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Betapapillomavirus infections : natural history and disease association

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CHAPTER 1

General introduction

Background and history of papillomavirus research

Human papillomavirus (HPV) is causally involved in the development of several benign and malignant hyperproliferative neoplasias originating from squamous epithelia, such as common warts on children's hands and carcinomas of the cervix. Involvement of a particular group of cutaneous papillomaviruses (PV), the so-called *betapapillomaviruses* (betaPV), in non-melanoma skin cancer development is currently under investigation. Detection and genotyping of this specific group of PV, as well as the natural history in healthy individuals and the possible associations with (pre)malignant diseases are the main subjects of this thesis.

Research in humans and animals concerning treatment and transmission of PV-associated genital and skin warts has a long history. The viral nature of human warts was first shown by Ciuffo in 1907 and confirmed by numerous inoculation experiments using unfiltered wart extracts and later cell/bacteria-free wart extracts, as reviewed by Rowson and Mahy in 1967 (97).

The carcinogenic nature of PV was first described in 1935, by inoculation experiments of domestic rabbits with PV from cottontail rabbits. Although the papillomas in the cottontails did not progress, inoculation of extracts of those lesions to domestic rabbits induced papillomas that frequently progressed to carcinomas and metastasized (90). A suggestion for a role of PV in human genital cancer was initially brought forward by Zur Hausen in 1976 (123) who recently has been awarded the Nobel Prize for his pioneering work in this area. The causal role of specific PV types in cervical cancer is now firmly established (16, 100). A role for PV in human skin carcinogenesis was suggested even earlier by Jablonska and co-workers (68) while working with patients suffering from a rare genodermatosis called *epidermodysplasia*

verruciformis (EV). A specific group of human PV (HPV) types was regularly detected in the macular lesions of these patients and has since then been commonly referred to as EV-HPV types (92). This disease was suggested as a model for viral oncogenesis, given that frequently occurring wart-like lesions progress into carcinoma, mostly of the Bowen's type (intraepithelial squamous cell carcinoma). Material from a verrucous EV lesion was inoculated in the skin of eight volunteering physicians. Within four months flat warts developed in one of these physicians (68). In addition, about one-third of EV patients develop multifocal skin cancer mainly localized on sun-exposed sites (79). Although ongoing research has consistently found associations between markers of EV-HPV infection and skin cancer, the exact role that these viruses play in skin carcinogenesis is still unclear.

Phylogeny and classification of papillomaviruses

The family of *papillomaviridae* was previously classified together with the family of *polyomaviridae* and simian vacuolating virus 40 (SV40) as *papovaviridae* (papillomavirus, polyomavirus, vacuolating virus). This classification was mainly based on the similarity in size, morphology and buoyant density of the non-enveloped virions, together with their double-stranded DNA genomes, intra-nuclear replication and tumourigenic properties (80). In 2001, the current classification of *papillomaviridae* and *polyomaviridae* as separate virus families was implemented (46), supported by dissimilarities between these families with respect to genome size, transcriptional and replication strategies and non-homologous proteins (14). Within the family of *papillomaviridae*, denomination occurred on the basis of host and order of discovery (e.g., bovine PV type 1). At the International Papillomavirus Workshop

carcinogenesis has also led to the development of different PCR-based systems detecting betaPV and other cutaneous HPV types.

BetaPV are detected at various frequencies in diverse clinical materials, such as non-melanoma skin cancer (NMSC) biopsies (62, 92, 105), skin swabs, plucked hairs and biopsies from normal skin (6, 19, 35, 56, 74). The great variety of testing methods probably contributed significantly to the observed strong differences in HPV DNA prevalence detected in these materials (105). Over the last two decades, several PCR-based methods have been developed to detect betaPV types in skin biopsies, plucked hairs and skin swabs (7, 12, 13, 19, 21, 31, 32, 34, 37, 55, 63, 104). These PCR methods can be divided into (A) type-specific PCRs, using HPV type-specific primers for the detection of a single HPV type, (B) multiplex type-specific PCR methods, in which multiple type-specific primer sets are combined in a single PCR reaction and (C) broad-spectrum PCR methods, using consensus primers, which permit simultaneous amplification of multiple types.

Detection of positive samples in the case of type-specific PCR methods can be performed by agarose gel electrophoresis. This method can give information about the prevalence or predominance of individual HPV types but is not useful when a large group of HPV types is investigated.

