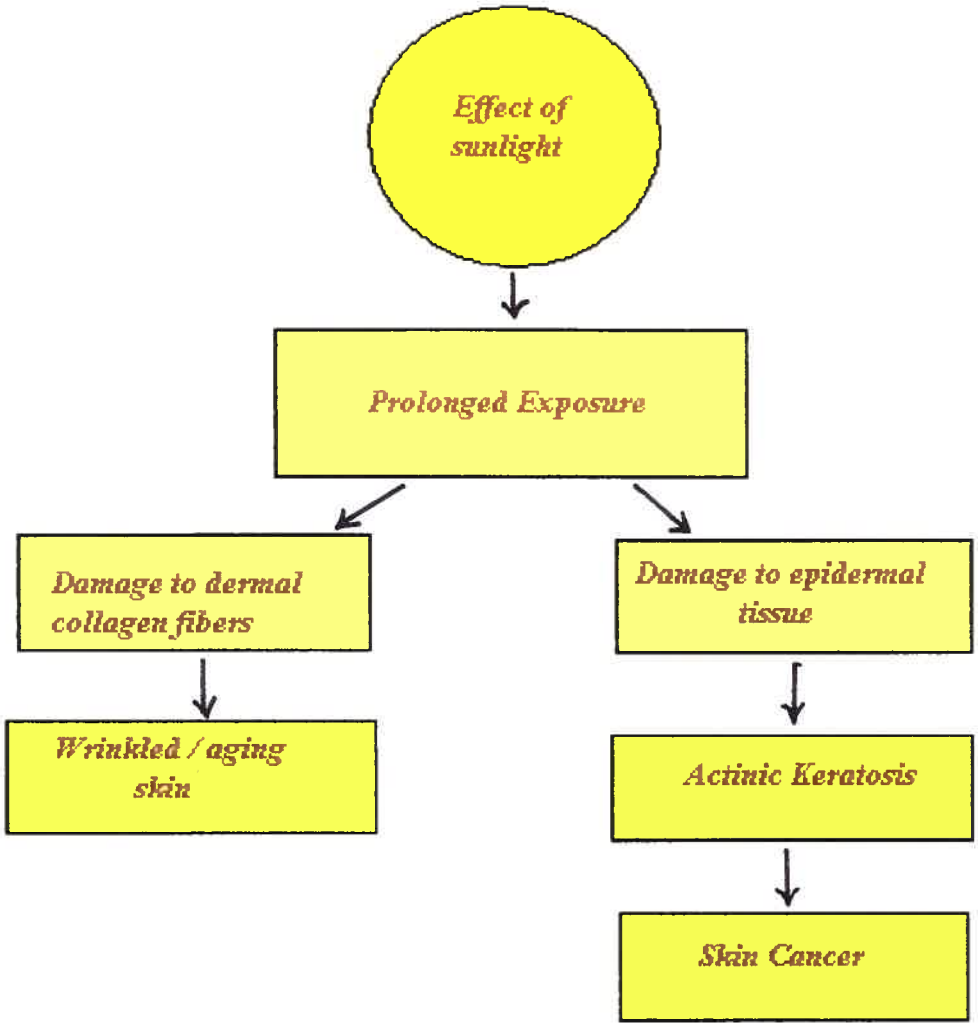

Figure (8): Effect of the sunlight on the skin

3.4 Measurement of UVR:

In cutaneous photobiology, radiant exposure is frequently expressed as 'exposure dose' in units of J/cm^2 (or J/m^2). 'Biologically effective dose', derived from radiant exposure weighted by an action spectrum, is expressed in units of J/cm^2 (effective) or as multiples of 'minimal erythema dose' (MED).

3.5 UVR in human skin

Responding to a variety of stimuli resulting in DNA damage, the sequence-specific transcriptional activator p53 is well known as a "guardian of the cell cycle" [124]. The E6 proteins of the high-risk HPV types, HPV-16 and HPV-18, bind to wild-type p53 *in vitro* and target its degradation via the ubiquitin pathway. Mutant p53 proteins which do not complex with E6 are not degraded [310], whereas those mutations leading to conformational change of the p53 protein, are degraded [311]. The E6 proteins of the low-risk genital HPV types do bind to p53, although degradation is not induced [32,312]. p53 mutations are frequently present in actinic keratosis and malignant tumors (squamous cell carcinoma and basal cell carcinoma) of the skin. The majority of the mutations are C-T or CC-TT transitions, with mutational hot-spots occur-ring predominantly in the DNA binding region of the p53 gene [188,313-317]. These mutations are mainly induced by UVB exposure, although similar mutations have been demonstrated in malignant tumors from PUVA-treated (psoralen and UVA) psoriasis patients [318]. The mechanism by which cutaneous HPV types interact with cellular proteins in the pathogenesis of NMSC is poorly understood. The transcriptional transactivation activity of p53 following DNA damage by UV radiation is inhibited by the HPV-18 E6 [319] as well as the HPV-1 E6, but not by the E6 of the cutaneous types, HPV-5, 8 and 47 [320] or HPV-77 [298].

On the other hand, little is known about the functional significance of the mutations present in the p53 proteins in NMSC. The clonal expansion of p53-mutated normal keratinocytes (as demonstrated by microdissection) indicates that the mutational event may be an early event [321,322]. An arginine substitution of a praline at the codon 72 of the p53 gene, resulting in electrophoretically distinct forms of the protein [323], was recently shown to be significantly more susceptible to high-risk HPV E6-mediated degradation [211]. This polymorphism was demonstrated in a large number of actinic keratosis samples [188] and squamous cell carcinomas of the skin [211], but a functional correlation with skin tumors remains to be determined. Codon 248 is regarded as one of the mutational hotspots in NMSC [314,316,317]. *In vitro* studies demonstrated that this p53 mutant was unable to transactivate a reporter gene, to inhibit cell proliferation or to

interfere with c-jun activity even though no change in its conformation was noted [324]. The introduction of human p53 proteins with mutations at codons 175, 248 or 273 into mouse cells with no endogenous p53 genes induces a tumorigenic potential in these cells and thus leading to a gain of function. p53 mutant proteins also act as a trans-dominant mutation influencing the oligmeric protein complexes with wild-type p53 proteins [325]. Wild-type p53 proteins are less stable with a half-life of approximately 20 min compared to several hours for the mutant p53 proteins. This results in a much higher level of mutant p53 in tumor tissue, the presence of which is demonstrated by specific antibodies [325].

Base substitutions in the tumour suppressor gene p53 found in human squamous cell skin carcinomas that had developed at sites exposed to the sun, were similar to those found in experimental systems exposed to UVR, and especially to UVB.

I. UVA radiation is mutagenic to prokaryotes and induces DNA damage in fungi. It is mutagenic to and induces DNA damage, chromosomal aberrations and sister chromatid exchange in mammalian cells. It also induces DNA damage and mutation in human cells *in vitro* [328].

II. UVB radiation is mutagenic to prokaryotes and induces chromosomal aberrations in plants. It is mutagenic and induces DNA damage, sister chromatid exchange and transformation in mammalian cells. It is also mutagenic and induces DNA damage and transformation in human cells *in vitro* and induces DNA damage in mammalian skin cells irradiated *in vivo* [328].

III. UVC radiation induces DNA damage and is mutagenic to prokaryotes, fungi and plants, induces DNA damage in insects and aneuploidy in yeast. It induces sister chromatid exchange in amphibian and avian cells *in vitro*. It is mutagenic and induces DNA damage, chromosomal aberrations, sister chromatid exchange and transformation in mammalian and human cells *in vitro*. It also induces DNA damage in mammalian skin cells irradiated *in vivo* [328].

3.6 UVR and skin cancer:

NMSC is the most frequently occurring malignancy worldwide in the Caucasian population [171]. Results from epidemiological studies suggest that exposure to sunlight increases the risk of NMSC. NMSC in a normal population occurs mainly on sun-exposed sites areas to UV radiation as a major environmental factor in the pathogenesis of these tumors [172]. Differences in incidences of 50-fold for BCCs and 100-fold for SCCs have been noticed among the white population in northern Europe and Australia. The rates observed in the Hawain white population are substantially lower than those for Australian whites, indicating that additional factors other than sun exposure may be important for the induction of NMSC [173]. This idea is substantiated by observations made in Finland where BCC could not be associated with outdoor occupations such as farming, fishing and forestry [174], as well as in cases of BCC developing on less-exposed areas [175]. Solar UVB radiation represents one of the major environmental impacts for humans [176] resulting in about 40,000 new cases of NMSC arising annually in the UK and 1,000,000 in the USA.

3.7 Other risk factors of non-melanoma skin cancer (NMSC):

There are many etiological factors that has been proposed to cause NMSC, chemical exposure (arsenic, tar, coal, paraffin), long-term or severe skin inflammation or injury, immunodeficiency, human papillomavirus, psoriasis patients treated with psoralen and ultraviolet light (PUVA), smoking, male gender, history of previous skin cancer, radiation therapy (such as radiation treatments for leukemia, goiters, ankylosing spondylitis), Xeroderma pigmentosum (very rare inherited condition that reduces the skin's ability to repair damage to DNA caused by sun exposure) and Basal cell nevus syndrome (rare congenital condition present at birth) and age.

3.8 Psoriasis:

Psoriasis is a chronic relapsing, inflammatory papulosquamous dermatosis. Psoriasis occurs worldwide affecting 2% of the population. Scalp involvement is a prominent feature (50%). It may appear at any age, but the age of onset has bimodal peaks in the teens and again in the sixties. The pathophysiology involves abnormal hyperproliferation of the skin with a rapid cell turnover time in the epidermis (3-4 days). This is probably secondary to activation of the cellular immune system producing a variety of cytokines (especially IL-1), eicosonoids (especially LT B4) and polyamines. The changes in epidermal cell kinetics result in alterations in the epidermal cell keratin cytofilament expression, and cellular differentiation.

There are five different types of psoriasis. The most common form of psoriasis is called "plaque psoriasis," which is characterized by well-defined patches of red raised skin. About 80 percent of people with psoriasis have this type of lesion. Plaque psoriasis can appear on any skin surface, although the knees, elbows, scalp, trunk and nails are the most common locations.

3.8.1 Psoriasis and UVR and HPV:

Psoriasis can be viewed as a hyperproliferative disorder of keratinocytes mediated by T cells. In contrast to lichenoid skin diseases, there is no microscopic evidence for the presence of apoptotic keratinocytes in psoriasis, despite Fas expression. One possible explanation is that Bcl-xL, shown to block apoptosis, is overexpressed in keratinocytes within lesional plaques [196]. In this respect, increased epidermal thickness in psoriasis can be explained by abnormalities in the apoptotic cell death pathway.

UVR has been used for several decades to treat skin diseases, notably psoriasis. A variety of sources of UVR are employed, and nearly all emit a broad spectrum of radiation. A typical dose in a single course of UVB phototherapy might lie between 200

and 300 times the MED. Psoralen and UVA (PUVA) photochemotherapy has been widely used in the treatment of inflammatory and malignant dermatoses for over 20 years. It is a highly effective and well tolerated treatment for many patients but its use is limited by the associated risk of developing NMSCs [197,198,199,200,201]. The risk of SCC in particular is increased up to 80-fold compared with the normal population. BCC also occur at a higher than expected frequency in PUVA patients [202], although the increased risk is considerably less than for SCC. The BCC: SCC ratio is then similar to that seen in immunosuppressed renal transplant recipients (RTR) [203].

Several factors are thought to determine the risk of skin cancer in PUVA patients, including, in particular, cumulative UVA dose and numbered treatment [204]. Sun sensitive skin type and past exposure to ionizing radiation, arsenic, and methotrexate may also be important factors. Psoralens intercalate between complementary strands of DNA, particularly at A-T rich sites, with formation of mono- and bi- functional adducts following photoactivation by UVA radiation [205]. PUVA has also been shown to induce cutaneous and systemic immunosuppression that may be an additional important determinant of its carcinogenic potential [205,206,207,208,209].

3.9 Epidermodysplasia verruciformis (EV):

Epidermodysplasia verruciformis (EV) is a rare, lifelong, autosomal recessive hereditary disorder affecting the skin. The disease usually begins in infancy or early childhood (about 7.5%), during childhood (in children aged 5-11 years) (61.5%), or at puberty (22.5%) with the development of various types of warts and plaques on the skin. The lesions may progress to form verrucous plaques and nodules, or they may transform into SCCs. The clinical course is protracted. As the disease progresses, some lesions disappear, while new lesions may appear on other areas of the body. The rate of appearance of new lesions varies considerably.

Individuals with EV have a specific impaired cellular immunity to EV-associated HPV types that increases their susceptibility to widespread viral infection. The disease is characterized by chronic infection with HPV. Widespread skin eruptions of flat-to-papillomatous, wart-like lesions and reddish brown pigmented plaques on the trunk, the hands, the upper and lower extremities, and the face are characteristic. The lesions may transform into malignant carcinomas, usually after age 30. Skin cancers initially appear on sun-exposed areas, such as the face and the ear lobes. Patients with EV are usually infected with multiple types of HPV. More than 30 HPV types, including types 3, 5a, 5b, 8-10, 12, 14, 15, 17, 19-21, 23-26, 37, 38, and 47, have been identified in EV tumors. Cells with early signs of malignant transformation have been found to be close of virus-infected epidermal regions. Although the exact mechanisms involved in the malignant transformation of keratinocytes in skin lesions of patients with EV are unknown, cooperation between HPV and UV is suspected.

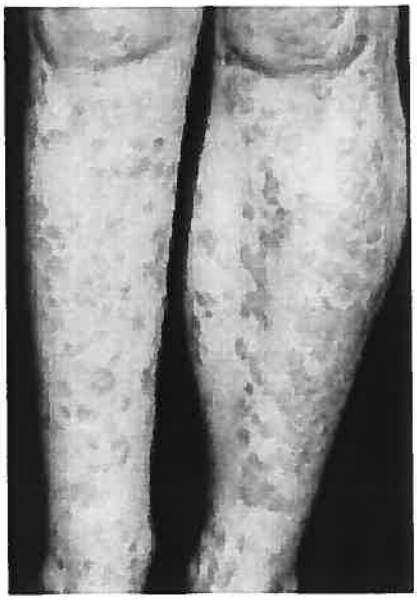

Figure (9): Epidermodysplasia verruciformis. Eruptive, polymorphic, warty papules and plaques on the lower extremities of a patient with epidermodysplasia verruciformis.

The progression on sun-exposed sites of benign to malignant lesions requires a lag phase of 20-30 years as was observed in skin autographs in these patients from non-sun-exposed and uninvolved skin [210]. Only benign lesions developed within the grafts, whereas premalignant and malignant changes occurred around the grafts during the subsequent observation over 20 years. These data suggest that additional factors are involved in the process of cutaneous carcinogenesis.

4 Cellular defense mechanisms against oncogenesis:

4.1 Apoptosis:

Apoptosis, or programmed cell death, is the major mechanism by which homeostasis of a number of physiological systems in the body can be regulated. Furthermore, recent studies have suggested that the failure of cells to undergo apoptotic cell death might be involved in the pathogenesis of a wide variety of human diseases, including cancer, autoimmune disease, and viral infections [177]. There is accumulating evidence that apoptosis occurs not only in the pathological conditions of the skin, but is an ubiquitous process that is important in regulating epidermal growth [178,179,180].

In the skin, cells dying by apoptosis have been found in a wide variety of conditions, such as inflammatory dermatoses and skin tumors [178,179,180]. Evidence is accumulating that apoptosis plays an important role not only in the pathogenesis of skin diseases, but is also involved in the homeostatic mechanisms in healthy skin. Apoptosis triggers a series of events leading to the efficient elimination of a cell. In actively proliferating tissues, such as the epidermis of the skin, apoptosis-like phenomena are often found, as seen in the regression of hair follicles [181,182] and in terminal differentiation [183,184,185]. For example, "sunburn cells", frequently observed in epidermis treated with UVB, have the apoptotic characteristic of condensed nuclei

[186,187], the response to UVB radiation being in part dependent upon the expression of p53 [188]. This p53-driven response, often termed cellular proofreading, eliminates rather than repairs, severely damaged cells. However p53-independent pathways have also been described [189,190].

