Deep sequencing extends the diversity of human papillomaviruses in human skin

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Most viruses in human skin are known to be human papillomaviruses (HPVs). Previous sequencing of skin samples has identified 273 different cutaneous HPV types, including 47 previously unknown types. In the present study, we wished to extend prior studies using deeper sequencing. This deeper sequencing without prior PCR of a pool of 142 whole genome amplified skin lesions identified 23 known HPV types, 3 novel putative HPV types and 4 non-HPV viruses. The complete sequence was obtained for one of the known putative types and almost the complete sequence was obtained for one of the novel putative types. In addition, sequencing of amplimers from HPV consensus PCR of 326 skin lesions detected 385 different HPV types, including 226 previously unknown putative types. In conclusion, metagenomic deep sequencing of human skin samples identified no less than 396 different HPV types in human skin, out of which 229 putative HPV types were previously unknown.

Analysis of metagenomes using deep sequencing has in recent years become a routine approach1. The bacterial community of the skin has been investigated through metagenomic sequencing targeting the 16S ribosomal DNA. The viral part of the microbiome has not been analyzed as thoroughly, because the viruses do not share a common consensus sequence that can be targeted by molecular methods2. However, metagenomic sequencing has revealed that >95% of the viral sequences present in skin samples belong to the Papillomavirus family, mostly to the β- and γ-genera3. There are now 197 different HPV types established at the International HPV Reference Center (www.hpvcenter.se, accessed on 2014-06-05), but putative novel HPV types are continuously being discovered4–9. The cutaneous HPV types have been found in healthy skin as well as in different skin lesions such as squamous cell carcinoma (SCC), actinic keratosis (AK) and keratoacanthoma (KA), in both immunocompetent and immunosuppressive patients10–18. Some mucosal HPV types cause cervical cancer19, as well as vulvar, anal and penile cancers20, whereas some cutaneous HPV types cause skin warts and others are associated with SCC in patients with a rare immunosuppressive disease21,22. Virus-associated cancers have an increased incidence among immunosuppressed patients23. This fact has spurred intensive efforts to search for viruses in cancers that have an increased incidence among the immunosuppressed patients, but that are not known to have a viral etiology. The cancer form that is most highly increased in incidence among the immunosuppressed is non-melanoma skin cancer23.

We previously performed metagenomic sequencing of 142 skin samples amplified only with whole genome amplification (that is, without prior PCR) using 454 and Ion Torrent sequencing technologies, where most of the viral sequences mapped to the HPV family and 7 and 12 HPV types were identified, respectively13. Classification of HPV is based on the sequence of the major capsid protein gene L1. The L1 sequence of a new HPV type should be >90% similar to the L1 gene in any known HPV type24. The HPV consensus PCR primer pair FAP59/64 amplifies both mucosal and cutaneous types25. As HPVs have been reported to be the most common viruses in the human skin3,13, we have also described the use of deep sequencing of general primer HPV PCR amplimers as a method to detect additional HPV types26. In this study, we wished to perform analysis with deeper sequencing using the much more powerful Illumina sequencing technology to investigate if there might be additional viruses present in human skin.

Results

Metagenomic sequencing of whole-genome amplified skin lesions (without prior PCR). Swab samples from 82 SCCs and 60 AKs skin lesions were subjected to whole genome amplification, pooled and sequenced using the
Illumina MiSeq sequencing platform. We found a total of almost 100,000 HPV reads (>99% of all viral reads) comprising 21 known established HPV types, 2 known putative types and 3 novel putative HPV types (Table I). The taxonomic definition of an HPV type is that the L1 sequence of a new HPV type should be less than 90% similar to the L1 gene in any known HPV type. Known putative HPV types refers to sequences that are present in GenBank, but have not yet been cloned and hence, no official HPV type number have been assigned to them by the International HPV Reference Center (www.hpvcenter.se). In addition, human polyomavirus 6, Merkel cell polyomavirus, torque teno virus and human endogenous retrovirus were detected with between 7 and 205 reads each (Table I).