Multiplex type-specific PCR and broad-spectrum PCR based methods amplify multiple HPV types in a single reaction. Subsequent genotyping in the case of amplification systems that target multiple HPV types is generally performed by sequence analysis, either directly or preceded by cloning of the amplicon. However, restriction fragment length polymorphism analysis of amplicons (87) and dot blot assay using type specific labeled oligonucleotide probes (2) have also been used for identification of specific betaPV genotypes.

More recently, three combined amplification genotyping systems have been described that theoretically allow for the simultaneous identification of all 25 characterised betaPV types.

The BGC-PCR is a broad-spectrum PCR using six forward and eight biotinylated reverse primers and amplifies a 72bp fragment from L1. Labelled PCR products are analysed by a reverse line blot (RLB) system identifying 19 betaPV types and 5 gammaPV types (22). This method has now been extended to include all 25 betaPV types (86).

The second system uses a multiplex PCR with type-specific and consensus primers to amplify betaPV E7 ORF targets ranging from 186 to 280bp. Amplification is followed by DNA microarray primer extension (APEX) for the identification of 25 betaPV types and a co-amplified human beta-globin sequence (59). The assay contains two probes for each betaPV type, a feature that could increase the specificity of the system and is enabled by the amplification of relatively large PCR fragments from a variable region of the HPV genome.

The third method is described in **Chapter 2** and concerns a broad-spectrum PCR (with biotinylated forward and reverse consensus primers) amplifying an 117bp region from the E1 ORF. The labelled amplicons are subsequently hybridised to nitrocellulose strips containing individual probes for the identification of 25 betaPV types (this thesis, **Chapter 2**). This system with high stringency hybridisation allows highly specific discrimination between nucleotide sequences that differ by just a single nucleotide.

Virion properties, genomic organisation and transcription

PV are naked icosahedral-shaped viruses with a diameter of ~60 nm (10) consisting of 12 pentavalent and 60 hexavalent capsomers (73) that show distinct five-fold axial symmetry. Both the penta-, and

hexavalent capsomers are pentamers of the major capsid protein L1 (10, 84). Recent evidence shows that the ratio of L1 to the minor capsid protein (L2) could reach 5:1 in a complete virion. Combined with cryoelectron microscopy and image reconstruction analysis, it was concluded that each of the 72 capsomers could contain one L2 protein but questions remain about the exact configuration of L2 within the virion (23).

The double-stranded circular PV genome is approximately 8 kb and varies in size between types. It is subdivided in an early (E) coding region, a late (L) coding region, and a long control region (LCR). The early region generally encodes for six non-structural viral regulatory proteins (E1, E2, E4, E5, E6, E7 in most PV types and an additional E8 in some PV types). The late region encodes for 2 structural proteins, namely L1 and L2 (121). Whereas all PV appear to have E1, E2, L1 and L2 ORFs the other ORFs are not consistently present in every PV (36). BetaPV have E1, E2, E4, E6, E7, L1 and L2 ORFs but lack the E5 ORF. E5 is considered as a PV oncogene, together with E6 and E7. The PV LCR contains the transcriptional control region and the origin of viral DNA replication. The early, late and LCR regions are separated by two polyadenylation (pA) sites: early pA (AE) and late pA (AL) (121). Two major promoters have been described for HPV16 (121). One (called P97 in HPV16) mainly drives the transcription of E6 and E7 and the other (designated P670 in HPV16) drives the transcription of the other ORFs.

The first transcription studies of betaPV have focussed on genome mapping and are limited to 3 of the 25 described types, namely HPV5, 8 and 47 (61, 67, 72, 110). More recent studies also have investigated HPV5, 8, 9, 14, 15, 20 and 23 transcription in squamous cell carcinoma (SCC) and revealed that DNA detection by PCR in most cases did not concur with RNA detection by *in situ hybridisation* (ISH) (95). In general, quantitative real-time

reverse transcription PCR showed low copy levels of transcripts (30). These findings suggest low transcriptional activity of betaPV in SCC and its precursor lesion, actinic keratosis (AK).