4.1.1 Bcl-2 family

The Bcl-2 is a proto-oncogene that was originally discovered as a result of its location at the site of a translocation between chromosomes 14 and 18. It is present in most human follicular lymphomas [191]. Although initially viewed as an oncogene, Bcl-2 has little mitogenic effect. Instead, its oncogenic potential has been attributed to its ability to inhibit apoptosis. Bcl-2 prolongs the survival of cells in the absence of required growth factors by blocking apoptosis, even in the presence of a variety of stimuli such as chemotherapeutic agents, irradiation, TNF, heat shock. Furthermore, the introduction of genes that inhibit Bcl-2 can induce apoptosis in a wide variety of tumor cell types, which suggests that many tumors continually rely on Bcl-2 to prevent cell death.

A number of Bcl-2 family members have been identified. Bcl-2, Bcl-xL, Bcl-w and Mcl-1 inhibit apoptosis, whereas others, such as Bax, Bik, Bak, Bad, and Bcl-xs activate apoptosis.

4.1.2 Bak protein

Bak, the pro-apoptotic effectors, a member of the Bcl2 family which activates apoptosis, is expressed in human epidermal keratinocytes [192,193] and is a target of the E6 protein of anogenital HPVs [194]. The impact of cutaneous HPV E6 proteins resulting in Bak dysfunction has important physiological implications with regard to skin cancer development [195].

UV irradiation of skin leads to a marked increase in the level of the Bak protein, a pro-apoptotic member of the Bcl2 family. Using a combination of *in vitro* and *in vivo* assays, the E6 proteins from phylogenetically diverse HPV groups was shown to

stimulate the degradation of Bak via the ubiquitin-mediated pathway, thereby inhibiting apoptosis.

One study showed that both normal human keratinocytes and HT1080 cells treated with UVB had dramatically increased levels of the Bak protein brought about by an increase in half-life, pointing to a role for Bak in promoting apoptosis in UVB-damaged skin. In contrast, HT1080 cells expressing the anogenital and cutaneous HPV E6 proteins showed no such increase in Bak levels following UVB damage. Bak is the first identified target of cutaneous E6 proteins. The mechanism of degradation, shared with anogenital HPVs, indicates that the E6 proteins from cutaneous types are able to discriminate between p53 and Bak as targets [195].

The protelytic degradation of Bak protein by E6 promotes leads to a decrease in apoptosis in UV-irradiated damaged cells, which could in turn promote tumor formation [195]. HPV E6 proteins may have the potential to inhibit Bak-induced apoptosis in skin following UVB damage, resulting in the accumulation of deleterious mutational changes, which further increase the genetic instability of HPV-containing lesions. Irradiation of epidermis regenerated from HPV-transfected keratinocytes showed that cells failed to accumulate Bak and did not undergo apoptosis [195]. They concluded that Bak function was to removes precancerous cells from the epidermis resulting from UVB damage. Evidence that HPV-positive NMSC lesions have undetectable levels of Bak protein, together with results suggesting that anogenital and cutaneous HPVs may possess the ability to use a common anti-apoptotic mechanism, raise the exciting possibility that the abrogation of Bak by the HPV E6 proteins is a common means of promoting the survival of infected cells. This may provide a useful target for intervention against skin lesions harboring a wide variety of HPV types [195]. Preliminary screening of HPV-positive and negative tumour biopsies revealed that the HPV-positive tumours consistently failed to express the Bak protein, whilst HPV-negative tumours frequently retained Bak expression. These findings point to a critical role of the Bak protein in eliminating UV damaged skin cells and suggest that the inhibition of this pathway by the E6 protein contributed towards malignant conversion.

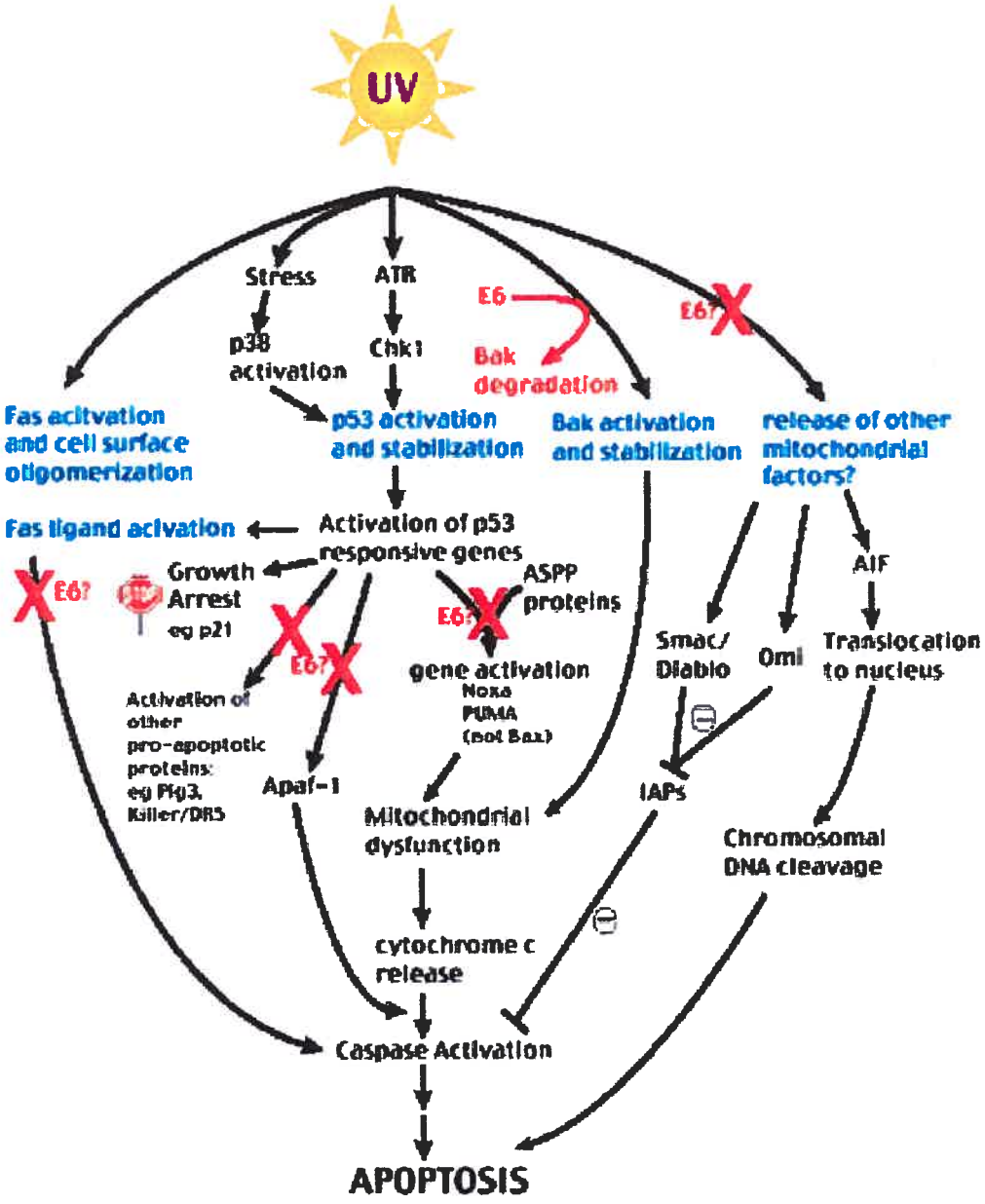

Figure (10): Apoptotic pathways in the skin and potential points of abrogation by the HPV E6 protein.

Several possible mechanisms of how HPV might act as a cofactor in cutaneous cell transformation have been proposed. The promoter activity of HPV-77 is stimulated by UV radiation and the response is mediated through a binding site for the p53 tumor suppressor protein in the upper regulatory region of the virus [298]. Moreover,

Ultraviolet-induced cytokines and interferons have been found to activate the promoter of the cuanteous HPV-20 [299]. The E6 proteins of cutaneous HPV types seem to harbour relatively low oncogenic capability, showing transformation and anchorage-independent growth of rodent cells, but without forming tumors in nude mice [39,300]. Recently, primary human cells were reported to be transformed by E6 of HPV-38 [301]; however, the E6 protein of the cutaneous HPV types appear to be unable to promote degradation of p53 [38,195], in contrast to that of oncogenic genital HPV types [303].

The E6 protein of cutaneous human papillomaviruses might promote genomic instability of infected cells since it was demonstrated that E6 of HPV types 1 and 8 (and HPV type 16) binds to the XRCC1 protein (required for the repair of DNA single-strand breads and genetic stability) [304]. Aslo, the E6 protein of cutaneous HPV-5, 10, and 77 binds the pro-apoptotic Bak protein [302]. This could inhibit Bak-induced apoptosis following ultraviolet radiation that could result in accumulation of deleterious mutational changes. Mutations are frequent in SCC of the skin and more than 90% of such lesions demonstrate mutations in the tumor suppressor gene p53 [305].

5 Prevalence of HPV on the skin:

5.1 Prevalence of HPV in RTR and Immunosuppressed patients:

Renal transplantation is a well-established procedure, with many graft recipients surviving 20 years or more. However, a major problem associated with long-term immunosuppression is the increased prevalence of various malignancies, especially in skin, anogenital tract and lymphoreticular system [214,215,216,217,218,219]. Moreover, renal allograft recipients (RARs) frequently develop a spectrum of cutaneous complications ranging from benign viral warts (VWs), to verrucous and AK exhibiting varying degrees of dysplasia, and SCC [220]. The prevalence and morbidity of such complications increase with duration time of immunosupression. A longer duration of immunosuppression for RARs increases the risk of developing multiple skin tumors

[221]. In RARs, the ratio of SCCs to BCCs is 15:1, a reversal of the 1:5 ratios normally observed in immunocompetent patients. It is well recognized that NMSC occurs 10-20 years earlier in renal transplant recipients compared to immunocompetent individuals [203].

Renal transplant recipients given immunosuppressive therapy for long periods of time have an increased incidence of cutaneous neoplasia [221,222,223]. Also, more than 90% of kidney recipients develop skin warts and 40% develop skin cancer within 15 years of transplantation, a 50-to 100-fold increase compared to the general population [224]. EV-associated HPV types have been found in skin tumors from such patients [2,225,226,227,228]. A number of factors have been implicated in the development of skin cancers in RARs. Ultraviolet (UV) radiation is known to be of considerable importance as the majority of tumors occur on sun-exposed skin [216,229,230,231]. It is well established that renal allograft recipients have an increased incidence of viral warts and premalignant and malignant cutaneous lesions. The risk for these lesions increases with the duration of graft survival. It has been postulated that, in addition to the effects of prolonged immunosuppression and previous sun exposure, HPV may also contribute to the carcinogenic process. Studies on HPV DNA detection on RTR has been somewhat controversial, with EV-associated types and a variety of cutaneous and genital HPV types being identified in some but not all studies [162,221,232,233,234,235]. Studies on samples from renal transplant recipients and immunosuppressed patients showed that the prevalence of HPV DNA ranged from 33% to 84.1% in SCC patients, 75% in BCC, 79% in warts and 42%-88.2% in actinic keratosis [212,223,236,237,238].

In one study [236], HPV DNA was detected in at least one of the samples from 94% (49/52) of the renal transplant recipients and from 82% (23/28) of the dialysis patients. A history of skin cancer (basal or squamous cell carcinoma) was common in the renal transplant recipient group (11.5%, 6/52), whereas no case of skin cancer had been noted in the dialysis patients or healthy controls [236]. Five of the six renal transplant recipients with a history of skin cancer were positive for HPV DNA. They used swab samples collected from five different sites on the skin of renal transplant recipients,

dialysis patients, and age- and sex-matched healthy controls. Most individuals were found to have asymptomatic HPV infections as we mentiend above in addition to 80% (64/80) of healthy controls were positive for HPV DNA [236]. There was a significant difference between immunosuppressed renal transplant recipients and non-immunosuppressed patients under dialysis and healthy controls with regard to the prevalence of HPV DNA ($p<0.05$) [236]. HPV types detected were 20 known types and 30 putatively new types [236]. HPV DNA has also recently been detected in plucked hairs from renal transplant recipients (92% positive) as well as from healthy volunteers (53% positive) [237,248].

5.2 Prevalence of HPV DNA in immunocompetent individuals with skin lesions:

In immunocompromised patients, warts and SCCs contain a diverse spectrum of HPV types, the virus being present in ~80% of lesions from immunocompromised patients and ~30% of lesions from immunocompetent patients [211,212]. The co-localization of warts and cancers at sun-exposed sites suggests a possible interaction between HPV and UVB irradiation [213]. There were several notable differences between lesions from immunocompetent compared with immunosuppressed patients. The overall prevalence of HPV DNA in squamous cell carcinomas, basal cell carcinomas and carcinoma in situ was significantly lower in immunocompetent individuals than in renal transplants immunosuppressed [212]. In immunocompetent patients, the prevalence of HPV DNA is 13%-27% in SCC, 100% in warts, 25% in actinic keratosis, 31%-36% in BCC, and 32%-33% in NMSC and about 13% in melanoma skin lesions [212,223,236,237,238],

5.3 Prevalence of HPV DNA in healthy people:

Human skin harbors a very large spectrum of HPV genotypes, most of them previously unknown [2,225,237,238,239,240]. Moreover, there could be a further substantial number of skin papillomavirus types that remains to be detected. Studies show

that the prevalence of HPV DNA ranges from 53%-80% of the healthy controls. For example in one study [236], HPV DNA was detected in 80% of healthy controls. HPV was detected more frequently on the forehead (76%) than on the arms (48%-49%) or thighs (38%-44%) [236]. In that study, a simple sampling method in which a saline-soaked cotton-tipped swab was gently drawn over a small area of skin was utilized to collect cells for HPV detection. In another study, skin surface swab samples from one or more sites on three of four healthy volunteers were found to contain HPV [241].

In another study [237], HPV DNA was detected in plucked hairs from healthy volunteers (53% positive) [248]. These hair samples were taken from different areas of the body. In the results reported in this study, HPV DNA was not only detected in the normal skin from the eyelids, but also in samples from other body sites (wrist, ear, forehead, temple, cheek, and leg) [237].

EV HPV types have been detected in hairs plucked from normal skin of 67% of healthy controls [242]. In another study [237], 35% of biopsy specimens from normal skin obtained during cosmetic surgery was positive for HPV DNA. EV-HPV types are frequently detected on the skin of healthy individuals, but also in skin cancer lesions of RTRs [2,225,227,239,243]. Therefore, it seems warranted to stop calling them EV-associated HPVs. Preferably, they should be classified according to phylogenetic super-groups [244], or, in a broader sense, simply referred to as cutaneous HPV types.

The presence of HPV, and specifically some EV-HPV types, on the normal skin supports that infection with a broad spectrum of HPV types occurs frequently [2,237]. The natural history of cutaneous HPV infection is unknown at present. Longitudinal studies are needed to delineate persistence of as well as the natural history of infection by cutaneous HPV types. Genomes of human papillomaviruses are common in biopsies from NMSCs but are also found on healthy skin. It is thus possible that HPV positivity in tumor biopsies by PCR may merely reflect contamination of the lesion by the skin. To investigate this issue, 229 immunocompetent patients were tested for HPV DNA in swab samples collected over skin tumors and in biopsies of the same tumors, obtained after

stripping with tape to remove superficial layers. HPV DNA was detected on the skin of 69% (159 /229) of lesions, and in 12% (28/229) of stripped biopsies. The difference was seen for all four types of tumours studied. Seborrhiec keratosis had 79% (34/43) HPV positivity on the skin versus 19% (8/43) in biopsies. Actinic keratosis had 83% (38/46) HPV positivity on skin versus 11% (5/46) in biopsies. Basal cell carcinoma had 63% (69/109) positivity on skin versus 8% (9/109) in biopsies and SCC had 58% (18/31) positivity on skin versus 19% (6/31) in biopsies. HPV DNA is common in superficial layers of lesions, but is not necessarily present throughout tumors [245].

