Shared and persistent asymptomatic cutaneous human papillomavirus infections in healthy skin

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ABSTRACT

Cutaneous human papillomavirus (HPV) types are commonly found in normal skin, and some of them have been suspected to play a role in the development of non-melanoma skin cancer. This present study is divided into three sections, the aims of this study were to examine if certain HPV-types persist over time and if HPV-types are shared within families.

From the first part of the study, swab samples from foreheads were collected for three longitudinal studies from one family with a newborn baby. Five specific HPV-types were isolated from the family with a newborn, with HPV-5 and FA67 being found at various time points and prevalence rates in all four members of the family.

Part two consisted of a followed up study from two families with a 6 years interval. Six of the family members were found to have at least one of the HPV-types identified in the family six years earlier. Many of the HPV-types identified were shared within the families studied.

Part three of this study involved weekly samples from four healthy females for four months. Among the four healthy individuals, 11%, 65% and 56% of the weekly samples were HPV-DNA positive with one individual HPV-negative.

All specimens were tested for HPV-DNA by PCR using the broad range HPV-type primer pair FAP59/64. The positive samples were HPV-type determined by cloning and sequencing. Specific cutaneous HPV-types persist over long periods of time in healthy skin in most individuals investigated and certain HPV are shared between family members.

INTRODUCTION

The human papillomaviruses (HPVs) are commonly associated with infections of cutaneous and mucosal epithelia [de Villiers et al., 2004]. They are recognized as the causative agents of warts, including condyloma and common skin warts. Some types of HPV have also been suspected to play a role in the development of non-melanoma skin cancer [Akgul et al., 2006; Harwood et al., 1999]. Many studies have been conducted to investigate the carcinogenic potential of HPV in non-melanoma skin cancer patients, but the correlation still remains controversial. Even though HPV is known to cause benign lesions in skin and suspected in the development of non-melanoma skin cancer, HPV is present at high frequency in clinically healthy skin that do not show any symptoms of papillomavirus infections [Alotaibi et al., 2006; Antonsson et al., 2003a; Antonsson et al., 2000; Astori et al., 1998; Chen et al., 2008; Hazard et al., 2007].

Previous reports have shown that normal skin of healthy adults and children is infected with many different HPV types, and that colonization of the skin with HPV starts very early in life [Antonsson et al., 2003b]. In the present study, the aim was to explore whether asymptomatic HPV infections in normal skin persists over time.

MATERIALS AND METHODS

Subjects

This study consisted of three parts: Part 1, an investigation of one newborn baby, his 3-year old brother and two parents; Part 2, a follow-up study 6 years later of two previously studied families [Antonsson et al., 2003b]; and Part 3, a longitudinal study of four women.

Part 1.

In total, 60 specimens were collected for a longitudinal study from a Caucasian family of four living in Brisbane, Australia. This family consisted of a newborn son, a 3-year old son, a mother (34 years old) and a father (33 years old). The first sample from the newborn baby was collected when the baby was less than one minute old. Samples from all four family members were collected daily for twelve days, followed by once a month for two months and final samples were collected when the baby was 1 year old.

Part 2.

Forehead swab samples for a longitudinal study were collected from two Caucasian families of three in 2001 [Antonsson et al., 2003b]. The two families consisted of a newborn baby that was sampled directly after birth. Both families lived in the Southern part (Skåne) of Sweden. The baby boy was followed up with weekly samples for 8 weeks, while the baby girl had samples taken at day 1, 3, 5, 8, 13 and 21. One swab sample per parent was collected. Six years later (2007), one new swab samples per individual were collected from both families, both of which now consisted of four members. In 2007, the followed up newborn babies were 6 years old, they each had a younger brother aged 3 or 4 years and all four parents were 36 years old.

Part 3.

