Subtype HPV38b[FA125] Demonstrates Heterogeneity of Human Papillomavirus Type 38.

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Abbreviations: *aa* amino acid; *AK* actinic keratosis; *BCC* basal cell carcinoma; *CV* coefficient of variation; *EV* epidermodysplasia verruciformis; *HPV* human papillomavirus; *NMSC* non-melanoma skin cancer; *ORF* open reading frame; *RCA* rolling circle amplification; *SCC* squamous cell carcinoma; *SK* seborrhoeic keratosis; *URR* upstream regulatory region.

Category: Infectious Causes of Cancer

We report that HPV38 is heterogeneous and have cloned and sequenced a major subtype (HPV38[FA125]).

Both prototype HPV38 and HPV38b[FA125] were more common in skin lesions than in healthy skin, although the viral loads were very low.

SUMMARY

The human papillomaviruses (HPVs) exist as more than 100 distinct types. While variants of HPV are common, only few HPV subtypes have been reported.

HPV type 38 has been proposed to be associated with non-melanoma skin cancer (NMSC), with reported prevalences of up to 55%. A subtype of HPV38 was cloned, completely sequenced and found to have a 96% sequence similarity to prototype HPV38 in the L1 open reading frame. The presence of prototype HPV38 and HPV38b[FA125] was examined in paired biopsies of tape-stripped skin lesions and healthy skin from 269 immunocompetent patients by real-time PCR. Prototype HPV38 and HPV38b[FA125] were present in seven (3%) and five (2%) lesions, respectively, in viral loads ranging from one copy per 150 cells to one copy per 70 000 cells. In summary, we found that HPV38 is heterogeneous and is one of so far only few HPVs that contain subtypes. The heterogeneity needs to be considered in studies of the biology of this virus.

INTRODUCTION

Non-melanoma skin cancers (NMSC) are the most prevalent malignancies in the Caucasian population [1] and an increase in prevalence has been seen in European countries during the last few years [2]. NMSC comprises basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). BCC has a relatively good prognosis, while SCC accounts for up to 20% of all deaths from skin cancer [3].

NMSC are often found on sun-exposed sites in patients with the rare inherited genetic disease epidermodysplasia verruciformis (EV) [4]. These patients are highly susceptible to human papillomavirus (HPV) infections [5, 6] mainly by the *Beta-papillomaviruses*, a phylogenetic subgroup of cutaneous HPVs (e.g., HPV5 and HPV8) [7]. These HPV types are detectable in skin carcinomas, as well as in control specimens of healthy skin from the general population [8-12].

There are more than 100 different HPV types that have been completely sequenced [7]. In addition, there appears to exist about a hundred additional putative HPV types, with sequence information so far available only from PCR amplimers [8, 13-16]. Variants of HPV types contain more than 98% sequence homology in the L1 open reading frame (ORF) [7]. Variants is a common finding that appears to associate with ethnic groups and sometimes even with biologic behaviour of the virus [17, 18]. By contrast, subtypes containing 90-98% sequence homology in the L1 region are very rare, with HPV 68a/HPV68b, HPV46, HPV55 and HPV64 (now known to be subtypes, originally thought to be different viruses) being the only fully characterised examples [7, 17]. Although the reasons for this are not known, existence of subtypes has profound implications for design of virus detection systems and for understanding of the biology of the viruses.

Epidemiological studies have established a causal association between UV-radiation and NMSC [3], but the role of HPV in these cancers remains to be elucidated.

A recent study [19] has demonstrated that the early proteins E6 and E7 of HPV38 are sufficient to substantially increase the lifespan of primary human keratinocytes by deregulation of the cell cycle control. Furthermore, HPV38 E7 targets the retinoblastoma protein (pRb) with about the same efficiency as HPV16 E7 [19, 20].

HPV38 has been proposed to be a skin cancer-associated HPV type based on reported prevalences of 7% to 55% in BCC, 13% to 46% in SCC and 17% to 43% in actinic keratosis (AK) [19, 21-23]. In comparison, HPV38 has been detected in 10% of healthy skin biopsies [19] and in 25% of plucked eyebrow hairs [24]. A possible reason for the variability in reported HPV DNA prevalences is the fact that the skin surface contains high amounts of viral DNA, whereas the skin lesions themselves contain less viral DNA [16]. For example, actinic keratosis demonstrated an HPV DNA prevalence of 70% in non-stripped biopsies [21] and 11% in stripped biopsies [16]. We have therefore proposed the use of tape stripping of the skin surface before taking biopsies as a method of increasing the likelihood that the reported presence of HPV DNA is indeed related to the skin lesion itself [16]. Two commonly used general primer PCR systems for detection of cutaneous HPVs are the FAP [13] and nested FAP [25]. Employing these PCRs, with subsequent cloning and sequencing of amplicons, a closely related HPV38 sequence, isolate FA125 (GenBank accession no. AY468422) [16], was found in 16 samples from 978 stripped skin biopsies, whereas the prototype HPV38 was not detected (Forslund, unpublished data).

