EXPLORING BETAPAPILLOMAVIRUS INFECTIONS AND THEIR ASSOCIATION WITH CUTANEOUS SQUAMOUS-CELL CARCINOMA DEVELOPMENT

Colophon

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Exploring betapapillomavirus infections and their association with cutaneous squamous-cell carcinoma development

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Als je goed om je heen kijkt zie je dat alles gekleurd is
-K. Schippers

Voor papa (1940-2002)

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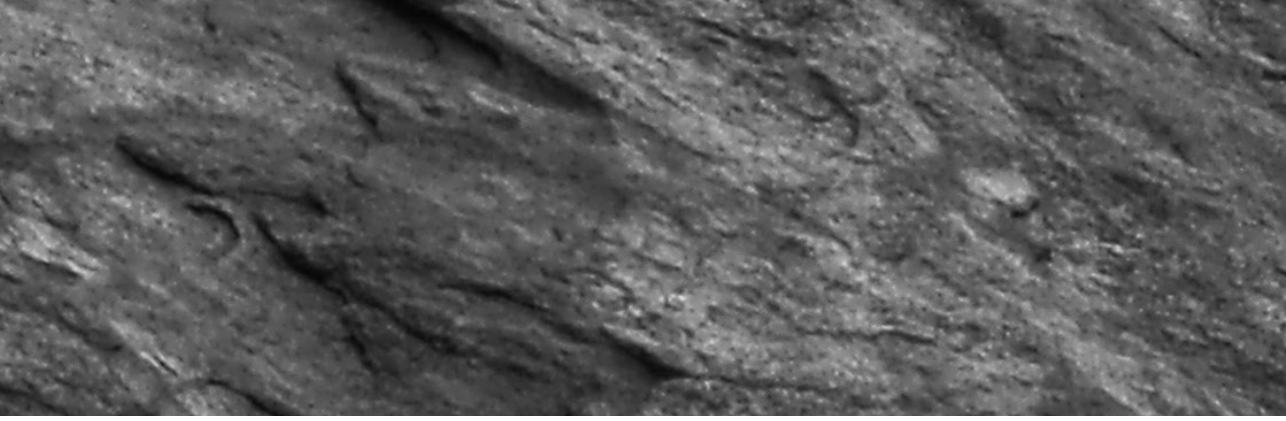

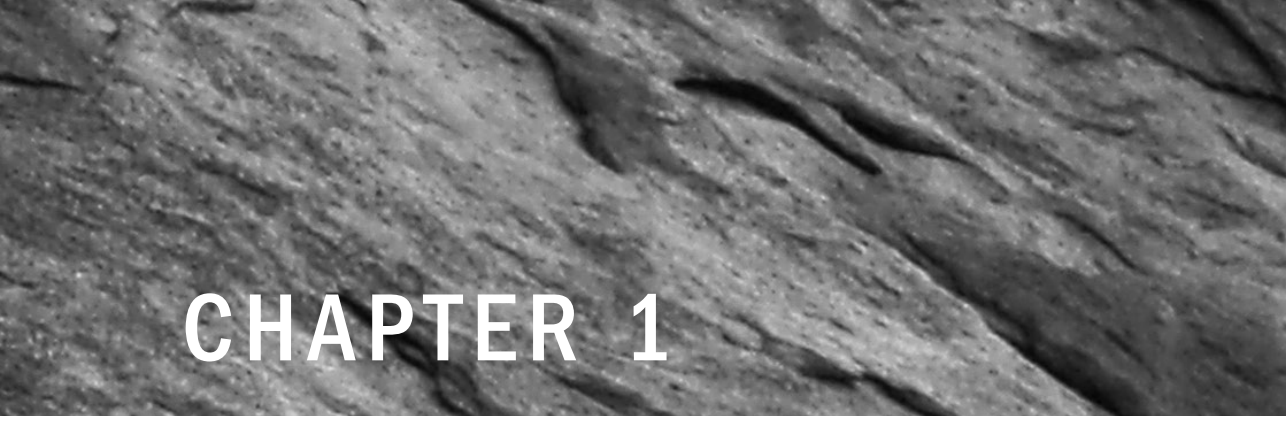

CHAPTER 1

GENERAL INTRODUCTION

Papillomaviridae

Papillomaviruses are small epitheliotropic DNA-viruses that can induce a wide variety of hyperproliferative lesions (papillomas, warts, carcinomas) in the skin and mucosa of mammals (rabbit, horse, dog, sheep, deer, elk, cattle, primates and humans) and birds. Papillomaviruses are subdivided into different genera (**Figure**): the human papillomaviruses (HPV) belong to the genera alpha, beta, gamma, mu and nu and include mucosal and cutaneous types.

In 1933, the etiologic agent of cutaneous warts in cottontail rabbits was identified by Richard Shope (1) as a transmittable virus later called the cottontail rabbit papillomavirus (CRPV). In 1949 Strauss and colleagues (2) were the first to detect viral particles in human warts by electron microscopy. Subsequently, at least 100 different full length HPV genomes have been described and new types are detected regularly (3-6). A new papillomavirus (PV) isolate is recognized as such if the complete genome has been cloned and the DNA sequence of the L1 open reading frame (ORF) differs by more than 10% from the closest known PV type (4).

HPV are known to be associated with benign anogenital and cutaneous warts (7), as well as to be involved in cancer development, in particular with anogenital cancer (8). Most notably the carcinogenic role of high-risk mucosal HPV in cervical cancer is well established and was first proposed in 1976 by Zur Hausen (9), who was recently awarded the Nobel Prize for his pioneering work in this area.

A role for HPV in human skin carcinogenesis was suggested even earlier by Jablonska and co-workers (10) while working with patients suffering from a rare genodermatosis called epidemodysplasia verruciformis (EV) who are at increased risk of cutaneous squamous cell carcinoma (SCC). Several HPV types have been found in EV-associated SCC and subsequently they have been associated with non-EV SCC in epidemiological as well as laboratory studies (11-14). Types from the betagenus (betaPV) appear to be the most likely candidates involved in skin carcinogenesis, especially SCC.

Betapapillomaviruses

At present, 31 betaPV-types have been fully sequenced (HPV5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, 92, 93, 96, 98, 99, 100, 104, 105 and 113). Based on partial sequences, probably more than 35 new types have to be added to this list

of known betaPV types (3;4;15) (Figure). BetaPV DNA can be found in plucked eyebrow hairs, skin swabs and skin biopsies, as well as betaPV antibodies being detected in serum.

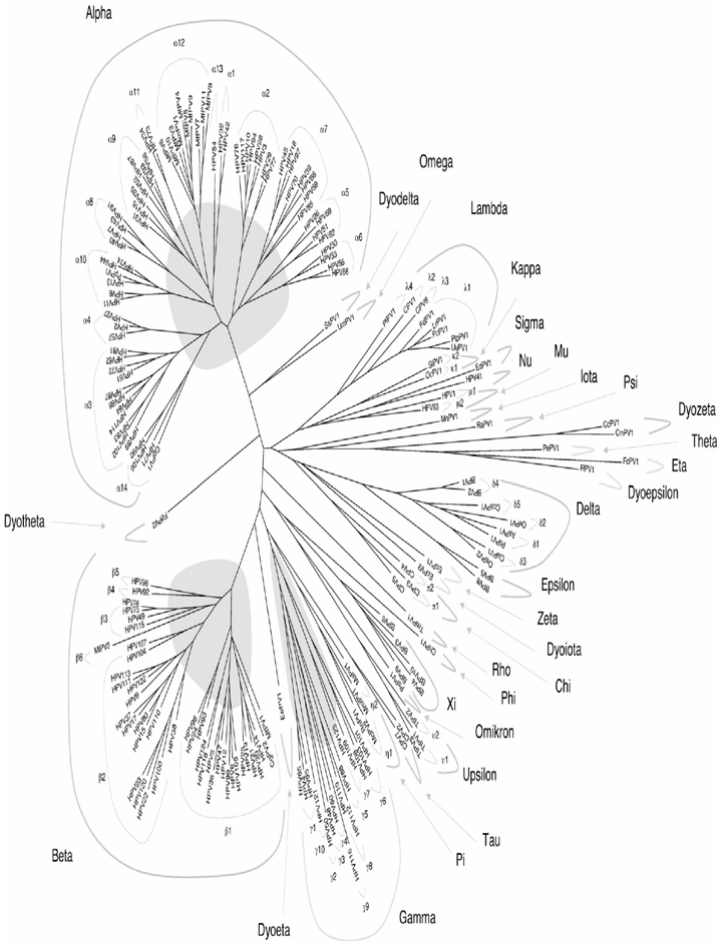

Figure. Phylogenetic tree inferred from the L1 nucleotide sequences of the currently known 189 papillomaviruses. Figure from and legend adapted from (6).

Detection methods

BetaPV DNA

The presence of betaPV in plucked eyebrow hairs has been used as a measure of betaPV infection in several epidemiologic studies. Detection of betaPV DNA in DNA extracted from plucked hairs, skin swabs or biopsies is usually performed with polymerase chain

reaction (PCR) whereby preferential areas of the genome can be amplified. Next to type-specific PCRs for betaPV genotypes, several broad-spectrum PCR methods have been developed to detect cutaneous HPV-types, species or genera: CPI/IIs (16), FAP59/64 (17), F/G (18), modified F/G (M^aH^a) (19), HPV-type specific PCR (20), degenerate nested PCR (21) and PM-PCR (22). Broad spectrum PCR methods can be combined with either cloning and sequencing or direct sequencing of the amplimer, but these methods are too laborious for large epidemiological studies. On the other hand the development of a reverse hybridization assay (RHA) in combination with the PM-PCR enables quick and simultaneous identification of 25 betaPV types (22). Other detection methods are the APEX (23) and the reverse line blotting (RLB) methods (24).

BetaPV antibodies

Antibodies against betaPV proteins can be detected to determine a person's betaPV serological status. These antibodies can be detected against the major capsid protein L1 and the non-structural protein E6 using HPV-virus like particle (VLP) or GST-HPV fusion proteins as antigen in ELISA (12;25) or multiplex (26). The latter method (Luminex®) is a new method based on fluorescent bead technology that allows simultaneous detection of antibodies against up to 100 different *in situ* affinity-purified recombinant HPV proteins (27).

Natural history

Acquisition and transmission

The betaPV life cycle is closely linked to the biology of the specific host cells, the keratinocytes, which are responsible for the renewal, cohesion and barrier function of pluristratified epithelia (28). The replication cycle of papillomaviruses is divided into two stages. First, the virus is maintained at low copy numbers within the initially infected, but still replicating cells. The viral proteins E1 and E2 are essential for this basal DNA replication. When the basal cells are pushed to the suprabasal compartment, they lose their ability to divide and instead initiate the terminal differentiation program. Papillomaviruses replicate in this compartment, and for their release into the environment, take advantage of the disintegration of the epithelial cells that occurs as a consequence of their natural turn-over at the superficial layers (reviewed in 29). By extrapolating research done in rabbits regarding cottontail Rabbit papillomavirus (CRPV) it is hypothesized that betaPV target stem cells are located in the basal layer of the epidermis and in the bulge of the hair follicles (30;31), the latter being considered an immune privileged region (30).

BetaPV can be found on different parts of the skin, as demonstrated by skin swabs of the forehead (17), arms and legs; and by plucked hairs from eyebrows, arms and legs (30;32). It is likely that betaPV infection is acquired early in life by close skin contact since children appear to be infected with the same cutaneous HPV types as their parents within months after birth (33). The exact transmission route of betaPV is unknown but it is hypothesized to be transmitted through skin and hair derivates (34). Recent studies have given contradictory results however, with one study suggesting that transmission between parents and children also occurs at later ages and in adulthood as well (35), while another has suggested that transmission occurs rarely between family members (36). The issue of betaPV transmission is the topic of Chapter 2, which suggests that close skin contact is the primary means of transmission.

Prevalence and persistence

DNA prevalence

The overall prevalence of betaPV DNA is high, but varies depending on the population, anatomic site assessed - whether eyebrow hairs, skin swabs or biopsies of normal skin are being sampled - and the method used for detection. Various studies showed a prevalence of betaPV, measured by either (multiple) skin swabs or plucked eyebrow hairs from multiple sites, to be between 45% and 80% (30;33;34;37). The largest study so far comprised 1405 persons without skin cancer (845 immunocompetent, 560 immunosuppressed) in 6 countries (38). The overall betaPV prevalence ranged from 84-91% between immunocompetent and immunosuppressed respectively, with HPV23 the most prevalent type. Multiple betaPV types per person were often found and there was no predominant type. Only age, and for immunosuppressed participants time of immunosuppression, was associated with betaPV (38). Sun exposure and skin type were not associated.

In biopsies from normal skin the prevalence varies between 50% and 80% (39;40). In Chapter 3 the intraperson distribution of betaPV DNA in normal skin, perilesional skin and SCC biopsies as well as plucked eyebrow hairs is addressed in detail.

Seroprevalence

In the healthy population the betaPV antibody prevalence is around 50-57% (41). Factors seen to influence betaPV seroprevalence are increasing age (41;42) and ethnicity (43). A Dutch case-control study showed an association between sunburn in the past, especially at

age 13-20 years and higher betaPV positivity (44). A higher lifetime sun exposure, however, was associated with decreased HPV infection. On the other hand, a US case-control study showed no significant relations between HPV seropositivity and age, skin sensitivity and number of sunburns (26). In Chapter 4 it is also shown that sunburn does not initiate the betaPV antibody response.

Persistence

Persistence of viral DNA is considered an important aspect of mucosal HPV infections in relation to cervical carcinogenesis (45). Recent studies indicate that also betaPV DNA infections persist. In a small cohort of 23 healthy adults it was demonstrated that the majority of detected betaPV infections persisted for up to 2 years (46). Eyebrow hairs were plucked at 8 time-points over 2 years and showed that 74% of the participants had at least one persisting infection. Another recent study showed persistent betaPV DNA positivity in 48% of the 42 healthy individuals after 7 years (37). It is unknown whether persistence plays a role in the betaPV related carcinogenesis and this issue is the topic of Chapter 5.

No previous studies have involved the persistence of betaPV L1 antibodies, while in Chapter 5 we saw that antibodies are stable over 8 years, with 89% of people remaining antibody positive or negative.

Disease associations

Epidermodysplasia verruciformis

EV was first described in 1922 by Lewandowsky and Lutz (47) as a disease where patients develop pityriasis versicolor-like lesions and flat warts as well as numerous SCC, but not basal cell carcinomas (BCC), on sun-exposed sites at a young age. In the SCC of EV patients mainly betaPV types 5 and 8 are found (48). Recently it was shown that EV-patients harbor multiple betaPV types in both eyebrow hairs and skin biopsies (49) with viral loads ranging from less than 1 betaPV copy per 100 cells up to 400 copies per cell.

Genetic studies in EV patients worldwide have shown mutations in two genes, EVER 1 and 2, to be involved. EVER genes are members of a transmembrane channel-like (TMC) gene family. The function of TMC proteins is still unknown, but it has been proposed that they could constitute a novel group of ion-transporters or channels or modifiers of such activities, and could be involved in signal transduction (reviewed in 28). Recent research

Table 1. Epidemiological studies about the association between betaPV DNA prevalence and SCC development

Author (ref)	Study type/ population	Infection marker	Method	HPV-types	Cases	Controls	Adjusted odds ratio (95% CI)	Comments
Boxman (19)	Nested case-control/ Australia	DNA in eyebrow hairs	Nested PCR	betaPV	64 NMSC* 51 BCC 25 SCC	64 51 25	0.8 (0.3-1.8) 0.6 (0.2-1.5) 2.0 (0.5-8.0)	*BCC/SCC/intra-epithial carcinoma/NMSC undefined
Boxman (56)	Cross-sectional/ Australia	DNA in eyebrow hairs	Nested PCR	betaPV	276 AK	231	3.4 (1.8-6.5) (M) 1.0 (0.6-1.8) (F)	Significant association between betaPV and AK only in men
Struijk (57)	Case-control/ Netherlands	DNA in eyebrow hairs	Type-specific PCR	2, 5, 8, 15, 16, 20, 24, 38	155 SCC	371	1.7 (1.1-2.7)	Association between betaPV and SCC with increasing age and male sex
Harwood (53)	Case-control/ UK	DNA in normal skin biopsies	Degenerate/ Nested PCR	betaPV	10 NMSC*	29	6.4 (1.8-22.9)	*BCC/SCC
Struijk (12)	Case-control/ Australia	DNA in eyebrow hairs	Type-specific PCR	5, 8, 15, 20, 24, 38	126 AK 64 SCC	57	1.6 (0.8-3.0) 0.9 (0.4-2.0)	
McBride (55)	Prospective/ Australia	DNA in eyebrow hairs	Nested PCR	betaPV	71 1-10 AK 41 > 10 AK	179	1.8 (0.7-4.4)	Association with having more than 10 AK. Significant associations with age over 60 years, fair skin color, high sun exposure

suggests that EVER-defects in zinc-metabolism may also play a role in the susceptibility of EV-patients to HPV-infections (50;51).

Keratinocyte skin cancer

Keratinocyte skin cancer is a common malignancy in mainly Caucasian populations, consisting of BCC and SCC and several epidemiological studies have investigated the association between markers of HPV infection, in particular betaPV infection, and keratinocyte skin cancer. Although basal cell carcinomas (BCC) are the most common keratinocyte skin cancer, no clear associations with betaPV DNA or antibodies have been found (16;25;26;41;52-54).

Studies that have investigated the role of betaPV DNA, detected in eyebrow hairs and skin biopsies, in the development of AK and SCC are summarized in **Table 1**. Associations have been found between the presence of betaPV DNA and AK (12;55;56) and SCC (12;19;53;57), but no specific high risk types were identified.

Studies investigating the association between antibodies against betaPV and AK or SCC are summarized in **Table 2**. Seroreactivity to betaPV L1 was associated with AK and SCC in a number of studies (12;26;53;54;58-61) and the presence of AK was inversely associated with seroreactivity to betaPV E6 (12). E6 and L1 antibodies were hardly ever found concomitantly, suggesting that antibody responses to the early (non-structural, intracellular) and late (structural, also extracellular) betaPV proteins take place at different times and phases during betaPV infection or betaPV-associated tumor development (12). It was also shown that HPV DNA positivity and L1 seropositivity were correlated, and E6 seropositivity was inversely correlated with HPV DNA positivity, somewhat in line with the hypotheses either that E6 antibodies partly protect against SCC or that SCC patients have difficulties inducing immune responses to cutaneous HPV E6 proteins (12;57).

Individuals in subtropical areas have an increased risk of actinic keratoses (AK) and keratinocyte skin cancer, since the principal causal factor is excessive exposure to solar UV radiation (62-64). Because betaPV is a possible cofactor in the development of AK and SCC in Queensland, Australia, where reported incidence rates are the highest in the world (65), a number of studies have been performed to investigate the role of HPV in the development of keratinocyte skin cancer (12;19;55;56). These were performed within the Nambour Skin Cancer study, a longitudinal cohort study in subtropical Queensland,

Table 2. Epidemiological studies about the association between betaPV seroprevalence and SCC development

Author (ref)	Study type/ population	Infection marker	Method	HPV-types	Cases	Controls	Adjusted odds ratio (95% CI)	Comments
Steger (59)	Case-control/ Germany	L1 serology	Western blot	8	11	445	10.7 (2.5-63.2)	
Stark (61)	Case-control/ Germany	L1 serology	ELISA	8	14 SCC	210	30.3 (7.4-142.5)	
Bouwes Bavinck (60)	Case-control/ Netherlands	L1 serology	ELISA	8	13 SCC	82	3.1 (0.7-13.3)	
Feltkamp (25)	Case-control/ Netherlands	L1 serology	ELISA	5, 8, 15, 20, 24, 38	540 SCC	333	1.4 (0.8-2.5)	
Masini (58)	Case-control/ Italy	L1 serology	ELISA	8, 15, 23, 36	46 SCC	84	3.2 (1.3-7.9) (HPV8) 0.4 (0.2-0.9) (HPV15) 1.0 (0.3-3.3) HPV23) 2.8 (0.8-10.0) (HPV36)	
Karagas (26)	Case-control/ USA	L1 serology	multiplex	betaPV	252 SCC	461	1.5 (1.1-2.1)	
Struijk (12)	Case-control/ Australia	L1/E6 serology	ELISA	5, 8, 15, 16, 20, 24, 38	126 AK 64 SCC	57	2.3 (0.9-4.9)(L1) 0.6 (0.3-1.3) (E6) 3.9 (1.4-10.7) (L1) 0.5 (0.2-1.1) (E6)	Associations between betaPV L1 and E6 serology and AK/SCC
Casabonne (26)	Nested case-control/ UK	L1 serology	multiplex	betaPV	39 SCC	80	0.5 (0.1-1.7)* 1.0 (0.4-2.5) **	Association between 1* or 2+** betaPV type(s) and SCC
Karagas (54)	Case-control/ USA	L1 serology	multiplex	betaPV	663 SCC	805	1.0 (0.7-1.3)* 1.4 (1.0-2.0)** 1.5 (1.0-2.2)*** 1.7 (1.1-2.6)****	Association between 1* or 2-3**, 4-8*** or >8**** betaPV type(s) and SCC

Australia, that started in 1986 with the enrollment of 2095 participants (66) who were then followed up until 2007. All studies described in this thesis have been performed in Australian participants, Chapters 2, 4, 5 and 6 as part of the Nambour Skin Cancer Study and Chapter 3 in a small cohort of SCC-patients in Northern Queensland.

Scope of this thesis

Chapter 2 describes the transmission of betaPV between opposite-sex partners as compared with age and sex matched controls.

Chapter 3 describes the distribution of betaPV as measured in eyebrow hairs and biopsies of normal skin, SCC tumor tissue and perilesional skin of 21 SCC-patients.

Chapter 4 describes the relation between two frequently used markers for betaPV research: betaPV DNA in eyebrow hairs and betaPV antibodies from serum, both cross-sectionally and longitudinally.

Chapter 5 describes the association between persistent betaPV infection as indicated by viral DNA in eyebrow hairs and the risk of AK on the whole body and on the face.

Chapter 6 describes the association between betaPV antibodies in serum and the development of SCC in a longitudinal study.

Chapter 7 comprises the general discussion and conclusions.