The transcription of the PV genome during productive PV infections in which virions are shed, has been linked to the differentiation of the infected epithelium (121). Evidence for differentiation-dependent transcription is limited for betaPV, but Haller and co-workers found that transcription of HPV5 is indeed associated with the differentiation of the epithelium in benign skin lesions (61). Recently, this differentiation-dependent transcription has also been confirmed *in vitro* for HPV8, 93 and 96, using transfected HaCaT cell lines, in which cell differentiation was induced by high Ca^{2+} (115). Only a few studies into betaPV transcription are available, and no studies investigating betaPV proteins in normal or lesional skin have been published. A major technical problem is that low levels of transcription result in extremely low levels of viral protein, which are difficult to measure.

Viral life cycle

Infection occurs when PV enters the basal layer of the epithelium and is present as a low copy-number episome. Entry to the basal cell layer is supposedly achieved by small abrasions of the epithelium. However, the body-wide distribution of betaPV infections suggests a direct route of infection bypassing the requirement of epithelial injury or abrasion (7, 19, 29, 48, 74). Life cycle studies have been mainly performed for the HPV types causing cervical cancer but the life cycle of several HPV types with a cutaneous tropism (Table 1) appears to be similar (83). E6 and E7 are mainly expressed in the (supra) basal layer of the epithelium and are associated with PV genome maintenance and cellular proliferation. E1 and E2 are involved in viral DNA replication and the

Table 1. The different genera from the family *Papillomaviridae* that contain HPV types and whether they infect mucosal or cutaneous epithelia is shown. Adapted from De Koning *et al.*, 2008 (33).

<i>Genus</i>	<i>HPV type</i>	<i>Tissue tropism</i>
<i>Alphapapillomavirus</i>	6, 11, 16, 18, 26, 30, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 74, 81, 82, 83, 84, 85, 86, 87, 89, and 90 7, 40, 43, and c91 2, 3, 10, 27, 28, 29, 57, 77, 78, and 94	Mucosal Mucosal and cutaneous Cutaneous
<i>Betapapillomavirus</i>	5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, 92, 93, and 96	Cutaneous
<i>Gamma</i> <i>papillomavirus</i>	4, 48, 50, 60, 65, 88, and 95	Cutaneous
<i>Mupapillomavirus</i>	1, and 63	Cutaneous
<i>Nupapillomavirus</i>	41	Cutaneous

regulation of E6 and E7 transcription. E6 and E7 proteins are primarily present in the upper part of the suprabasal layer and in the granular layer. E4 of some cutaneous HPV types (i.e., HPV1, 2, 63 and 65 investigated in cutaneous warts) appears to be expressed throughout the epithelium except in the basal cell layer (89) and presumably promotes viral DNA replication (43). Some studies have shown variability in viral life cycle between PV types (44, 89). For example genital HPV types 11 and 16 show genome replication and L1 expression in the upper part of the epithelium, whereas the genomes of cutaneous HPV types (i.e., HPV1, 2, 63 and 65) are amplified in the lower parts of the epithelium. However, the order of expression of viral genes throughout the differentiating epithelia is similar for these types, although the localisation of L1 expression is variable and starts either in the lower part of the epithelium (HPV1 and 2) or in the upper part (HPV63 and 65). Macroscopic betaPV lesions appear to be very rare which might be the cause of our limited knowledge on the life cycle of these types. Haller and co-workers (61) investigated HPV5 positive benign skin lesions of two EV patients, suggesting that betaPV also follow life cycle patterns directed by the terminal differentiation of the epithelium.

BetaPV and cell transformation

Transformation (i.e., immortalisation) of human cells has been studied in great detail for the HPV types causing cervical cancer (e.g., HPV16 and HPV18). In general, immortalisation requires long-term and deregulated expression of viral oncogenes E6 and E7. This process could be facilitated by integration of the viral episome into the cellular DNA, disturbing the E2 ORF, thereby causing a lack of control of E6 and E7 expression (43). High levels of E6 and E7 result in inhibition of apoptosis and persistent stimulation of S-phase entry. This mechanism acts through ubiquitination and subsequent degradation of the tumour suppressors p53 and pRb. Presumably, persistent infections are needed to accumulate sufficient mutations in the host cellular genome to generate a malignant cell. However, the proliferative phenotype of these malignant cells remains dependent on E6/E7 expression (124).