In the present study, our aims were to sequence the complete genome of the FA125 isolate, to determine its relatedness to HPV38, to design specific PCRs for HPV38 and FA125 with equal sensitivities, and to explore the presence of HPV38 and the putative subtype FA125 in skin lesions of SCC, BCC, AK, seborrhoeic keratosis (SK) and in paired healthy skin.

MATERIAL AND METHODS

Overlapping amplicons. (i) A stripped punch biopsy taken from the forehead of an immunocompetent 78-year-old male with the diagnosis of AK was positive for FA125, a putative subtype of HPV38 [16]. FA denotes an HPV sequence of about 450 nucleotides in the L1 ORF amplified with the FAP 59/64 primers [13]. **(ii)** The same sample was then used as template in a PCR using the forward primer FA125.205F (5'-ACAGGTCATCCCTTATTTA AC-3') (DNA Technology A/S, Aarhus, Denmark) and a degenerate reverse primer EVE7 (5'-GTRRCYTSTTTHCCAA TCAT-3') (DNA Technology). The 50 µl PCR solution contained 5 µl of sample, 0.5 µM of each primer, 0.2 mM of each dNTP (Amersham Pharmacia Biotech, Buckinghamshire, U.K.), 0.2% BSA (Fraction V, Sigma-Aldrich, Steinheim, Germany), 3.5 mM $MgCl_2$ (Expand High Fidelity PCR System, Roche, Mannheim, Germany), 1x HF buffer (Roche), and 1.2 U of Expand enzyme (Roche). The PCR was performed in an Eppendorf Thermocycler (Eppendorf, Hamburg, Germany) with simulated tube control using 2 min at 94°C, 10 cycles of 15 sec at 94°C, 30 sec at 50°C and 3 min at 68°C, followed by 30 cycles of 15 sec at 94°C, 30 sec at 50°C and 3 min at 68°C, with an extra 5 sec per cycle. The amplified DNA was separated by electrophoresis in a 1.5% agarose gel (Sigma) in TBE buffer [26], and a band of the expected size (2.2 kb) was excised, purified (QIAquick Gel Extraction kit, QIAGEN, Hilden, Germany) and cloned (TOPO TA Cloning® kit, Invitrogen, Leek, The Netherlands). **(iii)** To generate the remaining part of the genome (~5 kb), the sample was subjected to multiply-primed rolling circle amplification (RCA) with the TempliPhi 500 amplification kit (Amersham Biosciences AB, Uppsala, Sweden) according to the manufacturer's instructions, with an addition of extra dNTP (900 µM) [27].

The amplified DNA was then used as template in an Expand Long Template PCR System (Roche). The 25 µl PCR solution contained 2.5 µl of the RCA product diluted 1:10 in water,

0.3 µM of each primer: FA125.572F (5'-AAGCATTTCACAAGGTTAGAG-3') (Cybergene AB, Huddinge, Sweden) and FA125.154R (5'-CCACACCAACC TATGTTTATC-3) (DNA Technology), 0.35 mM of each dNTP (Amersham Pharmacia Biotech), 1x Buffer I, and 1.875 U of Expand Long Template Enzyme mix. The PCR was performed in a Hybaid PCR Express (Hybaid Limited, Middlesex, UK) with simulated tube control using 2 min at 94°C, 10 cycles of 10 sec at 94°C, 30 sec at 53°C and 6 min at 68°C, and then 20 cycles of 15 sec at 94°C, 30 sec at 53°C and 6 min at 68°C with an extra 20 sec per cycle, followed by a final incubation at 68°C for 7 min. The amplified DNA was separated by electrophoresis in a 1% IBI Agarose gel (Kodak Company, New Haven, CT, USA) in TBE buffer, and a band of approximately 5 kb was excised, purified (QIAquick Gel Extraction kit, QIAGEN), and cloned as described above.

Sequence analysis. All cloned fragments were sequenced according to ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, Foster City, California, USA) and analysed on a 3730 automated sequencer (Applied Biosystems).
The amplified DNA fragments were used as templates in primer walking, overlapping, sequencing reactions.

The ORF of each protein coded for by HPV38b[FA125] was identified using BioEdit version 7.0.1 software [28] and compared to available amino acid sequences in the GenBank database using the BLAST server. SignalScan version 4.0 software [29] was used to compare the binding sites for transcriptional regulatory factors [30] in HPV38 and HPV38b[FA125].