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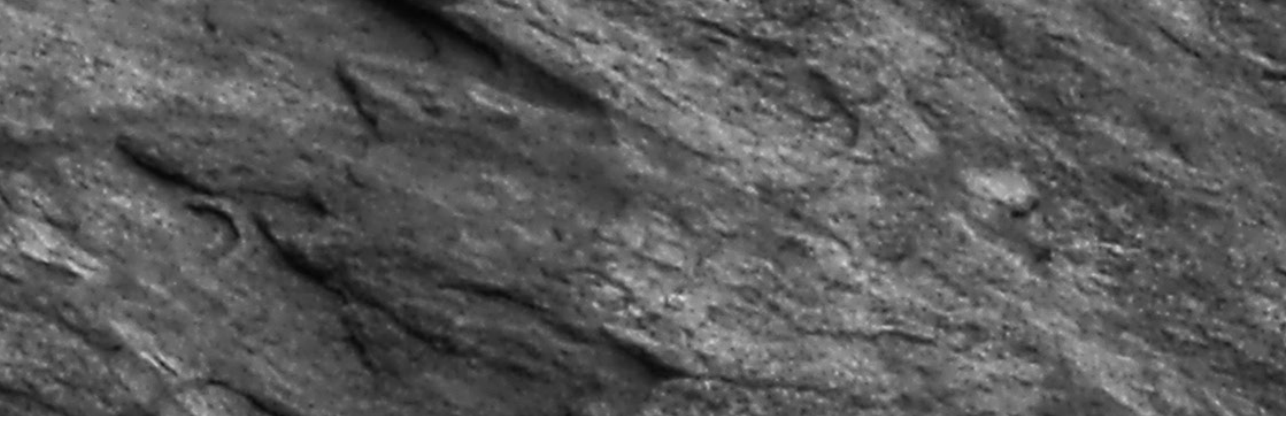

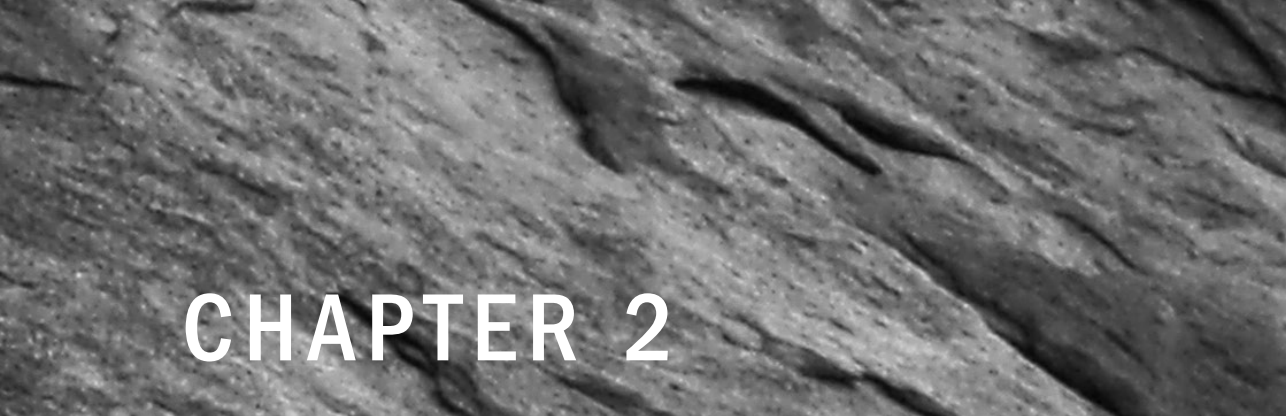

CHAPTER 2

TRANSMISSION OF BETAPAPILLOMAVIRUSES BETWEEN DOMESTIC PARTNERS IN AN AUSTRALIAN COMMUNITY

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Abstract

Betapapillomaviruses may be associated with the development of cutaneous squamous cell carcinoma but little is known about their transmission. One suggestion is that they are transmitted through close skin contact.

To test this hypothesis we assessed whether co-habiting opposite-sex couples were more or less likely to share betaPV types than each member of the couple and an age-matched, opposite-sex control. Betapapillomavirus was measured in eyebrow hairs of 57 couples and 114 age- and sex-matched controls. We compared the proportion of partners who shared at least one betaPV type with the proportion of control partnerships sharing a betaPV type. We further subdivided those who shared at least one type into those who shared only one and those who shared more than one. We tested the significance of differences in these proportions using Chi-squared tests. A case-wise concordance index was used to calculate the overall concordance of the partners and the control pairings.

At least one betaPV type was shared by 39% of the co-habiting couples and 26% of the control pairs ($p=0.10$). When restricted to all people with at least one virus infection (26 couples) 74% of the partners and 46% of the control pairs shared at least one type ($p=0.02$). The case-wise concordance index for partners was 0.28 (95% CI 0.21-0.35) and for the matched control pairs 0.16 (95% CI 0.12-0.20) ($p<0.001$).

Our results support the hypothesis that skin-to-skin contact is the primary means of beta-papillomavirus transmission.

Introduction

Human papillomaviruses of the beta-genus (betaPV) are cutanotropic viruses that are associated with cutaneous squamous cell carcinoma (SCC) (1). So far 31 different betaPV types have been fully sequenced and more than 100 types partially sequenced (2;3). Epidemiological studies have shown that all currently identified betaPV types are frequently found in hair bulbs of eyebrows and body hairs, normal skin swabs and biopsies from healthy controls and transplant recipients, as well as in tumour tissue from patients with SCC (4-6). Usually multiple infections are detected within a sample (7).

Little is known about the transmission of betaPV. In healthy people no clinical signs of initial infection are observed. We have found only 5 previous studies addressing transmission of betaPV, several of which are very small (8-12). The data about transmission between parents and children is ambiguous: one study involving 38 infants showed parents and babies as young as 4 weeks of age to share betaPV types (8) and another study showed that transmission between parents and children occurs frequently (13;14). However in a third study transmission between parents and children was observed rarely (15). In this cohort transmission between couples was also infrequently seen (15). A cohort of 23 participants showed that the 5 students sharing a household were not likely to obtain each other's betaPV, but instead kept their own infection profile (10). Despite different outcomes, all of these studies concluded that betaPV transmission probably takes place during close (skin-to-skin) contact.

To test this hypothesis we assessed whether co-habiting married or *de facto* opposite-sex couples (herein called 'partners') were more or less likely to share betaPV types than each member of the couple and an age-matched, opposite-sex control.

Material and methods

Study population and sample collection

Participants were an unselected subset of the study population of the Nambour Skin Cancer Study which has been described in detail previously (16;17). Briefly, in 1986, 2095 of 3000 randomly selected residents of Nambour, a subtropical township in Australia (latitude 26°S), who were aged 20-69 years, participated in a skin cancer prevalence survey. From 1992 to 1996, 1621 of these took part in a trial of sunscreen application and beta-carotene supplementation for the prevention of skin cancer. In 1996, 507 randomly selected members of the

cohort participated in a sub-study aiming to understand the association between HPV and skin cancer (18), and 10 eyebrow hairs were plucked from each participant and processed as described below. Participants' relationships with one another in 1996 were recorded. For the analysis described here we selected all 57 male-female co-habiting couples. For each of these 114 people, we randomly selected an opposite-sex control from the remaining 393 participants, matched to the age of his/her partner. For example, a 60-year-old man and his 50-year-old wife were matched to a 50-year-old woman and a 60-year-old man respectively.

DNA isolation, PCR and hybridization

DNA from eyebrow hairs was isolated according to a method described previously (19). BetaPV detection and genotyping were performed using a reversed hybridization assay as described previously (20). All amplimers generated with the broad spectrum PCR were analysed with a reverse hybridization assay (RHA) that permitted specific detection and identification of 25 established betaPV genotypes (i.e., 5, 8, 9, 12, 14, 15, 17, 19-25, 36-38, 47, 49, 75, 76, 80, 92, 93 and 96). The RHA was performed according to the manufacturer's instructions (skin (beta) HPV prototype research assay; Diassay BV, Rijswijk, The Netherlands).

Statistical analyses

We compared the proportion of partners who shared at least one betaPV type with the proportion of control partnerships sharing a betaPV type. We further subdivided those who shared at least one type into those who shared only one and those who shared more than one. We tested the significance of differences in these proportions using Chi-squared tests. These analyses were performed for all participants and for those in whom we identified at least one betaPV. In addition, case-wise concordance was calculated, which is defined as the conditional probability that one member of the matched pair is positive to a specific betaPV type given that the other member is positive. It was estimated as the ratio of the number of concordant pairs to the total of concordant and average discordant pairs. The standard error and 95% confidence interval were estimated according to methods documented by Huang and Tai (21). The totals for concordant and discordant pairs have been pooled across the 25 individual betaPV types. One estimate was calculated for partner pairs and a second for the pairs formed by matched control couples. Statistical analyses were performed in SAS 9.1.

Results

The mean age of the men was 55 years (SD 11) and of the women 51 (SD 11). Seventy-four percent of the male partners were betaPV-positive (median number of types: 2, range 1-12), compared with 86% of the male controls (p=0.07) (median number of types: 2, range 1-15), 70% of the female partners (median number of types: 2, range 1-11) and 74% of the female controls (p=0.65) (median number of types: 1, range 1-11).

At least one betaPV type was shared by 39% of the co-habiting couples (**Table**). For the control pairs this was 26% (p=0.10). Fourteen percent of partners versus 11% of control pairs shared more than one type (p=0.25). When we repeated the analyses for all people with at least one virus infection (26 couples) 74% of the partners and 46% of the control pairs shared at least one type (p=0.02), and 32% versus 19% shared more than 1 type (p=0.08) (Table). The case-wise concordance index for partners was 0.28 (95% CI 0.21-0.35) and for the matched control pairs 0.16 (95% CI 0.12-0.20) (p<0.001).

Table: Number of betaPV types shared by partners and by partners and their controls.

No. of shared types	**Partners, n=57**	**Control-pairs, n=114**
	N (%)	**N (%)**
Including all participants		
0	35 (61)	84 (74)
1+	22 (39)	30 (26)
Chi-square	2.71 (p=0.10)	
0	35 (61)	84 (74)
1	14 (25)	18 (16)
>1	8 (14)	12 (11)
Chi-square	2.79 (p=0.25)	
No. of shared types	**Partners, n=26**	**Control-pairs, n=52**
	N (%)	**N (%)**
Including only betaPV positive participants		
0	7 (26)	28 (54)
1+	19 (74)	24 (46)
Chi-square	5.08 (p=0.02)	
0	7 (26)	28 (54)
1	11 (42)	14 (27)
>1	8 (32)	10 (19)
Chi-square	5.08 (p=0.08)	

Discussion

In this study we found that participants more often shared at least one betaPV type with their opposite-sex domestic partner than with random controls of the same age and sex as their partner. This difference was significant when the analysis was restricted to people who had at least one betaPV infection. Partners also were likely to share more than one type than control pairs, although due to small numbers significant differences could not be observed. We found a highly significant difference in the concordance index. We assessed skin type, sun exposure and skin cancer rate as possible confounders and found no differences between the male and female partners and male and female controls. The borderline significant difference in betaPV prevalence between the male partners and male controls is most likely to be due to random sampling error and is not likely to have caused differences. The higher number of viruses seen in male controls than in male partners suggests that they would have an increased chance of sharing types with the female partner, so if anything, this variability may have led to an underestimation of the difference in shared types found.

The most likely explanation for our findings is the frequent close contact likely to occur between partners, which was also postulated to be the main cause of HPV transmission in babies (8). A study among tenants in a student share house showed that transmission was rare (10), suggesting that living in close proximity may not be sufficient for betaPV transmission and skin-to-skin contact may be required. A recent study about betaPV transmission in families with an overall HPV prevalence of 42% found that the frequency of shared types was higher among couples than among randomly selected individuals, but the frequency of sharing at least one type was only 21% and in all cases only one type was shared (15). We found a much higher proportion of couples with at least one shared type (39%), and 14% of these shared more than one type, possibly due to the higher overall prevalence of betaPV in our sample. The higher prevalence might be due to the fact that we used a different PCR and typing method than those used by Gottschling and colleagues (15). Furthermore, the mean age of our participants was higher (over 50 compared with 42 years), and age is independently associated with betaPV acquisition or detection (7). Weissenborn and colleagues studied the betaPV- spectrum in 10 families with up to 3 generations sampled over a period of time and found comparable results to ours with respect to partner transmission, despite using skin swabs rather than eyebrow hairs as the sample for viral DNA detection (22). Their longitudinal measures showed that persistent infections in one person of a family were shared by their family members in 30-50% of the cases.

Our data are cross-sectional and we therefore cannot address the issue of whether or not persistent betaPV types are shared between couples.

In conclusion, these cross-sectional data demonstrate that co-habiting partners of the opposite-sex share a greater number of betaPV types than with randomly selected matched members of the population. This finding supports the hypothesis that close contact is the primary means of betaPV transmission, probably through skin-to-skin contact. Larger, longitudinal studies are needed to confirm this finding and to give more insight into the sustainability of the shared infections.

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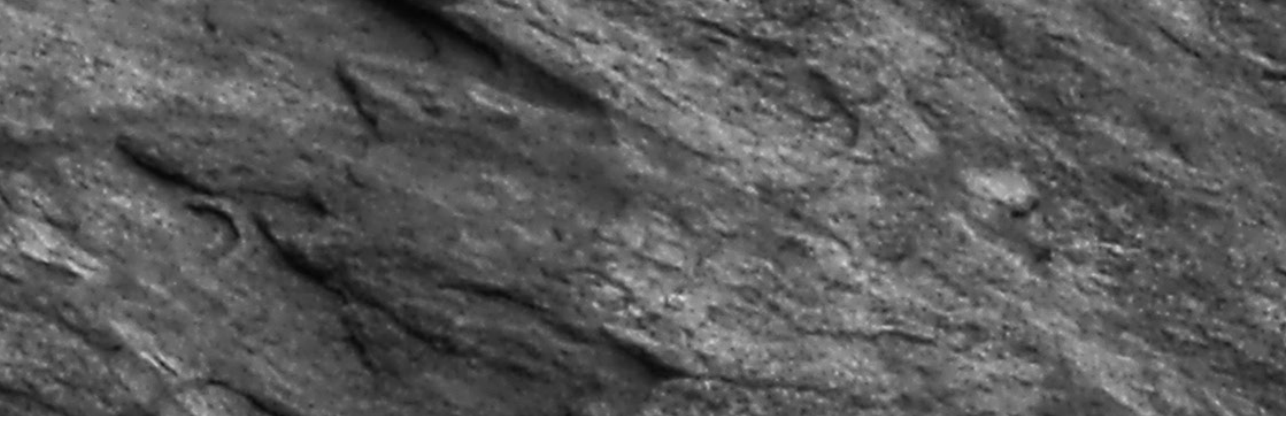

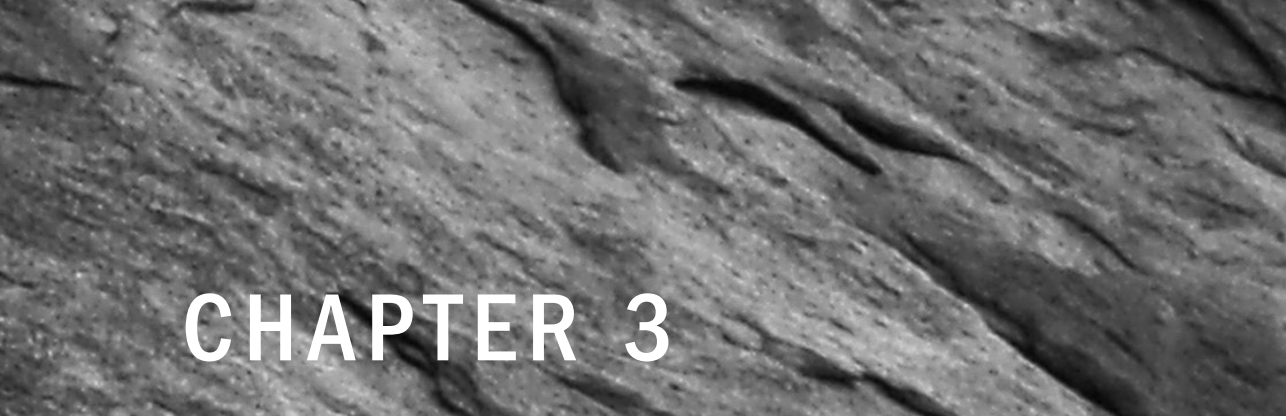

CHAPTER 3

BETAPAPILLOMAVIRUS INFECTION PROFILES IN TISSUE SETS FROM CUTANEOUS SQUAMOUS CELL-CARCINOMA PATIENTS

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Abstract

Human papillomaviruses from the genus beta (betaPV) are a possible cause of cutaneous squamous cell carcinoma (SCC). We compared the betaPV infections in SCC and in sets of cutaneous tissues collected from a series of individual SCC patients to determine concordance, and to assess the adequacy of eyebrow hairs as non-invasive markers of betaPV infection. Biopsies of SCC tumours, perilesional tissue, normal skin from the mirror image of non-facial SCC and plucked eyebrow hairs were collected from 21 patients with incident SCC living in Queensland, Australia. These were tested for the presence of DNA from 25 different betaPV types. Overall prevalence of betaPV was high in every sample type, ranging from 81-95%. The median number of types was significantly higher in the SCC tumour (6), perilesional skin (5) and eyebrow hairs (5) than in normal skin (2). Comparing SCC tissue with other sample types within patients showed 63 overlapping infections with eyebrow hairs (71%; 95%CI 60-80); 56 with perilesional skin samples (63%; 95% CI 52-73) and 23 with normal skin samples (26%; 95% CI 17-36). The sensitivity of eyebrow hair testing for detection of betaPV in the tumour was 82% (95%CI 57-96) with concordance defined as 50% of betaPV types in common and 29% (95%-CI 10-56) for 100% concordance.

Introduction

Infection with human papillomaviruses (HPV) from the beta-genus (betaPV) is associated with the development of actinic keratoses (AK) and squamous cell-carcinoma (SCC) in immune-competent persons as well as in organ transplant patients (1-7) The majority of people are infected with multiple betaPV (8), and a substantial proportion of these infections remains detectable over time, indicative of persistent infection (9;10).

Different mechanisms by which betaPV play a role in carcinogenesis have been proposed, for example the "hit-and-run" hypothesis, whereby betaPV act early in carcinogenesis and is not necessary for maintenance of the malignant phenotype (11;12). BetaPV may act within or contribute to field cancerisation where a discrete area of tissue is at increased risk of developing cancer (13), as seen for SCC of the oesophagus (14) and cutaneous actinic keratoses (15-18). A postulated mechanism of transformation is betaPV-mediated impairment of host cell defenses against excessive sun light exposure, such as inhibition of DNA repair and apoptosis (19-21).

In epidemiological studies, the presence of betaPV DNA in eyebrow hairs, skin swabs, and normal skin biopsies have all been used as markers of betaPV-infection (22-26). Which of these is the most appropriate indicator of the betaPV types found in the tumour and/or the surrounding area is currently unknown. It has been proposed that the hair follicle is the natural reservoir of cutaneous HPV (22;27) with support from studies showing that HPV is present in hair follicles obtained from different body sites such as scalp, eyebrow, arm, trunk, leg and pubic region (22;28).

Here we have investigated within a series of SCC patients the prevalence and distribution of 25 different betaPV types in sets comprising four sample types (SCC, perilesional skin, normal skin on the mirror image site of the SCC, and plucked eyebrow hairs) to gain possible insights into viral pathogenesis of SCC and assess if plucked eyebrow hairs are indeed sentinel for betaPV present in the tumour.

Material and methods

Study population and sample collection

This study took place in Townsville, a regional town in tropical Australia (latitude 19^0S). Patients, with diagnosis of histologically confirmed incident primary cutaneous SCC

between April 2002 and April 2003 were recruited from the Townsville Hospital by local specialist doctors and general practitioners. Ten eyebrow hairs were plucked from each participant using sterile tweezers and gloves, and biopsies were collected from the SCC, perilesional skin immediately adjacent to the SCC and normal skin from the mirror image site of the SCC. Because of ethical, cosmetically reasons, for the patients with a facial SCC (n=3) a biopsy of the forearm was used as normal skin. All samples were snap frozen and stored at -70°C. Age, sex and information about sun exposure were recorded for all participants. The study was approved by the ethics committee of James Cook University and by the Townsville Health Service District Institutional Ethics Committee.

DNA isolation, PCR and hybridization

DNA from eyebrow hairs and biopsies were isolated using a QIAamp DNA mini kit (Qiagen). Briefly, hairs and biopsies were pre-treated overnight with proteinase K solution according to the manufacturer's instructions. After lysis with 200 ul AL buffer, half of the volume was stored at -70ºC, whilst the other half was processed according to the manufacturer's instructions.

BetaPV detection and genotyping were performed using a reversed hybridization assay as described before (23). Briefly, PM-PCR was performed in a final reaction volume of 50 ul, containing 10 ul of the isolated DNA, 2.5 mM $MgCl_2$, 1x GeneAmp PCR buffer II, 0.2 deoxynucleotide triphosphates, 1.5 U AmpliTaq Gold DNA polymerase and 10 ul of the PM primer mix. The PCR was performed by a 9 min pre-heating step at 94ºC, followed by 35 cycles of amplification comprising 30 s at 94ºC, 45 s at 52ºC, and 45 s at 72 ºC. The PCR was ended by a final elongation step at 5 min at 72 ºC. All amplimers were subsequently analyzed with a reverse hybridization assay (RHA) that permitted specific detection and identification of the 25 established betaPV genotypes (i.e. 5, 8, 9, 12, 14, 15, 17, 19-25, 36-38, 47, 49, 75, 76, 80, 92, 93 and 96). The RHA was performed according to the manufacturer's instructions (skin (beta) HPV prototype research assay; Diassay BV, Rijswijk, The Netherlands).

Statistical analyses

We calculated the prevalence of betaPV DNA in each sample type, defining a sample as positive if it had at least one betaPV type detected. The number of viruses detected per sample was calculated. We calculated the Friedman test, to test for significant overall dif-

ferences in the median number of betaPV types between the four sample types. Wilcoxon tests were used to estimate the significance of differences between any two sample types.

Overall betaPV agreement was defined as the proportion of cases where both samples being compared were either betaPV-positive or -negative. To compare the number of types in common between the SCC tissue and other samples obtained from each patient, we derived the proportion of the total number of infections found in SCCs (type-specific per patient and summed across all participants) that were also found in normal skin, perilesional skin and hairs, respectively.

We estimated the sensitivity of testing hair follicles for betaPV DNA, using SCC tissue as the reference. We first calculated sensitivity assuming that for the test to be classified as 'positive', 100% of the types found in the SCC also had to be detected in the hair follicles. We then recalculated sensitivity with test concordance defined as 50% of the types in common between SCC and hair follicles. Finally we repeated the sensitivity analyses taking as the reference the perilesional field of skin adjacent to the SCC rather than the SCC itself.

Analyses were performed in SPSS 14.0 and SAS 9.1.

Results

Patients participating in this study were an unselected sample of 37 patients with SCC who had other tissue samples, next to the SCC, collected and available for analysis. We included only those 21 participants for whom complete sample sets were available. The mean age of these patients was 71 years (range 35-87) and 80% were males.

BetaPV presence and prevalence

An overview of all betaPV types found in the four different samples of all 21 SCC cases is shown in **Table 1**. Only one SCC patient (# 1) was entirely betaPV negative, with no betaPV DNA detected in any of his samples.

BetaPV DNA was found in the eyebrow hairs of 18 SCC patients (86%), and the median number of types found per patient was 5 (inter quartile range (IQR) 2-10) (**Table 2**). BetaPV DNA prevalence was 81% in biopsies of both normal skin and SCC, but more types were found in the tumour (median 6) and in eyebrow hairs (median 5) than in normal skin (median 2) (p values 0.016 and 0.001 respectively). Perilesional biopsies had the highest

Table 1. Individual sample listed with all betaPV types detected.

