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VIRUSES ASSOCIATED WITH SKIN AND MUCOSAL CANCERS
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Cutaneous HPV and skin cancer

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Classification of papillomaviruses

Human papillomaviruses (HPVs) belong to the family Papillomaviridae that infect the keratinocytes of skin and mucosa, and are widely prevalent among mammals and birds. To date, more than 170 HPV types have been isolated from different body sites and fully characterized, and this number is continuously growing [1]. Several recent studies have characterized new beta and gamma HPV types in the oral cavity [2–4]. Based on the sequences of the major capsid protein L1,
HPVs are classified into genera, species, and types [5]. HPV types are organized into five major HPV genera – alpha-, beta-, gamma-, mu-, and nu-papillomavirus – and are classified as cutaneous or mucosal according to their tropisms [1, 5, 6]. Most of the papillomaviruses belong to the alpha, beta, or gamma genus. Alpha papillomaviruses include mucosal HPVs, which are associated with anogenital cancer [7] and with a subset of head and neck cancer, particularly oropharyngeal cancer [8]. This genus also comprises some cutaneous HPVs such as HPV2, 3, and 10, which cause common warts [9]. The cutaneous HPVs are represented mainly by the beta and gamma genera, which are widely present in the skin of normal individuals [10]. More than 40 beta-HPV types and 50 gamma-HPV types have been isolated so far [1].

**Genomic organization of cutaneous papillomaviruses**

HPVs are small non-enveloped icosahedral viruses that are 50–60 nm in diameter [11]. The genome consists of double-stranded circular DNA and contains seven or eight open reading frames (ORFs) [12]. The HPV genome is circular double-stranded DNA of ~7000 to ~8000 nucleotide base pairs that is organised into three regions:

- the long control region (LCR), which contains the early promoter and regulatory elements involved in viral DNA replication and transcription;
- an early region, encoding five or six non-structural proteins (E1, E2, E4, E5, E6, and E7) required for viral gene expression, replication, and survival;
- a late region (L), encoding a major capsid protein L1 and a minor capsid protein L2, which are the structural capsid proteins (figure 1).

Most of the alpha-HPV types have an E5 ORF between the early and late genes, whereas this gene is missing from most of the beta- and gamma-HPV genomes [6] (figure 1). The E5 protein plays a role in the productive phase of the viral life-cycle and stimulates mitogenic signals of growth factors [13]. However, it is not yet clear what the consequences on the life-cycle are for those types lacking E5. Interestingly, as observed for some animal HPVs, the E6 ORF is absent from the genome of three HPV types (HPV101, 103, and 108) belonging to the gamma genus, recently isolated from cervicovaginal cells [14, 15].

**Figure 1**
The genome of HPV38 (GenBank: U31787), a member of the beta-HPV genus, is represented by a black circle. The positions of the early and late genes are represented by different colours. Features and functions are also described.
cultures, HPV108 E7 alone induces dysplasia and initiates tumorigenesis [15]. In this model, the HPV108 E7 protein may partially substitute for the functions of E6.

**Natural history of cutaneous HPV infections**

HPVs are transmitted through direct skin-to-skin contact. In order to establish an infection, the HPV particles need to reach the basal epithelial cell layer via micro-abrasions in the skin [16]. It is thought that cutaneous HPVs target the hair follicle bulge, which probably constitutes their reservoir [17,18]. Beta-HPV types colonize human skin within the first few days of life, and their prevalence increases with age [19,20]. Analyses of skin swab samples have revealed that after a few days of life, HPV DNA is present in 45% of babies [20]. A similar pattern of HPV types is shared among the members of the same family, which demonstrates intra-familial transmission [21]. Infections with beta-HPV types can persist. In fact, studies performed with sequential sampling from the same individual have shown that a specific spectrum of beta-HPV types could be detected for at least six months [19,21].

Beta-HPV types are widely spread within the general population. When plucked eyebrow hairs are tested, the prevalence of beta-HPV DNA reaches up to 91% [19,22]. Little is known about the natural history of antibodies to cutaneous HPV types. About half of people develop an immune response against cutaneous types, as observed for mucosal HPVs [23]. This can be explained by the HPV life-cycle. In fact, HPVs replicate in keratinocytes that are continually renewed, thus impairing the ability of the host system to respond to HPV infection [24]. A seropositivity to beta-HPV of up to 70% has been reported in the general population, and it increases with age [22,25]. Interestingly, some phenotypic and exposure factors (e.g., white skin in men, tobacco use, green eye colour in women) seem to be linked to seroconversion to cutaneous HPV types [26].