Subjects and samples. Stripped lesions and healthy skin biopsies [16] from patients with the diagnoses AK (n = 52), SK (n = 47), BCC (n = 118), or SCC (n = 52) were collected from 269 immunocompetent patients attending dermatology clinics in Sweden. The samples were selected from a larger series of 489 patients, in order to arrive at a matched subsample, where all four skin lesion groups were matched for age and sex. All four groups had an average age

of 77 years, ± 1 year. The distribution between men and women was 55% and 45% respectively, ± 1%, within each group, except for the SCC group that had slightly more men (58%).

The DNA from each biopsy was extracted and dissolved in 200 µl TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) using a simple phenol-free method [13]. To ensure the quality of the extracted DNA, all samples were analysed for the presence of the *β-globin* gene [16] and found to be positive. Swab samples from the tops of the lesions (before stripping) were collected in 1 ml saline [16].

Detectability of prototype HPV38 and HPV38b[FA125] by commonly used PCR primer systems. The sensitivities of the FAP and nested FAP PCRs for detecting HPV38 and FA125 were tested. Purified viral DNA from plasmids containing the complete genome of HPV38 and the FAP fragment of FA125 were used as templates. Serial logarithmic dilutions covering a range of 6 logs (100 000 copies to one copy per sample), were prepared in human placental DNA (10 ng/µl). Similar serial logarithmic solutions of HPV38 and the 2.2 kb fragment of FA125 were used as templates using the HPV38 type-specific primers presented by Caldeira *et al.* [19]. The 25 µl PCR solution contained 5 µl of sample, 0.5 µM of each primer, 0.2 mM of each dNTP (Amersham Pharmacia Biotech), 0.2% BSA (Sigma-Aldrich), 3.5 mM $MgCl_2$ (Applied Biosystems), 1x PCR Buffer II (Applied Biosystems), and 0.625 U of AmpliTaq Gold (Applied Biosystems). The PCR was performed in a Hybaid PCR Express machine (Hybaid) with simulated tube control using 10 min at 94°C, and then 45 cycles of 1 min at 94°C, 1 min at 49°C and 2 min at 72°C. The amplified DNA was separated by electrophoresis in a 1.5% agarose gel (Sigma) in TBE buffer [26].

Real-time PCR. Primers and probes for HPV38 and HPV38b[FA125] were designed in the L1 ORF, using the Primer Express 2.0 software program (Applied Biosystems).

Calculations of viral copy numbers for the standard curves were based on spectrophotometric measurement of purified viral DNA from a plasmid containing the complete genome of HPV38 and the 2.2 kb fragment of HPV38b[FA125]. For the establishment of standard curves, serial logarithmic dilutions covering a range of 6 logs (100 000 copies to one copy per sample) were employed using a solution of human placental DNA (10 ng/µl) as diluent. The HPV38 and HPV38b[FA125] primers and probes were tested for cross-hybridisation with closely related types within species two of the *Beta-papillomaviruses* [7]. The 25 µl PCR mixture contained 3.5 mM $MgCl_2$ (Applied Biosystems), 1x Reaction Buffer (Applied Biosystems), 0.2 mM of each dNTP (Roche Diagnostics GmbH), 0.2 µM of each primer (HPV38F: 5'- CAGGCCTATGTCCTCCACTAGAA-3', HPV38R: 5'- GTTTTGTTGTTTAT ATTGCCAAATCC-3' or FA125F: 5'-TTGAAGATGGAGACATGTTTGATATAGG-3', FA125R: 5'- TAACGATATCCAAACTTACATCAGATTG-3') (Cybergene), 0.04 µM Taqman probe (HPV38 Probe: 5'-FAM-GAAAAATAGTGTAATTGAAGACGGAGACAT GTTTGATAT-TAMRA or FA125 Probe: 5'-FAM-TGGCAACATAAACAACAAAACACT TTCT-TAMRA) (Cybergene), 0.625 U of AmpliTaq Gold™ polymerase (Applied Biosystems) and 2.5 µl of sample. The real-time PCR was carried out in a GeneAmp® 5700 SDS (Applied Biosystems), using the following parameters: 2 min at 50°C, 10 min at 95°C and then 50 cycles of 15 sec at 95°C and 1 min at 60°C (HPV38b[FA125]) or 63°C (HPV38). Human placental DNA (Sigma) (10 ng/µl) and water (Sigma) were used as non-template controls in each run. The same parameters were used for the patient samples, which were diluted 1:2 in TE buffer before analysis.