Compared to previous analysis of the same sample pool using 454GSFLX and Ion Torrent PGM technologies\textsuperscript{13}, we obtained a 260-fold and 35-fold larger number of viral reads using the Illumina MiSeq, respectively. The MiSeq analysis detected 26 established or putative HPV types, compared to 7 and 14 detected by GSFLX and PGM, respectively. 11 of the 14 HPV types detected by any of the formerly used technologies, the GSFLX and the PGM, were detected by the Illumina MiSeq. Only 3 previously detected types (HPV45, HPV59, FA73) were not detected by MiSeq. However, these 3 HPV types were all only detected with a single read each during previous sequencing runs with the 454 or Ion Torrent technologies\textsuperscript{13}.

For one of the known putative types, SE46, the complete sequence was obtained from a total of 4881 reads (maximum coverage of 273). From the previous GSFLX and PGM runs, SE46 was sequenced with 22 reads (maximum coverage of 5) and 132 reads (maximum coverage of 18), respectively with only a partial sequence obtained\textsuperscript{13}. In this study, the Illumina MiSeq sequencing increased the sequencing depth approximately 220 times for this particular virus and the complete genome of the type SE46 was obtained (Figure 1). The genomic organization was similar to that of established \( \gamma \)-papillomaviruses.

In addition, partial sequences of the 3 previously unknown putative HPV types (herein named SE355, SE356, SE357), were found in this study.

| Disease | SCC* and AK* | Number of patients | 82 SCCs and 60 AKs |
|---------|-------------|--------------------|-------------------|
| Sample type | Swab samples from the top of lesions |
| Pre-sequencing treatment | Whole genome amplification |
| Sequencing platform | GSFLX** | PGM 300 bp** | PGM 400 bp** | MiSeq |
| HPV8 | 189 | 1360 | 429 | 56896 |
| HPV12 | 1 | 8 | 4 | 381 |
| HPV20 | 2 | 1 | 0 | 149 |
| HPV22 | 0 | 0 | 0 | 1 |
| HPV24 | 0 | 0 | 0 | 2 |
| HPV28 | 0 | 0 | 0 | 15 |
| HPV36 | 0 | 0 | 0 | 45 |
| HPV38 | 0 | 1 | 0 | 2 |
| HPV45 | 0 | 1 | 0 | 0 |
| HPV59 | 0 | 1 | 0 | 0 |
| HPV76 | 0 | 0 | 0 | 18 |
| HPV93 | 0 | 0 | 0 | 2 |
| HPV104 | 7 | 32 | 28 | 1168 |
| HPV105 | 0 | 1 | 0 | 370 |
| HPV107 | 0 | 0 | 1 | 83 |
| HPV109 | 0 | 0 | 0 | 5 |
| HPV110 | 0 | 0 | 0 | 4 |
| HPV124 | 0 | 5 | 0 | 48 |
| HPV125 | 0 | 0 | 0 | 4 |
| HPV128 | 0 | 0 | 0 | 2 |
| HPV134 | 0 | 0 | 0 | 6 |
| HPV155 (SE42) | 156 | 1199 | 255 | 33668 |
| HPV161 | 0 | 0 | 0 | 4 |
| FA73 | 0 | 0 | 1 | 0 |
| HPV 915 F 06 007 FD1 | 1 | 3 | 0 | 94 |
| SE46 | 22 | 132 | 44 | 4881 |
| SE355 | 0 | 0 | 0 | 369 |
| SE356 | 0 | 0 | 0 | 42 |
| SE357 | 0 | 0 | 0 | 6 |
| Torque teno virus | 1 | 1 | 1 | 22 |
| Human polyomavirus 6 | 0 | 0 | 1 | 205 |
| Merkel cell polyomavirus | 1 | 2 | 1 | 36 |
| Human endogenous retrovirus | 0 | 3 | 0 | 7 |
| Total reads | 121752 | 912218 | 381017 | 23699142 |
| Total viral reads | 380 | 2750 | 765 | 98535 |
| Total HPV reads | 378 | 2744 | 762 | 98265 |
| Total established HPV types | 5 | 10 | 5 | 21 |
| Total known putative HPV types | 2 | 2 | 2 | 2 |
| Total novel putative HPV types | 0 | 0 | 0 | 3 |
using the Illumina MiSeq platform (Table I). For SE355 we obtained a 7080 bp long contig, assembled from 369 reads, representing almost the complete HPV genome. The GenBank accession numbers for the 3 novel putative types (numbered SE355 to SE357) are KJ506870 to KJ506872.