Four healthy volunteers (an Asian female, 21 years old; a Caucasian female, 27 years old; a Caucasian

female, 33 years old; and a Caucasian female 42 years old), living in Brisbane, Australia were studied longitudinally. Eighteen samples were collected weekly over four months.

Specimens

All samples were collected from the forehead with pre-wetted cotton-tipped swabs (Q-TIPS, Tyco Healthcare Group LP, Mansfield, MA, USA) soaked in sterile saline solution (0.9% NaCl) and drawn back and forth five times within an area of 3 cm by 5 cm, and then suspended in 1mL saline solution. Samples were frozen directly after collection and kept at -20°C until analysed.

PCR

FAP PCR

The 25 µL final volume of the PCR solution contained 0.75 or 1.0 µM of each primer (FAP59 and FAP64 [Forslund et al., 1999]; IDT, Coralville, IA, USA), 0.2 mM of each deoxynucleotide triphosphate base (dNTP; Roche, Mannheim, Germany), 0.2% bovine serum albumin (Fraction V, Sigma, Sydney, Australia), 2.5 µL of 10x PCR Gold buffer, 3.5mM MgCl₂, 0.625U AmpliTaq Gold DNA polymerase (Roche, Foster City, CA, USA), and 5µL of the sample or the control. Distilled H₂O was used as a negative control, and a HPV-18 positive clinical sample from a prostate cancer patient and a HPV-18 plasmid served as positive controls. The PCR reaction was carried out in a Thermo Hybaid Px2 PCR machine (Integrated Science, Sydney, Australia) with the following parameters: 10 minutes denaturation at 94°C, followed by 40 cycles of amplification at 94°C for 90 seconds, 50°C for 90 seconds, 72°C for 90 seconds and finally a hold step at 4°C.

Human L1 PCR

The 25 μL final volume of PCR solution contained 0.75 μM of each primer (Human L1 Forward and Reverse [Deragon et al., 1990]; Invitrogen, Auckland, New Zealand), 10 mM dNTP (Fisher Scientific, Perth, Australia), 1U NEB Taq DNA polymerase (New England Biolabs, Beverly, MA, USA), 2.5 μL of 10x Thermo Polymerase Buffer and 5 μL of the sample or control. For each PCR reaction, distilled H_2O was used as a negative control and DNA extracted HeLa cells as a positive control. PCR was carried out with the following parameters: 5 minutes denaturation at 94°C, followed by 40 cycles of amplification at 60°C for 30 seconds, 72°C for 30 seconds, and further elongation at 72°C for 5 minutes.

Gel electrophoresis

Amplified PCR products were analysed by electrophoresis in a 1.5% agarose gel containing TAE buffer and Ethidium Bromide (Sigma). PCR amplicons were size determined under UV light using the GelDoc software (Bio-Rad, Sydney, Australia).

HPV type determination

Cloning

Positive FAP PCR products were cloned using a TOPO TA cloning Kit Dual Promoter (Invitrogen, Carlsbad, CA, USA). Samples were ligated into pCR 2.1 TOPO cloning vector and transformed into Escherichia Coli TOP10 Competent Cells (Invitrogen) following manufacturer's specifications.

Insert PCR

Single bacteria colonies were picked using plastic loops from overnight LB-ampicillin plates and were diluted in 25 μL of H_2O , and the samples were then boiled at 99°C for 10 minutes. The clones with HPV DNA inserts were determined by insert PCR. A 25 μL reaction containing 2.5 μM of each primer (M13 Forward and Reverse) (Invitrogen), 10mM dNTP (Fisher Scientific), 2.5 μL of 10x Thermo Polymerase Buffer (New England Biolabs), 1U NEB Taq DNA polymerase and 5 μL of the boiled

sample was carried out with the following parameters: 4 minutes denaturation at 94°C, followed by 35 cycles of amplification at 94°C for 60 seconds, 45°C for 60 seconds, 72°C for 60 seconds and a final step at 30°C for 2 seconds.