6 Detection assays for cutaneous HPV primers pairs:

Until recently, it has been technically difficult to detect the 80 or more characterized HPV types in skin cancer, considerably hindering research in this area. In early studies, detection of HPV DNA in NMSC varied both in overall prevalence (from 0-64%) and in the spectrum of HPV types detected [213]. These discrepancies largely reflected the performance of detection methods used. Direct DNA-hybridization based techniques were generally employed using a limited number of HPV probes that were not informative for the majority of HPV types and were not sensitive. As a consequence, the true prevalence of HPV in cutaneous lesions was underestimated. Where polymerase chain reaction (PCR) was employed, early methods used type-specific primers capable of detecting only a limited range of HPV types [223,246,247].

PCR with primers targeting consensus DNA sequences in the L1 and E1 open reading frames (ORF) have been successfully used applied the detection of a wide range of genital HPV types [249,250,251,252]. PCR with consensus primers specially designed for cutaneous HPV types have also been developed [2,225,228,238,248,253]. However, current methods for the detection of cutaneous HPV types have disadvantages such as the use of nested PCR. Nested PCR predisposes to obtaining false positive results due to contamination. Other assays require combinations of several degenerate primers. The use

of degenerate primers allow for the amplification of several types in one reaction but results in a lower sensitivity than type-specific PCR or PCR assays using pools of non-degenerate primers [2,225,253]. Moreover, use of the different PCR approaches has resulted in discrepant reports of HPV DNA in skin lesions [254].

Table 5: Sequences of oligonucleotide used as a primers

HPV class	HPV types detected	ORF	Primer	Sequence (5'-3')*	Annealing site		Size(bp)	Degeneracy
					HPV type	Bases		
Cutaneous	General cutaneous	L1	FAP59	TAACYGTIGGICAWCCYTATT	8	5981-6001	478	8
			FAP64	CCYATATCYVHCATITCICCATC		6458-6436		36
Cutaneous	General cutaneous	L1	HVP2	TCNMGNGGNCANCCNYTNGG	18	5934-5953	650	16,384
			B5	AYNCCRTTRTTRTGNCCYTG		6561-6580		512
Cutaneous		L1	HVP7 =	AYXCCRTTRTTCATXCCYTG				
			HVP9	AYXCCRTTRTTXTGXCCYTG				
Cutaneous	1, 41, 63	L1	CN1F	AATARGTTWGATGATGCWGAA	1	5793-5814	309-328	8
			CN1R	AKRTARTCWGGATATTTGCA		6108-6128		16
Cutaneous	2, 27, 57	L1	CN2F	GGGGATATGGTTGAAACAGGT	2	6369-6360	294	0
			CN2R	CAGAGGACACCATAGAGCCA		6661-6681		0
Cutaneous	3, 10, 28, 29, 77	L1	CN3F	AACTCTAAYATWGCACATG	3	6140-6159	273	4
			CN3R	CAVGTRCSYTGGCAAATATC		6407-6427		24
Cutaneous	General EV	L1	CP62	GTWAATGAAAYTTGYAANTATCC	8	6520-6543	690	32
			CP69	GWTAGATCWACATTCCARAA		7231-7250		8
Cutaneous	General EV	L1	CP65	CARGGTCAYAAYAATTGGYAT	8	6832-6851	250	16
			CP68	GGDACRAAACCYARYTGCCA		7100-7120		48
			CP66	TGGTATTTTATGGGGCAATC	38	990-1010		0
	General EV		CP69	CAATTTTTCAGTCATGTCCACA		1375-1396		0
Cutaneous	General cutaneous	L1	FAP6085F	CCWGATCCHAATMRRTTTGC	8	6085-6104	235	48
			FAP6319R	ACATTTGIAITTGTTTDGGRTCAA		6319-6296		6
Cutaneous	General mucosal	L1	MY11	GCMCAGGGWCATAAYAAYTGG	6	6722-6742	450	16
			MY09	CGTCCMARRGGAWACTGATC		7150-7170		16
Cutaneous	5, 8, 12, 36, 47	L1	EN1F	TATTTCCCWACHGTHACTGGCTC	8	6753-6776	254	18
			EN1R	TCATAYTCYTCTACATGTCT		6987-7007		4
Cutaneous	14, 19, 20, 21, 25	L1	EN2F	CTGTCAGTGGCTCATTGGT	14	6524-6543	314	0
			EN2R	CATWGCATTAATTTGAGCTA		6818-6838		2
Cutaneous	9, 15, 17, 37, 22, 23, 38	L1	EN3F	ATGKCWAATGATGTHTATGG	23	6367-6387	293	12
			EN3R	TGRTTRYYCCAYAAAATRCCATT		6637-6660		32
Cutaneous	4, 48, 50, 60, 65	L1	C4R	GGAGATACAGAAAATCCT	4	5728-5746	330-335	0
			C4R	SHATCTCCATAGATATCTTT		6062-6082		6
General Mucosal	General Mucosal		GP5+	TTTGTTACTGTGGTAGATAC			150	
			GP6+	GAAAAATAAACTGTAAATCA				

* Degenerate base code: N=G,A,C,T; R=A,G; Y=T,C; W= A,T; M= A,C; D= G, A,T ; S=C,G; K=G,T; H=A, T,C; V=G,A,C; I=inosine
table modified from Harwood et al,1999 [268].

6.1 Overview of PCR assays for cutaneous HPVs:

6.1.1 FAP59/64 primer pair:

A pair of degenerate PCR primers (FAP59/64) was designed from two relatively conserved regions of the L1 open reading frame of most HPVs. The sensitivity and specificity of degenerate primers depend on their degeneracy, on the conditions used for amplification and on the region of the L1 open reading frame chosen [228]. Studies using degenerate primers have demonstrated a high prevalence of HPV DNA in NMSC in RARs. The size of the generated amplicon was ± 480 bp. The FAP59 primer (5'-TAACYGTIGGICAWCCYTATT-3') contained two inosines nucleotides and was degenerated at three positions, with a total degeneracy number of 8. The FAP64 primer (5'-CCYATATCYVHCATITCICCATC-3') also contained two inosines and was degenerated at four positions, from a total degeneracy number of 36 [241]. The positions of the primers corresponded to nucleotides 5981-6001 and 6458-6436 on the HPV-8 genome. These primers generated an amplicon of 478 bp. Primers FAP59 and FAP64 enabled amplification with PCR of a broad range of HPV types [241]. These primers showed high sensitivity, and were optimized to allow detection of less than 10 copies of cloned HPV genomes. The technique was also found to detect significantly higher numbers of HPV-positive skin samples compared to the nested PCR test described by Berkhout et al. which was aimed of detecting EV-HPV types (HPV-5b, 8, 9, 12, 14a, 15, 17, 19, 20, 21, 22, 23, 24, 36, 37, 38, 46, and 49) [225]. In the eight patients with various skin tumours, HPV was found in 63 % (5/8) of tumour and in 63% (5/8) of normal skin samples. In these eight patients, HPV-5, HPV-8, HPV-12, HPVvs20-4 and six putatively novel HPV types were identified. No correlation was found between specific HPV types and histology. Of the four healthy volunteers, three were found to harbor HPV on their skin. The overall HPV finding in skin samples was 50% (20/40) using FAP primers as compared to 18% (7/40) with another nested PCR test described by Berkhout et al. using CP65/CP70 and CP66/CP69 primer pairs for cutaneous types [225,241]. These results thus suggested the new method to be sensitive and generally applicable for detecting cutaneous HPV.

In another study using the same primer pair FAP59 and FAP64 [236], swab samples collected from five different sites on the skin of renal transplant recipients, patients undergoing dialysis, and age- and sex-matched healthy controls, were analyzed for HPV DNA. Most individuals were found to have asymptomatic cutaneous HPV infection, HPV DNA being detected in 85% (136/160) of all samples. Specifically, 94% (49/52) of the renal transplant patients, 82% (23/28) of the dialysis patients, and 80% (64/80) of the healthy controls were positive for HPV DNA. The multiplicity of the HPVs detected was astounding: 20 had been previously described and 30 were putatively new types as found by cloning and sequencing of 33 samples from 13 individuals [236]. This study also demonstrated the potential of identifying new types with FAP primers. It also suggested that cutaneous HPV types are ubiquitous.

6.1.2 HVP2/C and F14/B15 primer pairs:

The forward primer HVP2 with the sequence (5'-ctggatccTCNMGNGGNCANCCNYTNGG-3') was used and the backward primer being the primer mixture C. The primer combination HVP2 and C amplified the group of HPV type 4, 60 and 65 DNA poorly. HPV-4 and HPV-65 are closely related to each other, with HPV-60 being more distant [255]. To specifically amplify these types, additional primers were designed. The forward primer F14 (5'-ctggatccWGATGAYAAYAGAMWGGATG-3'), and backward primer B15 (5'-ctggatccATWCCRTTRTTYGYWCCYTG-3') corresponded to positions nt 5767-5768 and nt 6339-6320 on the HPV-4 genome, respectively, and generated a PCR product of 573 base pairs. In a total of 118 biopsies from skin lesions of 46 RAR, HPV DNA was detected in 62% (31/50), and 56% (14/25) from SCC and BCC biopsies, respectively. Nine putative new HPV types were detected with these primers [228].

In a subsequent study, these highly degenerate primers were split into a number of less degenerate primers [253]. The same samples analyzed above were tested using 16 different primer combinations followed by semi-nested amplification, cloning and

sequencing of the HPV-specific products. HPV DNA was demonstrated in 65% of SCC lesions and in 60% of BCC samples. A number of different HPV types, the majority belonging to the high-risk genital HPVs as well as EV-related HPV types were detected [253].

6.1.3 F and G primer sets:

Many primer pairs have been described for the detection of HPV in warts [326,327]. However, these primer sets have failed to consistently detect HPV DNA in skin cancer. Berkout et al. [225] designed a specific PCR method to detect the group of EV-associated papillomavirus types in SCC patients with different primers (F and G). The first primer set (F) consisted of two degenerate primers CP65/CP70, located in the late L1 ORF (CP65: 5'-CARGGTCAYAAYAATGGYAT-3', CP70: 3'-AAYTTTCGTCCYARAGRAWATTGRTC-5'). The annealing sites in the HPV-8 genomic sequence corresponded to positions nt 6832-6851 and nt 7273-7298 [256]. The second primer set (G) consisted of two degenerate primers CP66/CP69, located in the L1 ORF (CP66: 5'-AATCARYTGTTTRTTACWGT-3', and CP69: 3'-GWTAGATCWACATYCCARAA-5'). Their respective annealing sites were at positions nt 6862-6991 and nt 7231-7250 of HPV-8 [225]. In 53 biopsies with SCC from RTRs, HPV DNA was detected in 81% (43/53) of samples. The HPV types included EV-specific as well as 6 putative new EV-related HPV types [225].

6.1.4 HD and AM primer sets in human:

These two sets of primers covered adjacent sequence stretches within a highly conserved region of the L1 open reading frame of papillomaviruses [2]. The first set (HD) consisted of 16 different primer combinations described by Shamanin et al. (forward/backward) including A1/B1, F10/B5, F12/B5, A1/B6, F10/B6, F11/B6, F12/B6, F21/B11, F22/B11, F23/B11, F24/B11, F21/B12, F22/B12, and F24/B12 [253], as well as two additional combinations, HVP2/HVP7 and HVP2/HVP9, described by de Villiers

et al. [2,253]. The second set of primers (AM) designed to detect EV-specific HPV types consisted of primers CP65/CP70 for the initial amplification rounds, followed by a nested amplification with primers CP66/CP69 [225,237]. HPV-38 specific primers were also selected in the region of the L1 open reading frame in which the CP66/CP69 primer is located. The forward primer was 5'-ttggatccTGGTATTTTATGGGGCAATC-3' located at nt 990-1010 of HPV-38 and the backward primer was 5'-ttggatccCAATTTTTCAGTCATGTCCACA-3' located at nt 1375-1396 of HPV-38. A total of 68 skin biopsies collected from 25 RARs over a period of 12 years (15 warts, 7 actinic keratosis, 7 verrucous keratosis, 2 keratosis, 1 keratoacanthoma, 2 BCCs, 22 SCCs and 11 intra-epidermal carcinomas (IECs)) were analyzed using the same primers (HD, AM). Amplified DNA obtained using the first set of primer was obtained for 93% (14/15) of samples from warts [2].

Table (6): HPV DNA positive using the both sets of primers.

Lesions	HD primer (%)	AM primer (%)	Total
Warts (n=15)	14 (93)	15 (100)	15 (100)
Keratosis (n=17)	4 (24)	10 (59)	11 (65)
IECs* (n=11)	5 (45)	7 (64)	10 (91)
SCCs (n=22)	11 (50)	18 (82)	20 (91)

*IECs= Intra-epidermal carcinomas

From de Villier et al, 1997 [2].

The amplified DNA products obtained using the first set of 18 distinct primer combinations were cloned and sequenced. HPV DNA was detected in 14 of 15 (93%) samples from warts. The HPV types detected includes HPV-1, 2, 4, 10, 27, 57, 63, and vs102-4 (HPV 17-related [228]). Only 2 of the 7 biopsies from actinic keratosis samples contained detectable HPV DNA, i.e., HPV-57 and DL78, a putative new type. Of the 7 verrucous keratosis, one contained HPV-23 DNA and one seborrhoeic keratosis contained HPV-7. The keratoacanthoma was negative for HPV. Forty five percent (5/11) of IECs contained HPV DNA. Types detected in IEC included HPV-57 and 3 putative

new HPV types: DL40, DL78 and DL100. Fifty percent (11/22) of SCCs harboured HPV-1, 11, vs206-2 HPV 48-related [238] and 7 putative new HPV types: DL17, DL20, DL27, DL40, DL82, DL83 and DL84 [2]. The presence of HPV-1, 2, 7, 11, 23, 27, 57, and 63 DNA demonstrated with the first set of primers was confirmed after cloning and sequencing of the nested PCR products. Amplification with the second set of primers resulted in the detection of HPV DNA in all 15 samples from warts [2]. A combination of the results obtained with the 2 sets of primers demonstrated HPV DNA sequences in 100% (15/15) of warts, 59% (10/17) of keratosis, 91% (10/11) of IEC lesions and 91% (20/22) of SCC samples. No HPV DNA could be detected in either of the 2 BCC samples [2]. In opposite to FAP primers, nested PCR was necessary to reach these results.

In another study using the same primers [237], 21 samples from 14 immunocompetent patients were examined. Extracted cellular DNA was amplified by PCR using either one or both sets of degenerate primers HD and AM [237]. Nine percent (2/21) of samples were positive by HD and 38% (8/21) by AM (Table 7) [237].

Table (7): HPV DNA in tumors and per-lesional skin from immunocompetent patients using both primer sets.

Lesions	HD (%)	AM (%)
Self-healing epithelioma (n=1)	0 (0)	0 (0)
Sebaceous epithelioma (n=1)	0 (0)	0 (0)
Verrucous keratosis (n=1)	1 (100)	1 (100)
Actinic keratosis (n=2)	1 (50)	1 (50)
*IEC (n=6)	0 (0)	3 (50)
BCC (n=3)	0 (0)	0 (0)
SCC (n=1)	0 (0)	0 (0)
Uninvolved peri-lesional skin (n=6)	0 (0)	3 (50)

*IECs= Intra-epidermal carcinomas

From Astori et al. 1998 [237].