**Known and novel HPV types found after HPV consensus PCR.** Sequencing of the HPV-amplimers in the 3 pools of in total 326 skin lesions identified 159 known HPV types, out of which 52 were known established HPV types and 107 were known putative types (Table II). In addition, the deep sequencing identified 226 sequences of previously unknown putative novel HPV types. 65% of the identified previously known HPV types (104/159) belonged to the γ-genus, whereas 63% of the novel putative types (142/226) belonged to the β-genus (Table II). There were only small differences between the 3 pools. They had similar distribution of different kinds of HPV types, both known and novel. For all 3 pools, more than half of all HPV sequences detected were novel putative types. The GenBank accession numbers for the 226 novel putative types (herein numbered SE355 to SE357) are KJ506873 to KJ507098.

**Comparison of results from different samples.** The swabbing of the surface of human skin is intended to characterize the diversity of viruses present, but is less informative for making associations with skin diseases as viruses that are shed from other places on the body might be detectable in swabs of skin surfaces also on rather distant sites on the body. Skin surface swabs were used in the metagenomic analysis (pool D) as well as with deep sequencing of HPV general primer amplimers (pool C). The sequencing of amplimers was unquestionably more sensitive (352 different HPVs detected) compared to the metagenomic sequencing (26 different HPVs detected), but it is noteworthy that no less than 11 of the HPVs that were detected in the metagenomic sequencing were not detected when sequencing HPV general primer PCR amplimers.

**The new HPV phylogenetic tree.** 160 of the 226 novel putative HPV types identified by the MiSeq sequencing of the three pools of HPV amplimers contained >400 bp or >200 bp including the 3'-end of the HPV consensus PCR amplimers and are shown in the HPV tree together with all known established types and previously known putative types, labeled as SE-types (Figure 2). The novel putative types cluster together in the β- and γ-genera. One large group of 17 novel putative types and 2 known putative types forms a new branch in the tree, belonging to the γ-genus. Another group of 9 novel putative types forms a new branch belonging to the β-genus (Figure 2).
The aim of the present study was to investigate if deeper sequencing technology is a useful method to obtain the complete picture of the viruses that are present in human skin. 9 out of 12 virus types detected with only a single read using 454GSFLX were not confirmed by the deeper sequencing.

The whole genome amplification method used (GenomiPhiHigh-Yield) is intended for a random amplification of all DNA in a sample and is thus routinely used for amplifying linear DNA. However, the GenomiPhi reaction has a preference for circular templates, which will have made it easier to detect circular genomes in the pool amplified with WGA. The WGA amplified the human genome 25 times and a circular HPV plasmid an additional 25 times. Thus, although the WGA will have made it easier to detect the circular HPV genomes, it is unlikely that we would have missed linear viruses unless they were present only in very small amounts.

Three of the pools were amplified with an HPV consensus PCR before sequencing. Although the amplimer length is 450 bp, some sequences obtained were longer or shorter than expected. This could be due to the fact that these PCR primers are highly degenerate and may to some extent also bind unspecifically.

The majority of the HPV types and putative types detected in this study belonged to the β- and γ-genus, but 11 mucosal types from α-genus were also found, out of which one was a putatively novel HPV type. Both presence of anogenital oncogenic HPV types in skin samples and contamination of the skin by viruses originating from mucosal surfaces, mediated by the fingers, has been reported. In this study, biopsies from SCCs and AKs were taken after tape-stripping of the skin surface, to reduce the probability of detecting contaminating viruses.

Phylogenetically, the majority of the novel HPV sequences belonged to the β-genus (142/226) followed by the γ-genus (83/226). In previous studies, the majority of novel putative HPV types found belonged to the γ-genus. Possibly, the viruses in the γ-genus may exist at higher viral loads, making them easier to detect, whereas viruses of β-genus might be biologically different and be present in lower viral loads. It might intuitively be surmised that viruses present in high amounts are more pathogenic, but there is no data to support this notion. Alternative scenarios are possible where the many different viruses present may interact. A first step that will enable investigations of this issue is the basic knowledge that these viruses are present - the basic and fundamental discovery of the paper. Although 22 of the HPV’s detected appeared to be more common in either the SCC/AK or KA sample, the massive number of viruses detected suggests that the association may have been due to chance and that it needs to be investigated in further studies.