Several studies using eyebrow hairs have shown that they contain similar HPV pattern to other skin sites from the same individual, in both sun-exposed and non-sun-exposed areas [27]. Detection of viral DNA in eyebrow hairs or tumours, and antibodies to cutaneous HPVs are the main biomarkers used in epidemiological studies to investigate the role of HPV in non-melanoma skin cancer (NMSC).

**Clinical implications of cutaneous HPV types**

HPVs of the gamma, mu, and nu genera induce cutaneous papillomas or warts [6]; however, there are no findings that support their possible involvement in tumorigenesis. HPV types of the beta genus can induce various skin lesions, from warts to carcinomas [28], but their etiological role in the pathogenesis of NMSC remains controversial.

NMSC is the most common cancer in Caucasians, and the incidence is increasing worldwide [29]. Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the two most common subtypes of NMSC. The main cause of NMSC is exposure to ultraviolet radiation (UVR). However, cutaneous HPVs that belong to the beta genus may act as a co-carcinogen with UVR. Interestingly, it has been shown that cutaneous HPV DNA predominates in sun-exposed areas of the body [30]. This observation could be explained by an increase of cutaneous HPV replication in the presence of UVR. In fact, mechanistic studies have shown that UVR can trigger the promoter activity of the cutaneous types HPV5, 8, 20, and 77 [31,32]. Exposure to UVR could also favour replication of beta-HPV types by inducing local immunosuppression [33].

The association between beta-HPVs and skin cancer was first reported in patients with epidermodysplasia verruciformis (EV), an extremely rare autosomal recessive skin disorder that increases the risk of developing HPV-induced SCC [28]. EV patients develop pityriasis versicolor-like lesions and large numbers of atypical, flat-topped coalescing warts [28], which frequently progress to cutaneous SCC on sun-exposed areas of the body after 30–40 years [28]. Isolation of HPVs from these lesions suggested that HPVs might act as a co-carcinogen with UVR in EV patients [6]. HPV5 and HPV8 types of the beta 1 species have been found in 90% of SCC in EV patients [34]. These beta-HPV types have been classified by the IARC Monographs as “possibly carcinogenic” (group 2B) to patients with EV [7]. In these patients, the HPV infection persists because of mutation in EVER genes [35]. The products of the EVER genes seem to work as a natural barrier to beta-HPV DNA replication [36]. In fact, skin lesions from EV patients show high copy numbers of beta-HPV DNA [37–39]. However, to date, the exact role of EVER1 and EVER2 in the context of HPV infection has not been clearly elucidated.

The fact that the risk of developing cutaneous SCC increases by up to 100-fold in organ transplant recipients (OTRs) compared with the general population suggests a viral etiology for this malignancy [40]. Indeed, beta-HPV types have been found in up to 90% of SCC [41], and high beta-HPV viral load in eyebrow hairs is associated with risk of SCC in OTRs [17]. In addition, Proby et al. showed that the risk of SCC is higher in OTRs when both viral DNA in eyebrow hairs and antibodies to at least one beta-HPV type are detected, which supports a role of this virus in SCC [42].