All patient samples positive for HPV38 or HPV38b[FA125] in the real-time PCRs were verified in triplicate using undiluted samples. If a sample was positive in at least two of three verifying analyses, it was considered positive. The coefficient of variation (CV) for each

sample was based on these values. To confirm the results further, all amplicons from the verification PCRs were cloned and sequenced.

In order to determine the viral load relative to number of cells, the samples were analysed for the presence of the *β-globin* gene. Calculations assumed that 6.6 pg DNA is present per diploid cell. The quantitative PCR was performed as above, with an annealing temperature of 60°C using the PCO3 and PCO4 primers [31] and probe (*β-globin* Probe: 5'-FAM-TGCACCTG ACTCCTGAGGAGAA-TAMRA-'3). Serial logarithmic dilutions covering a range of 6 logs were used in the standard curve, as above.

For all biopsy samples positive for HPV38 or HPV38b[FA125], a swab sample taken from the top of the lesion before stripping was also analysed. The swab sample was only analysed for the presence of the same type or subtype as detected in the corresponding biopsy.

This study was approved by the Ethical Committees of Karolinska Institute and of Lund University, Sweden. All patients gave informed consent.

RESULTS

Complete genome of HPV38b[FA125]. The complete genome of HPV38b[FA125] (GenBank accession no. DQ090005) contained 7400 bp, which correlates with the size of HPV38. The E6 and E7 proteins showed 100% similarity to HPV38, whereas the E4 protein only showed 94% similarity (Table 1). Each of the other proteins encoded by HPV38b[FA125] showed high similarity to the corresponding proteins of HPV38 (Table 1). At the nucleotide level, the ORFs of L1 and L2 showed 96% sequence similarity to HPV38 (Table 1). The nucleotide sequence of the upstream regulatory region (URR) of HPV38b[FA125], located immediately downstream of the stop codon of the L1 protein to the first ATG in the E6 ORF, was 405 bp. The URR had 96% sequence similarity to the equivalent region of HPV38 (Table 1). Five of the 15 nucleotides at the 3'-end of the URR were mismatches between HPV38 and HPV38b[FA125]. However, this difference did not affect any known binding sites for transcriptional regulatory factors, as detected with the SignalScan software.

In the URR of HPV38b[FA125], there was an insertion of a guanine between positions 163 and 164 (numbered according to the prototype HPV38 genome). This insertion generated a shift in the open reading frames as compared to HPV38. There was also a deletion of a thymine in the HPV38b[FA125] genome, situated at position 4011, which was in a non-coding region between the ORFs of the E2 and L2 proteins. Because of this deletion, the L2 ORF of HPV38b[FA125] was shifted in relation to the early ORFs.

The complete L1 ORFs of HPV38 and HPV38b[FA125] have a coding capacity of 539 and 564 amino acids (aa), respectively. However, if we consider the typical splice acceptor site prior to the initiator ATG, described for some papillomaviruses [32], both L1 proteins would be the same length (510 aa).

Eight amino acids of the L1 ORF differed between HPV38 and HPV38b[FA125] (Table 2). Comparison with the three-dimensional structure of the L1 ORF of HPV16 [33], showed that six of the altered amino acids were most likely positioned in the hypervariable loops that extend towards the HPV capsid surface (Table 2). The non-conservative substitution at position 138 in HPV38 was in the position immediately before the surface-exposed hypervariable residue 138 in the DE loop of HPV16. Similarly, the substitution at position 291 was immediately before the surface-exposed hypervariable residue 285 in the FG loop of HPV16, which has been implicated as an important residue for binding of a major neutralising antibody [34, 35]. The possible surface exposure of the adjacent substitution at residue 288 in HPV38 is difficult to predict since the FG loop of HPV38 contains an insertion of seven amino acids seven residues before this position, compared to that of HPV16.

Real-time PCR. Plasmid titration series showed that the real-time PCR assays for both HPV38 and HPV38b[FA125] had equal, high sensitivities of approximately one copy per sample.

Plasmid titration series ranging from 100 000 copies down to one copy per sample of cloned plasmids of HVP types 9, 15, 17, 22, 23, and 37 were all negative in real-time PCR using the specific primers and probes for HPV38 and HPV38b[FA125]. Further, no cross-reactivity was detected between HPV38 and HPV38b[FA125] using the specific primers and probes.

HPV38 was detected in 7 (2.6%) of 269 lesions; three from SCC patients (viral loads: 1 copy/142 cells; 1/1107 cells; 1/12875 cells), one from BCC (1/67407 cells), two from SK (1/167 cells; 1/1960 cells) and one from an AK patient (1/349 cells) (Table 3).