The β-genus is already diverse with 45 completely sequenced HPV types established (www.hpvcenter.se). The γ-genus has been growing rapidly and has surpassed the β-genus, with now 61 completely sequenced γ types established (www.hpvcenter.se). It appears that some of the new HPV types detected in the present study form clusters outside the previously defined species. Two of them are particularly noticeable, a new branch in the β-genus formed from

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**Table II | The number of different HPV types or putative types identified in the 3 pools of HPV consensus PCR amplimers from 326 skin lesion samples.**

| Sample pool | Established (by genera α, β, γ*) | Known putative (by genera α, β, γ*) | Novel putative (by genera α, β, γ*) |
|-------------|----------------------------------|-------------------------------------|------------------------------------|
| A           | 52 [8, 26, 18]                   | 105 [1, 19, 85]                     | 197 [1, 125, 71]                   |
| B           | 51 [8, 26, 17]                   | 105 [1, 19, 85]                     | 176 [0, 106, 70]                   |
| C           | 51 [8, 26, 18]                   | 105 [1, 19, 85]                     | 206 [1, 133, 72]                   |
| A + B + C   | 52 [8, 26, 18]                   | 107 [1, 19, 87]                     | 226 [1, 142, 83]                   |

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*Note: Genbank and different (>90% nt similarity in the L1 gene) from all Established HPV types or HPV sequences present in Genbank.*
9 novel putative HPV types and a new branch in the γ-genus formed from 17 novel putative HPV types. The second branch also includes 2 previously detected putative HPV types. However, whether these new branches do indeed represent new HPV species needs confirmation by cloning, sequencing and deposition at the international HPV reference center (www.hpvcenter.se).

With increasing sensitivity and throughput of the sequencing technology, the possibility always exists that assembly algorithms may construct erroneous “chimeric” sequences by the assembly of two different sequences from different viruses. Genomic recombination has been described for cetacean papillomaviruses. Even though this has not been described for HPVs, multiple co-infections of related microorganisms can result in recombination. Both naturally occurring genomic recombination and PCR-mediated recombination may mislead phylogenetic analysis. We have previously developed a bioinformatics pipeline, which detects and removes putative chimeras that may have resulted from PCR-mediated recombination between related templates. The bioinformatics in this study was even stricter regarding chimera checking also for known viruses. Hence, some sequences reported in the previous studies did not pass the chimera checking step in the data analysis pipeline in this study. The phylogenetic tree was constructed only with the sequences that had passed our most strict chimera checking algorithm.

In conclusion, we demonstrate that the diversity of HPVs in human skin is far greater than previously known. Deep sequencing...
using the Illumina platform is effective to detect a plethora of HPV's that are present in skin samples. As deep sequencing technologies are continuously evolving with increasing throughput and decreasing cost per base pair, it is likely that an extraordinary and expanding diversity of cutaneous HPV's will continue to be revealed. As the HPV's are much more diverse than previously known, this needs to be taken into consideration in the design of detection methods and when designing studies of HPV biology or epidemiology.

### Methods

#### Samples

Swabs and biopsies of non-melanoma skin cancer lesions (SCC, AK and KA) from immunocompetent patients attending Swedish and Austrian hospitals and of KA lesions from both immunosuppressed and immunocompetent patients attending the Norwegian National Hospital in Norway were used. The swab samples used for pool A, B, and C were collected from the top of the lesion, and from normal skin adjacent to a skin biopsy by a pre-wetted (0.9% NaCl) cotton-tipped swab that was rolled on the lesion (within margins of the lesion) or on the normal skin respectively, and suspended in 1 mL of saline. Smaller diameter punch biopsies had been taken after tape-stripping from each lesion as well as from normal adjacent skin. Biopsies from SCC and AK had been HPV-positive in a previous study. The DNA was extracted with a phenol-free method for the Swedish/Austrian biopsies and with the QiAamp DNA MiniKit (Qiagen, Germany) for the Norwegian biopsies. Swab samples were not extracted, only frozen and thawed. Informed consent was obtained from participants. The study adhered to the declaration of Helsinki and was approved by the Ethical Review Committees of Karolinska Institutet and of Lund University (Sweden), the Regional Ethics Review Committees of Karolinska Institutet and of Lund University (Sweden), the Review Committees of Karolinska Institutet and of Lund University (Sweden), and the Institutional Review Board in Oslo (Norway). All methods were carried out in accordance with the approved guidelines.