6.1.5 GP5+/GP6 and CP65 / CP70 +CP66 /CP69:

Investigators analyzed skin samples from 54 psoriatic patients to define the spectrum of HPV types involved and to test if detection of HPV was influenced by psoralen ultraviolet A therapy compared with 42 healthy immuncompetent individuals undergoing nevus excisions (mean age 43.0 years range 16-79 years). Patients were all immunocompetent individuals with a mean age of 48.5 years (17-74 years). DNA preparations were examined for the presence of HPV sequences by PCR analysis with primers chosen to detect a broad spectrum of HPV types. The two primer sets used were: GP5+ (5'-TTTGTTACTGTGGTAGATAC-3') and GP6+ (5'-GAAAAATAAACTGTAAATCA-3') [258], designed for HPV types infecting the genital mucosa, and CP65 / CP70 and CP66 /CP69 (described above) [225] in a nested PCR for the detection of EV-associated papillomaviruses. They could detect HPV sequences in skin lesions of 83% of tested patients. In contrast, HPV DNA was only demonstrated in 19% of skin samples from 42 dermatologically healthy immunocompetent individuals.

Table (8): Detection of HPV DNA in samples of normal and psoriatic skin.

particpants	GP5+/GP+6 (%)	CP70/65-69/66 (%)	HPV5 Specific (%)	HPV36 Specific (%)	Total (%)
Psoriatic skin (n=54)	0 (0)	38 (70)	16 (30)	28 (52)	45 (83)
Healthy skin (n=42)	0 (0)	6 (14)	0 (0)	4 (10)	8 (19)

From Weissenborn et al. 1999 [259].

Sequence analysis of the PCR amplicons revealed 14 HPV types, all belonging to EV-related papillomaviruses. Only one case had a putatively new human papillomavirus type related to those of genital viruses. The most prevalent HPV type in this report was HPV-36, found in 62% of patients positive for human papillomavirus DNA, followed by HPV type 5 (38%) and HPV-38 (24%). Multiple infections with two to five different

HPV types could be detected in skin samples from 63% of the patients tested. The overall HPV detection rate did not differ significantly between patients subjected to psoralen ultraviolet photochemotherapy or locally treated with topical preparations (77% vs. 89%) [259]. Using the nested PCR approach with primers CP70/65 and CP69/66, papillomavirus sequences could be found in 70% (38 /54 studied biopsies) of patients, indicating a high prevalence of papillomavirus infections in psoriatic skin. No HPV DNA could be detected in psoriatic lesion using the single-step PCR test with primers GP5+/GP6+, specific for HPV types infecting the genital mucosa [259].

GP5+ and GP6+ were used in another study [260] as well as specific primers pair to amplify virus types HPV-11, 16, 18 and 33 by multiplex PCR (table 5). Another specific primers pair was used to amplify types 2, 5, 8, with another multiplex PCR [223,252,260,261]. They examined samples from 108 immunocompetent patients with benign and malignant skin lesions, and HPVs were detected in 27% (29/108) of samples. HPV-8 and HPV-18 were the most frequent types (detection rate of 62% and 48% respectively) (table 9,10) [260].

Table (9): HPV primer sequences used to detect HPV types 1, 2, 5 and 8.

HPV type	Primer	Sequence	Position	Size bp
1	P1	AGTCTTATGAGGTACCGGAAATAGAAG	383-409	136
1	P2	ATGCACTCTTTCTCCGTTTGACACAACCTC	520-490	
2	P1	ATGGTTTGGAGCTAGAGGATTTGCG	159-183	303
2	P2	AACTAGTAATGCCTCCTTCTCCTCC	463-438	
5	P1	CTCTAATACCAAATTCTGTGGCGT	616-640	279
5	P2	GAGGAACGCCTGGAAGGGAATCTG	894-870	
8	P1	CGGGCAGGACAAGGCTTCATATTTACACAC	200-230	220
8	P2	ACAACAACGACAACACGCAGTAACAAC	420-393	

From Dano et al 1982 [108].

Table (10): Detection of HPV in human skin lesions by PCR.

Lesions	HPV Positive (%)
BCC (n=72)	22 (31)
SCC (n=23)	3 (13)
Bowenoid lesions (n=5)	2 (40)
Precancerous (n=8)	2 (25)
Total (n=108)	29 (27)

From Biliris et al. 2000 [260].

6.1.6 MY09/11 and CP primers:

Analysis of HPV DNA on the skin has also been carried out using two different sets of L1 consensus primers and two sets of non-degenerate primers in nested PCR assays.

MY09/11 degenerate primers were shown to detect a broad spectrum of mucosal HPVs [249], while primers CP65, CP66, CP69 and CP70 (CP primers) allowed amplification of all EV-associated cutaneous HPVs [225]. Primer sets MY5-1/2, MY5-3/4, MY8-1/2 and MY8-3/4 were derived from the MY09/11 and MYN9/10 region of the HPV-5 and HPV-8 genomes, respectively [262].

Table (12): Frequency of HPV DNA detection in cutaneous SCC using different PCR.

Degenerate primer	Non degenerate primer	All PCR assays (n=32)
MY CP (n=32)	MY5 MY8 (n=20)	
50% (16)	45% (9)	69% (22)

From Meyers et al. 2000 [262].

32 cutaneous biopsies of SCC from 24 patients were analyzed for HPV DNA detection using both consensus PCR assays with degenerate primers and PCR assays with non-degenerate primers derived from HPV types 5 and 8. HPV DNA was found in 50% of SCC specimens using degenerate primers. The rate of HPV-DNA-positive specimens increased to 69% when PCR assays using non-degenerate primers were applied to the same set of samples [262].

Table (11): Sequences of oligonucleotides used as primers of specific PCR products.

Primer	Sequence	Length, bp
MY09	CGTCCMARRGGAWACTGATC	450
MY11	GCMCAGGGWCATAAYAATGG	
MYN9	GTTACTGTKGTWGAYACYAC	370
MYN10	TCYTTTARATYAACMTYCCA	
CP65	CARGGYCAYAAYAATGGYAT	460
CP70	AAYTTTCGTCCYARAGRAWATTGRTC	
CP66	AATCARMTGTTTRTTACWGT	380
CP69	GWTAGATCWACATYCCARAA	
MY5-1	GCCCAAGGTCATAATAATGG	464
MY5-2	CTGCCTAAGGAATATTGATC	
MY5-3	ATCACAGTGGTTGACAACAC	380
MY5-4	TCAGTTAAATCTACATCCCA	
MY8-1	GCCCAGGGTCATAATAATGG	464
MY8-2	CTGCCTAATGAATATTGATC	
MY8-3	GTCACTGTGGTAGACAACAC	480
MY8-4	TCTGTAAGGTCCACATTCCA	

* Degenerate code: Y=C,T; M=C,A; W=A,T; K=G,T; R=A,G; S=C,G.
From Meyers et al. 2000 [262].

6.1.7 HVP2/B5 and CP primers:

In one study [254], 4 established oligonucleotide primer pairs (HVP2/B5, F14/B15, MY09/11, CP62/69) described above, were compared [254]. These primers are all located within the conserved region of L1 [225,228]. The degenerate primer pair HVP2/B5 was described by Shamanin et al. to detect HPV from all groups with the exception of the phylogenetic clade comprising HPV-4, 48, 50, 60 and 65 for which the primer pair F14/B15 was used [228,253]. The primer MY09/MY11 was originally designed by Manos et al. for the detection of HPV in genital lesions [249] but has also been widely used to analyze skin lesions [263,264]. Finally, Berkhout et al. have described several primer pairs for nested PCR which were designed to detect EV HPV types [225,243]. Of these, one particular set comprising CP62/69 as an outer primer pair and CP65/68 as an internal nested pair was considered to be particularly sensitive for detecting HPV in skin biopsies from both immunocompetent and immunosuppressed individuals [225].

Table (13): Detection of HPV DNA with each degenerate primer sets.

Disease	HVP2/B5	FA14/B15	MY09/11	CP62/69
Warts (n=22)	15	0	12	10
SCC (n=19)	0	0	0	8
BCC (n=2)	0	0	0	0
Ano-genital carcinoma (n=5)	3	0	3	0
Normal (n=1)	0	0	0	0
Total (n=49)	18 (37%)	0 (0%)	15 (31%)	18 (37%)

From Surentheran et al. 1998 [254].

The sensitivity and specificity of three published degenerate primer sets (HVP2/B5 and F14/B15; MY09/MY11; CP62/69 outer and CP65/68 nested primer pairs) were evaluated in PCR reactions with serial dilutions of 12 representative cloned HPV

types. These primers sets were used to detect HPV DNA in 49 benign and malignant lesions of cutaneous and mucosal origin from immunosuppressed, immunocompetent, and EV patients. Results with each primer pair were compared [254]. HVP2/B5 was the best pair for detecting HPV in warts.

6.1.8 FAP6085/6319:

In this study, DNA sequences from the L1 gene were aligned within the region between the original FAP59/64 primers of known cutaneous HPV types described in the 1996 HPV Sequence Database compendia (Myers, 1996) and candidate HPV-92 and HPV-93 (Forslund et al., 1999). Conserved regions suitable for nested PCR were identified [256]. Two regions with a relatively high degree of nucleotide sequence homology were identified. A forward primer FAP 6085F (5'-CCWGATCCHAATMRRTTTGC-3') and a reverse primer FAP 6319R (5'-ACATTTGIAITTGTTTDGGRTCAA-3') were designed [256]. A total of 56 biopsies were tested from 51 patients: 64% (35/56) were positive for HPV using FAP6085/6319, including 62% (16/26) of BCCs, 43% (7/16) of SCC, 93% (13/14) of AK [256].

6.1.9 Others primers:

In one study [266], a panel of degenerate primers targeting L1 to detect mucosal, cutaneous, and EV HPV types with high sensitivity and specificity. These primers included MY09/MY11, GP5+/GP6+ for mucosal HPV types, HVP2/B5 for cutaneous HPV types [253] combined with three pairs of nested primers. The latter were designed from published sequence data to detect the following HPV groups [267]: A2 (CN3F/CN3R), A4 (CN2F/CN2R), and E (CN1F/CN1R). The cutaneous group B2 was detected using a single round of PCR with primer pair C4F/C4R. EV-associated HPV types of group B1 were detected with the nested primer pairs CP62/69 and CP65/CP68 as described above [225]. Three additional primer pairs were designed nested within CP62/CP69 amplicon to detect the major EV-HPV clusters a1 (EN1F/EN1R), a2

(EN2F/EN2R), and b1/b2 (EN3F/EN3R) (table 5) [226]. In this study, biopsies from benign and malignant cutaneous lesions were analyzed for the presence of HPV DNA from patients treated with high-dose of ultraviolet A. HPV DNA was detected in 75% (15/20) of NMSC, 41.2% (7/17) of dysplastic PUVA keratosis, 80% (4/5) of skin warts, and 33% (4/12) of PUVA-exposed normal skin samples [266]. This approach allowed for the detection of a wide spectrum of types but is fastidious, necessitating several reactions per specimen.

In general, FAP59/64 primer pair was shown to be the best primer pair to detect cutaneous HPV types. It is also the most frequently used primer pair in epidemiological investigations on cutaneous HPV infection [236,228,241]. These primers also demonstrated their potential to identify novel types. The second useful primer pair is HVP2/B5 as it showed to be the best for detection of HPV in warts [254].

Study objective:

- To describe the detection rate of cutaneous HPV types in individuals living in Canada.
- To describe the spectrum of HPV types infecting the normal skin.
- To determine the agreement between primer pairs to detect cutaneous HPV types.
- To compare the rate of detection of cutaneous HPV types between individuals with a renal allograft, squamous cell carcinoma, actinic keratosis and in healthy individuals.
- To assess the frequency of multiple infection on the skin of individuals with skin lesions.
- To describe novel types of HPV.
- To compare cloning and direct PCR sequencing for the typing of HPV amplicons.

ARTICLE

Declaration of the student in paticipation in this article

I participated in this project by taking the samples from the participants directly, proccess the specimens, PCRs, cloning, and analysing the PCR produts, wrote the methods section and analysing the results and completing this project and reviewed the article.

Cutaneous papillomavirus types are detected frequently in actinic keratosis and squamous cell carcinoma of the skin.

Running Head: Detection and typing of cutaneous HPV types with consensus PCR assays.

word count: 3586

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Abstract (word count = 250)

Objective. We compared the diagnostic yield of PCR using FAP59/64 and HVP2/B5 L1 consensus primers for the detection and typing of human papillomavirus (HPV) in skin swab specimens.

Design. Cross-sectional study.

Methods. HPV isolates were typed by sequencing amplicons directly and after cloning.

Results. Seventy-five (92.6%) of 81 subjects provided samples that could be analysed with PCR (34 healthy controls <50 years old, 13 healthy controls ≥50 years old, 12 with actinic keratosis (AK), 8 with squamous cell carcinoma (SCC), 8 renal transplant recipients). HPV DNA was detected more frequently with FAP59/64 (68/75, 91%) than with HVP2/B5 (9/75, 12%) ($p<0.001$). Agreement of typing results between PCR-sequencing directly or after cloning of FAP59/64-generated amplicons was fair (mean kappa 0.56±0.19, 95%CI: 0.46-0.65). HPV types were sometimes identified only by direct sequencing and not after cloning of FAP59/64 or HVP2/B5 amplicons. HPV species 1 and 2 of the Beta-papillomavirus genus were associated with the presence of AK (OR=24.8, 95%CI: 2.3-262.6). However, seven of eight participants with SCC were infected with putative novel types and only one with HPV species 1 and 2 of the Beta-papillomavirus genus. FA51 was detected more frequently in participants with AK or SCC than in healthy participants (OR=19.7, 95% CI: 2.1–186.5). A greater number of HPV types per sample was found in individuals with AK or SCC ($p=0.046$) or AK alone ($p=0.02$), than in healthy participants.

Conclusion. HPV infection in the skin could be better evaluated with a combination of primers and sequencing strategies. Novel putative types were frequently detected in SCC.

Introduction.

Human papillomaviruses (HPV) are ubiquitous viruses that infect the skin of the majority of immunocompetent and immunocompromised individuals (Antonsson et al., 2000; Astori et al., 1998). Although HPVs have been clearly shown to cause anogenital squamous carcinomas (Bosch et al., 2002), their role in the pathogenesis of non-melanoma skin carcinoma (NMSC) remains controversial (de Villiers et al., 1999; Kiviat, 1999). A broad spectrum of HPV types has been reported in NMSC, many of these being previously uncharacterised (Purdie et al., 1999; Shamanin et al., 1996; Antonsson et al., 2000). HPV DNA has been detected in up to 83% of immunocompetent individuals with NMSC (Sterling, 2005; Harwood et al., 1999; Forslund et al., 2003; Harwood et al., 2000). From 40% to 100% of actinic keratosis (AK) also contain HPV sequences (Harwood et al., 1998; Sterling, 2005; Harwood et al., 2000; Shamanin et al., 1996; Biliris et al., 2000). Differences across studies of prevalence rates and diversity of HPV types in skin lesions seem to reflect the lack of appropriate primers and probes to detect efficiently all cutaneous HPV types via PCR (Kiviat, 1999; Harwood et al., 1998; Kawashima et al., 1990).

Consensus PCR assays have been instrumental in demonstrating the complexity of cutaneous HPV infection and in detecting novel genotypes (Forslund et al., 2003; Astori et al., 1998; Forslund et al., 1999; Harwood et al., 1999; Harwood et al., 2004; Shamanin et al., 1996; Berkhout et al., 1995; De Jong-Tieben et al., 1995). The most commonly utilised consensus primers for cutaneous genotypes target highly conserved sequences in the HPV L1 gene (Forslund et al., 1999; Forslund et al., 2003; Forslund et al., 1999; Antonsson et al., 2003; Harwood et al., 2004; Antonsson et al., 2000; Shamanin

et al., 1996; Berkhout et al., 1995; Berkhout et al., 2000). FAP59/64 and HVP2/B5 consensus primers amplify a broad spectrum of HPV types infecting the skin, can detect as low as 1-10 copies of HPV DNA and have been utilised in several epidemiologic studies (Forslund et al., 1999; Forslund et al., 1999; Shamanin et al., 1994; Shamanin et al., 1994; Antonsson et al., 2003; Forslund et al., 2003; Antonsson et al., 2000; Shamanin et al., 1996; Surentheran et al., 1998). Few studies have compared the performance of consensus PCR assays for the detection and typing of cutaneous types and none have compared FAP59/64 with HVP2/B5 primer pairs (Harwood et al., 1999; Meyer et al., 2000; Surentheran et al., 1998; Forslund et al., 2003; Astori et al., 1998).