DNA Purification

Bacterial colonies with HPV DNA inserts were incubated in 5mL LB broth at 37°C and shaken at 225rpm for 18 hours. Plasmid DNA was purified using the Fast Plasmid Mini-Kit prep (Eppendorf, Hamburg, Germany) following the manufacturers specifications. DNA concentration was measured at 260 and 280nm absorption using a Nanodrop ND-1000 Spectrophotometer (Biolabs, Mulgrave, Australia).

Sequencing analysis

A sequencing reaction contained 600 ng DNA, 1 µL of 3.25 µM M13 forward or reverse primer (Invitrogen, Australia) and H₂O to make the total volume of 10 µL. The prepared reaction was sent to Australia Genome Research Facility at the University of Queensland (Brisbane, Australia) for sequencing. Between one to five clones per HPV positive sample were analysed. Also, some samples that we had difficulties to clone were sequenced directly using FAP PCR products. Obtained DNA sequences were compared with those already established by using the BLAST server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Putative new types

Since the FAP PCR amplicons only represent part of the L1 gene, the new HPV sequence identified in this paper is called a putative new HPV type instead of HPV type. The new putative HPV types were named FADI followed by a consecutive number (where FA stands for the primer pair used and DI for the institute, i.e. Diamantina Institute, Brisbane, Australia). The two putative new HPV types, FADI2 and FADI3, have been submitted to GenBank and has the accession number FJ407049 and FJ480954, respectively.

RESULTS

HPV type spectrum and persistence in a family with a newborn

Altogether, of the sixty samples analysed, thirty-one were HPV DNA positive and of those, twenty-five were successfully HPV type determined. Four out of fifteen samples from the newborn baby, eleven out of fifteen from the elder son, ten out of fifteen from the mother and six out of fifteen from the father were positive for HPV DNA when analyzed with the FAP primer pair. A total of five specific HPV types were detected from the family members (Fig. 1). All sixty samples were positive for human L1, suggesting that the samples did not contain any PCR inhibitory agents.

Three out of four HPV DNA positive samples from the newborn baby were successfully cloned and HPV type determined. The swab collected directly after birth was HPV DNA negative and samples collected the two following days continued to be negative. The first HPV DNA positive samples were from day four. Altogether, three HPV types were detected from the baby (Fig. 1), HPV-24 (day 4), HPV FA67 (2 months) and HPV-5 (1 year). Seven out of eleven HPV DNA positive samples from the 3-year-old son were successfully cloned and HPV type determined, and three different HPV types (HPV-5, HPV-24 and HPV FA67) were detected. Multiple types were also detected from the sample collected day 3, where all three different HPV types were identified. The samples from the mother contained HPV-5 and HPV FA67 and from the father; HPV-5, HPV-20, HPV FA67 and HPV FA123. The children shared two HPV types (HPV-5 and HPV FA67) with their parents, while HPV-24 was found in both the children, but not in their parents. HPV-20 and HPV FA123 were detected only in the father's samples.

Across this longitudinal study, specific HPV types were constantly detected from the same family members, which indicate persistence of cutaneous HPV types on normal skin. The most prevalent HPV types found from the older son, the mother and the father were, 40% of HPV-5, 60% of HPV

FA67 and 20% of HPV-5, respectively (Table I). The prevalence for each of the three different HPV types found on the newborn baby was 6.7%.

Persistence of HPV types that are acquired early in infancy

HPV types found in samples collected from two newborn babies and their families in 2001 [Antonsson et al., 2003b] were compared to new samples collected in 2007 (Fig. 2).