As described for OTRs, the incidence rate of NMSC increases in HIV-positive individuals, in whom it is approximately 2-fold higher compared with HIV-negative subjects [43]. The increased cancer risk in both OTR and HIV/AIDS populations is related to an immunodeficiency state, which strongly suggests that cutaneous SCC may be related to an infectious agent, such as HPV [44]. However, there is growing evidence that in immunocompetent populations, beta-HPVs may play a role in the pathogenesis of NMSC [45]. Beta-HPV DNA has been found in up to 65% of...
cutaneous SCC tumours [46,47] and in up to 50% of BCC tumours [48]. A positive association has been found between the presence of beta-HPV DNA in eyebrow hairs and a history of cutaneous SCC [49]. However, an association between beta-HPVs and BCC in immunocompetent hosts appears to be unlikely [50]. The presence of antibodies to cutaneous HPV types has been associated with SCC [50,51], with a risk that increases with the number of beta-HPV types to which antibodies have been found [52,53]. This association appears to be specific to beta-HPVs [52,54], in particular beta-HPV species 1 and 2 [51–53]. Concordantly, HPV DNA from beta-HPV species 1 or 2 predominates in SCC tumours [30,45]. However, further investigations are needed to identify cutaneous high-risk types. An association between the beta-HPV viral load in eyebrow hair follicles and risk of cutaneous SCC has been found in immunocompetent patients in Australia, whereas no association has been found in Italy [17]. Interestingly, several studies have established that the risk of SCC increases with the number of beta-HPV types in eyebrow hairs [46,55]. Coherently, SCC tumours show more beta-HPV types compared with BCC tumours [45]. Most of these assays used degenerate and/or consensus primers that result in under-detection of several HPV types due to primer competition. During the past decade, several highly sensitive and specific HPV detection systems have been developed for the detection of cutaneous HPVs in single and multiple infections [56,57]. The use of specific primers in a single reaction mixture increases the ability to detect multiple HPV infections and may improve understanding of the role of multiple infections in skin carcinogenesis [56]. The prevalence of beta-HPV types in plucked eyebrow hairs is higher in actinic keratosis (AK) cases compared with SCC cases [55]. Concordantly, HPV viral load decreases during skin carcinogenesis, being significantly higher in AK than in SCC [58], suggesting that the virus may play a role in the early stages of tumour development. In addition, unlike what it is known about cervical cancer [59], the expression of oncoproteins from cutaneous HPVs is not required to maintain the malignant phenotype of NMSC cells [58]. Very little is known about beta-HPV gene expression in SCC. So far, no messenger beta-HPV RNA has been identified in SCC [60]. The low viral load of beta-HPV types in skin cancer could explain this observation.

**Transforming activities of cutaneous HPV E6 and E7**

E6 and E7 proteins from high-risk (HR) mucosal HPV types have been extensively studied in *in vitro* and *in vivo* models [9, 61–63]. In contrast, E6 and E7 from beta-HPV types are a relatively novel area of investigation and seem to have a different mechanism of action than mucosal HPV E6 and E7 in promoting cancer development. In fact, unlike the mucosal proteins, cutaneous E6 and E7 are scarcely found in skin tumours and their expression is not required for the maintenance of the transformed phenotype.

**In vitro and in vivo studies of cutaneous HPV**

A classic *in vitro* experiment allowing evaluation of the transforming ability of HPV proteins is to test their capacity to either transform immortalized cells or immortalize primary cells, as these events require the inactivation of key cellular pathways. A recent study comparing several cutaneous HPV types highlighted the ability of E6 and E7 from HPV38 and HPV49 to immortalize primary keratinocytes, the natural host of the virus; in contrast, other types (e.g. cutaneous HPV types 10, 14, 22, 23, 24, and 36) did not display this ability [64]. In another study, Schmitt et al. compared the transforming ability of E6 and E7 from the cutaneous HPV type 1, 8 and cottontail rabbit papillomavirus (CRPV). Expression of E6 and E7 from those types failed to induce primary keratinocyte immortalization, however HPV8 E7 alone could transform primary rodent cells in cooperation with activated ras [65].

In agreement with the *in vitro* assays, transgenic (tg) mice co-expressing both viral genes from HPV38 or the whole early region of HPV8 under the control of keratinocyte-specific promoters exhibit epidermal hyperplasia and are susceptible to developing skin cancer either spontaneously, as observed for HPV8 tg mice, or promoted by various means, e.g. chemical carcinogens or UVR [66–68].