Individual	Age	Sex	Sample	betaPV types	no of types
1	**35**	**male**	eyebrow hairs	Negative	0
			normal skin	Negative	0
			perilesional skin	Negative	0
			SCC (forearm)	Negative	0
2	**58**	**male**	eyebrow hairs	**8* 9 15 22 24 36** 37 **38 49 93**	10
			normal skin	**9 22 24 38**	4
			perilesional skin	**9 24 36 38 93**	5
			SCC (forehead)	**8 9 15 22 24 36 38 49 93**	9
3	**59**	**male**	eyebrow hairs	8 **9 12 15 17** 19 **20 23 36 38 49 75 80** 92	14
			normal skin	**9 15 17 49**	4
			perilesional skin	**9 12 15 17 20** 22 **23 36 38 75 80**	11
			SCC (scalp)	Negative	0
4	**60**	**male**	eyebrow hairs	8 **15 22** 49 **80**	5
			normal skin	**36 80** 96	3
			Perilesional skin	**15 22 36 80**	4
			SCC (upper arm)	**15 22 36**	3
5	**61**	**male**	eyebrow hairs	**5 19 23** 37 **75 76** 80	7
			normal skin	Negative	0
			perilesional skin	**5 15** 17 **19 23** 36 **75 76** 92	9
			SCC (forearm)	**5** 9 14 **15 19 23 75**	7
6	**65**	**male**	eyebrow hairs	**20 36 38**	3
			normal skin	Negative	0
			perilesional skin	5 8 9 17 **20** 22 23 **36 38** 92	10
			SCC (lower leg)	93	1
7	**68**	**male**	eyebrow hairs	**9 17 20 22 80**	5
			normal skin	**20**	1
			perilesional skin	**9** 14 19 **20** 23 24 36 **38 80 93**	10
			SCC (hand)	**9 17 20 22 38 93**	6
8	**70**	**male**	eyebrow hairs	Negative	0
			normal skin	49	1
			perilesional skin	**20**	1
			SCC (arm)	15 **20** 92	2
9	**70**	**male**	eyebrow hairs	**9 15 23 92**	4
			normal skin	**9 15**	2
			perilesional skin	**9 15** 17 **23 92 96**	6
			SCC (hand)	**9 15 23** 49 **92 96**	6
10	**71**	**male**	eyebrow hairs	**5 8 9 14 15 17 19 21 23 25 36** 49 76 **92** 93 96	16
			normal skin	**5 8 23 25**	4
			perilesional skin	**5 8 9 14 15 17 19 21 23 25 36 92**	12
			SCC (shin)	**8 17**	2
11	**71**	**male**	eyebrow hairs	**15 23 24** 38 76 **96**	6
			normal skin	**23**	1
			perilesional skin	8 **15** 21 22 **23 24** 93 **96**	8
			SCC (thigh)	**96**	1
12	**72**	**female**	eyebrow hairs	15 **17 22 80 93**	5

Table 1 continued

Individual	Age	Sex	Sample	betaPV types	no of types
			normal skin	**22 80 93**	3
			perilesional skin	9 14 24 25 **80** 92 **93**	7
			SCC (upper arm)	5 **17 22** 23 **80 93**	6
13	**73**	**male**	eyebrow hairs	**17**	1
			normal skin	**15** 75	2
			perilesional skin	**9 15 17 23**	4
			SCC (forearm)	**9 15 17** 19 **23** 38	6
14	**76**	**female**	eyebrow hairs	**5 9 17** 23 **24 25** 36 37 38 **76 92 96**	12
			normal skin	**5 24** 49	3
			perilesional skin	**24 92**	2
			SCC (forearm)	**5 9 17 24 25 76 92 96**	8
15	**77**	**male**	eyebrow hairs	**15 23 75 93**	4
			normal skin	Negative	0
			perilesional skin	**5 15** 17 **23** 36 **75 93** 96	8
			SCC (forearm)	**5 15 23** 38 **75 93**	6
16	**77**	**male**	eyebrow hairs	**92**	1
			normal skin	**17** 36 **92 96**	4
			perilesional skin	**9 17** 19 **92 96**	5
			SCC (forearm)	negative	0
17	**78**	**male**	eyebrow hairs	9 **15 17** 22 **23 36 49** 93	8
			normal skin	**17**	1
			perilesional skin	**15 23 49** 96	4
			SCC (post ear)	**15 49**	2
18	**82**	**female**	eyebrow hairs	**5 15 17 23** 37 **93**	6
			normal skin	**5 17**	2
			perilesional skin	**5 17 23 93**	4
			SCC (ankle)	**5 15 17 23** 76 **93**	6
19	**82**	**male**	eyebrow hairs	**8 12 15 17 23 24 37 38 75 92 93**	11
			normal skin	**12 15 17** 22 **23 37 38** 49 **96**	9
			perilesional skin	**8** 9 **12 15 17** 21 **23 24 37 38 75 92 93 96**	14
			SCC (shoulder)	**12 15 17 23 38 75 92 93 96**	9
20	**83**	**male**	eyebrow hairs	**9 17 19 22** 36 38 49 **92 93 96**	10
			normal skin	15 **17**	2
			perilesional skin	**9 76 96**	3
			SCC (lower leg)	**19 21 22** 24 76 **92 93 96**	8
21	**87**	**female**	eyebrow hairs	Negative	0
			normal skin	**15** 23	2
			perilesional skin	**15**	1
			SCC (heel)	Negative	0

*Bold types are types shared between different samples of the same patient.

betaPV prevalence (95%) with a median number of types of 5, which was also significantly higher than in normal skin ($p<0.001$) (Table 2).

Table 2. Overall betaPV detection in samples of SCC-cases.

		(N=21)			
		eyebrow hairs	normal skin	perilesional skin	SCC
Detection of betaPV, n (%)					
	positive	18 (86)	17 (81)	20 (95)	17 (81)
median no betaPV types (IQR*)		5 (2-10)	2 (1-4)	5 (4-10)	6 (1-7)
	range	0-16	0-9	0-14	0-9

*IQR: Inter quartile range

Overall, the prevalence of most individual betaPV-types was lowest in normal skin, except for HPV37 and 80 where the prevalence was lowest in the SCC biopsies and for HPV 49 that was lowest in perilesional skin. The most prevalent types across all tissue samples were HPV15, HPV17 and HPV23 (**Table 3**).

Comparisons between SCC, perilesional and normal skin biopsies

We observed a high degree of overlap between the betaPV types in the different samples from each patient (Table 1). No type was found exclusively in any of the samples, including SCC, when betaPV type distribution was compared.

Overall betaPV agreement (having both samples concordant for the presence or absence of betaPV, irrespective of type) between tumour tissue and perilesional skin was 86% (18/21; 95%CI 64-97), and between tumour tissue and normal skin was 71% (15/21; 95%CI 48-89).

A total of 128 betaPV infections were found in the eyebrow hairs of the 21 participants, 48 in normal skin biopsies, 128 in perilesional biopsies and 89 in tumour tissue biopsies (Table 3). Of the 89 betaPV infections found in tumour tissue, 56 infections with the same betaPV type were also found in the perilesional skin of the same patient (56/89, 63%; 95%CI 52-73). In comparison, 23 overlapping infections were observed between SCC and normal skin, a proportion of 26% (23/89; 95% CI 17-36). There were 33 overlapping infections between perilesional skin and normal skin (26%, 33/128; 95% CI 19-34).

BetaPV in eyebrow hairs as marker of betaPV infection in SCC

Overall betaPV agreement was 86% between eyebrow hairs and tumour tissue and 90% between hairs and perilesional skin. Of the 89 betaPV infections found in SCC tissue, 63 type-specific infections were also found in the eyebrow hair follicles of the same patient 63/89, 71%; 95% CI 60-80) and 79 overlapping infections were detected between perilesional skin and eyebrow hairs (79/128, 62%; 95% CI 53-70).

Table 3. BetaPV prevalence in 21 SCC patients shown per sample.

HPV-type	(N=21)			
	eyebrow hairs	**normal skin**	**perilesional skin**	**SCC**
5	4	3	5	5
8	5	1	4	2
9	8	3	11	6
12	2	1	2	1
14	1	0	3	1
15	11	6	11	10
17	10	6	10	7
19	4	0	4	3
20	3	1	4	2
21	1	0	3	1
22	6	3	4	5
23	10	4	12	7
24	4	2	6	3
25	2	1	2	1
36	7	2	8	2
37	5	1	1	0
38	7	2	5	5
47	0	0	0	0
49	6	4	1	3
75	4	1	4	3
76	4	0	2	3
80	5	2	4	1
92	7	1	8	5
93	8	1	7	8
96	4	3	7	5
Total no infections	128	48	128	89

In three individuals SCC was present on the face (# 2, 3, 17 in table 1). In the two betaPV positive SCC (# 2 and 17) all types present in the SCC were also found in the eyebrow hairs of the corresponding individuals.

When we defined hair samples as being concordant if they contained all of the types found in the SCC biopsy, sensitivity was 29% (95%-CI 10-56). Using a less stringent definition of concordance whereby hair samples were classified as concordant if they contained 50% of the betaPV types found in the SCC, sensitivity was 82% (95% CI 57-96). When the perilesional skin was taken as the reference category (instead of SCC tissue), sensitivities for these comparisons were 25% (95% CI 9-50) and 65% (95% CI 41-85) respectively.

Discussion

In this study we systematically explored and compared type-specific betaPV prevalence and distribution in sets of four different tissue samples taken from 21 incident SCC patients.

The overall betaPV DNA positivity was high, ranging from 81% in normal skin and SCC tissue to 86% in the eyebrow hairs and 95% in perilesional skin. These percentages underscore the ubiquity of cutaneous betaPV infections that has been previously reported (8;9;29). The multiplicity of infections was also found to be high, with a median number of infecting types of 5 in eyebrow hairs and perilesional skin and 6 in the tumour, and up to 14 and 16 different betaPV types present in single samples from perilesional skin and eyebrow hair, respectively. The number of betaPV types detected in normal skin was considerably less than in the other tissues, in support of previous data showing that normal skin has fewer betaPV types than SCC tissue (30;31).

The lower number of betaPV types found in normal skin than in the tissue near the SCC supports the hypothesis that perilesional skin represents an area of field cancerisation from which the tumour arose (15-18). The localised presence of betaPV may have contributed to the field change, in conjunction with other factors such as sunburn or chronic sun exposure. Alternatively focal damage may have enhanced betaPV infection, increasing the viral load above the detection limit of the test, or less likely, may have rendered the affected skin more susceptible to infection with betaPV. We obtained the normal skin biopsies from the mirror image site of the SCC so that betaPV detection would be unconfounded by local photo immune suppression or to stimulation of viral replication by UV irradiation.

Although we found the same median number of betaPV types in perilesional and SCC tissue, a different spectrum of types was seen, as shown by the measures of type-specific agreement. Hypothetically the types present in the perilesional skin could partially represent commensal types, whereas those in the tumour could be associated with tumour formation. However, when we compared all tumours and non-tumour tissues no particular betaPV types stood out as occurring in the tumour alone or in healthy tissue alone, making the identification of specific oncogenic types unlikely. These results show, however, that it is unlikely that the betaPV DNA found in the perilesional skin was due to carry-over of viral DNA from the tumour, since distinct differences were found between these tissues. With this small study size, random variation may also have contributed to the differences in betaPV types between tissue types.

The detection of betaPV in eyebrow hairs has been used in epidemiological studies as a marker of infection, not only because the bulb is regarded as a reservoir of infection but also because of the ease of obtaining eyebrow hairs. The greater diversity of types found in hair follicles compared with normal skin lends support to the notion that hair follicle is a reservoir for betaPV (22), with the epidermal stem cells residing in the bulge as the probable main site of persistent infection.

We compared betaPV DNA in eyebrow hairs with biopsies of the SCC and perilesional skin to obtain information about the comparability of these samples. The type-specific agreement was slightly higher for SCC than for perilesional skin. To calculate the sensitivity of eyebrow hair follicle testing as a measure of relevant betaPV infection, we first took SCC-tissue as the reference tissue. The sensitivity ranged from 25-82% depending on the definition of concordance and whether the SCC or the perilesional tissue is used as the reference. The relevance of the high agreement in the two individuals with a betaPV positive SCC present on the face needs to be explored further in larger datasets given the small number of participants. Although eyebrow hairs are frequently used as markers of infection in betaPV studies, it is notable that substantial differences can exist between type-specific detection rates in SCC tumour and eyebrow hairs of individuals.

Two other studies have also compared HPV DNA in tumours of SCC patients with HPV DNA found in other specimens (32;33). Compared to the previous studies, our overall detection rate of HPV DNA was much higher for all skin samples, although the higher number of types in SCC biopsies than in normal skin was found in all three studies. The differences may be due to different DNA isolation and typing methods and different study populations. In contrast, the overall betaPV DNA detection rate in eyebrow hairs in our study and that conducted by Rollison and colleagues was similar (33). It may be that betaPV loads in eyebrow hairs exceed those in the skin samples, making analysis of eyebrow hair follicles less susceptible to differences in sensitivity due to differences in betaPV DNA isolation and detection methods. The proportion of participants in whom both tumour tissue and normal skin biopsies were betaPV positive was also similar between these two studies, at around 75%. However Rollison and colleagues did not appear to take individual betaPV type-specificity into account (33). Asgari and colleagues could not perform intra-patient comparisons between normal and affected skin, as control and SCC samples were obtained from different individuals (32).

A possible limitation of this study is the small patient group. However, based on the comprehensiveness of our sample sets, the high number of included HPV types and the unique study design, we believe the generated data provide valuable new information that is generalisable. Our study population was representative of SCC patients generally seen in the Townville area in terms of average age (70 versus 67 years) as the key risk factor for SCC in a high-risk population like Townsville, though it contained a higher proportion of males (80%) than seen overall (61%) (34).

In summary, this series of samples of SCC-patients show that perilesional skin is clearly different from normal skin with respect to betaPV infection, supporting the field change hypothesis for cutaneous SCC development. The contribution of betaPV to field cancerisation is unknown but might be related to its property to impair cellular defenses against UV-induced DNA damage (35;36). The clinical relevance, if any, of the difference in betaPV types is unknown. Since no specific types were identified in any of the particular biopsies that were not present in other samples, no obvious high-risk types emerged in this series. Possibly, the number of types or a combination of certain types increases the risk of developing a SCC (25;32). It is also possible that detection of more betaPV types represents higher viral loads accompanied by greater viral gene expression and an increased risk of SCC.

Similar analyses in larger datasets, ideally including measures of viral load, may help to elucidate the role of betaPV in cutaneous carcinogenesis, and to determine whether the betaPV status of eyebrow hairs is a sufficiently good marker of infection of the tumour field to warrant its continued use in epidemiological studies.

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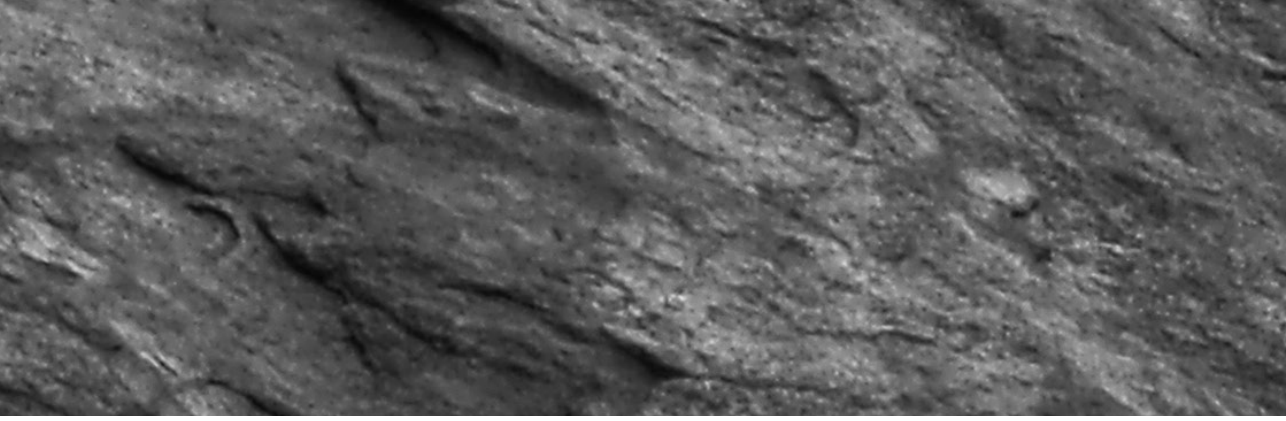

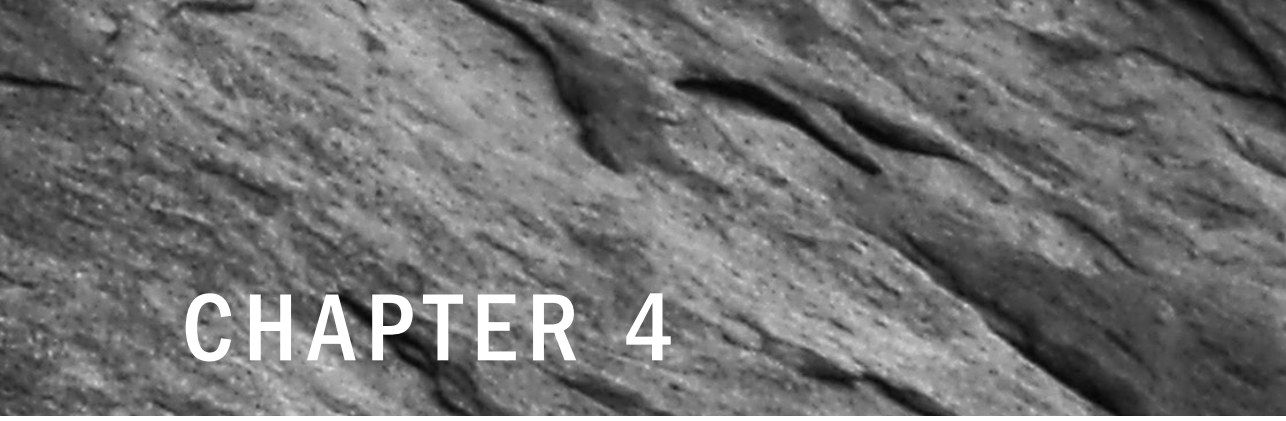

CHAPTER 4

LACK OF ASSOCIATION BETWEEN THE PRESENCE AND PERSISTENCE OF BETAPAPILLOMAVIRUS DNA IN EYEBROW HAIRS AND BETAPAPILLOMAVIRUS L1 ANTIBODIES IN SERUM

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Abstract

Betapapillomavirus (betaPV) DNA and seroresponses are highly prevalent in the general population and both are frequently used as infection markers in epidemiological studies to elucidate an association with cutaneous squamous-cell carcinoma (SCC). Little is known about the natural history of betaPV infection and the aspects of infection that drive antibody responses. To investigate the relation between these markers we assesed if the presence or persistence of betaPV DNA in eyebrow hairs and L1 antibodies of the same betaPV type co-occur more frequently than would be expected by chance in both a cross-sectional assessment and in a longitudinal study.

BetaPV DNA in plucked eyebrow hairs and L1 antibodies in serum were measured in 416 participants of the Australian community-based Nambour Skin Cancer Study in 1996. Similar data were available for a subset of 148 participants in 2003. Observed co-occurence of betaPV DNA and antibodies was compared with expected values based on prevalence. A case-wise concordance index was used to calculate the overall concordance of the betaPV DNA and antibodies of the same type.

No significant associations were found between the presence or persistence of betaPV DNA and antibody responses. Age and sex of the host did not influence the association, nor did SCC-status or a history of sunburns. We conclude that betaPV antibody responses in adults are not primarily driven by betaPV infection as measured in eyebrow hairs. Other factors, such as viral load, possibly play a more pivotal role in induction of detectable seroresponses.

Introduction

Human papillomaviruses of the beta-genus (betaPV) are non-enveloped cutanotropic DNA viruses that may be associated with the development of cutaneous squamous-cell carcinoma (SCC) (1). So far 31 different betaPV types have been fully sequenced (2;3).

Epidemiological studies have shown that betaPV DNA is frequently found in hair bulbs of eyebrows and body hairs (4), normal skin swabs (5) and biopsies from healthy people and transplant recipients without skin cancer (6), as well as in SCC tumor tissue (7-10). The presence of betaPV DNA has been associated with the presence of SCC-precursor lesions (actinic keratoses, AK) and SCC (11-14). Antibodies against the betaPV major capsid antigen L1 can be found in serum of healthy controls as well as patients with AK and SCC and have been associated with both tumor types in epidemiological studies (12;15;15-24).

Little is known about the association between betaPV DNA in hair follicles and serum antibodies. It may be expected that antibodies arise as a result of infection of the hair follicles with betaPV DNA, but the specific aspects of betaPV infection that drive antibody responses are currently unknown. For example, the location, load and persistence of infection, as well as inflammation at the site of infection may all be important in this respect (9;21).

Only two studies so far have investigated betaPV sero-prevalence among people with known betaPV DNA status (12;25). Struijk and co-workers reported a significantly higher prevalence of L1 seropositivity in those who were betaPV DNA positive in eyebrow hairs than in those in whom betaPV DNA was not detected (12;25). Andersson and co-workers reported that seropositivity was twice as common among people who had DNA detected for at least one betaPV type in a healthy skin or SCC biopsy, and that 20% of people with betaPV DNA were positive for L1 antibodies of the same type (25). In both studies, however, there was no statistical assessment of whether the associations found were higher than what would be expected on the basis of chance alone.

In comparison with betaPV, there is substantially more knowledge about alphapapillomaviruses. For example for HPV16, L1 capsid antibodies are known to be a valid measure of lifetime HPV16 exposure and the association between HPV16 DNA in cervical biopies and capsid protein antibodies is high (26). Within a few months after the acquisition of viral DNA a serological response is evoked in 50% of infected women. The majority of

HPV DNA-positive women clear the infection within 12 months (27-29). In women with persistent presence of HPV DNA in samples taken at two different occasions, the percentage of seropositive women is higher than in women with HPV DNA diagnosed on a single occasion (30). Although the pathophysiology of betaPV seems very different to that of the high-risk viruses with mucosal tropism, these data raise the possibility that betaPV persistence might also be linked to a serological response.

To elucidate the role of betaPV infection measured as the presence of viral DNA in the development of betaPV L1 antibodies, we aimed to assess firstly if detection of betapapillomavirus DNA in eyebrow hairs was associated with seropositivity for the same types in a cross-sectional assessment. Secondly, we evaluated to what extent persistent DNA infection determined seropositivity. Due to lack of information about long-term persistence of DNA, we measured betaPV DNA persistence from 1996 to 2003 on the assumption that this is indicative of infection prior to antibody formation. The study was performed in the context of an Australian longitudinal skin cancer study.

Material and methods

Study population and sample collection

Participants were an unselected subset of the study population of the Nambour Skin Cancer Study described in detail previously (31;32). Briefly, in 1986, 2095 of 3000 randomly selected residents of Nambour, a subtropical township in Australia (latitude 26°S), who were aged 20-69 years, participated in a skin cancer prevalence survey. From 1992 to 1996, 1621 of these participants took part in a trial of sunscreen application and beta-carotene supplementation for the prevention of skin cancer. In 1996, 507 unselected participants took part in a sub-study aiming to understand the association between HPV and skin cancer (33). Ten eyebrow hairs were plucked from each participant and blood was drawn where possible. For the present study we used the data from 416 people for whom betaPV DNA and antibodies from 1996 were both available (herein called the ‘cross-sectional group’). To analyse the association between persistent betaPV DNA and seropositivity, we used data from 148 people of whom betaPV DNA and serum antibodies from 1996 and also from 2003 were available (herein called the ‘longitudinal group’). Skin cancer follow-up took place until 31 December 2007. Ethical approval for all aspects of the study was obtained through the Bancroft Centre Human Research Ethics Committee, Queensland Institute of Medical Research.

DNA isolation, PCR and hybridization

DNA from eyebrow hairs was isolated according to a method described previously(34). BetaPV detection and genotyping were performed using a reversed hybridisation assay as described by De Koning and collegues (35). All amplimers generated with the broad spectrum PCR were analysed with a reverse hybridization assay (RHA) that permitted specific detection and identification of 25 established betaPV genotypes of which the following were used: 5, 8, 9, 14, 15, 17,20, 21, 22, 23, 24, 36, 38, 47, 49, 75, 76, 80, 92, 93 and 96. The RHA was performed according to the manufacturer's instructions (skin (beta) HPV prototype research assay; Diassay BV, Rijswijk, The Netherlands).

Multiplex serology

Serum samples were tested for the presence of antibodies to the major capsid antigen L1 of HPV 5, 8, 9, 14, 15, 17, 20, 21, 22, 23, 24, 36, 38, 47, 49, 75, 76, 80, 92, 93 and 96 by multiplex serology. This is an antibody detection method based on a glutathione S-transferase capture ELISA, in combination with fluorescent bead technology (17;25;36). Positive serology cut-offs were standardised at 200 MFI (Mean Fluorescence Intensity).

Statistical analyses

We calculated the prevalence of betaPV DNA and antibodies for any betaPV type overall and for each of the 21 genotypes tested both for DNA and antibodies. To test for significant differences between DNA and antibody prevalence of the same genotype we used McNemar tests. Persistent DNA was defined as having betaPV DNA detected for the same type in 1996 and in 2003, as we described previously (13).

To assess associations between betaPV DNA and antibody detection in the cross-sectional group, we calculated case-wise concordance, defined as the conditional probability that someone is positive for antibodies from a specific betaPV type given that s/he is betaPV DNA positive for that same type. It was estimated as the ratio of the number of concordant people (positive for DNA and antibodies for the same betaPV type) to the total of concordant and discordant (did not have betaPV DNA and antibodies for the same type) people. It can be interpreted similarly to a kappa statistic. The standard error and 95% confidence interval were estimated according to methods documented by Huang and Tai (37). We estimated concordance separately for each betaPV type and then calculated a pooled estimate across all types. To determine whether having persistent betaPV DNA was associated with the

presence of antibodies, we also calculated case-wise concordance between betaPV DNA persistence and the presence of antibodies in both 1996 and 2003.