The first family consisted of four members; a 6-year-old boy, a 3-year-old boy, a mother and a father. The specimen from the 6-year-old boy contained HPV-12 (Fig. 2A). HPV-12 was not detected in any of his nine samples collected weekly from birth, but HPV-12 was detected in a sample from his mother in 2001. The 6-year-old boy now had a 3-year-old brother, and HPV-17, HPV-50 and HPV-80 were identified in his sample. HPV-80 was found in his older brother when he was a baby, while HPV-17 and HPV-50 seem to have been introduced in the family since the first study was done. HPV-5, HPV-12, HPV-17, HPV-20 and HPV-80 was identified in the mother's samples. Detection of HPV-5 and HPV-12 seems to be a long term persistent infection since they were detected in the mother's sample in the first study, while HPV-20 and HPV-80 were found in samples from her oldest son in the previous study. HPV-80 was the only type that was identified in the samples obtained from the father.

The second family to be followed up comprised of a 6-year-old daughter, a 4-year-old son, a mother and a father. None of the three HPV types (HPV FA43, HPV FA89 and the putative new HPV type FADI3) that were found in the samples from the 6-year-old daughter were detected in the family previously or in any other of the family members in the 2007 samples (Fig. 2B). The 4-year-old boy's sample contained HPV-50 and HPV FA120. HPV-50 was identified in the first study (before he was born) in his sister and father. The mother was the only family member with a persistent HPV type (HPV FA50) in this study, a putative new HPV type FADI2 was also identified in her sample. The father in this family was negative for HPV DNA with FAP PCR in this follow up study.

Longitudinal HPV persistence study in four women

We wanted to determine the persistence of HPV in a single individual and to investigate if certain HPV types could be constantly detected from the same person over time. Therefore, eighteen weekly forehead skin swabs were collected from four healthy females over four months.

HPV DNA was not detected from any forehead skin swab sample from Participant 4 (aged 42 years), while Participant 1 (aged 21 years) was rarely positive for HPV DNA (11%; 2/18). On the other hand, Participant 2 (aged 27 years) and Participant 3 (aged 33 years) were more frequently HPV DNA positive, and HPV DNA was detected in 65% (11/17) and 56% (10/18), respectively in the skin swabs (Fig. 3). In order to determine the identity of each HPV type present in the samples, two to four HPV-positive FAP PCR products from each participant were HPV type determined. HPV FA22 was detected from one of the two HPV DNA positive forehead swab samples from Participant 1. From four out of ten HPV DNA positive forehead skin swab samples from Participant 3, HPV FA67 was constantly detected over the four-month period. Four of the eleven positive forehead samples from Participant 2 were HPV typed and, HPV FA38 was detected in one sample, while both HPV FA39 and HPV FAIMVS15.1 DNA were detected in three samples.

DISCUSSION

The data collected during the present study, suggest that initial HPV infection is acquired from family members and that specific cutaneous HPV types are shared among the members of a family. Furthermore, the results from the newborn baby confirm previous findings that acquisition of HPV occurs at an early stage of life and that HPV's are shared often with close family members [Antonsson et al., 2003b]. The newborn baby was positive for HPV DNA four days after birth. This newborn baby was placed in an incubator when he was seven hours old and stayed there for 72 hours. This might explain why HPV was not detected during the first days, since only momentary skin interactions took place. Apart from HPV 24, which is only shared between the two children, HPV 5 and HPV FA67 were detected in all four family members, suggesting particular HPV types are shared among family members. It would be interesting to observe the newborn baby over time to note whether an increased prevalence of HPV or more significant numbers of common types may be found.

A previous study [Hazard et al., 2007], have shown that cutaneous HPV types commonly persist for many years on normal skin. Another study [de Koning et al., 2007], conducted over a two year period with plucked eyebrow hairs from 23 healthy adults focusing on β -HPV infection, found that 22 individuals were β -HPV-positive at least once during follow-up. β -HPV type-specific persistence over a period of at least six months was observed in 74% of the participants, and 57% of them had multiple β -HPV types in the samples. These longitudinal studies suggest that multiple HPV types can persist concurrently in normal skin over months and even years. Specific HPV types were detected constantly on the same individual's forehead skin for months, and multiple HPV types were detected persistently from one of the participants. It is also interesting to note that individuals appear to acquire one dominant HPV type, as seen more evidently from the mother where HPV FA67 is detected more frequently than other HPV types. The reason for this remains unclear, but this could be because a virus-host "match" occurs that is different for each individual and HPV type and also explains the

variety of persistent HPV types even amongst families that live close together sharing the same environment.