In the present report, we compared consensus primer pairs FAP59/64 and HVP2/B5 for the detection and typing of HPV DNA in epithelial cells collected from the skin. We also evaluated the agreement between sequencing directly and after cloning HPV amplicons generated with each PCR assay, to identify HPV types detected in the skin. The detection rate of cutaneous HPV infection was also estimated in Canadians, a population with a high incidence of NMSC.

Materials and methods:

Study population. *Samples were collected from January 2003 to December 2004 from 81 subjects recruited in Montreal. Healthy individuals without current or past history of SCC or AK and ≥ 50 years old (n=13), immunocompetent individuals with AK (n=13), and immunocompetent individuals with cutaneous SCC (n=11) were recruited consecutively from an outpatient dermatology-oncology clinic. Healthy individuals without current or past history of SCC or AK and < 50 years old (n=36) and renal transplant recipients (n=8) were recruited from the Centre Hospitalier de l'Université de Montréal. All participants with AK, SCC were older than 50 years of age. Individuals with a renal allograft had been grafted for at least two years, had no current or past history of wart, SCC or AK, and were older than 50 years of age. The diagnosis of all cutaneous lesions was confirmed by histopathology. The Ethics Committee of the Centre Hospitalier de l'Université de Montréal approved the research protocol. Each participant provided informed consent at enrollment.*

Specimen collection and processing. Epithelial cells from the skin were collected with pre-wetted (sterile water) Dacron-tipped swabs (Fisher Scientific, Nepean, Ont, Canada) which were drawn back and forth four times over the forehead skin within an area of 5x15 cm for individuals without skin lesion and over the lesion within a similar surface for participants with cutaneous lesions (Antonsson et al., 2000; Antonsson et al., 2003). For individuals with a skin lesion, the sample was thus from the surface of the lesion and from perilesional normal skin. Each swab was immersed in tubes containing 500 µl of Preservcyt (Cytyc Corporation, Boxborough, MA), was agitated and then discarded.

Specimens in Preservcyt were centrifuged at 13,000 x g for 15 min at 22°C and resuspended in 300 µl of 20 mM Tris buffer (pH 8.3). DNA was purified using the Master pure procedure (Aho et al., 2004). Five µl of each sample was tested for the presence of human β-globin DNA with a PCR using PC04/GH20 primers (Bauer et al., 1991; Coutlée et al., 1997).

Consensus L1 PCR assays. Five µl of processed sample was amplified in each assay in a 9600 Thermal Cycler (Perkin-Elmer Cetus, Montreal, Canada) in a 100 µl reaction volume containing 10 mM Tris-HCl [pH 8.3], 50 mM KCl, 5 U of *AmpliTaq Gold* DNA polymerase (Roche Diagnostic Systems, Mississauga, Ont.), 3.5 mM $MgCl_2$, and 200 µM of each dNTP. For FAP59/64 PCR, purified DNA was amplified with 3 µM of each primer FAP59 (5'-TAACWGTIGGICAYCCWTATT-3') and FAP64 (5'-CCWATATCWVHCATITCICCATC-3') at 95°C for 10 min followed by 45 cycles of 1.5 min at 94°C, 1.5 min at 50°C and 1.5 min at 72°C (Forslund et al., 1999; Antonsson et al., 2003). For HVP2/B5 PCR, purified DNA was amplified with 0.75 µM of each primer HVP2 (5'-TCNMGNGGNCANCCNYTNGG-3') and B5 (5'-AYNCCRTTRTTRTGNCCYTG-3') (Shamanin et al., 1994; Shamanin et al., 1994; Shamanin et al., 1996) at 94°C for 10 min followed by 45 cycles of amplification for 1 min at 94°C, 2 min at 52°C and 1 min at 72°C, and a final step of 6 min at 72°C. For each PCR run, amplification buffer and human fibroblast DNA negative controls were included. Measures to avoid false positive reactions due to contamination were followed (Coutlée et al., 1997). HPV-5 or HPV-8 plasmids served as positive controls for both assays that reached an equal level of sensitivity in vitro (data not shown).

HPV DNA typing by PCR-sequencing. HPV amplicons were visualised by migration on a 2% agarose gel stained with ethidium bromide. The presence of HPV amplicons was suspected by size determination (amplicons migrating at ±650 bp for HVP2/B5 and at ±480 bp for FAP59/64). Amplicons were purified with the QIAquick gel extraction kit protocol (Quiagen Inc., Mississauga, Ont). Double-stranded PCR-sequencing was done directly on amplicons and also after cloning of amplicons in a vector. To this end, PCR products were cloned using the TOPO TA cloning kit (Invitrogen, Calrsbad, CA) (Mayrand et al., 2000). Five recombinant clones per sample and per primer pair were sequenced. To confirm results obtained when a HPV type identified by direct sequencing could not be identified in cloned HPV amplicons or when the first five clones yielded more than one type, 5 additional clones were sequenced. Plasmid DNA from transformed clones was then further purified using the QIAprep Spin Miniprep system (Quiagen Inc.) according to the manufacturer instructions. Double-stranded PCR-sequencing was done with forward and reverse primers using a fluorescent cycle-sequencing method (BigDye terminator ready reaction kit, Perkin-Elmer) on twenty ng of purified DNA (Yamada et al., 1997). Cycling parameters were 25 cycles at 96°C for 10 sec, 50°C for 5 sec and 62°C for 4 min. Sequence analysis was performed on an ABI Prism 3100 Genetic Analyzer system at the Centre de Recherche du CHUM.

HPV sequences were compared with sequences of known types and novel putative types in the EMBL and GenBank databases using the BLAST server (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). Isolates sharing ≥90% homology in the partial nucleotide L1 sequence were considered to belong to the same type. Guidelines

from the Papillomavirus Nomenclature Committee were followed in defining novel putative HPV types since only a region of L1 was analysed with both primer pairs (de Villiers, 2001). An isolate was considered as a new putative type if the partial L1 sequence displayed < 90% homology with known types. HPV types were classified into the various papillomavirus genus recently described (de Villiers et al., 2004). Novel putative types were classified into papillomavirus species when they shared between 71% to 89% nucleotide identity within the partial L1 sequence with a known type of that species (de Villiers et al., 2004). HPV L1 DNA sequences were all aligned using Clustal X 1.81 (Altschul et al., 1990).

Statistical analyses. The crude percentage of agreement (concordance) between sampling methods was the percentage of pairwise samples for which results were identical considering the presence of HPV DNA irrespective of type or the presence of each type individually. The modified Wald method was used to calculate 95% confidence intervals (CI) around binomial proportions (Agresti and Coull, 1998). The unweighted kappa (κ) statistic was calculated to adjust for chance agreement between sampling methods (Fleiss, 1981). Proportions were compared with the z statistical test. Categorical variables were compared with the Pearson's chi-square test or the Fisher's exact test, depending on the sample size. To identify potential associations, simple logistic regression analyses were performed to compute odds ratios (OR) and 95% confidence intervals (95% CI). The number of types per sample was compared between healthy participants and those with skin lesions using the Mann-Whitney rank sum test. Double-sided P values of ≤ 0.05 were considered significant.

Results

Comparison of FAP59/64 and HVP2/B5 primers for HPV DNA detection. β-globin DNA was detected in extracted DNA from 47 (95.9%) of 49 swab samples from healthy individuals, 12 (92%) of 13 samples from those with AK, 8 (72.7%) of the 11 samples from those with SCC, and 8 (100%) of 8 samples from renal transplant recipients ($p > 0.05$). Only participants with β-globin-positive samples were considered for the analyses described below. HPV DNA, irrespective of types identified by sequencing, was detected more frequently with FAP59/64 than with HVP2/B5 in all subject categories defined by lesion status (Table 1). Of the 47 swab samples from healthy individuals without skin lesion irrespective of age, 41 (87%) and 4 (9%) contained HPV DNA with FAP and HVP2 primers, respectively ($p<0.001$).

HPV typing with PCR-sequencing of FAP59/64 and HVP2/B5 amplicons. PCR-sequencing of amplicons generated with FAP59/64 primers revealed the presence of 77 different types (22 known types, 55 putative novel types) in 75 samples, whereas those generated with HVP2/B5 primers identified only 8 types (3 known types, 5 putative novel types) in the same set of samples ($p<0.001$). The list of the most frequently detected types with FAP59/64 and of all types detected with HVP2/B5 is provided in Table 2. Three putative novel HPV sequences (LIO1, LIO2, LIO3) were detected with FAP59/64 primers (Table 3). In 67 specimens, from 1 to 10 types (mean 2.3±1.8 types, median 2 types) were detected with FAP59/64 only. In two specimens, from 1 to 2 types (mean 1.5±0.7, median 1.5 types) were detected with HVP2/B5 only. However, the latter two

samples contained HPV types that were detected only by FAP59/64. FAP59/64 detected in at least one specimen one of the 77 types identified in this study while HVP2/B5 was negative (median 2.0 samples, mean 2.0±1.2 samples). Using PCR-sequencing of cloned amplicons generated with FAP59/64 primers, a median of 1 type per sample (range of 0-10, mean of 1.43±1,76, 95% CI 1.10-1.76) was identified in contrast to a median of 0 type (range of 0-2, mean of 0.06±0.31, 95% CI 0.01-0.12) with PCR-sequencing of cloned amplicons from HVP2/B5 primers ($p<0.001$). The difference between the number of types per sample detected with FAP primers with sequencing of HPV amplicons or after cloning was significant ($p<0.001$).

HPV amplicons migrating at the appropriate size were sequenced directly and also after cloning. Typing results obtained with both sequencing strategies were compared for each primer set. For each category of participants based on disease status (healthy, AK, SCC, immunosuppressed individuals), there was no difference in the detection of HPV DNA sequences by sequencing directly or after cloning amplicons generated with FAP59/64 or HVP2/B5 primers ($p>0.22$, data not shown). The percent agreement between direct sequencing of amplicons and sequencing after cloning of amplicons for each primer pair is provided in Table 2. Although agreement with FAP59/64 primers was above 93% for each type, the low kappa value between typing procedures suggested at most a fair agreement beyond chance (mean κ= 0.56±0.19, 95%CI: 0.46-0.65). PCR-sequencing after cloning was more efficient than direct sequencing for 15 types when amplicons had been generated with FAP59/64 (Table 2). In seven samples containing one (n=1) or several types (n=6), an HPV type was detected by direct cloning of FAP59/64 amplicons but only HPV-unrelated sequences were cloned

and sequenced (data not shown). Similarly, HPV types 25, FA23, FA112, VS92.1 were detected only by direct sequencing of amplicons generated by HVP2/B5 while sequencing of 10 clones did not reveal the presence of HPV DNA sequences.

Association between HPV infection and skin disease. The influence of age on the detection rate of HPV in skin swab samples was assessed in the 47 healthy individuals without skin lesions (Table 1). HPV DNA was detected as frequently in skin samples from healthy individuals younger and older than 50 years of age ($p=0.54$). In healthy individuals (Table 4), HPV FA127 was detected more frequently in participants > 50 years (23% versus 0%, $p=0.02$).

When individuals with a renal allograft, AK and SCC were compared to healthy individuals over 50 years old, there was no significant difference in the detection rate of HPV DNA (Table 1), healthy controls being frequently infected by HPV. HPV types within species 1 and 2 of the Beta-papillomavirus genus were the most common, being detected in 27 (36%) participants (Table 4). These two species considered together were significantly associated with the presence of AK (OR=24.8, 95%CI: 2.3-262.6). Nearly all skin swab samples obtained from participants with AK contained at least one type belonging to these two species. Only one participant with SCC was infected with an isolate belonging to the Beta-papillomavirus genus, all other samples contained putative novel types (table 4). Alpha papillomavirus species 2, usually found in benign lesion (de Villiers et al., 2004), were detected in only one participant with AK concurrently with an HPV-5 isolate, a type belonging to the Beta-papillomavirus genus.

As shown in Table 4, the most frequently detected HPV isolates in participants with AK or SCC were putative types FA51 and FA62 (Table 4). HPV FA51 was detected in samples from 3 (38%) of 8 individuals with SCC as opposed to 0 (0%) of 13 older healthy individuals (p=0.04). By combining results obtained for both skin diseases, FA51 was detected in 6 (30%) of 20 individuals with lesions as opposed to 0 (0%) of 13 healthy controls older than 50 years (p=0.06). Since HPV FA51 was detected only in young healthy participants, we combined all healthy participants irrespective of age and found FA51 was detected significantly more frequently in participants with AK or SCC (6 (30%) of 20) than in all healthy participants (1 (2.1%) of 47), a difference that was statistically significant (OR=19.7, 95% CI: 2.1–186.5;). However, the number of participants in our study was small and this association should be confirmed in a larger study. There was no clear association between skin disease status and HPV detection for the other types.

The association between the number of types identified by PCR per sample and skin disease or immunodeficiency status was then investigated (Table 5). Overall, median number of types was 1 (mean of 1.7±1.9, 95%CI: 1.3 – 2.0; range 0 – 11). The burden of HPV infection measured as the number of types per sample (Table 5) was significantly greater in renal transplant recipients than healthy participants older than 50 years (p=0.05). There was no difference between the burden of HPV infection in healthy individuals below or above 50 years of age (p=0.81). When healthy participants were compared to participants with AK or SCC, a greater burden of HPV infection was found in individuals with skin disease (p=0.046) or with AK alone (p=0.02).

Discussion

Our evaluation revealed that FAP59/64 primers detected a greater number of samples positive for HPV and also a greater number of HPV types per sample. The fragment generated with FAP primers (nucleotide positions 5981 to 6458 of HPV-8) is nested within the amplicon generated with HVP2/B5 (nucleotide positions 5835-6481 of HPV-8) (Shamanin et al., 1996; Forslund et al., 1999). Amplification efficiency may have been better with FAP59/64 primers because they amplified a smaller DNA fragment. FAP59/64 primers amplify less efficiently types 1, 2, 41, and 63 (Forslund et al., 1999) while HVP2/B5 amplify less efficiently types 4, 48, 50, 60 and 65 (Surentheran et al., 1998). The sensitivity of FAP59/64 and HVP2/B5 primers can be increased using nested PCR (Forslund et al., 2003; Shamanin et al., 1996), a strategy that was not used in this evaluation. In our study, even if more samples were found to be HPV-positive with FAP59/64 than HVP2/B5, the combination of results obtained with both primer pairs was more informative of the diversity of cutaneous HPV infection. Several studies have demonstrated that combined primer panels allow for a more comprehensive evaluation of cutaneous HPV infection (Astori et al., 1998; Tieben et al., 1993; Surentheran et al., 1998; Meyer et al., 2000; Harwood et al., 1999; Forslund et al., 1999; Pfister et al., 2003; Harwood et al., 2004; Shamanin et al., 1996; Boxman et al., 2000; de Villiers et al., 1997). Analysis of cloned amplicons generated with FAP identified more types per sample than direct PCR-sequencing. However, HPV DNA was identified repeatedly in several instances, especially with HVP2/B5 primers, by direct sequencing of amplicons while only HPV-unrelated sequences could be cloned and sequenced from these

specimens. Studies relying solely on PCR-sequencing after cloning for typing HPV sequences on the skin could thus have underestimated the prevalence of HPV infection.