HPV38b[FA125] was detected in 5 (1.9%) of the 269 lesions; two from SK (viral loads: 1/524 cells; 1/12336 cells) and three from AK (1/183 cells; 1/600 cells; 1/1849 cells) (Table 3). Two of 269 healthy skin biopsies (0.7%) were positive for HPV38b[FA125] (viral loads: 1 copy/133 cells; 1/254 cells), while none was positive for HPV38 (Table 3). Only one patient

was positive for the same virus type both in the lesion and in the healthy skin sample (Table 3). None of the samples were positive for both HPV38 and HPV38b[FA125].

Nine out of 12 swab samples, taken from the tops of lesions that were found to be HPV38 or HPV38b[FA125] positive, were positive for the same virus as in the lesion (Table 3).

The *β-globin* gene was detected in all lesion and healthy skin biopsies that were positive for HPV38 or HPV38b[FA125]. However, only 50% (6/12) of the swab samples from the tops of the lesions were *β-globin* positive (Table 3). Therefore, virus copies per µl sample solution were calculated, ranging from two viral copies per µl to 130 viral copies per µl (Table 3).

Detectability of prototype HPV38 and HPV38b[FA125] by commonly used PCR primer systems. Because testing with the FAP and nested-FAP primers, which were specifically designed to detect as many of the *Beta- and Gamma-papillomaviruses* as possible, had shown 16 of 978 samples positive for isolate FA125, but none for prototype HPV38, we evaluated the comparative sensitivity of these PCR systems using serial dilutions of cloned plasmids in the presence of human DNA. The FAP PCR system was capable of detecting approximately 10 copies per sample of HPV38b[FA125], but only around 100 copies per sample of prototype HPV38. The nested-FAP PCR, however, detected both HPV38 and HPV38b[FA125] at one copy per sample. The HPV38 type-specific PCR system of Caldeira *et al*. [19] was also tested and detected approximately 100 copies per sample of prototype HPV38, and around 1000 copies per sample of HPV38b[FA125].

DISCUSSION

In this study, we report that HPV38, one of the major HPV types suspected to be involved in NMSC, also exist as at least one subtype in addition to the prototype virus. The complete genome of this subtype, HPV38b[FA125], was sequenced and found to show 96% homology to HPV38.

Berkhout *et al*. have reported detection of two other putative subtypes of HPV38 [36]. The full genomic sequence of these putative subtypes was not reported, but by comparison of the reported PCR amplimer sequences, we can say that neither of them are identical to HPV38b[FA125]. In addition, one subtype has been reported for HPV36 [37], and HPV5 has been found to have several subtypes [38, 39], suggesting that subtypes may be more common among cutaneous HPV genotypes than among other HPVs. Such heterogeneity should be taken into account when designing new detection systems for a genotype.

Since the E6 and E7 proteins of HPV38 and HPV38b[FA125] were completely homologous, differences in possible carcinogenicity between these two types is not likely. Rather, our results indicate differences in the immunology and/or biology of the prototype and its subtype. The heterogeneity of HPV38 affected the E4 protein in particular, which is known to be expressed in very large amounts by some cutaneous HPV types [40]. The E4 protein is also thought to be a major target of CD4+ responses to HPV [41]. The fact that the amino acid substitutions in the L1 protein were clustered in the surface-exposed loops of HPV38b[FA125] are also suggestive of a possible immunological significance of the subtype heterogeneity. While it is well known that HPV variants within the same type constitute the same serotype [42] and that different HPV types typically constitute different serotypes [43], it is not well known whether subtypes are usually serologically distinct or not [39].

Even when combining the prevalences of HPV38 and HPV38b[FA125], we found considerably lower prevalences of these viruses in skin lesions than many other studies that

did not use stripping of the skin surface. For example, only 4 of 52 patients (7.6%) diagnosed with AK were positive for HPV38 or HPV38b[FA125] in our study, compared to previously reported HPV38 prevalences of 17% to 43% [19, 21, 22]. For SCC, we detected HPV38 in three of 52 biopsies (5.8%), compared to 13% to 46% in other studies [19, 21]. The greatest difference was found for patients diagnosed with BCC, where we only detected HPV38 or HPV38b[FA125] in two of 118 patients (1.7%), compared to previous reports with positivity rates ranging from 7% to as high as 55% [19, 21, 23]. This discrepancy is likely to be attributable to the removal of superficial cells with tape, which is known to reduce HPV DNA prevalences [16].

The virus DNA load was higher in the swab samples taken from the tops of the lesions than in the corresponding biopsies, indicating that most of the HPV38 DNA was present on top of the lesions and only to a minor extent in the lesions themselves. The fact that the *β-globin* gene was not detectable in several swab samples that had detectable HPV DNA suggests that the DNA in virions is more protected from degradation than human cellular DNA [8].