The function of the E6 and E7 proteins from a variety of betaPV types has been investigated. HPV38 E7 was shown to bind and degrade the tumour suppressor pRb similar as HPV16 E7 (25, 42). Furthermore, HPV38 E6 and E7 (in contrast to HPV10 and HPV20 E6/E7) are sufficient to deregulate the cell cycle and

senescence programs in primary human keratinocytes, thereby increasing the lifespan of human skin keratinocytes (25). HPV38 E6 (11), possibly in combination with E7 (57) induces telomerase, which plays a key role in immortalisation of human keratinocytes. Finally, HPV38 E6 and E7 appear to inhibit p53-induced growth suppression and apoptosis (1), altering the regulation of cell cycle checkpoints that are normally activated by UV radiation (40). In contrast to high-risk genital HPV E6 proteins which degrade p53, HPV38 E6 and E7 expression results in the accumulation of p53 which upregulates Δ Np73 which in turn hinders p53 transcriptional functions. Several studies investigating the effect of betaPV E6 and E7 in organotypic (raft) cultures showed that betaPV could delay cell differentiation and disturb keratinocyte outgrowth (4, 20).

Transgenic mice, containing the HPV8, HPV20 and HPV38 early region under control of a keratin promoter, showed papillomatosis sometimes accompanied by SCC, indicating carcinogenic effects of betaPV early proteins alone or in combination with UV irradiation (41, 82, 91, 99). Studies using complete betaPV genomes or ORFs under control of an autologous betaPV promoter have not been performed, but could be important, because they help excluding possible invalid results due to betaPV over-expression. BetaPV could confer its possible carcinogenic effect via inhibition of apoptosis in UV damaged cells. HPV5 E6 appeared to exert this effect via the degradation of Bak, a protein involved in induction of apoptosis (69, 70, 76). This function of HPV5 E6 was recently confirmed in a study showing that the E6 of HPV8, 20, 22, 38, 76, 92 and 96 can protect UV-treated keratinocytes from apoptosis (112). Struijk and co-workers (109) also reported UV-induced apoptosis inhibition in primary keratinocytes expressing HPV8 and 20 E6, which coincided with a down regulation of Bax, another protein involved in the

induction of apoptosis. Additionally, betaPV could also facilitate carcinogenesis by unscheduled proliferation, suboptimal activation of telomerase and subsequent extensive telomere shortening resulting in genomic instability (57).

Natural history of betaPV infections

Host and tissue specificity

A number of studies comprised inoculation of diverse human wart extracts into animals. These experiments did not provide evidence for transmission between species (reviewed by Rowson and Mahy (97)). The tight linkage of productive infection with the differentiation of the epithelium (121) is probably induced by species-specific host regulatory proteins. Binding studies with bovine PV virions and HPV virus-like particles attaching to and penetrating a wide variety of cell types originating from different tissues and species have indicated that the strict host specificity of PV is due to species-specific host regulatory proteins rather than host cell receptor mediated restriction (78, 85, 96).

Strong epithelial-specific HPV transcriptional enhancers were found for some *alphapapillomaviruses* (98) indicating that the epitheliotropism of PVs could be the result of epithelial-specific factors lacking in non-epithelial cells (78). However, several studies have now shown that this specificity is not absolute, as there is evidence for genital HPV infection and replication in trophoblasts (119, 120).

BetaPV reservoir

Initially, betaPV were only detected in skin lesions from EV patients, which raised the question about the reservoir of these viruses. Boxman and co-workers first showed the presence of betaPV DNA in plucked human hairs taken from different body sites (19). A possible reservoir for betaPV could be the epidermal stem cells present in the bulge region of the hair (19,

38) that remains attached to the hair when it is plucked (60). The presence of betaPV in stem cells would fit with the attributed role of these viruses in skin cancer development because a population of long-lived cells is generally required for cutaneous tumorigenesis (75). Additionally, betaPV are present throughout the skin and in desquamated skin cells as is shown by high betaPV prevalence in skin swabs and even in swabs taken from home and (laboratory) working environments (7, 52).

Viral load BetaPV loads are generally low, judging from the necessity of nested amplification systems for the detection of the virus DNA in plucked hairs (19). This was confirmed by more recent studies that applied quantitative PCR protocols for the detection of betaPV in actinic keratoses (AK), seborrhoeic keratoses, non melanoma skin cancer (NMSC), perilesional skin and healthy skin (51, 113, 114, 116). The tests performed by Weissenborn and co-workers revealed a lower viral load in SCC than in AK, which are regarded as precursor lesions, suggesting a role for betaPV in the early steps of tumour development. However, this finding could not be reproduced in a study (113) that used biopsies that had their superficial layers removed by adhesive tape (54) prior to sample taking. Furthermore, with respect to the possible causal link between betaPV infection and NMSC it should be noted that these lesions did generally contain far less than one betaPV copy per cell. Additionally, the detected betaPV genomes appeared to be derived from only a few positive nuclei per investigated biopsy section as shown by in-situ hybridisation experiments (116). In *epidermodysplasia verruciformis* (EV) patients however, viral loads are much higher (reaching in some cases more than 400 viral copies per cell) but again the virus appears to be restricted to only a few positive cells in the lesions (39).