The detection rate and spectrum of cutaneous HPV infection in healthy individuals and individuals with cancerous lesions of the skin was described here for the first time in Canadians. In agreement with other studies, we found a great diversity of HPV types on the skin, identifying 77 types or putative types in 75 samples (Surentheran et al., 1998; Antonsson et al., 2003; Shamanin et al., 1996; Shamanin et al., 1994; Harwood et al., 2004). Overall, types belonging to Beta-papillomavirus genus species 1 and 2 predominated in our population. Because FAP primers were used in our study, the prevalence of type 38 and candidate type 92 may have been underestimated (Forslund et al., 2003). Several of the cutaneous types undetected with FAP 59/64 (Harwood et al., 1999; Forslund et al., 1999) could be detected with HVP2/B5. A more complete evaluation of HPV type-specific prevalence could have been done by using nested primers with HVP2/B5 (Harwood et al., 1999; Harwood et al., 2004) or by adding another degenerate primer pair for EV types (Astori et al., 1998; Meyer et al., 2000). In our study, one of the putative HPV types was related to HPV-22 in the species Beta 2. The other two novel types shared homology with putative new types detected on the skin as mentioned in the table and were also related to known types HPV-47 for LIO2 (species Beta 1) and HPV-15 (species Beta 2) for LIO3. These latter species of Beta-papillomavirus contain several of the Epidermodysplasia verruciformis (EV)-related HPV types. In other reports, novel types frequently shared sequence homology with EV and cutaneous types (Forslund et al., 1999; Harwood et al., 1999; Astori et al., 1998; Harwood et al., 2000).

In our study, over 80% of healthy controls irrespective of age were infected by HPV, with sometimes up to eleven types per sample. HPV is ubiquitous in humans even without lesions (Forslund et al., 1999; Favre et al., 1998; Antonsson et al., 2003; Antonsson et al., 2000; Weissenborn et al., 1999; Astori et al., 1998; Boxman et al., 1999; Boxman et al., 0 AD; de Villiers et al., 1997; Wieland et al., 2000). HPV sequences can be found in 63% to 80% of skin swabs from healthy individuals (Forslund et al., 1999; Antonsson et al., 2000; Antonsson et al., 2003). Nearly half (46.8%) of samples from individuals without skin lesion contained more than one type in our study, similar to other studies on healthy individuals (Antonsson et al., 2000; Antonsson et al., 2003; Forslund et al., 1999). HPV types identified in skin swab samples depend on the site sampled on the body, the site most frequently positive being the forehead (Forslund et al., 1999; Antonsson et al., 2000). The same HPV type(s) is usually detected in multiple biopsies of tumours or normal skin obtained from the same individual, indicating a widespread colonisation by HPV types (Astori et al., 1998; De Jong-Tieben et al., 1995).

Types detected in perilesional swab samples from AK were different from those in SCC. Two studies also reported that the spectrum of HPV types detected in SCC and AK was also different (Pfister et al., 2003; Forslund et al., 2003). These results suggest that HPV may play at most the role of cofactor to other carcinogens implicated throughout carcinogenesis to invasive cancer. Beta-papillomavirus species 1 and 2 were associated with AK in our work as reported previously (Harwood et al., 2000; Pfister et al., 2003; Boxman et al., 0 AD). Participants with SCC were mainly infected with novel putative types in contrast with previous reports reporting the predominance of EV types (Harwood et al., 2000; de Villiers et al., 1997; De Jong-Tieben et al., 1995; Harwood et

al., 2004). Perilesional swab samples from individuals with AK or SCC contained a greater number of types than swab samples from healthy controls. Perilesional swabs have been shown to contain a greater number of types than biopsy of lesion (Forslund et al., 2003). HPV types detected in biopsies of skin tumours are usually found in the perilesional skin swab sample (Forslund et al., 1999). Perilesional swab samples are more often positive and contain more HPV types than biopsies. This could explain our higher rate of HPV infection in individuals with skin lesions compared to studies testing biopsy samples.

Putative novel types FA51 and FA62 were frequently detected in individuals with precancerous or cancerous skin lesions. HPV FA51 has been reported in two patients with basal cell skin carcinoma and AK (Forslund et al., 2003; Forslund et al., 2003) and infrequently in healthy individuals (Antonsson et al., 2003). The association between HPV infection and AK or SCC in our work should be investigated in a larger case-control study with enhanced power and a more systematic approach to accruing subjects so as to minimise potential biases. Such a study should include a more thorough evaluation of other factors associated with SCC such as exposure to UV light. Some have reported that HPV detection was increased in normal individuals at sun-exposed areas (Antonsson et al., 2000; Kiviat, 1999; de Villiers et al., 1999).

Renal transplant recipients were all infected by HPV as reported in a recent study (Forslund et al., 2003). The small number of participants with renal allograft precluded establishing differences in prevalence of HPV with non-immunosuppressed individuals. Several studies have demonstrated that immunosuppressed patients have a higher prevalence of cutaneous HPV infection (Forslund et al., 2003; Harwood et al., 2000;

Antonsson et al., 2000; Boxman et al., 1999). Despite the small number of participants, our study suggests that immunosuppressed individuals are infected with a greater number of types per sample than immunocompetent individuals of the same age group. One study had already reported that immunosuppressed individuals had more commonly multiple type infections (Harwood et al., 2000). HPV putative novel type FA23 was detected more frequently in renal transplant recipients than healthy individuals. This putative type had been described initially in renal transplant recipients and patients undergoing dialysis (Antonsson et al., 2000). It has also been detected in several countries in normal individuals (Forslund et al., 2003; Antonsson et al., 2003).

This study demonstrates that the ubiquity and type-diversity of cutaneous HPV infection reported in several populations are also present in our setting. It is possible that the HPV types involved in SCC are distantly related to actual known types and have not yet been described. This could explain the difficulty in obtaining concordant results on the role of HPV in skin cancer across studies. The fact that swabs from individuals with SCC contained only novel types in our population supports the concept that the types associated with these lesions have yet to be discovered. Our study supports an association between Beta-papillomavirus genus with AK. Prospective studies are needed to determine whether or not the role of certain HPV types is directly causal or as a cofactor to stronger carcinogens such as UV light. Studies of different populations at high-risk using several primer pairs and low-stringency PCR investigations could reveal types more strongly associated with these lesions. Large-scale epidemiological studies need to be conducted to better investigate the associations with Beta-papillomavirus and SCC as well as the potential role of novel types.

Table 1. HPV detection rates in swab samples from renal transplant recipients, individuals with various cutaneous lesions and healthy controls.

Skin lesion	No. of patients	No. of (%) of samples positive for HPV sequences		
		PCR FAP59/64	PCR HVP2/B5	Total
Healthy controls <50 yrs	34	30 (88)	4 (12)	30 (88)
Healthy controls ≥50 yrs	13	11 (85)	0 (0)	11 (85)
Renal transplant recipients	8	8 (100)	1 (13)	8 (100)
Actinic keratosis	12	12 (100)	2 (17)	12 (100)
Squamous cell carcinoma	8	7 (88)	2 (25)	7 (88)
All participants	75	68 (91)	9 (12)	68 (91)

Skin samples were amplified blindly in parallel with FAP 59/64 and HVP2/B5 primer pairs as described in the methods section. Six samples tested negative for β-globin: 2 from healthy controls, 1 from an individual with AK and 3 from individuals with SCC carcinoma. Statistical significance for each comparison between FAP59/64 and HVP2/B5 was $p<0.001$ except for individuals with SCC for whom a $p=0.04$ was obtained.

Table 2. Detection rates of HPV types in 75 β-globin-positive swab samples.

HPV Type	No. of samples positive by			% agreement (95% CI)	Kappa (95%)
	Both	cloning only	Direct only		
FAP59/64					
All types[1]	50	112	15	97.8 (97.4-98.0)	0.82 (0.69-0.94)
Frequent types[2]	30	59	8	87.9 (84.9-90.0)	0.92 (0.79-1.00)
Rare types[3]	20	53	7	97.8 (96.9-98.5)	0.85 (0.72-0.97)
5	0	3	0	96.0 (88.5-99.1)	nc[4]
8	2	2	1	97.3 (90.3-99.0)	0.55 (0.33-0.75)
12	2	2	2	94.7 (86.7-98.3)	0.47 (0.25-0.70)
17	0	3	0	96.0 (88.5-99.1)	nc
25	2	1	0	98.7 (92.2-99.9)	0.79 (0.57-1.00)
38	2	2	0	97.3 (90.3-99.0)	0.65 (0.44-0.87)
49	3	4	0	94.7 (86.7-98.3)	0.58 (0.37-0.78)
80	1	2	2	94.7 (86.7-98.3)	0.31 (0.07-0.53)
FA2	0	3	0	96.0 (88.5-99.1)	Nc
FA7	1	2	0	97.3 (90.3-99.0)	0.49 (0.30-0.68)
FA14	0	5	0	93.3 (85.0-97.5)	Nc
FA16	2	4	0	94.7 (86.7-98.3)	0.48 (0.29-0.67)
FA21	0	2	0	97.3 (90.3-99.0)	nc
FA23	2	1	0	98.7 (92.2-99.9)	0.79 (0.57-1.00)
FA35	0	3	0	97.3 (90.3-99.0)	nc
FA51	2	3	2	93.3 (85.0-97.5)	0.41 (0.19-0.63)
FA62	2	2	0	97.3 (90.3-99.0)	0.65 (0.44-0.87)
FA75	1	2	0	97.3 (90.3-99.0)	0.49 (0.30-0.68)
FA112	0	3	0	96.0 (88.5-99.1)	nc
FA118	1	3	0	96.0 (88.5-99.1)	0.39 (0.21-0.57)
FA127	2	2	0	97.3 (90.3-99.0)	0.65 (0.44-0.87)
LIO1	1	4	0	94.7 (86.7-98.3)	0.32 (0.15-0.48)
HVP2/B5					
All types[5]	2	3	6	98.8 (97.6-99.5)	0.31(0.28-0.33)
17	1	0	0	100 (94.2-100.0)	1.00 (0.77-1.00)
25	1	0	1	98.7 (92.2-99.9)	0.66 (0.45-0.87)
49	0	1	0	98.7 (92.2-99.9)	nc
FA51	0	1	2	98.7 (92.2-99.9)	nc
FA119	0	1	0	98.7 (92.2-99.9)	nc
FA23	0	0	1	98.7 (92.2-99.9)	nc
FA112	0	0	1	98.7 (92.2-99.9)	nc
VS92.1	0	0	1	98.7 (92.2-99.9)	Nc

The detection rates of HPV types in 75 β-globin-positive swab samples was assessed by direct sequencing of amplicons or by sequencing of cloned amplicons generated with FAP59/64 and HVP2/B5 primers as described in the methods section. Only types identified in >2 samples with FAP59/64 and all types identified with HVP2/B5 are shown in the table. 1: All typing results for 77 genotypes in 75 samples (5775 typing results). 2: selecting only types (n=24 types) detected in >2 samples (n=23 samples) for 552 typing results. 3: Selecting only types (n=53 types) detected in $\leq$ 2 samples (n=52 samples) for 2756 typing results. 4; nc is for not calculated. 5: all typing results for 8 genotypes in 75 samples (for a total of 600 typing results to be compared).

Table 3. New putative types and the closest related known HPV types

No. of participant	Putative new HPV	Closest related type	% homology	Fragment size (nt)
5	LIO1	22	88%	483
1	LIO2	FA121	81%	430
1	LIO3	FA57	83%	181

Sequences of putative new types.

Putative new type LIO1,

CGGCCGCGAATTCGCCCTTCCTATATCTCTCATGTCGCCATCCTCTATAACACTATTTTT
TAACTCTAGTGGAGGACATAAGCCCTCCTGACTGCCTGCATCTTCACACACTGGTGCTTT
ATCCCAATGTTCCCCTAAACAAGGTATGCAACCAATAATAAACATTTGTACCTGTTTAGG
ATCAAAAGAAACATTTCTTCTATCATCTGCTGTGCCTGTCTGGCGTTCACTAGGATTTTC
AGTGTCATGTAATTTATTAAATAATGGATGACCTGTGGTACCTACACCTATGGGCTGTCC
TCTCCCGATTTCTAAGCCTTTGCAAGCCCATACTAATCTGTACCTCTCAGGATCATGAAT
TGTCATATCTGCTAGAGCAAATTTATTAGGATCTGGAAAGGTTACTCTAAATGCTCTATA
CTGGTTCCCTGACACCTTTGGGACTTCTATTTTTGCTCCATCTGTTGCTGATCTTACATC
AAAATAAGGATGCCCCACAGTTAAAGGGCGAATTCGTTTAAACCTGCAGGACTAG

Putative new type LIO2,

GCGGCCTTCCCTATCAAGAAAAAACGCCATCTTGTATATTCTATTTACTAACTGAATTGG
TGGGCAATCACCCTTTCTAACTTGTACTCCTTCACAAGGTTTTGCTACATCCCAATGTTC
ACCAATTGGTGGAGCACAACCTACAATAAATAGCTGAGTTTGTTTAGGGTCAAAAGATAC
ATCCTGACGATTATCATCACTTTGTTTAATATATTTGTTAGGGTTTTCTGTATCACCTAG
TTTGTTATATAAAGGATGTCCAGTAGTACCTATTCCTAAAGGACCGCCTCTACCAACTTC
CAATCCTCTAAGTTTCCATACTAATCTTTGGTGTTCTGGATTATAACACCTTTCTCAATT
AAGGCAAAGCGATTAGGATCTGGTAATTTCATACGAAAAACTCTAAACTGACTTCCTGAG
ACATGAGGAACTAATATTTTAGTTCTATCTTGAACATCCCGAATAGGAAAATATGGATGC
CCCACAGTTAAAGGGCCGAATTCGCGGCCGCTTAAATTCAATTCGCCCCTATAGTGAGTC
GTATTACAAAT

Putative new type LIO3,

TATCTGACATGTCGCCATCCTCTATATATGAATTAACTAATTCTAAAGGAGGGCAATC
ACCATTCTTTACTTCTGTGGTAGCACAGGCCTTTGCAGCATCCCAATAGGCACCAGTACA
AGGTTCACAACCCACAATTAATAATTGGGTCTGTTTTGGGACCATGGACACATTTTGTCT
ATTATCAGTGCTGCTAGTAAAATATTGATTAGGATTTTCAGTGTCATTTAATTTATTTAG
TAAAGGATGACCTGTAGATCCTATGCCCAATGGGCCACCTCTCATTATTTCTATGCCTCT
TAGTTTCCATACTAGTCTTTCTGTATCAGGGTCATATATAGTGGGATCTACTAAAGCAAA
TCTATTAGGGTCAGGCAAAGTAAGTCTAAACACTCTATATTGATTTCCTGACACTTTTGG
TACAAGTTCGTTTCCAGTTGGATCTTTTACAGGAAAATAAGGATGCCCCACAGTTAAAGG
GCGAATTCGCGGCCGCTAAATT

Table 4. Detection rates of HPV types in 75 β-globin-positive skin samples selecting HPV types detected in more than two samples.