#### DNA amplification and pooling

The samples were mixed into 4 pools. Pool A, B, and C were used in a previous study, where pool A constituted fresh frozen biopsies from 29 SCC lesions and 31 AK lesions, pool B fresh frozen biopsies from 91 KA lesions and pool C of lesion swabs from 84 SCCs and 91 AKs. Prior pooling, pools A, B, and C, the samples were PCR amplified with the Illumina consensus primer pair FAP59/64 targeting the L1 gene of α-, β-, and γ-HPVs, as described previously, using 5 µL of each sample per reaction. For SCC and AK biopsies, only samples that were HPV positive by PCR were used (SCC, n = 29 and AK, n = 31). A plasmid including the sequence of HPV type 16 was used as a positive control and a detection limit of one copy per microliter was found. The PCR amplifications from the three pools were purified using the MinElute spin column kit from Qiagen according to manufacturer’s guidelines. Two columns per sample pool were used with a sample volume of 120 µL per column. The elution volume was 10 µL of EB-buffer. The two elutions from each sample were mixed, generating a purified PCR product of 20 µL. Pool D, also used in a previous study, was a mix of swab samples from 82 SCC lesions and 60 AK lesions. The 142 swab samples were subjected to random whole genome amplification using the GenomiPhi High Yield Kit (GE Healthcare, UK) and combined into pool D, where upon the DNA was ethanol precipitated and dissolved in 100 µL water. The 4 pools were quantified using the QuantiFluor ST (Promega, USA), a fluorometric assay quantifying dsDNA, according to manufacturer’s guidelines. The concentrations were 22.4 ng/µL, 17.8 ng/µL, 19.6 ng/µL and 56.4 ng/µL for pool A, B, C, and D respectively.

#### Sample library preparation

DNA libraries for the 3 HPV consensus PCR amplified pools, A, B, and C, were prepared using the TruSeq Nano DNA Sample Preparation kit according to the user guide revision A (Illumina) with the following modifications: as deep sequencing technologies are present in skin samples. As deep sequencing technologies are continuously evolving with increasing throughput and decreasing cost per base pair, it is likely that an extraordinary and expanding diversity of cutaneous HPV’s will continue to be revealed. As the HPV’s are much more diverse than previously known, this needs to be taken into consideration in the design of detection methods and when designing studies of HPV biology or epidemiology.

For pool A, B and C, the samples were amplified with the TruSeq Nano DNA Sample Preparation kit as described above. The DNA was extracted, only frozen and thawed. Informed consent was obtained from participants. The study adhered to the declaration of Helsinki and was approved by the Ethical Review Committees of Karolinska Institutet and of Lund University (Sweden), the Review Committees of Karolinska Institutet and of Lund University (Sweden), and the Institutional Review Board in Oslo (Norway). All methods were carried out in accordance with the approved guidelines.

The sequencing flow cell cluster density for pool B, first to be sequenced, was 422 K/mm², the total yield was 6.2 Gb, 68% > Q30 and 98% of reads were passing filter. The cluster densities for pool A and C were 1113 K/mm² and 1339 K/mm², the total yields were 15.6 Gb and 18.6 Gb, 66% and 67% > Q30, 95% and 94% of reads passing filter - an improvement compared to pool B presumably due to the adjusted input DNA for library preparation.

The deduplication flow cell size was estimated to be 1–1.5 kb and the conversion formula 1 ng/µL = 1.5 nM DNA was used.