We found that the HPV38 type-specific primers used by Calderia (2003) and Forslund (2003) also detected HPV38b[FA125], although with a lower sensitivity than for HPV38. This difference is probably due to two mismatches in the reverse primer to HPV38b[FA125], whereas a complete match exist for HPV38. The nested-FAP PCR contains one mismatch in each of the reverse primers to HPV38, but was equally sensitive for HPV38 and HPV38b[FA125].

Of the 16 samples (of 978 tested) that were positive for FA125 in the previous study using general FAP and nested FAP PCRs (Forslund, unpublished data) 12 samples were also analysed in this study. However, only seven (58%) of these 12 samples were positive for HPV38b[FA125] in the specific real-time PCR. A possible explanation is the fact that the

viral load in these samples is so low that there is a considerable likelihood that different aliquots of the same sample may or may not contain a viral genome copy merely by chance. In conclusion, our characterisation of HPV38b[FA125] demonstrates that HPV38 shows heterogeneity. Both prototype HPV38 and HPV38b[FA125] were more commonly found in skin lesions than in healthy skin. However, there were higher copy numbers on top of the skin lesions than in the lesions themselves. Since both HPV38 and HPV38b[FA125] were found at low prevalences and with low viral loads, the role of HPV38 in the development of NMSC remains elusive,

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REFERENCES

1. Stern RS. The mysteries of geographic variability in nonmelanoma skin cancer incidence. Arch Dermatol 1999;135:843-4.
2. MacKie RM. Awareness, knowledge and attitudes to basal cell carcinoma and actinic keratoses among the general public within Europe. J Eur Acad Dermatol Venereol 2004;18:552-5.
3. Ramos J, Villa J, Ruiz A, Armstrong R, Matta J. UV dose determines key characteristics of nonmelanoma skin cancer. Cancer Epidemiol Biomarkers Prev 2004;13:2006-11.
4. Jablonska S, Majewski S. Epidermodysplasia verruciformis: immunological and clinical aspects. Curr Top Microbiol Immunol 1994;186:157-75.
5. Orth G, Jablonska S, Favre M, Croissant O, Jarzabek-Chorzelska M, Rzesa G. Characterization of two types of human papillomaviruses in lesions of epidermodysplasia verruciformis. Proc Natl Acad Sci U S A 1978;75:1537-41.
6. Kremsdorf D, Favre M, Jablonska S, Obalek S, Rueda LA, Lutzner MA, Blanchet-Bardon C, Van Voorst Vader PC, Orth G. Molecular cloning and characterization of the genomes of nine newly recognized human papillomavirus types associated with epidermodysplasia verruciformis. J Virol 1984;52:1013-8.
7. de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H. Classification of papillomaviruses. Virology 2004;324:17-27.
8. Antonsson A, Erfurt C, Hazard K, Holmgren V, Simon M, Kataoka A, Hossain S, Hakangard C, Hansson BG. Prevalence and type spectrum of human papillomaviruses in healthy skin samples collected in three continents. J Gen Virol 2003;84:1881-6.
9. Pfister H, Ter Schegget J. Role of HPV in cutaneous premalignant and malignant tumors. Clin Dermatol 1997;15:335-47.
10. Harwood CA, Proby CM. Human papillomaviruses and non-melanoma skin cancer. Curr Opin Infect Dis 2002;15:101-14.
11. Astori G, Lavergne D, Benton C, Hockmayr B, Egawa K, Garbe C, de Villiers EM. Human papillomaviruses are commonly found in normal skin of immunocompetent hosts. J Invest Dermatol 1998;110:752-5.
12. Li YH, Chen G, Dong XP, Chen HD. Detection of epidermodysplasia verruciformis-associated human papillomavirus DNA in nongenital seborrhoeic keratosis. Br J Dermatol 2004;151:1060-5.
13. Forslund O, Antonsson A, Nordin P, Stenquist B, Hansson BG. A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. J Gen Virol 1999;80 (Pt 9):2437-43.
14. Antonsson A, Forslund O, Ekberg H, Sterner G, Hansson BG. The ubiquity and impressive genomic diversity of human skin papillomaviruses suggest a commensalic nature of these viruses. J Virol 2000;74:11636-41.
15. Antonsson A, Karanfilovska S, Lindqvist PG, Hansson BG. General acquisition of human papillomavirus infections of skin occurs in early infancy. J Clin Microbiol 2003;41:2509-14.
16. Forslund O, Lindelof B, Hradil E, Nordin P, Stenquist B, Kirnbauer R, Slupetzky K, Dillner J. High prevalence of cutaneous human papillomavirus DNA on the top of skin tumors but not in "Stripped" biopsies from the same tumors. J Invest Dermatol 2004;123:388-94.
17. Calleja-Macias IE, Kalantari M, Allan B, Williamson AL, Chung LP, Collins RJ, Zuna RE, Dunn ST, Ortiz-Lopez R, Barrera-Saldana HA, Cubie HA, Cuschieri K, et