**Transforming properties of E6**

E6 proteins of cutaneous and mucosal HPVs are cysteine-rich proteins consisting of two zinc-binding domains (CXXC) named E6N and E6C. One main difference in structure between HR alpha-HPV types and beta-HPV types is the lack of a 4-amino-acid motif at the C-terminal PDZ motif, which is able to bind to PDZ-domain-containing proteins to promote cell invasion. However, a recent study showed that the C terminus of beta-HPV types 5 and 8 would contain a different motif, but with similar functions as it would promote cell migration [69]. HPV E6 has the ability to bind to several cellular proteins (reviewed in [70]). One of the best-characterized properties of HR-HPV16 E6 is its ability to induce degradation of the tumour suppressor protein p53 via the ubiquitin pathway. p53 is a transcription factor that guarantees the integrity of the cellular genome by regulating the cell cycle, DNA repair machinery, and apoptosis. Several cellular stress signals can trigger the activation of p53 via post-translational modifications (e.g. phosphorylation, acetylation, or sumoylation). p53 inactivation is a frequent event in cancer, and it is a key event in virus-induced transformation, as demonstrated by the redundancy of strategies evolved by oncogenic viruses to inactivate it. In order to induce p53 degradation, E6 needs to bind to the conserved
LXXLL motif present on E6-associated protein (E6AP), a cellular protein with an E3 ubiquitin protein ligase function. The E6/E6AP complex binds to p53, which becomes rapidly ubiquitinated and degraded by the proteasomes. The ability to bind to E6AP is relatively well conserved among the different E6s. In fact, independent groups have shown that E6 from beta types HPV8, 24, and 38 binds to E6AP in *in vitro* assay. However, this binding does not result in E6AP-mediated p53 degradation. Notably, a study from our group, comparing the transforming ability of E6 and E7 from different beta types (HPV14, 23, 24, 36, 38, and 49), showed that only expression of E6/E7 from HPV49 can induce degradation of p53 in an E6AP-dependent manner [64] (see figure 2).

An additional mechanism of E6-mediated inactivation of p53 transcriptional function requires E6 interaction with the transcriptional co-activators CBP and p300 [71,72]. p300/CBP modulates p53 activity via regulation of degradation by mdm2, co-activation of p53-regulated genes, and acetylation of p53. Like HR-HPV E6 proteins, some beta-HPV E6 proteins (from HPV5, 8, and 38) can bind to p300. The interaction of HPV38 E6 with p300 results in the prevention of p53 acetylation and the inhibition of its transcriptional activities (figure 2). This activity is important for HPV38-mediated immortalization, as a mutant of HPV38 E6 lacking the domain responsible for p300 binding failed to induce immortalization of primary keratinocytes if co-transduced with wild-type HPV38 E7 [73]. HPV5 and 8 E6 blocks phosphorylation of p300 by AKT, resulting in its destabilization [74]. The degradation of p300 leads, in turn, to a reduction in ATR protein levels, and, as a consequence, to an increased thymine dimer persistence and increased UVB-induced double-strand breaks [75]. In addition to hampering p53 response to UVR-induced damage, the ability of E6 to inactivate p300 also contributes to accumulation of chromosomal abnormalities such as supernumerary centrosomes or polyploidy [76]. UVR exposure induces p53 phosphorylation and stabilization. E6 from beta-HPV types seems to have evolved different strategies to prevent UVR-induced p53 activation by interfering with specific p53 kinases. In fact, HPV23 E6 hampers UVR-mediated phosphorylation of p53 at serine 46, by interacting with and inhibiting the activity of HIPK2 [77]. Moreover, Wallace et al. recently showed a reduction of UVR-induced p53 phosphorylation and stabilization in cells expressing E6 from HPV5, 8, and 38 due to their ability to inactivate the p53 kinases ATM and ATR [78] (figure 2). Similar to alpha-HPVs, HPV E6 proteins of beta-HPVs target the mitochondrial protein Bak for degradation, thereby maintaining mitochondrial integrity and function and preventing the release of pro-apoptotic mitochondrial factors. HPV E6-mediated Bak degradation requires the interaction of E6 with E6AP [79]. HR-HPV E6/E6AP interaction is also required for the transcriptional activation of *hTERT* (human telomerase reverse transcriptase), the catalytic subunit of the telomerase complex. Somatic cells lack telomerase activity therefore undergo telo-

**Figure 2**

Different mechanisms of beta-HPV-mediated inactivation of p53 [64,74,75,77,78,91]
mure shortening and, consequently, replicative senescence (reviewed in [80]). In contrast, in cancer cells, due to activation of telomerase expression, telomere length is stable. Similarly to HPV16 E6, the E6 protein of beta-HPV types (HPV5, 20, 22, and 38) is able to induce hTERT expression, even though to a less extend [81,82]. Another activity of HPV6s from the beta genus is to bind to the transcriptional co-activator of Notch, MAML1, via its LXXLL motif [83]. The downstream effect of this interaction is an inhibition of MAML1-mediated Notch signalling that has a key role in skin differentiation [84]. This ability is not shared by the alpha E6s but is specific to the cutaneous HPV types, possibly as a consequence of their skin tropism [85].