For each betaPV type we multiplied the proportion of participants positive for antibodies by the proportion positive for DNA to calculate the proportion that would be expected to be concordant for both measures purely by chance. To calculate the number of people that would be expected to be concordant for at least one type, these 21 proportions were summed and the product multiplied by the number of people measured. We compared the number observed with that expected, using a Chi-squared test to determine statistical significance. Because age, sunburns and SCC-status have all been shown to influence betaPV-seroreactivity (17;20;21;24), stratified analyses were performed for sex, age below and above the mean age, lifetime sunburns (0, 1-4, 5+) and SCC (detected 1986-1996). The same approaches were used to determine associations between persistent DNA and antibodies detected in 1996 and 2003 (n=148). Because seroprevalence was very stable (overall, 89% of people remained either seropositive or seronegative) and analyses with 2003 antibodies showed the same results as with the 1996 antibodies, we present in this paper only the results of the 1996 betaPV antibodies. Statistical analyses were performed with SAS 9.1.

Results

Population characteristics

The mean age of the 416 participants in the HPV DNA data and L1 antibody data from 1996 (the cross-sectional group) was 51 years, and 50% were male. In total 60 people (14%) had never had a painful sunburn, 184 people (44%) had experienced 1 to 4 painful sunburns in their life and 172 people (41%) had had 5 or more painful sunburns. Eighteen people (4%) were newly diagnosed with a SCC between 1986 and 1996.

The mean age of the 148 people for whom HPV DNA data was available from both 1996 and 2003 (the longitudinal subgroup) was 50 years, and 47% were male. Painful sunburns had the same distribution as in the whole cohort and 4 (3%) people in this group developed a SCC between 1986 and 1996.

Association between betaPV DNA presence and L1 antibodies (cross-sectional group)

The prevalence of betaPV DNA in the cross-sectional group was 74%, with 53% of people being positive for more than one type (**Table 1**). There were 288 people (69%) with betaPV antibodies and 51% had antibodies against more than one type. The prevalence of individual types ranged from 0 to 23% for DNA, and 0 to 33% for antibodies.

Table 1: Prevalence of betaPV DNA in eyebrow hairs and betaPV antibodies, overall and per betaPV type

	cross-sectional group (n=416)		longitudinal group (n=148)		
	1996 DNA	**1996 antibodies**	**1996 DNA**	**1996 antibodies**	**1996-2003 persistent DNA**
	N (%)	**N (%)**	**N (%)**	**N (%)**	**N (%)**
Overall	308 (74)	288 (69)	107 (72)	95 (64)	73 (49)
HPV5	46 (11)	36 (9)	17 (11)	14 (9)	11 (7)
HPV8	44 (11)	137 (33)*	17 (11)	47 (32)*	5 (3)
HPV9	52 (13)	68 (16)	17 (11)	29 (20)*	11 (7)
HPV14	20 (5)	4 (1)†	3 (2)	2 (1)	1 (1)
HPV15	73 (18)	102 (25)*	22 (15)	38 (26)*	7 (5)
HPV17	70 (17)	118 (28)*	26 (18)	41 (28)*	10 (7)
HPV20	42 (10)	47 (11)	16 (11)	16 (11)	7 (5)
HPV21	10 (2)	86 (21)*	5 (3)	35 (24)*	2 (1)
HPV22	35 (8)	51 (12)	10 (7)	17 (11)	4 (3)
HPV23	94 (23)	62 (15)†	27 (18)	21 (14)	10 (7)
HPV24	69 (17)	68 (16)	21 (14)	25 (17)	12 (8)
HPV36	73 (18)	53 (13)†	22 (15)	20 (14)	11 (7)
HPV38	83 (20)	139 (33)*	27 (18)	49 (33)*	15 (10)
HPV47	0 (0)	56 (13)*	0 (0)	21 (14)*	0 (0)
HPV49	53 (13)	104 (25)*	19 (13)	33 (22)*	11 (7)
HPV75	11 (3)	64 (15)*	3 (2)	23 (16)*	0 (0)
HPV76	35 (8)	63 (15)*	8 (5)	24 (16)*	3 (2)
HPV80	42 (10)	78 (19)*	13 (9)	25 (17)*	9 (6)
HPV92	27 (6)	56 (13)*	9 (6)	21 (14)*	7 (5)
HPV93	70 (17)	13 (3)†	27 (18)	7 (5)†	14 (9)
HPV96	33 (8)	79 (19)*	12 (8)	27 (18)*	10 (7)
No of types					
0	108 (26)	128 (31)	41 (28)	53 (36)	75 (51)
1	86 (21)	76 (18)	35 (24)	22 (15)	31 (21)
2	66 (16)	44 (11)	20 (14)	17 (11)	20 (14)
3+	156 (37)	168 (40)	52 (34)	56 (38)	22 (14)

* Significant difference in prevalence where antibody>DNA
†Significant difference in prevalence where DNA>antibody

We found no association between being betaPV DNA and antibody positive. Among the 308 people with detectable betaPV DNA in 1996, 217 people (70%) had betaPV antibodies, while among the 108 people without betaPV DNA, 66% had betaPV antibodies detected (p=0.30). For 12 of the 21 betaPV types the antibody prevalence was significantly higher than DNA prevalence (p <0.05), while for 4 of the 21 types the DNA prevalence was significantly higher than for antibodies (Table 1). Although no person in the cohort had HPV47 DNA detected, 56 people (18%) had antibodies against HPV47.

The pooled case-wise concordance index between betaPV DNA and antibodies for the cross-sectional group was 0.18 (95% CI 0.16-0.20). Examining individual betaPV type concordance, we found all case-wise concordance indices to be below 0.28 (**Table 2**). Of the 217 people with both betaPV DNA and L1 antibodies, 114 people (53%) were positive for at least one betaPV DNA and antibody of the same type, whereas 140 (65%) was expected on the basis of chance (p=0.15). Of the 308 people with at least one betaPV DNA type, 114 people (37%) were positive for at least one of the corresponding betaPV antibodies, compared with 138 expected (p=0.045) (**Table 3**).

Table 2: Type-specific concordance between betaPV DNA and antibodies measured in 1996 in the cross-sectional group (N=416).

	N (%) DNA positive	N (%) of DNA+ who are AB +	N (%) DNA negative	N (%) DNA - who are AB +	case-wise concordance index
HPV5	46 (11)	10 (22)	370 (89)	26 (7)	0.24
HPV8	44 (11)	18 (41)	372 (89)	119 (32)	0.20
HPV9	52 (13)	7 (13)	364 (88)	61 (17)	0.12
HPV14	20 (5)	0 (0)	396 (95)	4 (1)	0.00
HPV15	73 (18)	20 (27)	343 (82)	82 (24)	0.23
HPV17	70 (17)	25 (36)	346 (83)	93 (27)	0.27
HPV20	42 (10)	11 (26)	374 (90)	36 (10)	0.25
HPV21	10 (2)	2 (20)	406 (98)	84 (21)	0.04
HPV22	35 (8)	7 (20)	381 (92)	44 (12)	0.16
HPV23	94 (23)	14 (15)	322 (77)	48 (15)	0.18
HPV24	69 (17)	10 (14)	347 (83)	58 (17)	0.15
HPV36	73 (18)	14 (19)	343 (82)	39 (11)	0.22
HPV38	83 (20)	31 (37)	333 (80)	108 (32)	0.28
HPV47	0 (0)	0 (0)	416 (100)	56 (13)	0.00
HPV49	53 (13)	17 (32)	363 (87)	87 (24)	0.22
HPV75	11 (3)	3 (27)	405 (97)	61 (15)	0.20
HPV76	35 (8)	6 (17)	381 (92)	57 (15)	0.12
HPV80	42 (10)	12 (28)	373 (90)	66 (18)	0.20
HPV92	27 (6)	7 (26)	389 (94)	49 (13)	0.17
HPV93	70 (17)	3 (4)	346 (83)	10 (3)	0.07
HPV96	33 (8)	5 (15)	383 (92)	74 (19)	0.09

When we stratified by age (below 50 years and 50 years and over) no significant differences were found in the proportion of people who were betaPV DNA and antibody concordant for at least one type (p=0.62). In those aged below 50 years, 42 people were concordant compared with 52 expected (p=0.15), and in those older than 50 years, 72 were observed and 86 expected (p=0.14) (Table 3).

Table 3: Number of betaPV types observed (OBS) and expected (EXP) to be in common for DNA and antibodies for all people with DNA for at least one betaPV type

	Cross-sectional group (n=416)				Longitudinal group (n=148)			
	betaPV prevalence and antibodies				betaPV persistence and antibodies			
	n	OBS N (%)	EXP N (%)	p-value	n	OBS N (%)	EXP N (%)	p-value
All DNA+ participants	308	114 (37)	138 (45)	0.05	73	28 (38)	25 (34)	0.61
Age < 50 years	119	42 (35)	52 (45)	0.15	33	13 (39)	12 (33)	0.61
Age ≥ 50 years	189	72 (38)	86 (46)	0.14	40	15 (38)	12 (30)	0.48
Males	157	67 (43)	79 (51)	0.14	36	14 (39)	13 (36)	0.81
Females	151	48 (32)	60 (40)	0.15	37	14 (38)	12 (32)	0.63
no sunburns	50	18 (36)	22 (44)	0.41	9	4 (44)	2 (22)	0.32
1-4 sunburns	139	56 (40)	65 (47)	0.28	37	15 (41)	15 (41)	1.00
5+ sunburns	116	41 (35)	51 (44)	0.18	27	9 (33)	10 (37)	0.58
no SCC	293	106 (36)	127 (43)	0.09	70	26 (37)	23 (33)	0.60
SCC	15	9 (60)	10 (67)	0.71	3	2 (66)	1 (33)	0.40

Stratification by SCC-status did not show any significant differences in the proportion of people that were DNA-antibody concordant (p=0.70). In the group with a SCC detected, 9 people were concordant compared with 10 expected (p=0.71), and in the group without SCC 106 were observed and 127 expected (p=0.09). There was also no difference according to sex or number of lifetime sunburns (Table 3).

Association between betaPV DNA persistence and L1 antibodies (Longitudinal group)

The prevalence of betaPV DNA at baseline was 72%, with 48% of people being positive for more than one type (Table 1). Ninety-five persons (64%) had betaPV antibodies at baseline and 49% had antibodies against more than one type. The prevalence of antibodies for individual betaPV types ranged from 0 to 18% for DNA and 0 to 33% for antibodies. 73

people (49%) had persistent DNA detected for at least one betaPV type in both 1996 and 2003 (Table 1).

The pooled concordance index describing the association between persistent DNA and antibodies was 0.13 (95% CI -0.005-0.27) and all individual case-wise concordance indices were below 0.32 **(Table 4)**. Twenty-eight participants (38%) who had persistent betaPV DNA also had L1 antibodies detected in 1996 for at least one betaPV type, which was not significantly different from the number expected to occur by chance (N=25, p=0.61) (Table 3). There were no significant differences in relation to age, sex, history of sunburns or SCC-status among study participants (Table 3).

Table 4: Type-specific concordance between DNA persistence and antibodies in the longitudinal group (N=148).

	N (%) persistence positive	N (%) of persistent+ who are 1996 AB +	N (%) persistence negative	N (%) of persistent- who are 1996 AB +	case-wise concordance index
HPV5	11 (7)	4 (36)	137 (93)	10 (7)	0.32
HPV8	5 (3)	1 (20)	143 (97)	46 (32)	0.04
HPV9	11 (7)	3 (27)	137 (93)	26 (19)	0.15
HPV14	1 (1)	0 (0)	147 (99)	2 (1)	0.00
HPV15	7 (5)	1 (15)	141 (95)	37 (26)	0.04
HPV17	10 (7)	4 (40)	138 (93)	37 (26)	0.16
HPV20	7 (5)	2 (29)	141 (95)	14 (10)	0.17
HPV21	2 (1)	1 (50)	146 (99)	34 (23)	0.05
HPV22	4 (3)	1 (25)	144 (97)	16 (11)	0.10
HPV23	10 (7)	3 (30)	138 (93)	18 (13)	0.19
HPV24	12 (8)	4 (33)	136 (92)	21 (15)	0.22
HPV36	11 (7)	2 (18)	137 (93)	18 (13)	0.13
HPV38	15 (10)	7 (47)	133 (90)	42 (32)	0.22
HPV47	0 (0)	0(0)	148 (100)	21 (14)	0.00
HPV49	11 (7)	4 (36)	137 (93)	29 (21)	0.18
HPV75	0 (0)	0 (0)	148 (100)	23 (16)	0.00
HPV76	3 (2)	0 (0)	145 (98)	24 (17)	0.00
HPV80	9 (6)	4 (44)	139 (94)	21 (15)	0.24
HPV92	7 (5)	2 (29)	141 (95)	19 (13)	0.14
HPV93	14 (9)	1 (7)	134 (91)	6 (4)	0.10
HPV96	10 (7)	2 (20)	138 (93)	25 (18)	0.11

Discussion

This study assessed the associations between both prevalence and persistence of betaPV DNA, and L1 antibodies, and found that neither DNA measure was predictive of antibody detection.

The overall betaPV DNA prevalence of 74% we measured at baseline was lower than has been reported previously in an Australian population (91%) (6). BetaPV detection has been shown to increase with age (6), so this difference is probably due to the younger age of our participants. The overall seroprevalence of 69% is higher than previously reported for Australia (17). As betaPV seropositivity increases with age (17;38), the relatively high seroprevalence in our study is not due to age and remains unexplained.

We found antibodies to be very stable over 8 years, with only 16 people (11%) changing their overall betaPV serology status between 1996 and 2003, and they had MFI values very close to the cut-off. We analysed our data using antibody status in 2003 and, due to the stability of these antibodies, found no difference in the results.

HPV47 DNA was not found in eyebrow hairs of any of the participants but 13% were seropositive for HPV47. Although sero cross reactivity, possibly with unknown betaPV types, cannot be excluded, it could be possible that the reference HPV47 type represents a regional variant (39). So far HPV47 DNA has only been found in an ongoing study in organ transplant patients using this method (M.N.C de Koning, unpublished observation), and other betaPV typing methods targeting different genomic regions of HPV47 have also detected this type (40;41). However, removal of HPV47 from the analyses did not change the results of this study, because the proportions of DNA- antibody concordance as well as the case-wise concordance remained unaffected.

Neither the presence nor persistence of betaPV in eyebrow hairs was associated with the detection of L1 antibodies. All type-specific and pooled concordance indices were low and there were no consistent differences in the number of people expected to be concordant and the number observed. Stratification by age, sex, history of sunburns or SCC did not alter these findings.

Two other studies have addressed the relation between betaPV DNA and antibodies. One study found a significantly higher prevalence of L1 positivity in those who were betaPV

DNA positive than in those in whom betaPV DNA was not detected (12). Of the 37 participants in that study with both betaPV DNA and L1 antibodies, 32% were positive for the same type (12). However we would expect some people to be positive by chance alone, and without knowledge of this expected number, results are difficult to interpret. We found that 53% of people who were DNA and antibody positive had at least one virus type where both measures were positive, but this was not higher than expected based on the prevalence of DNA and antibodies. A second study found that seropositivity was twice as common among people who had DNA detected for at least one betaPV type in a healthy skin or an SCC on biopsy, and that 20% of people with betaPV DNA were positive for L1 antibodies of the same type (25), but again there was no reference to an expected value. Although we found that 37% of people with betaPV DNA in eyebrow hairs were antibody positive for at least one type detected, this was not higher than expected by chance.

There are several possible explanations for our observed lack of association between betaPV DNA and L1 antibodies. It may be that the presence and/or persistence of betaPV DNA we measured was not indicative of infection many years many years prior to 1996 and that the antibody response was provoked earlier in life when the DNA was not present. However, a previous study showed that antibody responses against betaPV types are rare in childhood and have their peak prevalence between 40 and 60 years for women and 50 and 70 years for men suggesting that antibodies arise at a time more proximal to our measure of infection (38). Secondly it is possible that antibodies are detected for multiple types due to cross-reactivity, without there being infection of the skin with those types, and true type-specific seroresponses may be lower than measured. The high multiplicity of L1 antibodies and the significantly higher prevalence of antibody positivity than betaPV DNA positivity for 12 types may support this hypothesis. Alternatively, betaPV antibody responses may be associated with the betaPV load rather than simply with presence or absence of viral DNA. Loads of betaPV shown as the number of HPV copies per infected cell are known to be much lower when compared with the alpapapillomavirus types (9), but increases in load might be important in evoking an antibody response.

BetaPV DNA detection in eyebrow hairs has been used as a convenient marker of infection in epidemiological studies, but it is unclear to what extent this is indicative of pathologically relevant skin infection. Although we and others have shown some association between betaPV DNA found in eyebrow hairs and in biopsies of cutaneous SCC and the perilesional skin (7;8;10), the prevalence in eyebrow hairs is substantially higher than in other tissues.

Thus it seems likely that a high proportion of infections in eyebrow hairs do not evoke an antibody response.

As the induction of immune response often requires additional signals, inflammation accompanying betaPV infections might be an important factor in evoking seroresponses. Favre and colleagues showed the induction of HPV5 seroresponses upon second degree burns of the skin, as well as in patients with autoimmune bullous diseases and psoriasis, all diseases with prominent inflammation (21). BetaPV infections as detected in eyebrow hairs are not known to be accompanied by inflammation. However, if eyebrow hairs are indicative of infection at other sites, we might expect that our association between betaPV and antibody detection would be altered by a history of sunburns or SCC. We did not find an effect however, of previous sunburns or SCC on the association between betaPV DNA and L1 antibodies. Possibly, inflammation in the context of most SCC and AK is small compared with those conditions examined by Favre et al (21).

In conclusion, we did not find a meaningful association between betaPV DNA presence or persistence in eyebrow hairs and betaPV-L1 antibodies in serum. This lack of association highlights the need to better understand the natural history of betaPV infection, and the ways in which infection induces an immune response, before associations between measures of betaPV infection and disease can be elucidated.

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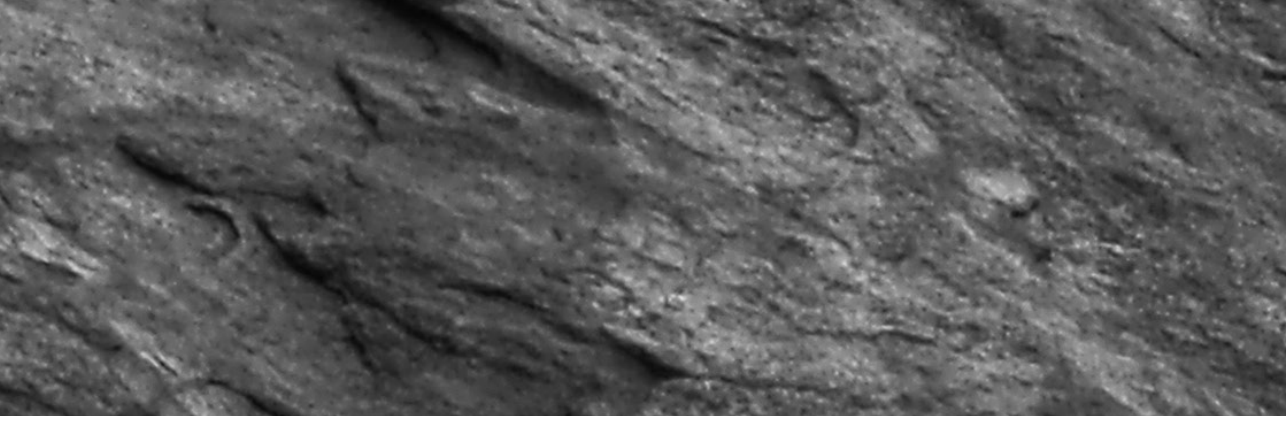

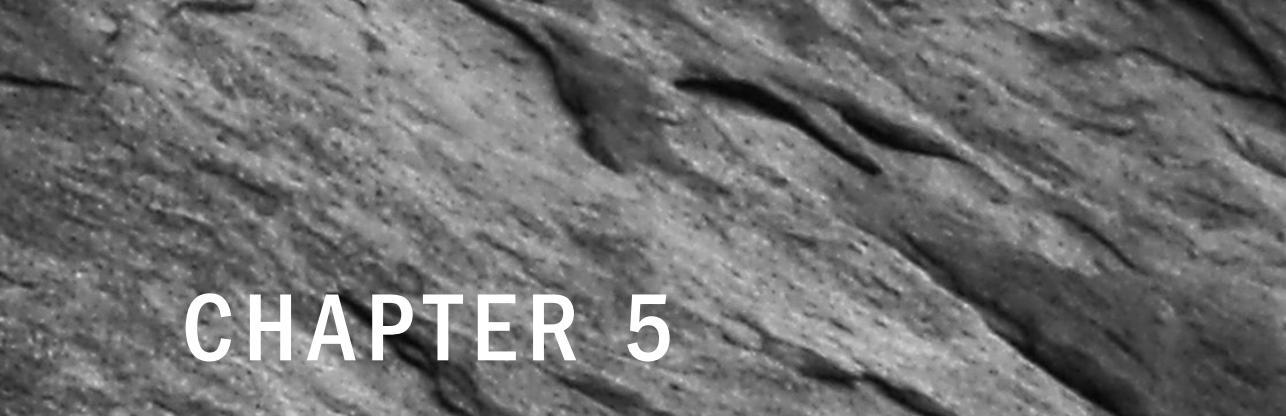

CHAPTER 5

PERSISTENCE OF BETAPAPILLOMAVIRUS INFECTIONS AS A RISK FACTOR FOR ACTINIC KERATOSES, PRECURSOR TO CUTANEOUS SQUAMOUS-CELL CARCINOMA

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Abstract

Human papillomaviruses from the beta genus (betaPV) are a possible cause of cutaneous squamous-cell carcinoma (SCC). We assessed the extent to which betaPV infections persisted long-term in a subtropical Australian community and whether betaPV persistence is positively associated with actinic keratoses (AK), precursor for SCC.

Eyebrow hairs were collected from 171 participants of the community-based Nambour Skin Cancer Study in 1996 and 2003. Hair samples were tested for the presence of DNA from 25 different betaPV types and assessed in relation to AK presence in 2007.

In 1996 a total of 413 betaPV infections were found in 73% of participants, rising to 490 infections among 85% in 2003. Of the total betaPV infections detected, 211 (30%) were found to persist. Age was significantly associated with betaPV persistence: those over 60 years had 1.5 fold (95% CI 1.1-1.9) increased risk of type-specific viral-persistence than those under 40. After accounting for AKs at baseline, persistence of betaPV DNA resulted in a 1.4 fold (95% CI 1.0-1.9) increase in risk of having AKs on the face in 2007.

In conclusion, persistent betaPV infections in this population were associated with an increased occurrence of AK. Additional studies are needed to determine the possible association of betaPV persistence with SCC.

Introduction

Human papillomaviruses (HPV) have oncogenic capacities and persistent infection with a high-risk mucosal HPV type from the alpha genus causes the development of cervical cancer (1;2). HPV from the beta genus (betaPV) have cutaneous rather than mucosal tropism. They were first found to cause flat, wart-like lesions in epidermodysplasia verruciformis (EV) patients and so were formerly known as EV-types. Currently 25 betaPV-types have been fully sequenced (HPV5, 8, 9, 12, 14, 15, 17, 19-25, 36-38, 47, 49, 75, 76, 80, 92, 93 and 96) (3), but many more new types can probably be added to this list based on partial sequences (4).

BetaPVs are detected frequently in actinic keratoses (AKs) and cutaneous squamous-cell carcinomas (SCCs), but have also been found in biopsies from normal skin, eyebrow hairs and skin swabs from people with and without skin cancer (5-7). A number of studies have shown statistically significant associations between markers of betaPV infection (viral DNA in eyebrow hairs or skin biopsies, and antibodies in serum) and SCC (8-12), but no betaPV types have been found to cause SCC in experimental systems.