Transmission & route of infection

It is thought that PV infections are established when an infectious virus particle successfully reaches the basal cell layer of the epithelium. For genital HPVs (that are sexually transmitted), the availability of the basal cells seems to depend on micro traumata or the transition zone between two types of epithelium (43). Warts can be transmitted via small wounds as well as via close contact as demonstrated by the presence of warts on opposing skin surfaces of the hand (97). Transmission of the betaPV genus is less well studied, due to the lack of clinically apparent skin lesions confirmed to be caused by a single betaPV type. The occurrence of the same betaPV type(s) in hairs taken from different body parts (7, 19, 74) suggests that the virus has a different way of causing subclinical infections bypassing the requirement of small wounds. Recently developed techniques now enable transmission studies by providing very sensitive detection and genotyping of multiple betaPV types often present in clinical samples (this thesis, **Chapter 2** (22, 59)). It could now be shown that the rate of betaPV transmission is probably low (this thesis, **Chapter 3**) and requires close contact between individuals (e.g., family members) while betaPV type-specific susceptibilities might exist (this thesis, **Chapter 5**). PV primarily contact epithelial cells by attaching to specifically modified heparan sulfate proteoglycans (HSPG) that might be present in the extracellular matrix. This is followed by interaction with a secondary HSPG receptor and subsequent transfer, probably to a non-HSPG receptor (102). The process of PV cellular entry might occur within 30 minutes after virus attachment via endocytosis and complete virions are transported through the cell in phagosomes (122). Transport of the virions to the nuclear membrane occurs via associated microtubules and at the nuclear membrane both complete and disrupted virions are

seen (77). Finally, the L2 protein facilitates transport to the nucleus (118) via two nuclear localisation signals, which mediate nuclear import via high-affinity binding to karyopherin β nuclear import receptors and binding to the viral DNA (15).

Prevalence

Studies investigating the prevalence of cutaneous HPV (betaPV and gammaPV) were carried out via sequence analysis of amplimers generated with broad spectrum amplification systems. In immunocompetent persons the prevalence of cutaneous HPV as measured by either (multiple) skin swabs or plucked hairs from multiple sites was between 45% and 80% (7, 8, 19, 65). The prevalence was higher in immunosuppressed patients from the same studies, ranging from 71% to 100%. Furthermore, multiple infections were reported irrespective of the immune status of the study participants. The largest study to date included 248 healthy individuals from 5 different countries (6) with a cutaneous HPV prevalence ranging from 42% to 70% as measured in skin swabs. HPV5 was one of the most prevalent types (6.5%), and the only type that was detected in all countries. Multiple genotypes were present in at least 39% of the HPV positive samples. Importantly, 10 recently reviewed studies revealed that apart from the 25 already characterized ones at least 36 putative betaPV types exist that are not yet included in any betaPV genotyping test (50).

Persistent and transient infection

Persisting cervical infections with certain types of genital HPV types will increase the risk of subsequent cervical pre-malignant lesions (117), sometimes leading to cancer development. The HPV type-specific ability of causing persisting infections may allow this type to convey an increased risk for carcinogenesis, but it should be stressed that for genital HPVs the ability to persist and the ability to cause cancer are two separate properties (24,

101). For betaPV little is known about the likelihood of infections to persist and the possible associated carcinogenic potential of these longer lasting infections. Berkhout and co-workers (12) first showed indications of persistence of betaPV infections in lesional skin of renal transplant recipients (RTR) for periods up to 5 years. Similar results are obtained from forehead skin swabs from healthy individuals and RTR (65). A systematically conducted cohort study investigating persistent presence of betaPV DNA in eyebrow hairs showed (multiple) persistent betaPV infections in 17 of the 23 participants (this thesis, **Chapter 3**). Systematic cohort studies, especially in populations at higher risk of skin cancer, such as RTR and those highly exposed to sunlight, are of interest as they could reveal possible differential abilities of betaPV types to cause persistent infections in relation to skin carcinogenesis.