al. Papillomavirus subtypes are natural and old taxa: phylogeny of human papillomavirus types 44 and 55 and 68a and -b. J Virol 2005;79:6565-9.
18. Xi LF, Koutsky LA, Galloway DA, Kuypers J, Hughes JP, Wheeler CM, Holmes KK, Kiviat NB. Genomic variation of human papillomavirus type 16 and risk for high grade cervical intraepithelial neoplasia. J Natl Cancer Inst 1997;89:796-802.
19. Caldeira S, Zehbe I, Accardi R, Malanchi I, Dong W, Giarre M, de Villiers EM, Filotico R, Boukamp P, Tommasino M. The E6 and E7 proteins of the cutaneous human papillomavirus type 38 display transforming properties. J Virol 2003;77:2195-206.
20. Dong WL, Caldeira S, Sehr P, Pawlita M, Tommasino M. Determination of the binding affinity of different human papillomavirus E7 proteins for the tumour suppressor pRb by a plate-binding assay. J Virol Methods 2001;98:91-8.
21. Forslund O, Ly H, Reid C, Higgins G. A broad spectrum of human papillomavirus types is present in the skin of Australian patients with non-melanoma skin cancers and solar keratosis. Br J Dermatol 2003;149:64-73.
22. Pfister H, Fuchs PG, Majewski S, Jablonska S, Pniewska I, Malejczyk M. High prevalence of epidermodysplasia verruciformis-associated human papillomavirus DNA in actinic keratoses of the immunocompetent population. Arch Dermatol Res 2003;295:273-9.
23. Wieland U, Ritzkowsky A, Stoltidis M, Weissenborn S, Stark S, Ploner M, Majewski S, Jablonska S, Pfister HJ, Fuchs PG. Communication: papillomavirus DNA in basal cell carcinomas of immunocompetent patients: an accidental association? J Invest Dermatol 2000;115:124-8.
24. Termorshuizen F, Feltkamp MC, Struijk L, de Gruijl FR, Bavinck JN, van Loveren H. Sunlight exposure and (sero)prevalence of epidermodysplasia verruciformis-associated human papillomavirus. J Invest Dermatol 2004;122:1456-62.
25. Forslund O, Ly H, Higgins G. Improved detection of cutaneous human papillomavirus DNA by single tube nested 'hanging droplet' PCR. J Virol Methods 2003;110:129-36.
26. Sambrook J, Russell DW. Molecular Cloning A Laboratory Manual, Third ed., vol. 1. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001.
27. Rector A, Tachezy R, Van Ranst M. A sequence-independent strategy for detection and cloning of circular DNA virus genomes by using multiply primed rolling-circle amplification. J Virol 2004;78:4993-8.
28. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. ed., vol. 41, 1999.
29. Prestridge DS. SIGNAL SCAN: a computer program that scans DNA sequences for eukaryotic transcriptional elements. Comput Appl Biosci 1991;7:203-6.
30. Wingender E, Chen X, Hehl R, Karas H, Liebich I, Matys V, Meinhardt T, Pruss M, Reuter I, Schacherer F. TRANSFAC: an integrated system for gene expression regulation. Nucleic Acids Res 2000;28:316-9.
31. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985;230:1350-4.
32. Pettersson U, Ahola H, Stenlund A, Moreno-Lopez J. Organization and Expression of Papillomavirus Genomes. In: Salzman NP, Howley PM. The Papovaviridae: The Papillomavirusesed., vol. 2. New York: Plenum Press, 1987:67-107.