There is limited knowledge about persistence of cutaneous betaPV infection over extended periods of time. Studies of betaPV persistence in eyebrow hairs so far have involved relatively few participants followed over limited time periods (two to six years) (13;14), and no studies have examined betaPV persistence in relation to future occurrence of AK or SCC. Furthermore, most epidemiological studies have been cross-sectional (11;15), assessing both betaPV detection in an individual's eyebrow hairs and their skin cancer status at the same point in time.

One of the few cohorts available for estimation of the risk of skin cancer in regard to HPV infection has been the Nambour Skin Cancer Study cohort (16;17). Boxman and colleagues performed a cross-sectional study with 518 individuals sampled in 1996 and found a strong association between betaPV infection and AKs in men, but not women (17). McBride and colleagues studied the prevalence of AKs in 291 participants of the Nambour Skin Cancer Study 7 years after single measurement of betaPV DNA in eyebrow hairs. They found that betaPV-positivity was associated cross-sectionally with the presence of more than 10 AKs, especially in those over 60 years, those who had fair skin or those who had experienced high sun exposure (16).

In the present more detailed and longer-term study of the Nambour Skin Cancer Study cohort, we assessed the persistence of 25 different betaPV types in eyebrow hairs over an 8-year period, and then examined the longitudinal association between betaPV persistence and the disease outcome, actinic keratoses, over a total period of 11 years.

Materials and Methods

Study population and sample collection

Participants comprised an unselected subset of the study population of the Nambour Skin Cancer Study (**Figure**), described in detail previously (18-20). Briefly, in 1986, 2095 of 3000 randomly selected residents aged 20-69 years, from Nambour, a subtropical township in Australia (latitude 26°S) participated in a skin cancer prevalence survey. From 1992 to 1996, 1621 of these participants then took part in a trial of sunscreen application and beta-carotene supplementation for the prevention of skin cancer, and follow-up continued until 2007.

In 1996, 507 participants took part in a sub-study aiming to understand the association between HPV and skin cancer (17). Ten eyebrow hairs were plucked from each participant and processed as described below. In 2003, 291 of these 507 who were still actively participating in the follow-up of the Nambour Skin Study took part in a second phase of the HPV study when eyebrow hairs were plucked again (16). At baseline and in 2003 standardized questionnaires were used to elicit information about skin type, and sun exposure.

In 1992, 1994 and 1996 specialist dermatologists conducted full-body skin examinations of study participants and documented the degree of telangiectasia and nuchal elastosis and the number of AKs on each anatomical site. A proportion of participants were fully examined for actinic keratoses again in 2007 (Figure).

Ethical approval for all aspects of the study was obtained through Bancroft Centre Ethics Committee, Queensland Institute of Medical Research.

DNA isolation, PCR and hybridization

DNA from eyebrow hairs collected in 1996 was isolated according to a method described previously (21). The 2003 eyebrow hairs were treated using the QIAamp DNA mini kit (Qiagen). Briefly, hairs were pre-treated overnight with proteinase K solution according

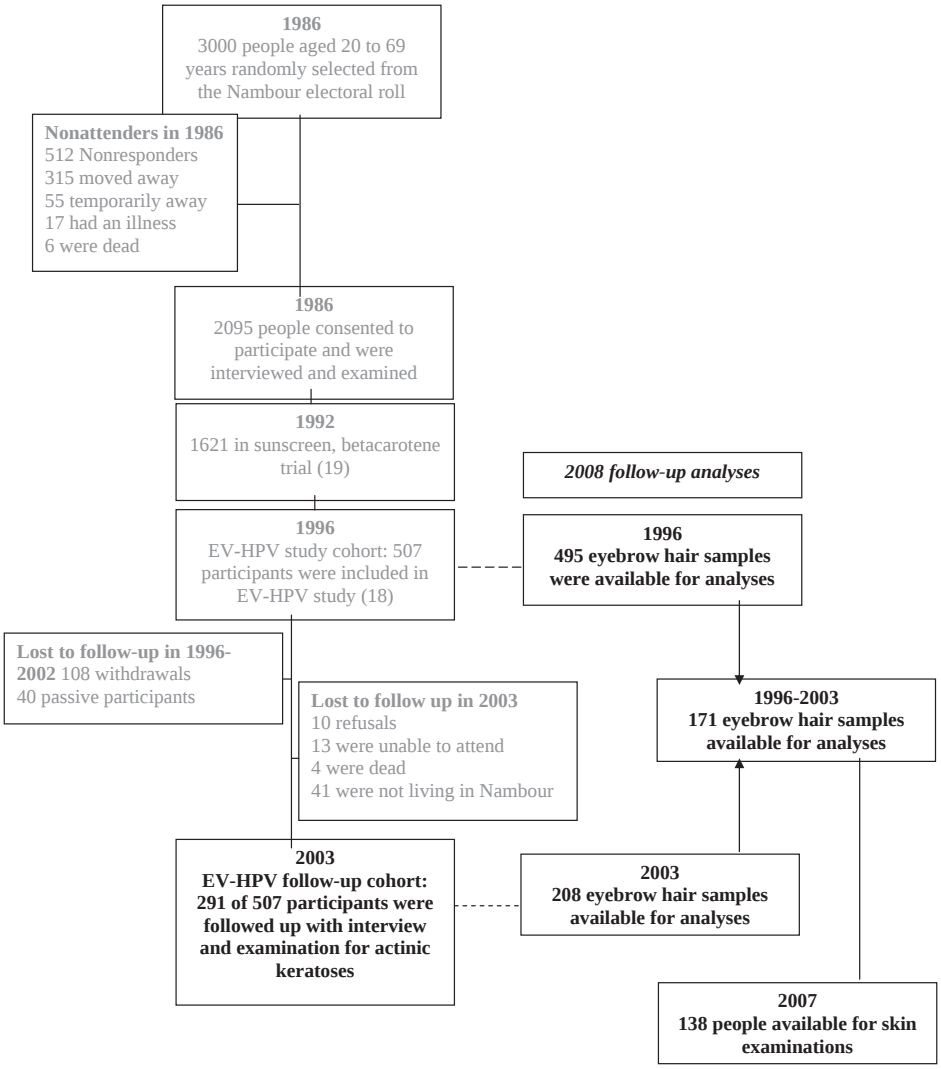

Figure. Nambour Skin Cancer Study flow diagram, December 1986 to August 2007. (adapted from **(16).**

to the manufacturer's instructions. After lysis with 200 µl AL buffer, half of the volume was stored at -70ºC, whilst the other half was processed according to the manufacturer's instructions.

BetaPV detection and genotyping of 1996 and 2003 samples was performed using a reversed hybridization assay as described previously (22). Briefly, PM-PCR was performed

in a final reaction volume of 50 µl, containing 10 µl of the isolated DNA, 2.5 mM $MgCl_2$, 1x GeneAmp PCR buffer II, 0.2 deoxynucleotide triphosphates, 1.5 U AmpliTaq Gold DNA polymerase and 10 µl of the PM primer mix. The PCR was performed by a 9 min pre-heating step at 94ºC, followed by 35 cycles of amplification comprising 30 s at 94ºC, 45 s at 52ºC, and 45 s at 72 ºC. The PCR was ended by a final elongation step at 5 min at 72 ºC. As the positive PCR control, a betaPV plasmid clone was included at an amount approximately 100 times the limit of detection of the assay. All positive controls were detected. Each tenth sample was a negative isolation control that was processed parallel to the other samples of which 12 percent was positive with no specific betaPV type standing out.

All amplimers were subsequently analyzed with a reverse hybridization assay (RHA) that permitted specific detection and identification of the 25 established betaPV genotypes (i.e., 5, 8, 9, 12, 14, 15, 17, 19-25, 36-38, 47, 49, 75, 76, 80, 92, 93 and 96). The RHA was performed according to the manufacturer's instructions (RHA Kit Skin (beta) HPV, Diassay BV).

Statistical analyses

Chi square tests were used to analyze univariate associations. To examine factors associated with persistence, and the association between betaPV infection/persistence and AKs, binomial models were used to compute relative risks (RRs) and 95% confidence intervals (CI). A range of possible confounding factors were explored but none of the following: skin type, occupational sun exposure, sunburns, smoking, use of sunglasses and randomized sunscreen /beta-carotene interventions, altered the effect estimates and therefore were not included in the models. The final model describing associations with betaPV detection over time was adjusted for age; the final models for assessing association with AKs were adjusted for age, sex and the presence of AK at baseline. Statistical analyses were performed in SAS 9.1.

Results

Baseline

BetaPV DNA detection and typing information for the participants from 2003 was available for 171 of the 507 participants (34%) whose data were first analyzed in 1996, and these therefore formed the basis of this study. The characteristics of the 171 participants, whose mean age in 1996 was 50 years, were not significantly different to the remainder seen

at baseline (data not shown). Half of the participants reported their skin type as fair and most had mainly indoors occupations. On dermatologic examination in 1996, moderate to extensive photo ageing of the skin of the neck (nuchal elastosis) was prevalent in at least 50% of participants.40% of participants had AK present at baseline, ranging from 23% in the group under 40 to 61% in the older than 60 years group (**Table 1**). Of the 171, 138 (81%) were still active in the study in 2007 and underwent the final skin examination.

Table 1: Baseline characteristics of (N=171) participants of the Nambour Skin Cancer study followed-up in 2003.

	<40 years in 1996	40-60 years in 1996	>60 years in 1996
	N (%)	N (%)	N (%)
Sex			
male	27 (68)	44 (45)	12 (36)
female	13 (32)	54 (55)	21 (64)
skin color (self-reported)			
medium/olive	25 (63)	51 (52)	12 (36)
fair	15 (38)	47 (48)	21 (64)
occupational sun exposure			
mainly indoors	18 (45)	49 (50)	14 (42)
both indoor and outdoor	10 (25)	35 (36)	12 (36)
mainly outdoors	12 (30)	14 (14)	7 (21)
number of lifetime sunburns			
0	3 (8)	11 (11)	6 (18)
1-4	7 (18)	50 (51)	17 (52)
>5	30 (75)	37 (38)	10 (30)
nuchal elastosis			
limited	17 (47)	22 (23)	0 (0)
moderate	18 (50)	59 (62)	20 (54)
extensive	1 (3)	14 (15)	17 (46)
telangectasia face			
low	12 (30)	15 (15)	6 (18)
moderate	18 (45)	55 (56)	20 (61)
high	10 (25)	28 (29)	7 (21)

BetaPV detection and typing

Of the 25 who were betaPV negative in 2003, 11 (6% of all participants) were also betaPV negative in 1996. Of the 171 subjects whose betaPV was measured, 11 (6%) were betaPV negative in 1996 and 2003. In 1996, 47 of 171 participants (27%) had no betaPV DNA

detected and were therefore deemed to be betaPV negative. The 124 participants who were betaPV positive (73%) had between one and 12 different types detected (median 2), with 100 people (81% of those positive) having multiple betaPV types (**Table 2**).

In 2003, betaPV was detected in 146 of 171 participants (85%) and the median number of detections was 2 (range 1-14). Of those positive, 109 (75%) had more than one virus type detected. The total number of betaPV type-specific detections in all 171 participants in

Table 2: Detection of betaPV DNA per type in eyebrow hairs of 171 participants of the Nambour Skin Cancer Study in 1996 and 2003

	1996, N (%)	2003, N (%)	1996 and 2003, N (%)	proportion persistent
Any betaPV type	124 (73)	146 (85)	83 (49)	
Total no. of infections	413	490	211* (30)	
HPV5	20 (12)	26 (15)	14 (8)	0,70
HPV8	23 (13)	23 (13)	10 (6)	0,43
HPV9	19 (11)	27 (16)	13 (8)	0,68
HPV12	13 (8)	15 (9)	7 (4)	0,54
HPV14	3 (2)	5 (3)	1 (1)	0,33
HPV15	26 (15)	25 (15)	8 (5)	0,31
HPV17	30 (18)	41 (24)	11 (6)	0,37
HPV19	10 (6)	17 (10)	5 (3)	0,50
HPV20	17 (10)	18 (11)	7 (4)	0,41
HPV21	5 (3)	5 (3)	2 (1)	0,40
HPV22	10 (6)	5 (3)	4 (2)	0,40
HPV23	32 (19)	33 (19)	12 (7)	0,38
HPV24	26 (15)	32 (19)	15 (9)	0,58
HPV25	6 (4)	4 (2)	3 (2)	0,50
HPV36	26 (15)	29 (17)	14 (8)	0,54
HPV37	9 (5)	12 (7)	5 (3)	0,56
HPV38	31 (18)	44 (26)	19 (11)	0,61
HPV47	0 (0)	0 (0)	0 (0)	0,00
HPV49	23 (13)	22 (13)	12 (7)	0,52
HPV75	3 (2)	4 (2)	0 (0)	0,00
HPV76	11 (6)	11 (6)	4 (2)	0,36
HPV80	17 (10)	31 (18)	12 (7)	0,71
HPV92	10 (6)	14 (8)	8 (5)	0,80
HPV93	30 (18)	28 (16)	15 (9)	0,50
HPV96	13 (8)	19 (11)	10 (6)	0,77

*The total number of betaPV type-specific infections detected (692) was calculated by adding up the total numbers of betaPV infections in 1996 (413) and 2003 (490) minus the total number of infections that were detected at both time-points (211).

1996 was 413. Of these, 211 were also detected in 2003 (Table 2) and 202 were no longer detectable. In 2003, 279 viruses were detected which had not been found in 1996.

The most prevalent types in 1996 were HPV23 (19%), 17 (18%), 38 (18%) and 93 (18%). HPV47 was not found in any samples from 1996 or 2003. The types detected most frequently in 2003 were HPV38 (26%), 17 (24%), 23 (19%) and 24 (19%) (Table 2), and for the persistent types HPV38 (11%), HPV24 (9%) and HPV93 (9%) were among the most prevalent types. HPV17, with prevalences of 18% and 24% in 1996 and 2003 respectively, was detected on both occasions in only 6% of participants. HPV-types 92, 96, 80, 5 and 9 had the highest probabilities of persistence (Table 2).

Type-specific detection of betaPV DNA in 1996 and 2003, was seen in 83 of 171 (49%) of participants. Of these, 32 (39%) had one type persisting and 51 (61%) more than one (range 2-11).

The presence of betaPV DNA in 1996 and type-specific persistence both increased with age, with those over 60 having a 1.5 fold (95% CI 1.1-1.9) increased risk to have betaPV DNA detected in 1996 and a 1.6 fold (95% 1.0-2.8) increased risk to have at least one persistent betaPV type as those under 40. Sex, skin type, occupational sun exposure, number of lifetime sunburns, use of sunglasses, elastosis of the neck and telangiectasia of the face were not associated with the presence of betaPV DNA in 1996, nor with persistence in 2003 (**Table 3**). No interaction was observed between betaPV persistence and traditional risk factors for AK, although with the modest sample size the power to detect interactions was inadequate.

Actinic keratoses

In 2007, 76 of 138 participants (55%) who underwent a skin examination had at least one actinic keratosis on the head and neck, and 94 of the 138 participants (68%) had at least one AK on the whole body. Twenty-seven of 138 participants (20%) had more than 10 AKs on the whole body. The presence of AKs in 2007 was associated with being aged over 60 years (RR 1.7 95% CI 1.2-2.4), male sex (RR 1.4, 95% CI 1.1-1.8), with the presence of AK at baseline (RR 1.9, 95% CI 1.5-2.5), having moderate (RR 1.8, 95% CI 1.1-2.9) or extensive (RR 2.4, 95% CI 1.5-3.8) solar elastosis of the neck, and high degree of telangiectasia of the face (RR 1.5, 95% CI 1.0-2.3).

Table 3: BetaPV detection in 1996, 2003 and at both time points in relation to participants characteristics (RR adjusted for age).

	1996 (N=171)		2003 (N=171)		1996 and 2003 (N=171)	
	betaPV-positive, N (%)	RR (95% CI)	betaPV-positive,N (%)	RR (95% CI)	betaPV-positive, N (%)	RR (95% CI)
Age, y						
<40	25 (63)	1.0	33 (83)	1.0	14 (35)	1.0
40-60	69 (70)	1.1 (0.9-1.5)	83 (85)	1.0 (0.9-1.2)	50 (51)	1.5 (0.9-2.3)
>60	30 (91)	1.5 (1.1-1.9)	30 (91)	1.1 (0.9-1.3)	19 (58)	1.6 (1.0-2.8)
Sex						
female	65 (74)	1.0	76 (86)	1.0	43 (49)	1.0
male	59 (71)	1.0 (0.8-1.2)	70 (84)	1.0 (0.9-1.1)	40 (48)	0.9 (0.7-1.3)
Skin colour						
fair	65 (74)	1.0	72 (82)	1.0	46 (52)	1.0
medium/olive	59 (71)	1.1 (0.9-1.3)	74 (89)	0.9 (0.8-1.1)	37 (45)	1.3 (0.9-1.7)
Occupational sun exposure						
mainly indoors	27 (82)	1.0	29 (94)	1.0	18 (55)	1.0
both indoor and outdoor	40 (70)	0.9 (0.8-1.1)	50 (88)	1.0 (0.8-1.1)	25 (44)	0.8 (0.5-1.1)
mainly outdoors	57 (70)	0.9 (0.8-1.1)	67 (83)	0.9 (0.8-1.1)	40 (49)	0.9 (0.6-1.3)
Sunglasses						
never	20 (80)	1.0	21 (84)	1.0	11 (44)	1.0
2-4 hours/day	40 (74)	1.0 (0.8-1.2)	49 (91)	1.1 (0.9-1.3)	30 (56)	1.4 (0.8-2.2)
5+ hours/day	63 (69)	1.0 (0.8-1.2)	75 (82)	1.0 (0.8-1.2)	42 (51)	1.2 (0.7-1.9)
Number of lifetime sunburns						
0	16 (80)	1.0	17 (85)	1.0	10 (50)	1.0
1-4	58 (78)	1.0 (0.8-1.3)	64 (86)	1.0 (0.8-1.3)	41 (55)	1.0 (0.6-1.7)
>5	50 (65)	0.9 (0.7-1.2)	65 (84)	1.0 (0.8-1.3)	32 (42)	0.8 (0.5-1.4)
Nuchal elastosis						
limited	24 (67)	1.0	28 (78)	1.0	12 (33)	1.0
moderate	67 (71)	0.9 (0.7-1.1)	87 (92)	1.1 (0.9-1.3)	50 (53)	1.4 (0.8-2.3)
extensive	30 (81)	1.0 (0.8-1.3)	29 (76)	0.9 (0.7-1.2)	20 (54)	1.3 (0.7-2.4)
Telangectasia face						
low	24 (73)	1.0	27 (81)	1.0	16 (48)	1.0
medium	66 (71)	0.9 (0.8-1.2)	80 (86)	1.0 (0.8-1.2)	45 (54)	0.9 (0.6-1.4)
high	34 (77)	1.0 (0.7-1.2)	39 (87)	1.0 (0.9-1.3)	22 (49)	0.9 (0.6-1.5)

Table 4 shows the associations between actinic keratoses diagnosed in 2007 and the presence of betaPV DNA in 1996, 2003 and at both time points, adjusted for age, sex and AK diagnosed at baseline. Having betaPV detected at a single time point (in either 1996 or 2003) was not associated with AK in 2007. BetaPV type-specific persistence however, was associated with AKs on head and neck (RR 1.4, 95% CI 1.0-1.9). When we repeated the analyses with only the 77 AK-free people at baseline the results were essentially identical, although with slightly wider confidence intervals. Associations between AKs diagnosed

Table 4: Actinic keratoses in 2007 of 138 Nambour Skin Cancer Study participants of which lesion data were available in relation to betaPV detection in 1996, 2003 and at both time points (RR adjusted for age, sex and AK diagnosed at baseline).

	AK total body 2007			AK head and neck 2007		
	AK +	AK-	RR (95% CI)	AK+	AK-	RR (95% CI)
	N (%)	N (%)		N (%)	N (%)	
BetaPV 1996 (all) (n=138)						
-	22 (58)	16 (42)		14 (37)	24 (63)	
+	72 (72)	28 (28)		62 (62)	38 (38)	
BetaPV 1996 (single detection) (n=71)*						
-	22 (58)	16 (42)	1.0	14 (37)	24 (63)	1.0
+	20 (61)	13 (39)	1.0 (0.8-1.2)	16 (48)	17 (52)	1.1 (0.6-1.7)
BetaPV 2003 (all) (n=138)						
-	9 (56)	7 (44)		6 (38)	10 (62)	
+	85 (70)	37 (30)		70 (57)	52 (43)	
BetaPV 2003 (single detection) (n=71)*						
-	9 (56)	7 (44)	1.0	6 (38)	10 (62)	1.0
+	33 (60)	22 (40)	1.0 (0.8-1.2)	24 (44)	31 (56)	1.0 (0.6-1.7)
BetaPV 1996&2003 (persistence) (n=138)						
-	42 (59)	29 (41)	1.0	30 (42)	41 (58)	1.0
+	52 (78)	15 (22)	1.0 (0.9-1.2)	46 (69)	21 (31)	1.4 (1.0-1.9)
BetaPV 1996&2003 (persistence) (n=77)**						
-	27 (56)	21 (44)	1.0	32 (67)	16 (33)	1.0
+	13 (45)	16 (55)	1.0 (0.7-1.5)	14 (42)	15 (52)	1.5 (0.9-2.5)

*In this category only participants with a virus detected at one time point are counted
**People that were AK negative at baseline (1996)

in 2003 and betaPV DNA persistence were of similar magnitude, but non-significant (data not shown). Strength of associations did not vary with increasing numbers of prevalent AK (data not shown).

Discussion

We studied the persistence of betaPV DNA in eyebrow hairs over an 8-year period in a broadly representative sample of adults from an Australian community. We then assessed prospectively whether such long-term betaPV persistence was a risk factor for the development of AKs, after adjustment for presence of AKs at baseline. Among 171 people aged 30-79 years in 1996, 73% were betaPV-positive for at least one type in 1996 and 85% at follow up in 2003. These prevalences were higher than found in earlier studies in this cohort (16;17), probably because of the newer, more sensitive method of betaPV detection used here. Half of the participants had at least one betaPV type-specific persistent infection, 67% of the people who tested positive in 1996. Of the total betaPV infections at baseline in all

participants, half of the infections appeared to persist over the 8-year follow-up period. The likelihood of betaPV DNA being both present and persisting 8 years later increased with age. Except for older age, no other factors were associated with betaPV persistence. We postulate that this association of betaPV infection and persistence with older age reflects natural deterioration of the immune system with age, known as immune senescence (23). Alternatively, contraction of de novo betaPV infections with increasing age might cause the observed association, but we consider this option less likely.

McBride and colleagues performed a study in the same population, assessing AK risk 7 years after baseline betaPV status had been established (16). We have not only extended this period to 11 years but have also assessed association between persistent betaPV infection and AKs. In the current study, we did not find the effect of betaPV presence in 1996 on the risk of AKs that McBride found, nor associations between AK and risk factors other than age. We also did not find different associations between betaPV infection and AKs in men and women, as Boxman and colleagues found in 1996 (17). Again these differences may be due to our use of a betaPV test that was able to detect the common low copy number infections. The different cohort sizes and time-intervals measured may also have played a role.

Indeed a potential limitation of this study was the different methods used for DNA isolation of the 1996 and the 2003 samples. Since recent research shows that different isolation methods can give different end-results (24), it is possible that the newer and more sensitive method used in 2003 partly also explains the higher number of infections that were found in 2003 than in 1996 (490 versus 413). On the other hand, based on previous findings it was to be expected that the prevalence of betaPV increased with age (15;25). Further, while we have referred to the presence of a virus at both time points as “persistent” it is possible that infections are labile and the virus was not present through the entire time period. The fact that 12% of the negative control samples tested betaPV positive, with no dominant type, did not affect our conclusions as this would have resulted in underestimation of betaPV persistence. Finally betaPV can occur in very low copy numbers (26), so it is conceivable that some viruses were still present in 2003 but were below the detection limit.