Immunological response

PV propagate in terminally differentiating epithelia and viral protein expression in the basal layer is restricted to low levels of E7 (49). The accumulation of viral L1 and L2 proteins is delayed until the cell reaches the upper epithelial layers. During the infection apoptosis is reduced and finally the virions are shed without cell lysis. These combined features may cause the low immunogenicity of HPV infections (43). T-cell mediated immunity is probably involved in clearance of PV induced lesions, since patients with conditions that affect T-cell immunity such as HIV infection and immunosuppressive treatments (3, 111) have a higher prevalence of HPV infections and associated lesions. Genital HPV infections generally resolve within one to two years (66, 94). Longer lasting infections have been reported in older women and are probably related to a general age-associated decrease in immune responsiveness (27, 58). The situation appears to be different for betaPV as

clinically unapparent infections are detected early in life and persist already in children and young adults (this thesis, **Chapters 3 and 5** (8)). These infections, as measured by DNA presence in plucked eyebrow hairs do not always lead to a serological response (47, 107). In EV patients humoral immunity is normal, whereas cell-mediated immunity is depressed in most EV patients (79). This finding probably indicates that T-cell mediated immunity is important for controlling betaPV infections. In a recent study in the German general population, seroprevalence increased with age, irrespective of the specific betaPV type (81). What precisely determines the induction and intensity of a betaPV seroresponse is unknown (48).

The role of betaPV in skin (pre)malignant disease

Epidermodysplasia verruciformis

EV is considered to be a rare autosomal recessive disease that has initially been described by Lewandowsky and Lutz in 1922 and has been proposed as a model for PV-mediated skin carcinogenesis (68). A review of 135 case reports describing 147 EV patients has provided some basic knowledge of the disease (79). The onset of EV takes place at an average age of nine years and is accompanied by flat warts in almost all patients. In 75% of the patients the pityriasis-like warts which are scaly macules or patches that are not elevated from the skin surface, arise after the onset of flat warts. Skin cancers develop in one-third of the patients, mainly on sun-exposed sites. They may already occur at the age of 13, but on average they follow the onset of the disease by 24 years. Whether these cancers arise from apparent normal skin or from wart like lesions is unclear and both possibilities have been reported. Remarkably, these patients appear to be specifically susceptible to betaPV and not to other viral, fungal or

bacterial infections (79). It has been reported that especially betaPV types 5 and 8 and to a lesser degree betaPV types 14, 17, 20 and 47 are found in SCC of EV patients and are therefore regarded as high risk betaPV types (28, 92).

Non-melanoma skin cancer

NMSC is a common malignancy in mainly Caucasian populations that is associated with sun exposure and consists of basal-cell (BCC) and squamous-cell carcinomas (SCC). The ratio between SCC and BCC in the normal population is 1:4 and reverses in immunosuppressed transplant patients due to an exponential increase in the number of SCC whereas the number of BCC increases linearly (45). This disproportional increase of SCC in organ transplant recipients (OTR) under influence of chronic immunosuppression suggests that besides sun exposure an infection might be involved in the aetiology of this tumour. The high prevalence ($\geq 75\%$) of betaPV in these lesions and precursor lesions suggested that similar to EV patients, SCC development in OTR could possibly also be caused by betaPV infection (13, 18, 31, 37, 64, 93, 103, 106). With the development of betaPV detection techniques with increasing sensitivity it also became evident that SCC and its precursor lesion of immunocompetent individuals contained betaPV DNA (9, 88). Associations of betaPV infection with SCC development in immunocompetent patients were furthermore confirmed by epidemiological studies investigating both the presence of viral DNA and anti-PV antibody responses (5, 47, 53, 71, 93, 107, 108).