Certain cutaneous HPV types including HPV-5, HPV-8, HPV-20 and HPV-77 have been shown to be activated by UV radiation [Akgul et al., 2005; Michel et al., 2006; Purdie et al., 1999], and higher cutaneous HPV prevalence has been detected in healthy males who work outdoors in strong UV radiation (Queensland, Australia) [Chen et al., 2008]. These results suggest that multiple cutaneous HPV types persist in normal skin in countries with strong UV radiation (e.g. Australia), as well as in countries with less UV radiation (e.g. Sweden). It is also noted that HPV types persists over seasonal change. Therefore, cutaneous HPV types seem to persist in healthy individuals disregarding differences in amount of sun exposure.

To date, the interaction between the virus and the host is vaguely understood, although previous studies have shown that uptake of the virus particles take place within two hours [Day et al., 2003; Muller et al., 1995]. The duration for an HPV infection to be established and how the virus particles are shed to enter the skin remains unknown. It has been demonstrated that multiple types of HPV DNA can be detected from plucked eyebrows [Boxman et al., 1997], however, questions still remains as to whether hair follicles are reservoirs for HPV leading to persistent infection of the virus.

Due to the limited information about the transmission of cutaneous HPV types and the mechanisms of cutaneous HPV in their hosts, there is a need to determine the transmission pathway of cutaneous HPV, and to understand the virus-host interactions between cutaneous HPV and humans.

The majority of individuals positive for cutaneous HPV infections are not recognized to manifest any clinical symptoms, which may imply that HPV is part of the microbiological flora on healthy human skin just like bacteria. The exact nature of the lingering relationship of the virus on the skin is still unclear, therefore it is difficult to conclude if the relationship is mutual, harmful or both.

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Figure legends

Fig. 1. HPV types found in the four family members at different time points. No samples were on collected day 8. Filled box indicates a determined HPV type.

Fig. 2. HPV types detected in a follow-up study of two newborn babies and their families. HPV types in samples collected in early infancy are compared to samples collected when the children are 6-years-old. Families analysed a) samples from a boy that was sampled weekly from birth. b) samples from a girl that was sampled at day 1, 3, 5, 8, 13 and 21. Results from 2001 has been summarised from [Antonsson et al., 2003b]

Fig. 3. HPV DNA findings in the four female participants from whom weekly swabs were obtained for four months

Coloured box indicates a HPV positive sample

Non-coloured box indicates a HPV negative sample

- no sample was collected on the time point

+ the sample was cloned and sequenced

Tables

TABLE I. The most prevalent HPV types found in each individual family member.
n=15 samples/subject

HPV type/subject	Newborn	3-year-old son	Mother	Father
HPV 5	7 % (1/15)	40% (6/15)	13% (2/15)	20% (3/15)
HPV 20				13% (2/15)
HPV 24	7 % (1/15)	13% (2/15)		
HPV FA67	7 % (1/15)	13% (2/15)	60% (9/15)	13% (2/15)
FA123				13% (2/15)

Fig. 1

Fig. 2A and B

Fig. 3

	21 yr old female	27 yr old female	33 yr old female	42 yr old female
21-Feb		+		-
28-Feb				
7-Mar			+	
14-Mar	+			
21-Mar	-	-	-	
29-Mar				
4-Apr		+	+	
11-Apr				
18-Apr				
25-Apr				
2-May				
9-May				
16-May		+	+	
23-May				
30-May				
6-Jun	+			
13-Jun		-		-
20-Jun				
27-Jun		+	+	