33. Chen XS, Garcea RL, Goldberg I, Casini G, Harrison SC. Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. Mol Cell 2000;5:557-67.
34. Christensen ND, Cladel NM, Reed CA, Budgeon LR, Embers ME, Skulsky DM, McClements WL, Ludmerer SW, Jansen KU. Hybrid papillomavirus L1 molecules assemble into virus-like particles that reconstitute conformational epitopes and induce neutralizing antibodies to distinct HPV types. Virology 2001;291:324-34.
35. Carter JJ, Wipf GC, Benki SF, Christensen ND, Galloway DA. Identification of a human papillomavirus type 16-specific epitope on the C-terminal arm of the major capsid protein L1. J Virol 2003;77:11625-32.
36. Berkhout RJ, Bouwes Bavinck JN, ter Schegget J. Persistence of human papillomavirus DNA in benign and (pre)malignant skin lesions from renal transplant recipients. J Clin Microbiol 2000;38:2087-96.
37. Mahe E, Bodemer C, Descamps V, Mahe I, Crickx B, De Prost Y, Favre M. High frequency of detection of human papillomaviruses associated with epidermodysplasia verruciformis in children with psoriasis. Br J Dermatol 2003;149:819-25.
38. Deau MC, Favre M, Jablonska S, Rueda LA, Orth G. Genetic heterogeneity of oncogenic human papillomavirus type 5 (HPV5) and phylogeny of HPV5 variants associated with epidermodysplasia verruciformis. J Clin Microbiol 1993;31:2918-26.
39. Favre M, Majewski S, Noszczyk B, Maienfisch F, Pura A, Orth G, Jablonska S. Antibodies to human papillomavirus type 5 are generated in epidermal repair processes. J Invest Dermatol 2000;114:403-7.
40. Doorbar J, Campbell D, Grand RJ, Gallimore PH. Identification of the human papilloma virus-1a E4 gene products. Embo J 1986;5:355-62.
41. Steele JC, Roberts S, Rookes SM, Gallimore PH. Detection of CD4(+)- and CD8(+)-T-cell responses to human papillomavirus type 1 antigens expressed at various stages of the virus life cycle by using an enzyme-linked immunospot assay of gamma interferon release. J Virol 2002;76:6027-36.
42. Pastrana DV, Vass WC, Lowy DR, Schiller JT. NHPV16 VLP vaccine induces human antibodies that neutralize divergent variants of HPV16. Virology 2001;279:361-9.
43. Dillner J, Brown DR. Can genital-tract human papillomavirus infection and cervical cancer be prevented with a vaccine? Expert Rev Mol Med 2004;2004:1-21.

Table 1. Homology between HPV38b[FA125] and HPV38

Protein	Amino acid	Nucleotide sequence (pre stop codon)
E6	100% (141/141)	99% (417/423)
E7	100% (100/100)	99% (297/300)
E1	99% (595/604)	98% (1768/1812)
E2	97% (429/441)	97% (1285/1323)
E4	94% (175/186)[a]	97% (544/558)
L2	99% (521/527)	96% (1524/1581)
L1	98% (502/510)	96% (1462/1530)
URR	-	96% (390/405)

[a]Comparison made from the first ATG in the open reading frame.

Table 2. Amino acid differences of the HPV38 and HPV38b[FA125] L1 proteins.

Position[a]	HPV38	HPV38b[FA125]	Structural location[b]
97	Tyr	His	B-pleated sheet D
138	Asn	Gly	D-E loop
146	Asn	Ser	D-E loop
288	Asn	Thr	F-G loop
291	Gly	Ala	F-G loop
367	Ser	Ala	H-I loop
368	Ala	Thr	H-I loop
394	Asn	Thr	Helix 2

[a]Numbered according to the HPV38 L1 protein.

[b]Locations in predicted 3-dimensional structures based on sequence comparison with the 3-dimensional structure of the HPV16 L1 protein (Chen *et al.* Mol Cell. 2000 Mar;5(3):557-67)

Table 3. Presence and viral loads of HPV38 and HPV38b[FA125] in skin lesions. (CV calculated on three values).

Diagnosis	HPV type	Viral copy/cells Lesion (CV %)	Viral copy/cells Healthy skin (CV %)	Viral copy/cells Top of lesion (CV %)	Viral copy/µl Top of lesion (CV %)
AK[a]	HPV38	1/349 (76)	-	12/1 (58)	30 (58)
(n=52)	[FA125]	1/600 (39)	-	-	38 (26)
	[FA125]	1/1849 (0.6)[b]	-	-	-
	[FA125]	1/183 (41)[b]	-	9/1 (31)	26 (31)
SK[a]	HPV38	1/1960 (75)	-	-	3 (17)
(n=47)	HPV38	1/167 (37)[b]	-	NA	NA
	[FA125]	1/12336 (41)[b]	-	-	-
	[FA125]	1/524 (37)	1/133 (63)	-	9 (40)
BCC[a]	HPV38	1/67407 (1.5)[b]	-	1/29 (38)	2 (38)
(n=118)	[FA125]	-	1/254 (44)	1/20 (51)	91 (51)
SCC[a]	HPV38	1/142 (27)	-	-	3 (34)
(n=52)	HPV38	1/12875 (21)[b]	-	9/1 (3.6)	62 (3.6)
	HPV38	1/1107 (64)	-	1/2 (18)	130 (18)

[a]AK=actinic keratosis, SK=seborrhoeic keratosis, BCC=basal cell carcinoma, SCC=squamous cell carcinoma.

[b]CV calculated on two values.

NA = not analysed (sample missing)