#### Sequencing on the Illumina MiSeq

Denatured libraries at 20 pm from the FAP-PCR amplified pool A, B and C were spiked with 5% PhiX control and individually sequenced by paired-end 301 + 501 cycles on the MiSeq instrument using version 3 reagent kit (Illumina, US). For the multiple displacement amplified pool D, 10 pm denatured library was spiked with 1% PhiX control and sequenced by paired-end 251 + 251 cycles on the MiSeq using version 2 reagent kit. The sequencing preparations were made according to the user guides Preparing Libraries for Sequencing on the MiSeq revision C, Reagent Preparation Guide revision A and MiSeq System User Guide revision K.

The sequencing flow cell cluster density for pool B, first to be sequenced, was 422 K/mm², the total yield was 6.2 Gb, 68% > Q30 and 98% of reads were passing filter. The cluster densities for pool A and C were 1113 K/mm² and 1339 K/mm², the total yields were 15.6 Gb and 18.6 Gb, 66% and 67% > Q30, 95% and 94% of reads passing filter - an improvement compared to pool B presumably due to the adjusted input DNA for library preparation.

#### Analysis of sequences

Sequences from the MiSeq (Illumina) instrument. Indexes, included in the Illumina adaptors, were used to assign the sequences obtained to the originating sample. The bioinformatic analysis started with quality checking, where sequences were trimmed according to their Phred quality scores. Quality checked reads were then screened against the human reference genome hg19 using BWA-MEM and SOAP aligner (http://soap.genomics.org.cn) and reads with >95% identity over 75% of their length to human DNA were removed from further analysis. The non-mapped sequences were normalized (http://ged.msu.edu/papers/2012-diginorm) to discard redundant data and reduce sampling variation and sequencing errors. The normalized dataset was then processed for assembly using the Trinity (http://trinityrnaseq.github.io) assemblers into contiguous sequences (contigs). Reads before assembly were re-checked and the resulted contigs and sequences per each assembled contig. The use of several assembly algorithms and re-mapping of all singleton reads to assembled contigs were used to validate assembly results.

Assembled contigs were then subjected to taxonomic classification by comparing them against GenBank nucleotide database using paracel blast (www.ncbi.nlm.nih.gov/BLAST). Singleton reads to assembled contigs were used to validate assembly results.

Phylogenetic analysis

BEAST v1.8.0 was used to construct a Bayesian Phylogenetic tree. 4 independent Markov chain Monte Carlo (MCMC) tests were run under a coalescent tree prior and relaxed uncorrelated lognormal molecular clock for 100 000 generations, with a tree longer every 1000th generation. A maximum credibility tree was constructed by a tree annotator after discarding the initial 25% generations as a conservative generalization of the “burn-in” phase. The analyses were restricted to sequences that were >400 bp or contained >200 bp of the 3’-end of the amplifier. This restriction was necessary in order to avoid the possibility that non-overlapping sequences might derive from the same virus.

#### Amplification of viral and human DNA by whole genome amplification (WGA)

Because the samples pool D were amplified by WGA before sequencing, we quantified the amount of amplification of human DNA and of HPV DNA. To this aim, we added 10 nM DNA of HPV16 plasmid and amplified the samples with WGA using the GenomiPhi High Yield Ready-to-go kit (GE Healthcare) in the same manner as for the clinical samples. The amounts of human DNA and viral DNA were quantified using real-time PCR for Betaglobin and for HPV16, respectively. The human DNA was found to be amplified 26-fold, whereas the HPV16 DNA was amplified 679-fold.

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**Acknowledgments**

We thank Carina Eklund for excellent technical assistance.

**Author contributions**

E.H. and J.D. conceived the project. E.H., J.E. and O.F. designed the experiments. E.H. performed the experiments. D.B. performed bioinformatic analyses and prepared figures. E.H., L.S.A.M. and J.E. interpreted the data. E.H., D.B. and J.D. wrote the manuscript.

**Additional information**

Competing financial interests: The authors declare no competing financial interests.

**How to cite this article:** Bzhalava, D. et al. Deep sequencing extends the diversity of human papillomaviruses in human skin. *Sci. Rep.* **4**, 5807; DOI:10.1038/srep05807 (2014).

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