Type	No. of positive samples (%)					
	Normal <50yrs (n=34)	Normal >50yrs (n=13)	Normal (n=47)	AK (n=12)	SCC (n=8)	Allograft (n=8)
5	1 (2.9)	0 (0)	1 (2.1)	2 (16.6)	0(0)	0 (0)
8	1 (2.9)	1 (7.7)	2 (4.3)	2 (16.6)	0 (0)	1 (12.5)
12	2 (5.9)	2 (15.4)	4 (8.5)	0 (0)	0 (0)	1 (12.5)
17	1 (2.9)	1 (7.7)	2 (4.3)	1 (8.3)	0 (0)	0 (0)
25	1 (2.9)	0 (0)	1 (2.1)	2 (16.6)	0 (0)	0 (0)
38	3 (8.8)	0 (0)	3 (6.4)	1 (8.3)	0 (0)	0 (0)
49	5 (14.7)	1 (7.7)	6 (12.8)	1 (8.3)	0 (0)	0 (0)
80	1 (2.9)	1 (7.7)	2 (4.3)	1 (8.3)	1 (12.5)	0 (0)
FA2	1 (2.9)	1 (7.7)	2 (4.3)	1 (8.3)	0 (0)	0 (0)
FA7	0 (0)	1 (7.7)	1 (2.1)	1 (8.3)	0 (0)	1 (12.5)
FA14	1 (2.9)	1 (7.7)	2 (4.3)	1 (8.3)	1 (12.5)	1 (12.5)
FA16	1 (2.9)	3 (23.1)	4 (8.5)	1 (8.3)	0 (0)	1 (12.5)
FA18	1 (2.9)	2 (15.4)	3 (6.4)	0 (0)	0 (0)	0 (0)
FA23	1 (2.9)	0 (0)	1 (2.1)	0 (0)	0 (0)	2 (25)
FA35	2 (5.9)	1 (7.7)	3 (6.4)	0 (0)	0 (0)	0 (0)
FA51	1 (2.9)	0 (0)	1 (2.1)	3 (25)	3 (37.5)	0 (0)
FA62	0 (0)	1 (7.7)	1 (2.1)	0 (0)	3 (37.5)	0 (0)
FA75	0 (0)	1 (7.7)	1 (2.1)	1 (8.3)	0 (0)	1 (12.5)
FA85	2 (5.9)	0 (0)	2 (4.3)	1 (8.3)	0 (0)	0 (0)
FA112	0 (0)	2 (15.4)	2 (4.3)	0 (0)	1 (12.5)	0 (0)
FA118	2 (5.9)	0 (0)	2 (4.3)	1 (8.3)	0 (0)	1 (12.5)
FA119	1 (2.9)	0 (0)	1 (2.1)	0 (0)	1 (12.5)	1 (12.5)
FA127	0 (0)	3 (23.1)	3 (6.4)	1 (8.3)	0 (0)	0 (0)
LIO1	3 (8.8)	1 (7.7)	4 (8.5)	1 (8.3)	0 (0)	0 (0)
Beta 1-2	11 (32.4)	4 (30.8)	15 (31.9)	11 (91.7)	1 (12.5)	4 (50)
Beta 3	5 (14.7)	1 (7.7)	6 (12.8)	1 (8.3)	0 (0)	0 (0)
Beta 5	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (12.5)
Alpha 2	1 (2.9)	0 (0)	1 (2.1)	1 (8.3)	0 (0)	0 (0)
Gamma	1 (2.9)	2 (15.4)	3 (6.4)	0 (0)	0 (0)	1 (12.5)

Results obtained with FAP and HVP2 primer pairs with direct sequencing or after cloning were combined for this table. HPV types 3, 15, 20, 24, 47, 94, 96, 19 , FA12, FA13, FA15, FA20, FA22, FA39, FA41, FA43, FA46, FA48, FA56, LIO3, FA61, FA64, FA65, FA67, FA70, FA74, FA108, FA109, FA114, FA120, LIO2, FA121, FA138, FA139, RTRX7, FA12, FA32, were detected in only one sample while types 4, 23, 50, 93, FA1, FA21, FA25, FA26, FA27, FA28, FA37, FA45, FA53, FA83, FA90, FA91, FA98, VS92.1, were detected in two samples each.

Table 5. Burden of HPV infection in 75 β-globin-positive skin samples measured as the number of types detected per sample and underlying disease.

Disease	No. of participants	Median	Range	Mean (95% CI)
Normal<50 yrs	34	1.0	0-5	1.9±1.5 (1.4-2.4)
Normal ≥50 yrs	13	1.0	0-11	2.2±2.8 (0.5-3.9)
Normal	47	1.0	0-11	2.0±1.9 (1.4-2.5)
AK	12	3.0	1-6	2.8±1.4 (1.9-3.7)
SCC	8	2.0	0-5	2.3±1.7 (0.9-3.6)
AK+ SCC	20	2.5	0-6	2.6±1.5 (1.9-3.3)
Allograft recipient	8	3.5	1-7	3.6±2.1 (1.9-5.4)

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DISCUSSION

Of the approximately more than 100 different types of human papillomavirus identified hitherto, 40 are known to infect the genital tract, and some have been identified as the causative agent of cervical cancer [281]. Over 20 HPV types have been identified in patients with epidermodysplasia verruciformis (EV), though only a few of these types, mainly HPV-5 and 8, have been found to be associated with skin cancer [269]. Most of the HPV types infecting the skin have yet to be characterized [1,241,270]. PCR with primers chosen in conserved sequences in the L1 and E1 open reading frames (ORF), have been successfully used for the detection of a wide range of genital HPV types [249,250,251,252]. The same strategies have been applied for the detection of cutaneous types in epidemiological studies.

6.1.10 FAP 59/64 primer pair:

PCR with universal primers specially designed for cutaneous HPV types has been developed [2,225,228,238,248,253]. However, methods for the detection of cutaneous HPV types have disadvantages such as nested PCR or combinations of several degenerate primers. Forslund et al. designed a single pair of degenerate PCR primers FAP59/64 primarily aimed at the amplification of cutaneous HPV types. Two regions with a relatively high degree of nucleotide homology were found in the 5'end of the L1 ORF. The positions of the primers corresponded to nucleotides 5981-6001 and 6458-6436 of the HPV-8 genome, yielding an amplicon of 478 bp [241].

Studies using this primer pair showed high sensitivity, the assay being optimized to allow the detection of less than 10 HPV DNA copies per test [241]. In that study, HPV DNA was detected in 50% of all skin samples while in another study the detection rate was 85% [236]. In our study, we were able to detect HPV DNA in 91% of all B-globin positive skin samples. This primer pair was also found to detect significantly greater numbers of HPV-positive skin samples as compared to HVP2/B5 primer pair. In one study where the investigators used FAP primers, 30 new types were identified that we detected also in our study [1]. We could also detect three new types and revealed the presence of 77 different types (22 known types, 55 putative novel types) in 75 samples.

HVP2/B5 primer pair:

The degenerate primer pair HVP2/B5 was described by Shamanin et al to detect HPV from all groups with the exception of the phylogenetic clade comprising HPV-4, 48, 50, 60. Generally, the copy number detected by HVP2/B5 was high 5-5x10^4 copies which is likely to be a consequence of the high degree of degeneracy of these primers [228,253,254].

HVP2/B5 primer pair detected all cloned HPV types in one study and in up to 100% of wart lesions in opposite to primers designed specifically for EV types, with the exception of types 4, 48, 50, 60 and 65 [254]. The original degenerate primer pair included primer C instead of primer B5. To improve sensitivity of the assay, less degenerate primers were derived from the original degenerate HVP2/C pair. B5 was the most efficient of these less degenerate primers [238]. In one study, HVP2/B5 proved to be the most sensitive primer pair to detect the presence of HPV DNA in skin samples but was unable to detect HPV DNA in SCC [254] while CP62-69 detected the presence of HPV DNA mainly in SCC and could not detect HPV DNA in warts and vulvar cancer [254].

In our study, HVP2/B5 primer was less sensitive than FAP59/64 primer. Of the 47 swab samples from healthy individuals without skin lesion irrespective of age, 4 (9%) contained HPV DNA with HVP2/B5 primers. Another study that used HVP2/B5 reported a detection rate of HPV DNA of 75% in wart samples while it was 0% SCC samples [254]. We could detect HPV DNA in 25% and 17% of SCC and AK samples, respectively. PCR-sequencing of amplicons generated with HVP2/B5 primers identified 8 types (3 known types, 5 putative novel types) in the same set of samples.

Comparison between FAP59/64 primer pairs versus HVP2/B5 primer pair:

Until recently, it has been technically difficult to detect the HPV types involved in skin cancer compared to genital HPVs, considerably hindering research in this area. In early studies, detection of HPV DNA varied both in overall prevalence and in the spectrum of HPV types detected [213]. As a consequence, the true prevalence of HPV in cutaneous lesions was unknown. A limited number of studies have demonstrated differences between efficiency of degenerate primer pairs to detect cutaneous HPV types [256,266,279]. Degenerate primers AM were found to be more sensitive that degenerate primers HD in one study [237]. HVP2/B5 primers had not been compared with the most efficient primers for detection of cutaneous HPV types, the FAP59/64 primers. Our evaluation revealed that FAP59/64 detected a greater number of cutaneous HPV infection as well as more types per sample in healthy controls and individuals with various skin lesions. Amplification efficiency may have been better with FAP59/64 primers because they amplified a smaller DNA fragment (478 bp versus 650 bp). Both degenerate primer pairs amplify the 5' end of the L1 open reading frame, the most conserved gene within the papillomavirus genome [110]. The fragment generated with FAP primers (nucleotide positions 5981 to 6458 of HPV-8) is nested within the amplicon generated with HVP2/B5 (nucleotide positions 5835-6481 of HPV-8) [238,241]. Both primer sets have been shown to amplify a broad spectrum of types, including novel HPV types [238,241]. FAP59/64 reaches excellent sensitivity endpoints for several cutaneous types down to 1-10 copies per test maybe because of higher primers, length of the amplicons and due to cycling parameter [241]. HVP2/B5 and FAP59/64 have been utilized in several epidemiologic studies [1,34,212,238,241,254,270,277,278]. The sensitivity of FAP59/64 and HVP2/B5 primers can be increased using nested PCR [256,285], a strategy that was not used in this evaluation. Several studies have demonstrated that combined primer panels allow for a more comprehensive evaluation of cutaneous HPV infection [2,237,238,241,254,279,280,284,285]. In our study, even if FAP59/64 detected more types than HVP2/B5, the combination of results obtained with both primer pairs reported

a greater number of positive samples. As found in this study, types identified by two assays applied on the same samples can sometimes be different [237,238,254]. Using only one degenerate primer pair will likely underestimate HPV prevalence on the skin.

Comparison between direct sequencing and sequencing after cloning:

Cloning of amplicons has revealed additional HPV types often matching the type detected with direct PCR-sequencing. Infections with more than 2 types were identified in 62% of samples. HPV DNA sequences amplified by FAP were identified in 88% of samples using sequencing of cloned amplicons compared to 80% by direct sequencing of amplicons. The same sequence was detected by direct sequencing and sequencing of cloned FAP amplicons for 42% of samples. HPV DNA was detected by HVP2/B5 in 5% of samples by sequencing of cloned products compared to 12% by direct sequencing. With HVP2/B5, the same type was identified in 22% of samples with both sequencing procedures.

It is possible that sequencing many more clones might have detected additional HPV types. We could have sequenced systematically 25 clones for all samples. Budget and time restriction did not allow to pursue this strategy. The yield would have been minimal for samples with only 1-2 types per 5 to 10 clones. Sequencing of cloned PCR products sometimes revealed different types than direct sequencing. More types were identified after cloning which makes the cloning of PCR product necessary for a complete evaluation of mixed HPV type infections (Figure 11).

The agreement between PCR-sequencing of amplicons directly and after cloning was also investigated in this study. Analysis of cloned amplicons generated with FAP identified more types per sample than direct PCR-sequencing. However, especially with HVP2/B5 primers, HPV DNA was identified repeatedly in several instances with direct sequencing but only human sequences could be cloned and sequenced from these

specimens. PCR-sequencing after cloning thus did not reveal any HPV infection in these samples. Studies relying solely on PCR-sequencing after cloning of amplicons for detection and typing of HPV sequences on the skin could thus have underestimated the prevalence of HPV infection. Our study not only supports combining results obtained with different primer pairs but also with different sequencing strategies.

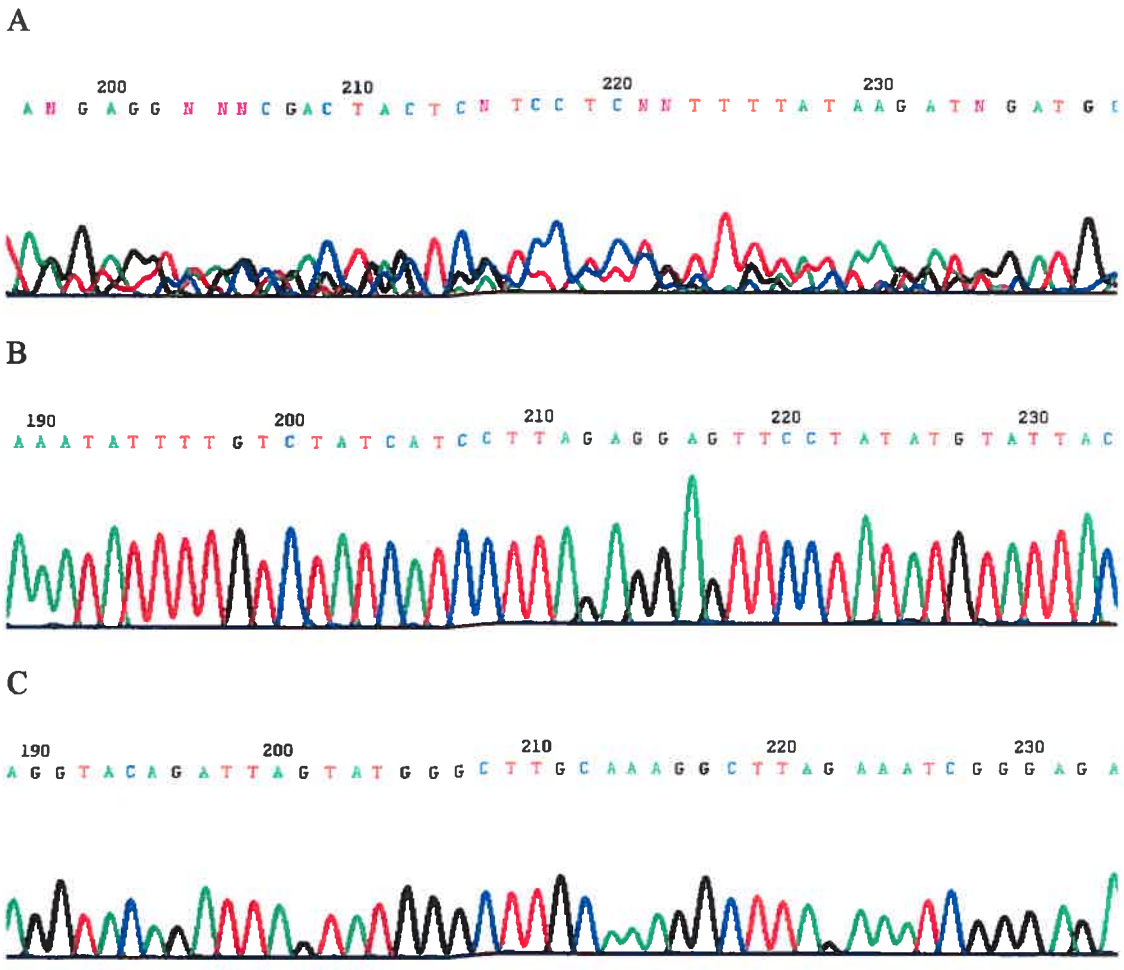

Figure (11): Mixed HPV types are detected with FAP primers. The electropherograms in panels A to C were from skin swab of normal person age above 50 years. (A) the sequence was obtained by direct sequence shows features suggestive of the presence of at least more than one distinct HPV types.(B) and (C) the sequence was obtained after cloning appears to be a single type in each colony different than other colonies.

Clinical assessment of the skin samples:

Methods of collecting samples:

For most of the studies on cutaneous HPV infection, biopsies of the skin were analyzed. A biopsy is the process of removing a portion of skin for diagnostic examination. This invasive method increases the risk of infection because the skin barrier has been broken and sutures are sometimes needed according to depth of the biopsy. Simple methods for collecting skin cells include using wetted cotton-tipped swabs that are drawn back and forward 4-5 times over an area of the skin and immersed in a tube containing 500ml of Preservcyt. Swabbing of the skin by using this simple method has been successfully used for detection of cutaneous HPV infection [1,241,270]. Perilesional swab samples are more often positive and contain more HPV types than lesion biopsies, explaining our higher rate of HPV infection in individuals with skin lesions compared to studies testing biopsy samples that obtained HPV detection rates from 22% to 84% in immunocompetent individuals [212,238,254,260,271,278,282]. Skin swabbing had been utilised previously to estimate the rate of cutaneous HPV infection in the immunocompetent host. Studies showed that 63%-80% of swabs from healthy individuals or individuals with skin lesions contained HPV sequences [1,241]. Types detected varied according to the site sampled on the body and varied over time, the site most frequently positive being the forehead [1,241]. Of a total of 160 participants, the prevalence of HPV in skin samples from renal transplant recipients, patients undergoing dialysis and healthy controls was 51% when the left arm was swabbed, 51% from the right arm, 49% from the left thigh, 49% from the right thigh, and 80% from the forehead [1]. HPV types detected in tumours were also frequently found in skin swab samples [241]. Forehead skin samples contained less frequently HPV EV types than when plucked eyebrow hairs were tested [242]. Other investigators have used invasive samples, such as plucked hairs [242,248] and skin biopsy specimens [226,237,239,243], but they obtained lower rates of detection of HPV than ours.