There is little knowledge about the long-term persistence of betaPV infections. A previous study on betaPV persistence in eyebrow hairs among 23 healthy volunteers aged 21 to 64 years in The Netherlands showed that 48% of betaPV type-specific infections in 74% of

the study participants were persistent over a period of six months (13). We found lower persistence (30% of betaPV infections in 49% of the participants) after 8 years of follow-up. Apart from our longer follow-up time, the smaller number of betaPV measurements, the different DNA purification method and the different geographical region of the Australian study group compared with the Dutch, are all likely to have influenced the comparability of these results.

A small study in 42 immunocompetent people found that 48% who were positive at baseline had a persistent infection after 5 to 7 years of follow up (14). In our study the viral persistence among those who were betaPV-positive at baseline was 67% (83/124). This difference again might be explained by our larger study group, the different geographical region, and the different method used for betaPV detection.

With regard to specific betaPV types, we observed that the more prevalent a betaPV type was, the more likely it was to persist, although one frequently occurring type (HPV17) was only found to persist in 6% of participants. Our overall finding accords with studies concerning high-risk mucosal HPV persistence in women (27), where the more prevalent HPVs persist more often. While comparisons between mucosal and betapapillomaviruses are interesting, the lack of knowledge about the pathophysiology of betaPV infections and about the role of the immune system in clearance of these infections does limit conclusions that can be drawn. In general however, betaPV persistence appears much more common than persistence of mucosal HPV infections in the (ano) genital tract (27). To what extent betaPV persistence increases the risk of cutaneous SCC was not determined in this study, because the number of incident SCC cases was too small. Similarly, we could not identify any betaPV types which unequivocally increased the risk of developing AK.

The finding that long-term betaPV infection in eyebrow hairs is related to subsequently developing AKs on the skin of the head and neck has not been previously reported. We observed no association with whole-body AKs in those with the long-term persistent betaPV infections. The association between betaPV persistence and AKs on the head and neck but not with AKs on the whole body might indicate that betaPV in eyebrow hairs is a better marker of infection on the face, neck and scalp than on the rest of the body, despite a recent study showing that eyebrow hairs are a good marker of cutaneous infection on the body (5;28-30). Previous studies of the association between AK and betaPV did not examine head and neck AK in isolation and therefore comparisons cannot be made.

Overall, we conclude that persistence of betaPV infection in eyebrow hairs over a long period of time is common. Our results indicate that persistent infection with betaPV is a risk factor for the development of AK; whether it is a risk factor for SCC needs to be determined. In contrast, transient infection did not alter the risk of AK. Although these findings accord with persistent mucosal HPV infections and increased risk for cervical intraepithelial neoplasia and cervical cancer, there are many uncertainties and dissimilarities between mucosal and cutaneous HPVs and their role in tumorigenesis. The role of betaPV in the development of cutaneous tumors and the importance of duration of betaPV infection requires ongoing investigation.

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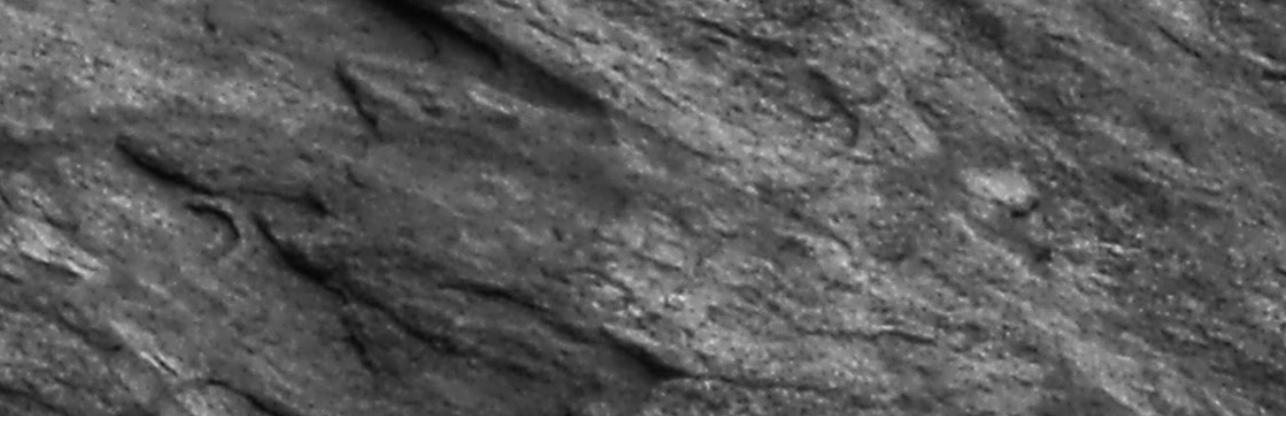

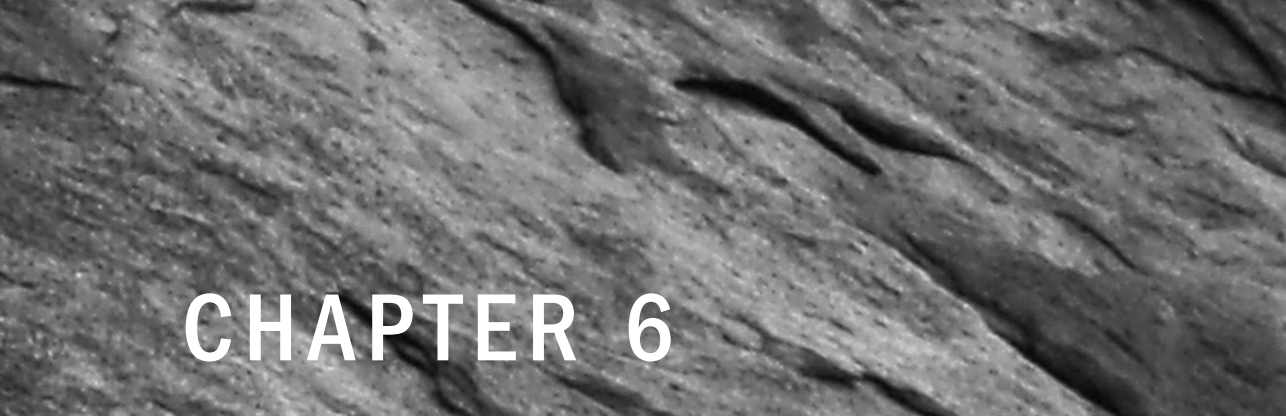

CHAPTER 6

THE ASSOCIATION BETWEEN CUTANEOUS SQUAMOUS CELL CARCINOMA AND BETAPAPILLOMAVIRUS SEROPOSITIVITY: A COHORT STUDY

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Abstract

Betapapillomavirus (betaPV) serum antibodies are frequently used as a marker of infection in epidemiological studies aiming to elucidate the possible association between betaPV and cutaneous squamous-cell carcinoma (SCC). Most previous studies have been case-control and their collective results are inconclusive. Therefore we have investigated the relation between betaPV antibodies and SCC in a population-based cohort study.

Serum samples were collected in 1992 and/or 1996 from 1311 participants of the community-based Nambour Skin Cancer Study. These were tested for the presence of L1 antibodies from 21 different betaPV types and an age- and sex-adjusted Cox proportional hazards model was used to analyse the relation with subsequent SCC occurence from 1992 until 2007.

During follow-up SCC was newly diagnosed in 150 people. No associations were found between the presence of betaPV L1 antibodies and the occurrence of SCC in longitudinal analyses overall (HR for any betaPV infection 1.0), and stratification by sex, skin colour and sunburn propensity did not affect these results. However among people who were less than 50 years old in 1992, the presence of betaPV antibodies was associated with a two-fold increased risk of SCC. There was no significant association between antibodies to any individual betaPV types examined (HPV5, 8, 9, 15, 20, 23, 24, 36, 38) and the later development of SCC.

Whether betaPV infection of the skin, and indirectly betaPV antibodies, are involved in the oncogenic process in the general population remains unclear, but this longitudinal study provides some limited support.

Introduction

Cutaneous squamous cell carcinoma (SCC) is among the most commonly diagnosed cancers in people with fair skin. Human papillomaviruses of the beta-genus (betaPV) are non-enveloped cutanotropic DNA viruses that have been associated with the development of SCC (1). At present, 31 different betaPV types have been fully sequenced (2-4).

Epidemiological studies have shown that antibodies against the betaPV major capsid antigen, L1, can be found in the serum of patients with SCC or the precursor lesion actinic keratosis (AK), but also in people unaffected by these lesions. (5-10). BetaPV L1 antibodies have been associated with both SCC and AK in case-control studies (6;10-20) although the findings are somewhat inconsistent (reviewed in (14)). The most recent and largest case-control study found an association between betaPV antibodies and SCC, with an increasing risk for antibodies to multiple betaPV types as well as in people using glucocortcoids (20). It is difficult to assess the independent effect of specific viruses, due to the very high frequency of multiple infection (21), but to date associations have mostly been identified between HPV8 and HPV38 and prevalent SCC (11-13;16;17;19). A more recent study also found associations with HPV15 and 17 as well as with gammaPV types (18).

Case-control studies assess betaPV exposure at the same time as, or even after, diagnosis of the skin cancer. This prevents assessment of temporality, so the direction of any observed association cannot be determined. Longitudinal studies overcome this issue but there has been only one report of a prospective pilot study published, in which there was no association found between baseline HPV antibodies and subsequent SCC in 39 patients (14). However, as the authors of this paper pointed out, their study did not have adequate power to assess the association.

To further explore the issue we aimed to assess the association between betaPV L1 antibodies and cutaneous SCC using data from a population-based cohort study in Nambour, Australia.

Material and methods

Study population and sample collection

Participants were a subset of the study population of the Nambour Skin Cancer Study described in detail previously (22-24). Briefly, in 1986, 2095 of 3000 randomly selected

residents aged 20-69 of Nambour, a subtropical township in Australia (latitude 26°S), participated in a skin cancer prevalence survey. From 1992 to 1996, 1621 of these took part in a trial of sunscreen application and beta-carotene supplementation for the prevention of skin cancer. All participants received full-body skin examinations by a dermatologist in 1992, 1994 and 1996 to ascertain the presence of actinic keratoses, skin malignancies, telangiectasia on the face and elastosis of the neck. Skin lesions arising between these examinations, and from 1997 to 2007, were ascertained through the local pathology laboratories. Participants completed standardised questionnaires about sun exposure and other possible risk factors for skin cancer including past history of skin cancer. Blood was collected from a randomly selected subsample of participants in 1992 and from all consenting participants in 1996. Participants who were known to have had an SCC prior to their first serum measurement were excluded from the study. Ethical approval for all aspects of the study was obtained through the Bancroft Centre Human Research Ethics Committee, Queensland Institute of Medical Research.

Multiplex serology

We tested serum samples for the presence of antibodies to the major capsid antigen L1 of HPV 5, 8, 9, 14, 15, 17, 20, 21, 22, 23, 24, 36, 38, 47, 49, 75, 76, 80, 92, 93 and 96 by multiplex serology. This is an antibody detection method based on a glutathione S-transferase capture ELISA, in combination with fluorescent bead technology (25-27). Positive serology cut-offs were standardised at 200 MFI (Mean Fluorescence Intensity) (8).

Statistical analyses

We calculated the prevalence of betaPV antibodies for any betaPV type overall and for each of the 21 genotypes tested in 1992 and 1996. We estimated hazard ratios for the association between SCC and the presence of any betaPV antibodies, the number of different antibody types and selected specific antibody types (HPV5, 8, 9, 15, 20, 23, 24, 36, 38) using a Cox proportional hazards model, adjusted for age and sex. The date of entry into the cohort was the date at which serum antibodies were first measured (1992 or 1996), and the date of censoring was either the date of first SCC diagnosis, the date participant was lost to follow-up or 31 December 2007, whichever occurred first. If participants had both 1992 and 1996 serum measurements available, we used both records to allow for changing antibody status. A robust sandwich covariance matrix was used to account for intra-person correlation using the method described by Lin et al (28). We conducted additional analyses, within strata of age, sex, skin colour, and tanning ability. Statistical analyses were performed using SAS 9.1.

Table 1: Baseline characteristics of participants of the Nambour Skin Cancer Study (n=1311) with betaPV antibodies measured in 1992 and/or 1996

	N (%) (n=1311)	Person years	Number who developed SCC (%)	Incidence rate /100.000/ person years	adjusted HR*
Sex					
F	740 (56)	7805	71 (11)	910	1
M	571 (44)	5845	79 (12)	1352	1.2 (0.9-1.7)
age in 1992 (Y)					
< 50	730 (56)	8190	38 (5)	464	1
50+	581 (44)	5459	112 (19)	2052	4.4 (3.1-6.4)
skin colour					
olive	89 (7)	1008	3 (3)	298	1
medium	499 (38)	5091	49 (10)	962	3.7 (1.2-11.6)
fair	722 (55)	7539	98 (14)	1300	5.5 (1.8-17.0)
when exposed to sun					
only tan	144 (11)	1416	9 (6)	636	1
burn than tan	911 (69)	9627	88 (10)	914	1.3 (1.1-4.4)
always burn, never tan	256 (20)	2594	53 (21)	2043	5.0 (2.5-9.8)
occupational sun exposure					
mainly indoors	584 (45)	6264	57 (10)	910	1
both indoor and outdoor	483 (37)	4938	62 (13)	1256	1.0 (0.7-1.5)
mainly outdoors	244 (19)	2436	31 (13)	1273	1.1 (0.7-1.7)
recreational sun exposure					
mainly indoors	198 (15)	2029	18 (9)	887	1
both indoor and outdoor	573 (44)	5944	64 (11)	1077	1.4 (0.9-2.4)
mainly outdoors	540 (41)	5665	68 (13)	1200	1.2 (0.7-2.1)
Nuchal elastosis					
Limited	292 (22)	3322	7 (2)	211	1
Moderate	639 (49)	6709	63 (10)	939	2.7 (1.2-6.0)
Extensive	376 (29)	3598	78 (21)	2168	4.3 (1.9-9.9)
Telangiectasia face					
Low	384 (29)	4113	30 (8)	729	1
Moderate	628 (48)	6529	71 (11)	1087	1.1 (07-1.7)
Extensive	294 (23)	2962	48 (16)	1621	1.3 (0.8-2.2)
Smoker					
life-long non smoker	800 (61)	8171	78 (10)	955	1
current smoker	148 (11)	1508	15 (10)	995	1.6 (0.8-3.2)
ex-smoker	363 (28)	3970	57 (16)	1436	1.0 (0.6-1.6)

*Hazard ratio adjusted for age and sex

Results

Baseline characteristics

1311 people were included in this analysis, 176 with only a 1992 blood sample, 655 only a 1996 blood sample and 480 with both 1992 and 1996 samples. Their mean age was 49 years (SD 13, range 25-75) and 44% were men (**Table 1**). Being aged over 50 years, having medium or fair skin colour, a propensity to burn when exposed to the sun and having

Table 2: Detection of betaPV antibodies overall and for specific betaPV types in 1992 and 1996, for the whole cohort and restricted to those people with both 1992 and 1996 sera available.

	1992 antibodies (n=656)* N (%)	1996 antibodies (n=1135)** N (%)	1992 antibodies (n=480)*** N (%)	1996 antibodies (n=480)*** N (%)
Any betaPV type	409 (62)	748 (66)	300 (63)	329 (69)
HPV5	61 (9)	109 (9)	43 (9)	46 (10)
HPV8	220 (34)	346 (30)	159 (33)	173 (36)
HPV9	109 (17)	179 (16)	79 (16)	93 (19)
HPV14	11 (2)	17 (2)	6 (1)	8 (2)
HPV15	155 (24)	253 (22)	112 (23)	119 (25)
HPV17	149 (23)	283 (25)	109 (23)	129 (27)
HPV20	97 (15)	134 (12)	69 (14)	61 (13)
HPV21	119 (18)	207 (18)	82 (17)	97 (20)
HPV22	82 (13)	137 (12)	60 (13)	60 (13)
HPV23	82 (13)	139 (12)	54 (11)	63 (13)
HPV24	107 (16)	174 (15)	77 (16)	84 (18)
HPV36	86 (13)	136 (12)	56 (12)	57 (12)
HPV38	214 (33)	347 (31)	160 (33)	176 (37)
HPV47	85 (13)	134 (12)	56 (12)	59 (12)
HPV49	170 (30)	269 (24)	130 (27)	138 (29)
HPV75	113 (17)	174 (15)	79 (16)	84 (18)
HPV76	103 (16)	149 (13)	75 (16)	72 (15)
HPV80	110 (17)	208 (18)	74 (15)	89 (19)
HPV92	84 (13)	142 (13)	60 (13)	66 (14)
HPV93	21 (3)	29 (3)	15 (3)	12 (3)
HPV96	126 (19)	199 (18)	92 (19)	98 (20)
Number of types				
0	247 (38)	387 (34)	180 (38)	151 (31)
1-3	214 (32)	416 (37)	156 (32)	167 (35)
4+	195 (30)	332 (29)	144 (30)	162 (34)

* All people with 1992 serum sample

** All people with 1996 serum sample

*** All people with 1992 and 1996 serum sample

a high degree of nuchal elastosis were significantly associated with the development of SCC (Table 1). SCC was diagnosed in 150 participants during follow-up. A single SCC developed in 97 people, and 53 participants developed more than one (range 2-20).

BetaPVL1 antibody prevalence

The overall prevalence of betaPV antibodies in 1992 was 62%, and 46% of participants were positive for multiple types (**Table 2**). The type-specific prevalance was highest for HPV8 (34%), HPV38 (33%) and HPV49 (26%). In 1996, 66% of participants were betaPV seropositive and 48% were positive for more than one type (Table 2). Type-specific prevalence varied from 31% for HPV38 to 1% for HPV14. The overall and type-specific prevalances were similar among the 480 people with 1992 and 1996 sera available (table 2). The betaPV antibody prevalence of the 483 people with serum samples collected in 1992 and 1996 was generally stable in the intervening 5 years, with only 13% of people changing their antibody status between 1992 and 1996.

BetaPV antibody-SCC associations

No association was found between the presence of betaPV L1 antibodies to at least one betaPV type and the development of SCC (RR 1.0, 95% CI 0.7-1.4), or with antibodies to multiple types (**Table 3**). However among people who were less than 50 years old in 1992, the presence of betaPV antibodies was associated with a two-fold increased risk of SCC. This association was not evident in those older than 50 years. Stratification by sex, skin colour and burning ability showed no differences in assocations between betaPV risk factors and SCC-risk. There was no significant association between any of the individual betaPV types examined (HPV5, 8, 9, 15, 20, 23, 24, 36, 38) and the development of SCC (**Table 4**).

Discussion

The majority of previous studies finding associations between betaPV and SCC of the skin have been case-control studies (6;11-13;16;17;19;20;29). In this longitudinal study of 1311 adults followed over 10 to 15 years we did not observe any association with overall antibody positivity, antibodies to multiple betaPV types or to specific types previously shown to be associated with SCC. There was however an association among younger adults: those under 50 years showed a two-fold increased risk of SCC in the presence of betaPV antibodies.

Table 3: Relative risks for SCC for the overall population as well as stratified by sex, age, skin type and propensity to burn

	N (%)	Person years	Number of participants with SCC (%)	Incidence rate/100.000/ person years	adjusted HR
All* (n=1311)					
betaPV AB					
-	437 (33)	4727	44 (10)	931	1,0
+	874 (67)	8894	106 (12)	1192	1.0 (0.7-1.4)
number betaPV AB					
0	437 (33)	4727	44 (10)	931	1,0
1-3	478 (37)	4911	61 (13)	1242	1.0 (0.7-1.5)
4+	396 (0)	3983	45 (11)	1130	0.9 (0.6-1.4)
Men** (n=571)					
betaPV AB					
-	157 (27)	1715	20 (13)	1166	1,0
+	414 (73)	4118	51 (12)	1238	0.7 (0.4-1.1)
number betaPV AB					
0	157 (27)	1715	20 (13)	1166	1,0
1-3	202 (35)	2052	25 (12)	1218	0.7 (0.4-1.2)
4+	212 (38)	2066	26 (12)	1258	0.7 (0.4-1.3)
Women** (n=740)					
betaPV AB					
-	280 (38)	3012	24 (9)	797	1,0
+	460 (62)	4776	55 (12)	1152	1.3 (0.8-2.0)
number betaPV AB					
0	280 (38)	3012	24 (9)	797	1,0
1-3	276 (37)	2859	36 (13)	1259	1.4 (0.9-2.4)
4+	184 (25)	1917	19 (10)	991	1.0 (0.6-1.8)
< 50 years*** (n=730)					
betaPV AB					
-	255 (35)	2949	8 (3)	271	1,0
+	475 (65)	5224	30 (6)	574	2.1 (1.0-4.6)
number betaPV AB					
0	255 (35)	2949	10 (4)	271	1,0
1-3	256 (35)	2871	15 (6)	522	2.3 (1.0-5.0)
4+	219 (30)	2353	16 (7)	637	2.1 (0.9-4.9)
50+ years*** (n=581)					
betaPV AB					
-	182 (31)	1778	45 (24)	2025	1,0
+	399 (69)	3670	123 (29)	2071	0.8 (0.5-1.2)
number betaPV AB					
0	182 (31)	1778	45 (24)	2025	1,0
1-3	222 (38)	2040	65 (28)	2206	0.9 (0.6-1.3)
4+	177 (31)	1630	58 (31)	1902	0.7 (0.4-1.1)

Table 3 continued

	N (%)	Person years	Number of participants with SCC	Incidence rate/100.000/ person years	adjusted HR
Fair skin* (n=722)					
betaPV AB					
-	238 (33)	2650	25 (11)	943	1,0
+	484 (67)	4873	73 (15)	1498	1.3 (0.8-2.0)
number betaPV AB					
0	238 (33)	2650	25 (11)	943	1,0
1-3	259 (36)	2639	39 (15)	1478	1.3 (0.8-2.1)
4+	225 (31)	2234	34 (15)	1522	1.2 (0.7-2.0)
Medium/olive skin* (n=588)					
betaPV AB					
-	199 (34)	2078	19 (10)	914	1,0
+	389 (66)	4021	33 (8)	821	0.6 (0.3-1.1)
number betaPV AB					
0	199 (34)	2078	19 (10)	914	1,0
1-3	219 (37)	2272	22 (10)	968	0.7 (0.4-1.3)
4+	170 (29)	1749	11 (6)	629	0.4 (0.2-1.0)
Always burn* (n=256)					
betaPV AB				1233	
-	79 (31)	892	11 (14)	2475	1,0
+	177 (69)	1697	42 (24)		1.1 (0.6-2.1)
number betaPV AB					
0	79 (31)	892	11 (14)	1233	1,0
1-3	89 (35)	865	22 (25)	2543	1.4 (0.7-2.7)
4+	88 (34)	832	20 (23)	2404	0.9 (0.5-1.9)
Burn-tan/only tan* (n=1054)					
betaPV AB					
-	358 (34)	3836	33 (9)	860	1,0
+	696 (66)	7197	64 (9)	889	0.9 (0.6-1.3)
number betaPV AB					
0	358 (34)	3836	33 (9)	860	1,0
1-3	388 (37)	4046	39 (10)	964	0.9 (0.6-1.5)
4+	308 (29)	3151	25 (8)	793	0.8 (0.5-1.3)

* Hazard ratio adjusted for age and sex
** Hazard ratio adjusted for age
*** Hazard ratio adjusted for sex

The overall prevalence of betaPV antibodies of 62% in 1992 and 66% in 1996 was slightly higher than the 51% previously found in a similar population where the same laboratory technique and cut-off were used for ascertainment of antibodies (27). As betaPV sero-positivity increases with age (8;27) and the mean age was lower in this study than in the previous Queensland study (27), the relatively high seroprevalence here is not due to older age and remains unexplained. The betaPV antibody prevalence of the 480 people with

Table 4: Associations between betaPV type-specific seropositivity and SCC

	N(%) (n=1311)	Person years	Number of participants with SCC (%)	Incidence rate/100.000/ person years	adjusted HR*
HPV5					
-	1174 (90)	12344	136 (12)	1102	1,0
+	137 (10)	1278	14 (10)	1095	1.0 (0.5-1.7)
HPV8					
-	888 (68)	9329	95 (11)	1018	1,0
+	423 (32)	4293	55 (13)	1281	1.1 (0.8-1.5)
HPV9					
-	1092 (83)	11387	129 (12)	1133	1,0
+	219 (17)	2235	21 (10)	940	0.7 (0.5-1.2)
HPV15					
-	1002 (76)	10522	112 (11)	1064	1,0
+	309 (24)	3100	38 (12)	1226	1.0 (0.7-1,5)
HPV20					
-	1131 (86)	11895	133 (12)	1118	1,0
+	180 (14)	1727	17 (9)	984	0.6 (0.3-1.0)
HPV23					
-	1138 (87)	11973	136 (12)	1136	1,0
+	173 (13)	1649	14 (8)	849	0.7 (0.4-1.1)
HPV24					
-	1011 (77)	11455	128 (13)	1117	1,0
+	300 (23)	2167	22 (7)	1015	0.8 (0.5-1.3)
HPV36					
-	1133 (86)	11887	134 (12)	1127	1,0
+	178 (14)	1735	16 (9)	922	0.7 (0.4-1.1)
HPV38					
-	900 (69)	9386	102 (11)	1087	1,0
+	411 (31)	4236	48 (12)	1133	0.9 (0.6-1.3)

* Hazard ratio calculated using Cox proportional hazards model and adjusted for age and sex

serum samples collected in 1992 and 1996 was quite stable in the short-term (5 years), with only around 10% of people changing their antibody status in that time.