**Transforming properties of E7**

E7 is a protein of approximately 100 amino acids. Similarly to E6, it contains zinc-binding motifs. E7 is divided into three domains, named conserved regions 1–3 (CR1–3). The E7 CR2 domain contains an LXCXE motif that mediates the interaction with the pocket proteins: retinoblastoma (pRb1) and its related proteins, p107 and p130. A second motif (QLN), located immediately after this domain, is essential to promote pRb degradation [86]. This interaction is the best-characterized function of E7 and is required for HPV-mediated deregulation of the cell cycle. In fact, the tumour suppressor pRb1 is responsible for maintaining the cell in a quiescent state during the G0/G1 phase by negatively regulating the activity of members of the E2F family (E2F1–3). Binding of HR-HPV E7 to pRb leads to its degradation via proteasome pathways, and to the release and activation of E2F, which in turn induces the transcription of a panel of genes (such as cyclin A and CDC2) whose products are involved in S-phase entry and cell-cycle progression [87]. Low-risk HPV E7s, such as HPV6 E7, that have the LXXCLE motif can bind to pRb but do not induce its degradation. Few studies have addressed the ability of cutaneous HPV E7s to degrade pRb. The expression of HPV8 E7 in primary human adult keratinocytes has been shown to cause decreased pRb levels [88]. Studies from our group have shown that the E7s from the beta-HPV types HPV14, 22, 23, 24, 36, 49, and 38 bind to pRB in vitro. Moreover, E7 proteins from the beta types HPV38, 24, and 49 induce degradation of pRB in rodent fibroblasts, with different efficiency according to their ability to increase the lifespan of primary keratinocytes. Intriguingly, when expressed in human keratinocytes, the same E7 proteins do not target pRB degradation. Nevertheless, in keratinocytes expressing E6 and E7 from beta types HPV38 and 49, E2F-regulated transcription is activated, most likely due to the ability of the viral proteins to induce phosphorylation of pRB, an event that leads to the release of E2F1 [64]. The variety of mechanisms evolved by HPV to interfere with the function of pocket proteins highlight the importance of this event for the completion of the HPV life-cycle.

It is noteworthy that E7 from HPV38 also interferes with the pro-apoptotic transcriptional activity of p53, by inducing the transcription of a dominant-negative isoform of p73, ∆Np73α, that is able to inhibit both p53- and p73-regulated pathways [89]. Moreover, HPV38 E7 is able to trigger nuclear accumulation of hXb kinase beta (IKKβ), which, in turn, phosphorylates and stabilizes ∆Np73α [90]. In keratinocytes expressing HPV38 E7, ∆Np73α and IKKβ are part of an inhibitory complex that includes two epigenetic enzymes, DNA methyltransferase 1 (DNMT1) and enhancer of zeste homolog 2 (EZH2) [91] (figure 2). This multiprotein complex specifically binds to the promoters of a subset of p53-regulated genes whose products play a role in apoptosis.

In addition of their ability to deregulate the cell cycle and inhibit apoptosis, beta E7s can also play a role in cellular differentiation. It has been shown that the expression of E7 from HPV8 in keratinocytes pushes the cells into an undifferentiated or “stem cell-like” state [92]. In conclusion, E6 and E7 from certain cutaneous HPV types, similarly to the oncoproteins from mucosal HR-HPV types, display transforming activities as they target the same cellular pathways, even though, in some cases, by using different mechanisms.

**Conclusion**

In the past years, growing lines of evidence, coming from both epidemiological and molecular studies, have reinforced our knowledge of the biology of cutaneous HPVs and highlighted some cutaneous HPV types (species 1 and 2 of the beta genus) as potential HR types. However, the low HPV viral load combined with the lack of expression of E6 and E7 from those HPV types in NMSC, has made it difficult to confirm the association of beta-HPV types with cancer development in the general (immunocompetent) population. Nevertheless, it seems increasingly plausible that cutaneous E6 and E7 expression is required only in the initial step of skin carcinogenesis, for example by inhibiting the UVR-induced DNA damage repair machinery, therefore facilitating the accumulation of DNA breaks and mutations; the two proteins would not be required at later stages for the maintenance of the cancer phenotype (the “hit-and-run mechanism” hypothesis). Moreover, our observation that expression of HPV38 E7 protein can modulate the activity of the cellular epigenetic machinery [91] further supports the hit-and-run hypothesis, as epigenetic changes are stable and heritable and could persist also after loss of expression of the viral proteins [93].

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