In our evaluation, epithelial cells were collected with pre-wetted swabs and were resuspended in Preservcyt. This is the first study to use Preservcyt to preserve the integrity of DNA between collection and processing of samples for the detection of cutaneous HPVs. A majority of samples tested positive for human DNA indicating the excellent quality of samples. The high prevalence of HPV DNA infection even in healthy controls also supports the high quality of the DNA analysed.

Ubiquity of HPV infection of the skin:

The detection rate and spectrum of cutaneous HPV infection in healthy individuals and individuals with cancerous lesions of the skin was also described here for the first time in Canadians. HPV can be detected on normal uninvolved skin and plucked hair samples, including EV-related types, and is widely spread in humans without lesion [1,237,241,259,270,273,274,276]. HPV DNA sequences have been detected in 35% to 63% of individuals with normal skin biopsies, supporting the concept that cutaneous infection by HPV occurs frequently [2,237,241,287]. Such a high prevalence rate complicates the investigation of association between HPV infection and NMSC [254]. Commensal infection by HPV on the skin has a worldwide distribution [270]. In our study, over 80% of healthy controls irrespective of age were infected by HPV, with sometimes up to 11 types per sample. In a recent international study, from 42% to 70% of participants without skin lesion in different countries were infected by HPV on the skin [270]. The prevalence of HPV DNA in samples of healthy skin from Bangladesh was 68%, Japan 54%, Ethiopia 52%, Zambia 42% and Sweden 70% [270]. In fact, in that study up to 62% of samples was negative for human DNA sequences [270]. Our higher rate could be explained by the use of Preservcyt to maintain the integrity of cellular DNA, the use of a greater quantity of *Taq* DNA polymerase for amplification and possibly by the population studied. Northern countries evaluated in that work had a 70% prevalence rate of HPV infection, a rate near that found in our study. Our study shows that the previously reported high prevalence and type diversity of skin papillomavirus in healthy also applies to individuals living in Canada [1,2,237,241,270,287]. These findings emphasize the ubiquity and impressive diversity of genotypes amongst the skin

papillomaviruses, both in human [1,270] and in animals [282]. Adapted to their hosts presumably over hundreds of millions of years, these viruses are the first viral skin commensal to be described.

Multiple type infections on each sample:

More than one HPV type can be detected within an individual skin lesion or in exfoliated skin cells [225,238]. As in our study, another group reported that nearly 40% of skin samples collected with a pre-wetted swab contained more than one type [241]. We report here that FAP could detect significantly a greater number of HPV types per sample than HVP2/B5. Nearly half (46.8%) of samples from individuals without skin lesion contained more than one type in our study, similarly to an international study on healthy individuals in which 39% of samples were multiple type infections [270]. Another study also revealed the multiplicity of HPV infection in healthy hosts without skin lesion [1]. We found no difference in the number of types per sample between younger and older participants, although the small number of individuals above 50 years old limited the power of our analysis.

As demonstrated in other studies, we found a great diversity of infection by cutaneous types demonstrated by the detection of 77 types or putative types in 75 β-globin-positive samples [238,259,270,280,285]. Three studies have reported the presence of 88 different types from 142 samples, 50 types from 33 samples and 45 types from 49-HPV-positive samples, respectively [1,270,278]. The same HPV type(s) is usually detected in multiple biopsies of tumours or normal skin obtained from the same individual, indicating a widespread colonisation by HPV types [237,243].

Specific HPV types:

Putative novel HPV types predominated in perilesional swabs from individuals with SCC. Because FAP primers were used in our study, the prevalence of hpv type 92

may have been underestimated [278]. Several of the cutaneous types undetected with FAP 59/64 such as types 1, 2, 10 and 41 [241,279], can be detected with HVP2/B5 primers. A more complete evaluation of HPV type-specific prevalence could have been done by using nested primers with HVP2/B5 [279,280] or by adding another degenerate primer pair for EV types [237,283]. Putative novel types FA51 and FA62 were the most frequent types detected in individuals with cancerous lesions. HPV FA51 was associated with the presence of AK or SCC in our population. HPV FA51 has been reported in two articles in two patients with basal cell carcinoma of the skin and solar keratosis [256,278]. These two putative types were also reported infrequently in healthy individuals [270]. A recent study on AK lesions revealed that 80% of frozen biopsies in contrast to 40% of fixed tissue specimens contained HPV DNA sequences [284]. As in the latter study, types 5, 8, 25 and 38 were frequent known types identified in AK lesions. Unfortunately, primers used in that study were different from FAP primers and targeted another region of the HPV genome. It is thus impossible to know if they also detected putative types FA51 and FA62 in their patients.

In our study, HPV-49 was found in 6 healthy controls while HPV FA127 and FA16 were detected more frequently in older participants. In opposite to other studies, the most frequent type was not HPV-5 but HPV-49 [270]. HPV-5 is more frequently found in patients with psoriasis and none of our participants was afflicted by this disease [259].

Novel HPV types:

Forslund, Antonsson et al. in 1999 designed FAP primers for the first time. They were able to detect five known HPV types and 12 novel HPVs types from eight patients [241]. The novel HPVs types were FA1-FA9, FA11-FA13 [241]. One year later, they studied of 160 participants and were able to detect 20 known HPV and 30 novel HPV types [1]. The novel HPV types were FA14-FA43 [1]. Three years later by using samples from four different countries, they were able to detect more HPVs types that had not been detected before [270]. Almost all of the putatively novel HPV types they described it

their studies were detected in our study, in addition to three novel HPV types not described before: LIO1, LIO2, and LIO3.

Most consensus PCR protocols have revealed the presence of novel types on the skin. We need to better characterise novel types in order to develop optimised HPV detection assays [238]. There are now 96 HPV genotypes that have been fully characterised and several hundreds putative papillomavirus types have been partially identified in the form of short DNA fragments [110]. HPV genotypes have been recently classified into 5 genus, each further divided into 1 to 15 species [110]. HPV types detected on the skin belong to the alpha-, beta,- gamma-, Mu- and Nu-papillomavirus genus [110]. In our study, one of the putative HPV types was related to HPV-22 in the specie 2 of the Beta-papillomavirus genus. The other two novel types shared homology with putative novel types detected on the skin and were related to type 47 for LIO2 (specie 1 of the Beta-papillomavirus genus) and to type 15 (specie 2 of the Beta-papillomavirus genus) for LIO3. These two species of Beta-papillomavirus contain several of the old EV-related types. In other reports, novel types frequently shared sequence homology with EV and cutaneous types [212,237,241,279].

Cutaneous disease and HPV types:

Cutaneous warts and benign epithelial tumours induced by some cutaneous HPV types (HPV-1, 2, 3, 4, 7, and others), occur frequently in up to 20% of the population. The highest prevalence is measured in children and adolescents at the ages of 11-16. These lesions undergo spontaneous resolution within two to three years [289].

Non-melanoma skin cancer represents the most frequent cancer occurring among the Caucasian population world-wide [171,283]. Various attempts have been made to determine the factors involved in the etiology of nonmelanoma carcinomas of the skin. It is widely accepted that the UV component of solar radiation is a major environmental cause [172]. To study the mechanism by which papillomavirus infections may contribute

to the etiology of NMSC in patients without EV, the HPV types associated with such lesions have to be identified. Different groups of investigators have screened various numbers of samples of non-melanoma carcinomas of the skin from immunosuppressed and non-immunosuppressed populations for the presence of HPV DNA sequences. A diverse spectrum of HPV types was reported [228,225,284-288]. No single HPV type was specifically identified in SCC and AK and RTR in some studies [235,263], while other studies showed specific HPV types associated with some cutaneous diseases like SCC or AK. HPV-5 and 8 are strongly associated with non-melanoma skin cancer in EV patients while they are harmless for the general population [260]. HPV-20, 23, 38 were predominant in another study being found in 73% of all malignant lesions, in 35%of solar keratosis and in 13% of the warts [2].

In our study, HPV types belonging to Beta-papillomavirus genus species 1 and 2 predominated. The latter types were previously designated as EV-types. Beta-papillomavirus species 1 and 2 were significantly associated with AK in our work as reported previously [212,274,284]. Participants with SCC were mainly infected with novel putative types in opposite to previous studies reporting a predominance of EV types [2,212,243,280]. The associations between HPV infection and AK or SCC in our work should be investigated in a larger case-control study to increase power of analysis and include a more thorough evaluation of other factors associated with SCC such as exposure to UV light. In our study, we did not control for sampling at sun-exposed or non-exposed sites. Some have reported that HPV detection was increased in normal hosts at sun-exposed areas [1,275,282]. However, all healthy controls were sampled at the same sun-exposed site, the forehead. HPV prevalence in swab samples from individuals with lesions has also been shown to be similar when obtained from sun-exposed or non-sun-exposed sites [278]. Another group reported in Poland that EV-types were less frequently detected in SCC than in AK [284]. The spectrum of HPV types detected in individuals with SCC and AK was also different in another study conducted in Australia [278]. This could suggest that HPV could play a role as a cofactor in the early steps of carcinogenesis. An anti-apoptotic E6 activity may serve to allow cells with cellular damage induced by UV to survive and facilitate the persistence of UV-induced genetic

changes. Reduced apoptosis was demonstrated in cells transfected by the HPV E6 gene of some EV or cutaneous types [281]. The Bak protein is involved in signalling apoptosis in the skin exposed to UVB and is up regulated in skin exposed to UVs [195]. In vivo, cells exposed to UV light showed a marked increase in p53 and Bak in all cell layers with many apoptotic cells being present, while those containing HPV E6 did not show an increase in Bak levels [195]. E6 protein from cutaneous HPV types targeted and abrogated Bak function by promoting its proteolytic cleavage both in vitro and in regenerated epithelium [195]. HPV-positive skin cancers have undetectable levels of Bak protein in contrast to HPV-negative cancers that expressed Bak [195]. Perilesional swab samples from individuals with AK or SCC contained a greater number of types than swab samples from healthy controls or biopsy of lesion [278]. The current evaluation is the first to compare the burden of HPV infection between healthy individuals and those with skin lesions.

Renal transplant recipients and HPV:

Renal transplantation has been performed for more than 35 years with a large success. However, the survival of the graft is associated with the induction and the maintenance of an immunosuppressive status which predisposes to the development of multiple skin disorders. These disorders include warts, hyperkeratosis, keratoacanthomas, and skin malignancies. Skin cancers are considered to be the most frequent tumours in transplant patients, usually developing in sun-exposed areas [291]. The incidence of skin cancer is increased in transplant recipients as compared to the general population and varies according to the country studied. Thus, Hartevelt et al. [290] found a cumulative incidence of skin cancer of 10% and 40% at 10 and 20 years, respectively, after transplantation. This incidence is higher in Australians (45% and 70% at 10 and 20 years, respectively, after transplantation). This difference could be related to more intense sun exposure in Australia.

Various attempts have been made to determine the factors involved in the aetiology of nonmelanoma carcinomas of the skin. It is widely accepted that the UV

component of solar radiation is a major environmental cause [172]. As described before, this is reflected by the body site distribution of lesions. Papillomaviruses play a crucial role in the development of certain malignant tumors [292]. This has been demonstrated clearly in the case of genital tumors such as cervical carcinomas. Specific types of HPVs (high-risk HPV) have been implicated in neoplastic transformation. The transforming function of the HPV E6/E7 gene products depends on the prior modification and functional elimination of cellular tumor suppressor genes and their products [293]. Certain types of HPV have the potential to induce malignant and benign genital tumors [294]. The HPV types from supergroup B (HPV-5 and related HPV types originally isolated from EV patients) have cutaneous tropism but their possible role in the aetiology of NMSC is not clear. The DNA of cutaneous HPV types is found both in solar actinic keratosis and NMSC, especially among immunosuppressed organ transplant recipients [295,296,297].

Renal transplant recipients were all infected by HPV as reported in a recent study [278]. The small number of participants in our study with renal allograft precluded establishing differences in prevalence of HPV with non-immunosuppressed individuals. Several studies have demonstrated that immunosuppressed patients have a higher prevalence of cutaneous HPV infection [1,212,273,278]. Despite the small number of participants, our study demonstrates clearly that immunosuppressed individuals are infected with a greater number of types per sample than immunocompetent individuals of the same age group. One study had already reported that immunosuppressed individuals had more commonly multiple type infections [212]. HPV putative novel type FA23 was detected more frequently in renal transplant recipients than healthy individuals older than 50 years of age. This putative type had been described initially in renal transplant recipients and dialysis patients [1]. It has also been detected in a study conducted in Australia at equal frequency in immunosuppressed and normal individuals [278]. HPV FA23 has also been reported in samples from Japan in nearly 10% of healthy participants without skin lesion and in Sweden [270].

So far, the vast majority of studies on cutaneous HPV infection have used sensitive PCR methods. Less sensitive methods, such as Southern blot, are only infrequently positive [284], indicating very low amounts of viral genomes [239,306]. But common skin warts appear to have higher amounts of viral genomes, since HPV DNA (predominantly HPV-1 and 2) is readily detectable by the Southern blot technique [307].

A significant problem for investigations of the association between cutaneous HPV and nonmelanoma skin cancer is that cutaneous HPV is part of the microbiological flora of healthy human skin as shown in our study and other studies [1,237,248,256,257,270,278]. The site of replication of cutaneous HPV DNA might be stem cells of the hair follicle [242,248,272] or of eccrine ducts [308]. These sites could also be the origin for non-melanoma skin cancer [308,309]. However, it is not known whether cutaneous HPV are involved in the development of skin cancers.

If viral particles are produced and shed from infected healthy skin, they are likely to be contaminating large areas of the body surface, including the surface of skin tumors. If so, punch biopsies of tumors might be scored as HPV positive by PCR, although the viral DNA is not present throughout the tumor. Forslund, et al. [245], investigated specifically whether the HPV DNA prevalence on the surface of skin tumors differed from the prevalence in biopsies from the same tumors. The prevalence of HPV DNA was much higher (69%) in the perilesional swab samples than in biopsies taken after removal of superficial cell layers by repeated tape stripping (12%) [245]. This result demonstrates that cutaneous HPV DNA is commonly present over skin lesions but to a lesser extent throughout the lesion. The fact that swab samples form healthy skin sites were found to be HPV positive to about the same extent as swab samples from the tumors, suggests that presence of virus is not specifically related to the tumor below [1,237,248,256,257,270,278]. Whether the presence of viral DNA in superficial skin is due to viral particles or to episomal HPV genomes of infected cells is not known.

Conclusion

This study demonstrates that the multiplicity and wide spectrum of cutaneous HPV infection reported in several populations are also presents in Canadians, a population at high-risk for SCC. It is possible that the HPV types involved in SCC are distantly related to actual known types and have not yet been described. This could explain the difficulty in obtaining concordant results on the role of HPV in skin cancer across studies. The fact that perilesional swabs from individuals with SCC contained only novel types in our population supports the concept that the types involved in this pathology have yet to be discovered. Our study supports an association between Beta-papillomavirus genus with AK. Prospective studies are needed to determine if this relationship is one of causality and assess if HPV is central to SCC or is acting merely as a cofactor to stronger carcinogens such as UV light. Normal skin is constantly repairing UV-induced damage through normal DNA repair mechanisms but in case of SCC and AK, HPV may play role of a cofactor and enhance transformation by a mechanism not yet clear. Further evaluation on these mechanisms needs to be done. Studies on different populations at high-risk using several primer pairs and low-stringency PCR investigations could reveal types more strongly associated with these lesions. Large-scale epidemiological studies need to be conducted to better investigate the associations with Beta-papillomavirus and SCC as well as the potential role of novel types. Prospective studies are also needed to define the natural history of cutaneous HPV infection.

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