This is the largest longitudinal study to evaluate the relationship between betaPV antibodies and SCC, and it is consistent with a small prospective pilot study (cases=39) in the United

Kingdom in that both found no association between baseline serology and incident SCC (14). However we did find an association in people diagnosed with SCC who were less than 50 years at entry into the study. It is possible that SCC diagnosed at younger ages is less strongly related to cumulative ultraviolet radiation exposure with other HPV more likely to play a role than in older people. No previous studies have reported results stratified by age, so we do not know if stronger associations have been apparent in younger age groups in previous studies.

Apart from the association in younger people, our overall results are not in accord with those from recent large case-control studies which have showed generally positive associations (10-20). Understanding the relation between HPV and skin cancer is hampered by lack of knowledge about the appropriate measure of infection. BetaPV are almost ubiquitous on the skin, with much higher prevalence of betaPV DNA than of antibodies, so some factor(s) other than simply the presence of betaPV on the skin must influence the development of antibodies. It has been hypothesised that the presence of SCC (or its precursor lesions, actinic keratoses) may result in increased viral load or local inflammation, resulting in presentation of the virus to the immune system and seroconversion (6;10;19). If this is the case, it is possible that the results of case-control studies do not indicate a causal association but are due to "reverse causality". In support of this, in the previous small longitudinal study in the United Kingdom, there was a tendency towards higher seroprevalence in 15 prevalent cases than in 39 incident cases, although this was not statistically significant. However we did not find that a diagnosis of SCC between 1992 and 1996 increased the likelihood of seroconversion in our study, and there is also no evidence that the diagnosis of AK leads to seroconversion in people without a diagnosis of SCC (Antonsson, unpublished data). Furthermore, the lack of association between the presence of antibodies and BCC in case-control studies argues against the reverse causality hypothesis (20).

There may be other biases or uncontrolled confounding that have led to the associations observed in case-control studies. Assuming that neither bias or reverse causality is responsible and that there is a causal relation, the possible reasons for the overall lack of association in our longitudinal study need to be considered. It is possible that very high levels of sun exposure in Queensland overwhelm any detectable effect of betaPV. However we have previously shown that the prescence of persistent betaPV DNA in eyebrow hairs is associated with the development of AK in this population (30), providing support for an effect of long-term infection. We measured antibody status in mid-adulthood, and although

antibodies appear to be relatively stable over a short time period, it is possible that this does not reflect the status at the time of initiation of SCC many years earlier. (This limitation would also be true of case-control studies.) Finally, a high proportion of controls in this study had been diagnosed with AK. As these lesions are frequently not histologically diagnosed, we could not ascertain all AKs occurring during the follow-up period, so could not adjust for the presence of AK. The fact that AKs are on the causal pathway between sun exposure and SCC, and possibly also between betaPV infection and SCC, may have made adjustment inappropriate even had it been possible. The complex interplay between betaPV, sun exposure, AK and SCC therefore may be responsible for the lack of association between betaPV and SCC in people over 50 at entry into this cohort.

In conclusion, whether betaPV infection of the skin, and indirectly betaPV antibodies, are involved in the oncogenic process in the general population remains unclear, but this study provides some limited support. Research incorporating measures of immune function and genotype, along with multiple measures of betaPV infection and sun exposure, may help to elucidate the role of betaPV in cutaneous carcinogenesis.

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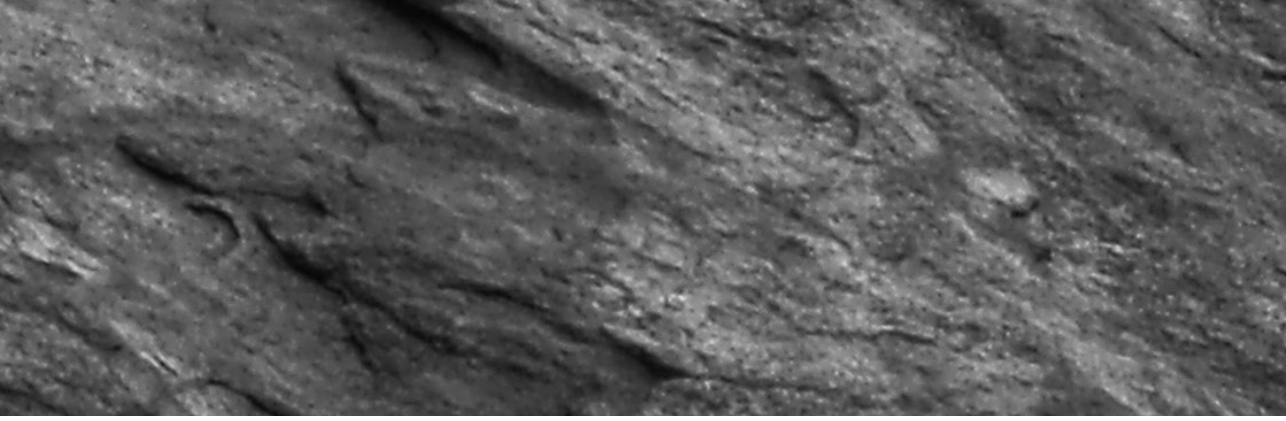

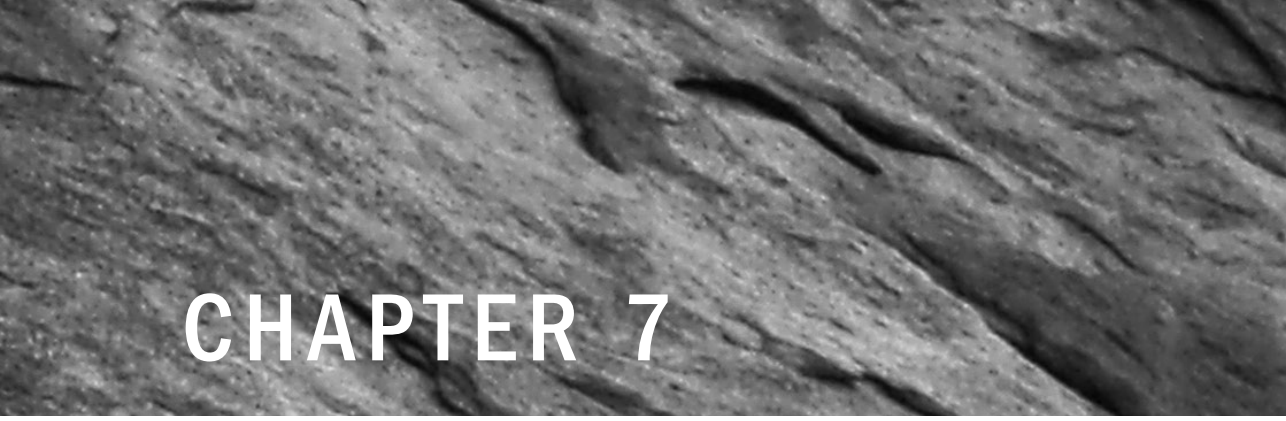

CHAPTER 7

GENERAL DISCUSSION

Betapapillomaviruses

It is widely believed that betapapillomaviruses (betaPV) are associated with the development of actinic keratoses (AK) and cutaneous squamous cell carcinoma (SCC), but to date many deficiencies exist in the fundamental knowledge about the natural history of betaPV infections as well as about their possible role in keratinocyte skin cancer development. In this last chapter, aspects of the natural history of betaPV infections and their association with cutaneous SCC development are discussed in view of the new evidence described in this thesis and recent findings by others.

Natural history: Acquisition and transmission

Little is known about the transmission of betaPV. In healthy people no clinical signs of initial or ongoing infection are observable. Prior to the present study described in **Chapter 2**, only 5 other studies, several of which were small with ambiguous findings, had addressed the question of transmission of betaPV (1-5). Despite different methods however, all of these studies concluded that betaPV transmission probably takes place during close (skin-to-skin) contact. One of these studies (3) was among tenants in a student share house and showed that under these conditions transmission was rare, suggesting that simply living in close proximity is not sufficient for betaPV transmission; closer contact is probably required in addition.

A more recent study of betaPV transmission in families with an overall HPV prevalence of 42% found that the frequency of shared types was higher among couples than among randomly selected individuals, but the frequency of sharing at least one type was only 21% and in all instances only one type was shared (5). Weissenborn and colleagues (6) studied the betaPV-spectrum in 10 families with up to 3 generations sampled over a period of time and found comparable results to ours with respect to partner transmission, despite using skin swabs instead of eyebrow hairs as the sample for viral DNA detection. Their longitudinal measures showed that persistent infections in one person of a family were shared by their family members in 30-50% of the cases (6). Thus consistent with other findings, **Chapter 2** (7) showed that cohabiting married or *de facto* opposite-sex couples more often shared betaPV DNA with their opposite-sex domestic partner than with random controls of the same age and sex as their partner, drawn from the same population. Again the most likely explanation for this finding is the frequent close contact likely to occur between partners, as between mothers and babies (1).

In **Chapter 2** a much higher proportion of couples with at least one shared type (39%) was found than in the German study (5), and 14% of these shared more than one type (7), possibly due to the higher overall prevalence of betaPV in the Australian sample. The higher prevalence might be due to the fact that different PCR and typing methods were used in the present study (7) compared with the study by Gottschling and colleagues (5). Furthermore, the mean age of participants was higher than in the present study (over 50 years (5) compared with 42 years (7)), and age is independently associated with betaPV acquisition and detection (8).

In view of all the now-available data, it can be concluded that transmission of betaPV DNA starts at a very young age and continues through life because of close skin-to-skin contact.

Natural history: presence and persistence

Other aspects of the natural history of betaPV infection are the site distribution of infection as indicated by the presence of betaPV DNA in different body-tissue samples, the persistence of viral DNA and the presence and persistence of betaPV antibodies. In epidemiological studies, the presence of betaPV DNA in eyebrow hairs, skin swabs, and normal skin biopsies have all been used as markers of betaPV-infection (9-13). Which of these is the most appropriate indicator of the betaPV types found in the tumour and/or the surrounding area was unknown prior to the study described in **Chapter 3.** The type-specific betaPV prevalence and distribution in 21 sets of four different tissue samples (SCC, perilesional skin, normal skin on the mirror image site of the SCC, and plucked eyebrow hairs) taken from incident SCC patients were systematically explored and compared. The overall betaPV DNA positivity as well as the multiplicity of infections was high and this underscores the ubiquity of cutaneous betaPV infections that has been previously reported (3;8;14). The number of betaPV types detected in normal skin was considerably less than in the other tissues, in support of previous data showing that normal skin has fewer betaPV types than SCC tissue (15;16).

BetaPV is present in hair follicles obtained from different body sites such as scalp, eyebrow, arm, trunk, leg and pubic region (9;17). The detection of betaPV in eyebrow hairs has been used in epidemiological studies as a marker of infection, not only because of the ease of obtaining eyebrow hairs, but also because the bulb is regarded as a reservoir of infection (9;18). The greater diversity of types found in hair follicles compared with normal skin

lends support to this notion (9), with the epidermal stem cells residing in the bulge as the probable main site of persistent infection.

However, there is limited knowledge about persistence of cutaneous betaPV infection over extended periods of time. Furthermore, most epidemiological studies (19;20) have been cross-sectional, assessing both betaPV detection in an individual's eyebrow hairs and their skin cancer status at the same point in time. To better address these issues, in **Chapter 5** the persistence of betaPV DNA in eyebrow hairs was studied over an 8-year period. Half of the infections present at baseline appeared to persist over the follow-up period and the likelihood of betaPV DNA being both present and persisting 8 years later increased with age. Except for older age, no other factors were associated with betaPV persistence and it is postulated that this association of betaPV infection and persistence with older age reflects natural deterioration of the immune system with age, known as immune senescence (21).

With regard to specific betaPV types, it was observed that the more prevalent a betaPV type was, the more likely it was to persist. This finding accords with studies concerning high-risk mucosal HPV persistence in women (22), where the more prevalent HPVs also persist more often. In general, betaPV persistence appears much more common than persistence of mucosal HPV infections in the (ano-)genital tract (22). Within a few months after the acquisition of viral DNA, a serological response to mucosal HPVs is evoked in about half of infected women. In the latter case, the majority of HPV DNA-positive women clear these infections within 12 months (23-25). In women with persistent presence of mucosal HPV DNA in samples taken at two different occasions, the percentage of seropositive women is higher than in women with HPV DNA diagnosed on a single occasion (26). While comparisons between mucosal and betapapillomaviruses are interesting, it is recognised that the lack of knowledge about the pathophysiology of betaPV infections including the role of the immune system in their clearance, limits the conclusions that can be drawn from such comparison.

The above mentioned data further raise the possibility that betaPV persistence might also be linked to a serological response. Little is known about the association between betaPV infection of hair follicles and antibodies in serum. It may be expected that antibodies arise as a result of infection of the hair follicles with betaPV DNA, but the specific aspects of betaPV infection that drive antibody responses are currently unknown. For example, it is feasible that the location, load and persistence of infection, as well as inflammation at the

site of infection may all be important in this respect (27;28). In **Chapter 4** the associations between both prevalence and persistence of betaPV DNA in plucked eyebrow hairs, and L1 antibodies in serum were assessed, and it was found that neither DNA measure was predictive of antibody detection. Stratification by age, sex, history of sunburns or SCC did not alter these findings. Several possible explanations for the observed lack of association between betaPV DNA and L1 antibodies can be proposed. Firstly, it may be that the presence and/or persistence of betaPV DNA that was measured was not indicative of infection many years prior to 1996 and that the antibody response was provoked earlier in life. Secondly, it is possible that a high proportion of infections in eyebrow hairs do not evoke an antibody response because not all may be indicative of pathologically relevant skin infection. Some association has been shown between betaPV DNA found in eyebrow hairs and in biopsies of cutaneous SCC and the perilesional skin (**Chapter 3**) (29;30), but the prevalence in eyebrow hairs is substantially higher than in other tissues. Alternatively, or in addition, betaPV antibody responses may be associated with the betaPV load rather than simply with presence or absence of viral DNA. Lastly, it is possible that antibodies are detected for multiple types due to cross-reactivity rather than infection of the skin with those types, and thus true type-specific seroresponses may be lower than measured. The high multiplicity of L1 antibodies and the significantly higher prevalence of antibody positivity than betaPV DNA positivity for 12 types as found in **Chapter 4** supports this hypothesis.

In line with the limited data available on betaPV DNA persistence, stability of betaPV antibody status had not been assessed previously. In **Chapters 4** and **6** is described that betaPV L1 antibody status is very stable when measured over 4 to 8 years. In **Chapter 4** only 11% of people changed their overall betaPV serology status between 1996 and 2003, and they had MFI values very close to the cut-off. In **Chapter 6** people were followed over 4 years and only 13% of people changed their serostatus.

In the view of the data described in this thesis, combined with previous literature it can be concluded that eyebrow hairs can still be used as a convenient marker of betaPV infection, however it must be acknowledged that only a proportion of the types found might give a relevant skin infection. Perilesional skin biopsies can be more useful in that respect, but are absent by definition in healthy controls. Although betaPV DNA is often present and persistent over years in humans, the presence or persistence of betaPV DNA per se is not always sufficient to evoke an antibody response in the infected person.

Disease associations

BetaPVs are detected frequently in actinic keratoses (AKs) and cutaneous squamous-cell carcinomas (SCCs), but have also been found in biopsies from normal skin and eyebrow hairs and skin swabs from people with and without skin cancer (1;9;11). Although a number of studies have shown statistically significant associations between markers of betaPV infection (viral DNA in eyebrow hairs or skin biopsies, and antibodies in serum) and SCC (12;19;31-34), and experimental studies have found some evidence of the transforming effect of viral (onco)genes of betaPV types (35-40), so far the role of betaPV in skin carcinogenesis in vivo has not been fully elucidated.

Different mechanisms by which betaPV play a role in carcinogenesis have been proposed, firstly the "hit-and-run" hypothesis, whereby betaPV act early in carcinogenesis and is not necessary for maintenance of the malignant phenotype (41;42). Secondly, betaPV may act within or contribute to field cancerisation, where a discrete area of tissue is at increased risk of developing cancer (43), as seen for SCC of the oesophagus (44) and cutaneous actinic keratoses (45-48). This might be explained by betaPV-mediated impairment of host cell defences against excessive sun light exposure, such as inhibition of DNA repair and apoptosis (37;49;50). As already described (in **Chapter 3**), type-specific betaPV prevalence and distribution in different tissues from each of 21 incident SCC patients were systematically explored and compared. The lower number of betaPV types found in normal skin compared to the tissue near the SCC supports the hypothesis that perilesional skin represents an area of field cancerisation from which the tumour arose (45-48). The localised presence of betaPV may have contributed to the field change (possibly by its property to impair cellular defences against UV-induced DNA damage (51;52)), in conjunction with other factors such as sunburn or chronic sun exposure. Alternatively, focal damage may have enhanced betaPV infection, increasing the viral load above the detection limit of the test or, less likely, may have rendered the affected skin more susceptible to infection with betaPV. However, this study was small and cross-sectional, and therefore has limitations that have to be taken into account when interpreting its findings.

In **Chapter 5** long-term betaPV persistence (described above) was further assessed prospectively, as a risk factor for the development of AKs. No association was observed with whole-body AKs in those with long-term persistent betaPV infections, however the finding that long-term betaPV infection in eyebrow hairs is related to subsequently developing AKs on the skin of the head and neck had not been previously reported. The association between

betaPV persistence and AKs on the head and neck but not with AKs on the whole body might indicate that betaPV in eyebrow hairs is a better marker of relevant infection on the face, neck and scalp than on the rest of the body (9;30;53;54).

To date mainly cross-sectional analyses within case-control studies had been performed to assess the relationship between betaPV L1 antibodies and SCC development (20;28;31;32;34;55-58). In **Chapter 6** this relationship was assessed in a large longitudinal study. While no overall associations were found between betaPV L1 antibodies and the later development of SCC, a significant association with betaPV was found in people diagnosed with SCC who were less than 50 years old at baseline. Apart from the association in younger people, our overall results are not in accord with those from recent large case-control studies which have showed generally positive associations (12;31;32;34;55-61).

Understanding the relation between HPV and skin cancer is hampered by lack of knowledge about the appropriate measure of infection. BetaPV are almost ubiquitous on the skin, with much higher prevalence of betaPV DNA than of antibodies, so some factor(s) other than simply the presence of betaPV on the skin must influence the development of antibodies. It has been hypothesised that the presence of SCC (or its precursor lesions, actinic keratoses) may result in increased viral load or local inflammation, resulting in presentation of the virus to the immune system and seroconversion (12;28;32). If this is the case, it is possible that the results of case-control studies do not indicate a causal association but are due to "reverse causality". This is supported in the previous small longitudinal study in the United Kingdom (59). However in **Chapter 6** it was not found that a diagnosis of SCC between 1992 and 1996 increased the likelihood of seroconversion in our study, and there is also no evidence that the diagnosis of AK leads to seroconversion in people without a diagnosis of SCC (Antonsson, unpublished data). Furthermore, the lack of association between the presence of antibodies and BCC in case-control studies argues against the reverse causality hypothesis (34). Besides this, other biases or uncontrolled confouding could have played a role in not finding the associations foudn before, for example the very high level of sun exposure in Queensland or the high frequenty of AK in the population. The fact that AKs are on the causal pathway between sun exposure and SCC, and possibly also between betaPV infection and SCC, may have made adjustment inappropriate even had it been possible. The complex interplay between betaPV, sun exposure, AK and SCC therefore may be responsible for the lack of association between betaPV and SCC in people over 50 at entry into this cohort.

Concluding remarks

This thesis studied the natural history of betaPV and its possible co-carcinogenic effect with sun exposure in the formation of cutaneous SCC and AK. The present evidence supports earlier assumptions that transmission takes place via close skin contact. Secondly persistence of betaPV DNA was shown to be a common occurrence, as was stability of antibody status, in the general population studied. Current antibody responses however appeared not to directly reflect either current presence of betaPV DNA, or the betaPV DNA that were present and persistent in the recent past (in the previous decade).

On balance the detailed, community-based studies described in this thesis support the hypothesis that betaPV does play a role in the formation of a proportion of AK and SCC. The evidence could have important implications for skin cancer prevention, while recognizing the established fact that in fair-skinned populations in general, sun exposure remains the predominant cause of these tumours. That betaPV DNA is found in different samples of skin and eyebrow hairs of SCC cases underscores the ubiquity of background infection; the type-specific differences in prevalence between perilesional and normal skin further suggest a possible additional effect of betaPV in the field of tumorigenesis (for example by inhibiting apoptosis and enhancing cell growth). Persistent betaPV DNA was associated with the later development of AK, strongly suggesting that viral persistence is important in early skin neoplasia in a proportion of affected people, but not all, since so many healthy people with high sun exposure were also seen to have persistent betaPV infections. BetaPV antibodies seem not to be directly driven by betaPV DNA and were associated with SCC development only in people under 50 years.

The precise elucidation of the existence and nature of the association under study, and answers to other important questions are yet to be understood nearly forty years after the first description in the literature (62) of the possible role of betaPV in cutaneous squamous cell carcinoma development. Such questions are, for example, why only some betaPV infections are potentially pathogenic, and what are the immunological (and other) characteristics of those who may be susceptible to the co-carcinogenic effects of this virus in the skin. From an epidemiological viewpoint, large prospective cohort studies over several decades, involving all betaPV measurements (eyebrow hairs, skin biopsies and serum) and complemented by in vitro searches for a mechanism whereby betaPV could help activate cutaneous neoplasia, are required to resolve the issues definitively. In view of design and costs however, the likelihood that such studies can take place in the near future is probably

low, but they are needed because only then will we have a basis for believing that, alongside sun protection, prevention of betaPV infection may be a useful strategy, as it is in prevention of ano-genital cancers by the high-risk mucosal HPV types, in skin cancer prevention.