Not all tumour cells of immunocompetent, immunosuppressed and EV patients contain betaPV DNA (39, 116) and the detection rate in lesions dropped if tape-stripping was used prior to taking a biopsy (54). This suggests that there is no proof that HPV is necessary for the maintenance of the cutaneous tumour in contrast to the

role of genital HPV in cervical cancer. The available data are more supportive of a 'hit and run' mechanism (92). Such a mechanism has been described for bovinePV type 4 (BPV4) and alimentary canal carcinogenesis of cattle where infection by BPV4 is a necessary step but the continued presence of the viral DNA is not required for the progression to, or maintenance of, the malignant state (26). In human cutaneous SCC, inhibition of apoptosis that could allow UV induced mutations to accumulate was suggested as a possible underlying mechanism (17) which is supported by recent studies as described above. Conflicting data have however are available showing associations between specific betaPV species and SCC as species 1 DNA (HPV5, 8, 12, 14, 19, 20, 21, 24, 25, 36, 47 and 93) was more prevalent in SCC than BCC (88) and species 2 DNA (HPV9, 15, 17, 22, 23, 37, 38 and 80) was more prevalent in SCC than in healthy skin (9, 53). Overall, there is no conclusive epidemiological evidence for the existence of specific 'high-risk' betaPV types.

Outline of the thesis

Chapter 2 is a technical chapter describing the evaluation of a sensitive PCR and genotyping method that allows detection and identification of all 25 characterised betaPV types. This method also allows the easy detection of multiple infections. The assay was designated the PM-PCR RHA method. Its analytical sensitivity, specificity, reproducibility, genotype inclusivity and performance in different clinical materials compared to the established MaHa PCR are analysed.

Chapters 3 to 5 focus on the natural history of betaPV infections. Very little is known about this subject and more knowledge could support future studies into the clinical relevance of these viruses.

Chapter 3 describes the type-specific persistence of betaPV DNA in eyebrow hairs from 23 healthy individuals during a cohort study lasting 2 years. It is concluded that the detection of betaPV DNA in eyebrow hairs is a valid method to determine ongoing betaPV infection. Furthermore, (multiple) persistent betaPV infections were found in 17/23 participants and transmission of viral infection appeared low in this group as 5/23 participants shared a common household for at least 3 years but each participant had his/her own specific betaPV spectrum.

Chapter 4 This chapter describes the prevalence of, and possibly associated factors with, betaPV infection. Eight hundred forty-five participants without a history of cutaneous SCC from The Netherlands, Italy and Australia and 560 OTR without a history of cutaneous SCC from The Netherlands, Germany, UK, France and Italy are included. The frequency of betaPV-positive participants ranged from 84% - 91% in the immunocompetent population with HPV23 as the most prevalent type, and from 81% - 98% in the immunosuppressed population with HPV23 as the most or second most prevalent type. The median number of infecting betaPV types ranged from 4-6 in the immunocompetent and from 3-6 in the immunosuppressed population. Increasing age in the immunocompetent participants and (duration of) immunosuppression in the immunosuppressed patients were associated with betaPV infection whereas characteristics related to sun exposure and skin type were not.

Chapter 5 describes betaPV distribution and persistence in 10 families including children aged 10 days to 8.6 years. In conclusion, family members typically displayed similar spectra of betaPV types indicating that BetaPV transmission mainly results from long lasting close contact between family members. However, regular exposure to cutaneous

HPV did not always lead to the establishment of a persistent infection, which may point to type-specific susceptibilities of different individuals.

Chapters 6 to 8 focus on possible associations of betaPV infection with three different (pre)malignant diseases.

Chapter 6 describes the distribution of betaPV and genital HPV in relation to p16^{INK4a} expression in 39 cases of vulvar carcinoma. All cases of basaloid/warty carcinomas, but none of the remaining tumors, overexpressed p16^{INK4a} protein. Of these p16^{INK4a} positive lesions all but one contained a genital high-risk HPV type. The study reaffirms the role of genital HPVs in the pathogenesis of vulvar carcinoma but does not support a role for betaPV in these sometimes wart-like lesions.

Chapter 7 describes the distribution of betaPV in 121 skin tumours and 11 normal skin samples. In immunocompetent patients, betaPV was detected in 50.8% of the tumours and 18.2% of normal skin samples. In immunosuppressed (IS) patients, betaPV was found in 43.5% of tumours but no normal skin samples were available for comparison. Multiple infections were most common in tumours from IC patients (70.0%) compared with those from IS patients (25.9%).

Chapter 8 describes the distribution of betaPV and genital HPV types in 81 cases of conjunctival squamous cell neoplasia and 29 controls. No evidence of an association between genital HPV types and this disease was found. The prevalence of betaPV was significantly higher among cases as compared to controls which is consistent with results from the two other case-control studies performed to date. The relatively low prevalence of betaPV among cases however, indicates that there remains considerable uncertainty about a role of betaPV in the aetiology of this tumour.

Chapter 9 General conclusions and discussion

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