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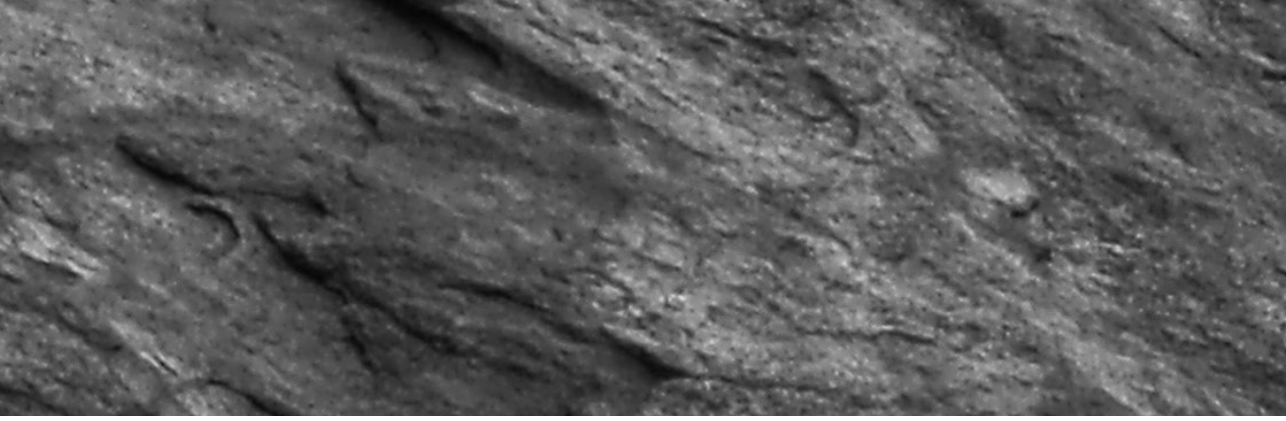
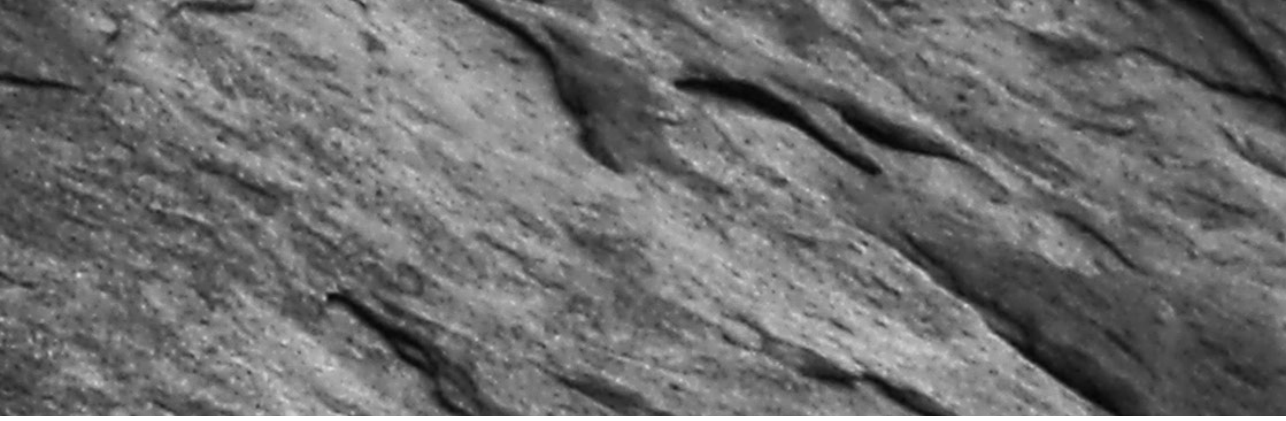




SAMENVATTING

Dit proefschrift beschrijft onderzoek naar de relatie tussen betapapillomavirussen (betaPV) en het cutane plaveiselcelcarcinoom (PCC) en diens voorloper actinische keratose (AK).

BetaPV maken deel uit van de papillomavirusfamilie, die meer dan 100 humane papillomavirussen (HPV) omvat en meerdere animale. HPV is geassocieerd met anogenitale carcinomen en cutane wratten. In 1972 werd een associatie tussen betaPV en PCC beschreven in patiënten met de zeldzame erfelijke huidziekte Epidermodysplasia Verruciformis (EV). Momenteel zijn 31 typen BetaPV bekend (HPV5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, 92, 93, 96, 98, 99, 100, 104, 105 en 113) en nieuwe types worden regelmatig beschreven. Associaties zijn beschreven tussen AK en PCC en de aanwezigheid van betaPV DNA in wenkbrauwharen, huiduitstrijken en L1 antilichamen.

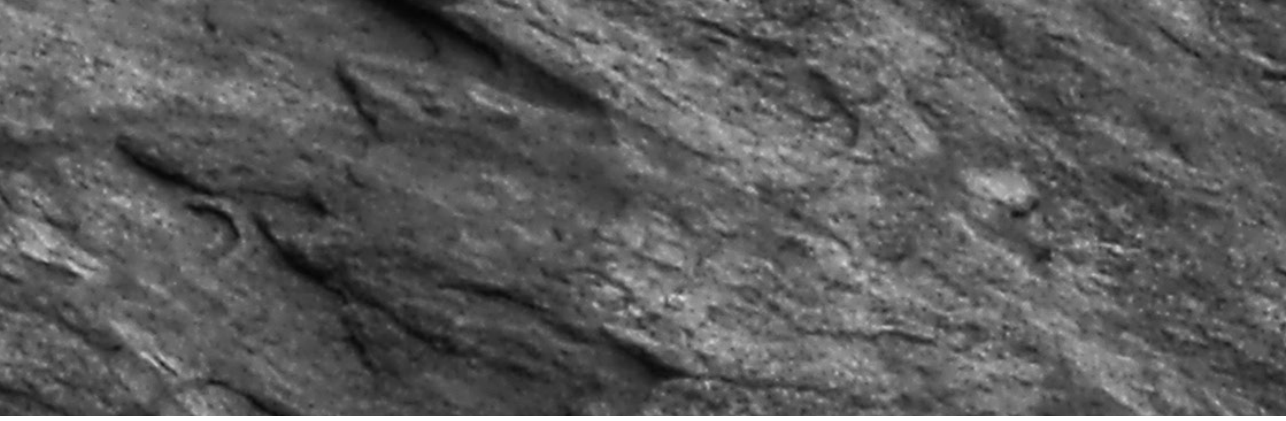
Voor dit onderzoek zijn wenkbrauwharen gebruikt om betaPV DNA uit te isoleren en bloed om antilichamen tegen betaPV te isoleren. Hoofdstuk 2, 4, 5 en 6 zijn uitgevoerd binnen de context van de Nambour Skin Cancer Study, een prospectieve cohortstudie naar het voorkomen van huidkanker in Nambour, een stad met 10.000 inwoners op 2 uur rijden van Brisbane, Australië. Huidkanker komt hier veel voor vanwege de tropische ligging en voornamelijk blanke bevolking. Hoofdstuk 3 is uitgevoerd onder PCC-patiënten in Townsville, Australië. Ook hier zorgt de hoge UV-belasting en de blanke bevolking voor een hoog huidkanker cijfer.

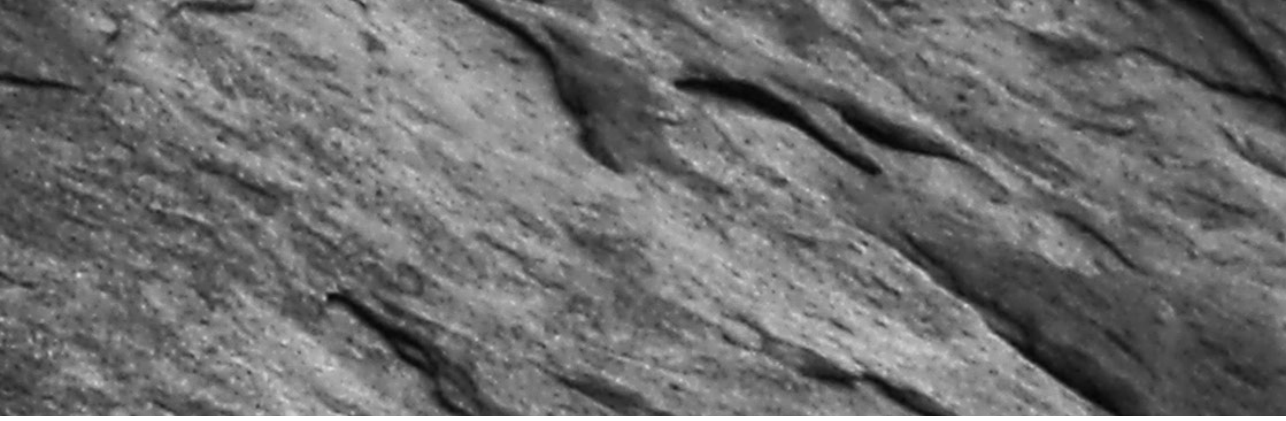
In **Hoofdstuk 2** is gevonden dat samenwonende partners vaker dezelfde types betaPV hebben wanneer ze vergeleken worden met leeftijd- en geslacht-gematchte controles. Dit is een aanwijzing dat betaPV overgedragen wordt via nauw contact. In **Hoofdstuk 3** is onderzocht hoe vaak betaPV DNA voorkomt in biopten van PCC, de huid rondom de tumor (perilesionale huid), gezonde huid en wenkbrauwharen van 21 PCC-patiënten. Gevonden werd dat het gemiddelde aantal betaPV types significant hoger was in biopten van PCC en perilesionale huid dan in de biopten van normale huid. Dit suggereert dat er sprake is van een “field change” van waaruit het PCC ontstaat. Of betaPV daarbij een rol speelt kan hieruit niet worden opgemaakt. Ook werd gevonden dat betaPV types in wenkbrauwharen redelijk goed overeenkomen met de types die in de tumor worden gevonden. Wenkbrauwharen kunnen dus gebruikt worden als maat voor betaPV infectie in de tumor. In **Hoofdstuk 4** werd gevonden dat de reactie van het lichaam om betaPV L1 antilichamen aan te maken niet alleen bepaald wordt door de aanwezigheid van betaPV in gezonde huid. Ook langdurige aanwezigheid van betaPV (gedurende 8 jaar), was niet geassocieerd met betaPV

L1 antilichamen. Waarschijnlijk zijn additionele stimuli nodig om tot antilichaamproductie over te gaan, zoals een overmaat aan aanwezig virus. In **Hoofdstuk 5** is onderzocht hoe vaak persistentie van betaPV voorkomt onder gezonde Australiërs en of het hebben van persistent betaPV het risico op het krijgen van AK verhoogd. Gevonden werd dat 30% van de virussen na 8 jaar nog aanwezig zijn (persisteren). Mensen die ouder zijn dan 60 jaar hadden tweemaal zoveel kans op een persistente betaPV infectie. BetaPV persistentie was geassocieerd met het hebben van AK in het gelaat. In **Hoofdstuk 6** is onderzocht of L1 antilichamen geassocieerd zijn met PCC in een longitudinale studie, aangezien eerder alleen dwarsdoorsnede onderzoek is uitgevoerd. Gevonden werd dat alleen in de groep patiënten onder de 50 jaar een associatie bestond tussen betaPV en het optreden van PCC. Associaties binnen de gehele patiëntengroep of met individuele types werden niet gevonden.

Alles in overweging nemend ondersteunen de studies in dit proefschrift de hypothese dat betaPV geassocieerd zijn met de ontwikkeling van AK en PCC. Dit zou consequenties kunnen hebben voor de preventie van PCC, hoewel moet worden benadrukt dat UV-expositie in de blanke populatie de belangrijkste risicofactor blijft. Persistentie zou de rol van betaPV in de oncogenese kunnen versterken, echter persistente infecties worden ook vaker bij gezonde personen gevonden.

Bijna 30 jaar na de eerste publicatie over de associatie tussen betaPV en PCC blijven belangrijke vragen bestaan. Deze zijn bijvoorbeeld waarom maar een klein deel van de infecties potentieel pathogeen lijkt te zijn en welke overige (immunologische, genetische of andere) factoren een rol spelen bij de individuele vatbaarheid van een persoon voor een co-carcinogeen effect in de huid. In het algemeen is meer *in vitro* onderzoek naar het mechanisme hoe betaPV kan bijdragen aan de ontwikkeling van cutane tumoren van groot belang. Idealiter worden daarnaast grote epidemiologische studies met diverse materialen (wenkbrauwharen, huidbiopten en serum) uitgevoerd om meer duidelijkheid te verkrijgen over de rol van betaPV in het ontstaan van huidkanker.





PUBLICATIONS

Plasmeijer EI, Neale RE, O'Rourke P, Mallitt KA, de Koning MN, Quint WG, Buettner PG, Pawlita M, Waterboer T, Green AC, Feltkamp MC. Lack of association between the presence and persistence of betapapillomavirus DNA in eyebrow hairs and betapapillomavirus L1 antibodies in serum. J Gen Virol. 2010 Aug;91(Pt 8):2073-9.

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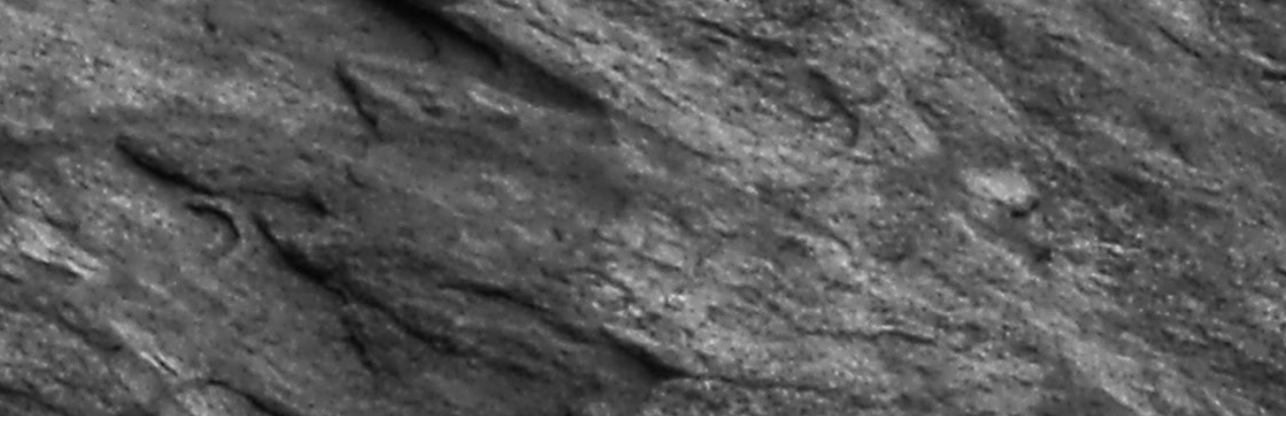

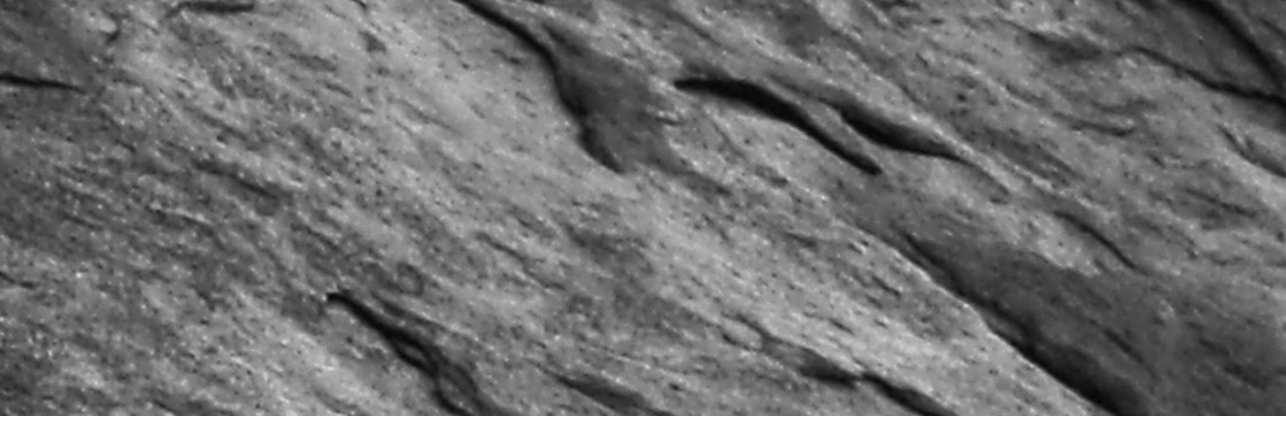

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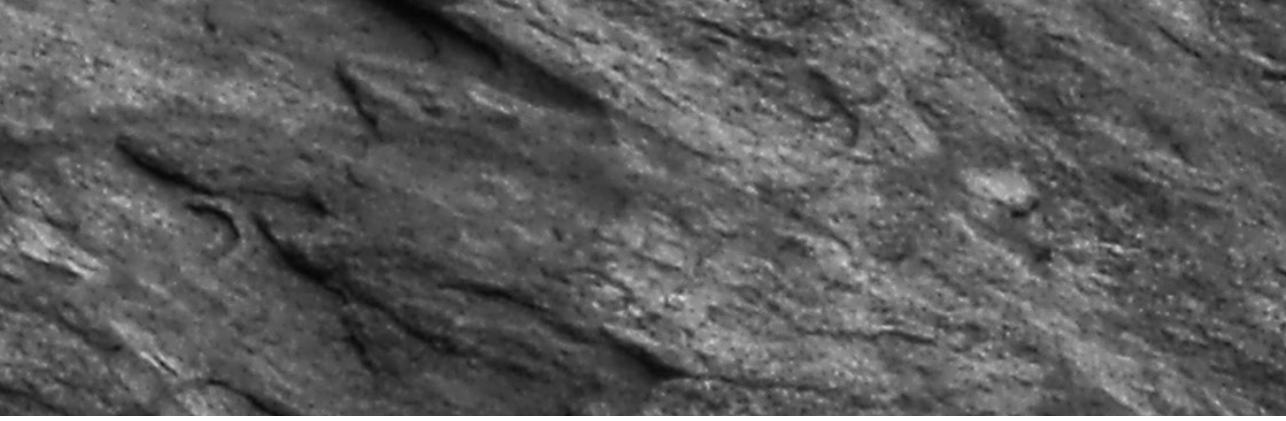
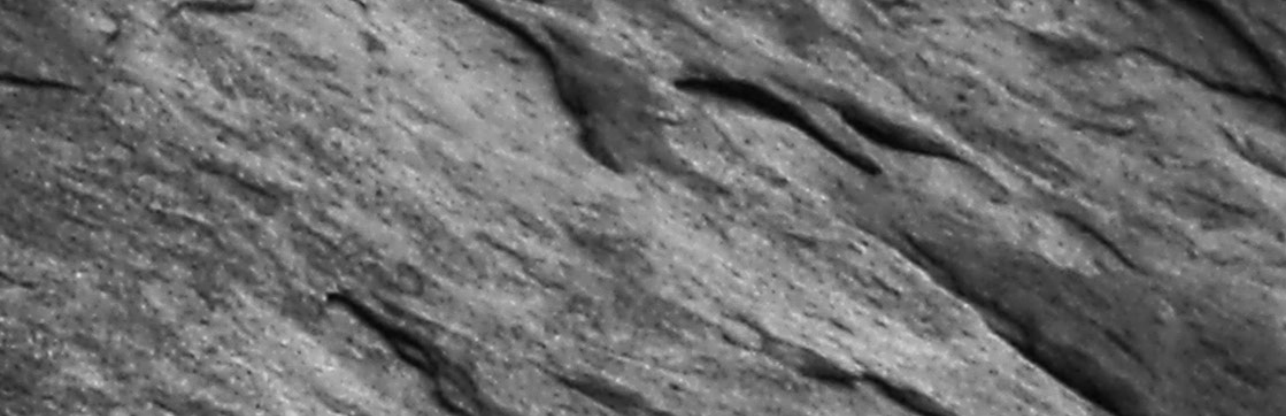


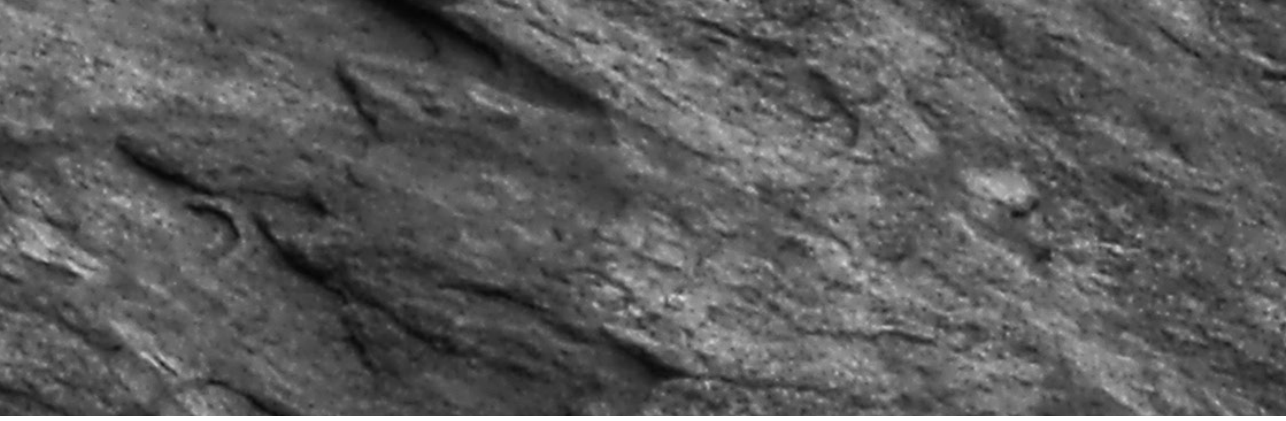


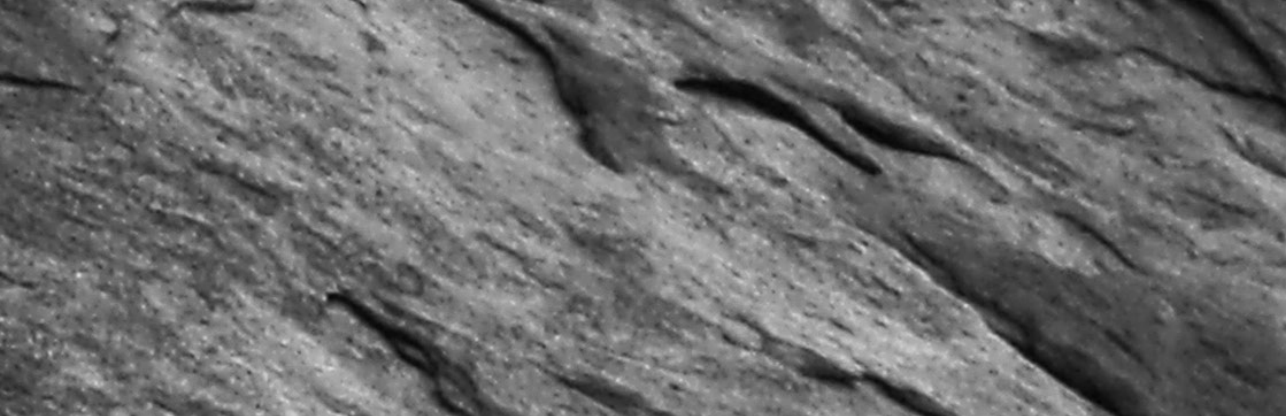
CURRICULUM VITAE

The author of this thesis was born on 21 December 1979 in Almelo, The Netherlands. After graduating from 'Het Noordik' (VWO) in 1998 she started with the study Biomedical Sciences at the Leiden University. After finishing the first year she entered the Medical School of the same university in 1999. As part of her study she performed research internships in Nalerigu, Ghana, supervised by Dr. Ziem and Dr. Polderman of the department of Parasitology of the Leiden University Medical Center (LUMC) at the Department of Dermatology of the LUMC, supervised by Dr. Verdijk and Dr. Tensen. During her study she worked as a assistant histology teacher at the Department of Moleculair Cell Biology of the LUMC. She received her Master's degree cum laude in 2003 and graduated as a Medical Doctor in 2005.

In January 2006 she started as a PhD-student at the Department of Medical Microbiology of the LUMC under supervision of Dr. M.C.W. Feltkamp, Prof. Dr. A.C.M. Kroes and Prof. Dr. A.C. Green. As part of this project she worked from October 2008 until December 2008 and from September 2009 until November 2009 at the Cancer and Populations Studies Group of the Queensland Institute of Medical Research in Brisbane, Australia, under supervision of Prof. Dr. A.C. Green and Dr. R.E. Neale. The results of her studies are described in this thesis.

From April 2010, Elsemieke Plasmeijer is working as a resident at the Department of Dermatology of the LUMC under supervision of Prof. Dr. R. Willemze. She also works as a volunteer at the Kruispost (Cross Aid Post) in Amsterdam providing medical care to patients who are uninsured and have no residence permit.





DANKWOORD - ACKNOWLEDGEMENTS

And I thank you
For bringing me here
For showing me home
For singing these tears
Finally I’ve found
That I belong here

-Depeche Mode, Home

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