Three novel papillomaviruses (HPV109, HPV112 and HPV114) and their presence in cutaneous and mucosal samples

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Abstract

To expand our knowledge of the genomic diversity of Human Papillomaviruses (HPVs), we searched for new HPVs in squamous cell carcinomas of the skin (SCC) and seemingly HPV-negative, otherwise typically HPV-associated lesions.We describe the characterization of three novel human papillomavirus (HPV) types. HPV109 was isolated from an SCC, HPV112 from a condyloma and HPV114 from a low-grade cervical lesion. Pairwise alignment of the L1 sequences classified HPV114 to genus *alpha* species 3 whereas HPV112 defined a new species in the genus *gamma*. HPV109 had uncertain classification because of a low and about equal similarity to different genera. Type-specific real-time PCRs of cervical samples, a majority from women with low grade atypical cytology, (n=2856) and various cutaneous samples (n=538) found HPV114 in 1.7% (48/2856) of the genital samples, whereas both HPV109 and 112 were rare viruses that were found at high viral loads only in their index samples.

Keywords: Real-time PCR, multiple displacement amplification, phylogeny, Luminex, universal probe, complete genome

Introduction

The human papillomaviruses (HPVs) comprise a large and diverse group of viruses that infect keratinocytes in mucosa and skin. There are more than 100 completely characterized HPV types and new types are continuously found (Chen et al., 2007a; Chen et al., 2007b; Nobre et al., 2009; Vasiljevic et al., 2008; Vasiljevic et al., 2007). A novel HPV type has less than 90% similarity of the L1 gene to any known HPV type, HPV types within a species have between 60 to 70% similarity to HPV types in other species, and HPV types within a genus show less than 60% similarity to HPV types in other genera (de Villiers et al., 2004). The five major HPV genera are *alpha*, *beta*, *gamma*, *mu* and *nu*. The *alpha*-PVs are mainly associated with mucosal infections with the exception of HPV *alpha* -types belonging to the species 2 and 4, which can also be found in benign skin lesions (de Villiers et al., 2004). *Beta*-types are most frequently found in cutaneous lesions, but were in a recent report also found in oral mucosal lesions (de Villiers and Gunst, 2009). HPV types of genera *gamma*, *mu* and *nu* have to our knowledge hitherto been found only in cutaneous samples (Forslund, 2007).

HPV is the major cause of cervical cancer with close to 100% prevalence in cervical tumors (Walboomers et al., 1999). HPV also causes other types of mucosal cancer e.g. anal, vulvar and oral cancers (IARC, 2007) and benign genital tumors such as condylomas (Greer et al., 1995). The cutaneous types are found both in benign skin warts (Pfister and Ter Schegget, 1997) and in non melanoma skin cancer (NMSC), including both in squamous cell carcinoma of the skin (SCC) and in basal cell carcinoma (BCC) (IARC, 2007) and on top of healthy skin (Antonsson et al., 2000; Forslund et al., 2004). Most NMSCs contain only very low viral loads, usually less than 1 copy/1000 cells (Weissenborn et al., 2005; Hazard et al., 2006; Vasiljevic et al., 2008; Vasiljevic et al., 2007). The possibility that very low amounts of cutaneous HPV may represent a contamination of the skin surface rather than an infection has been discussed (Forslund et al., 2004). However, occasional NMSC can indeed contain very

high amounts of virus, up to $1.3*10^6$ copies/cell (Forslund et al., 2003; Kullander et al., 2008). For the few HPV-negative cervical cancers that exist, four possible explanations (in addition to true negativity) have been suggested: (1) specimen inadequacy, (2) loss of L1 gene due to integration (3) insensitivity of the detection method, (4) the existence of still unidentified HPV types that are not detectable by the method (Walboomers and Meijer, 1997). As it is also possible that NMSCs that contain very low amounts of known viruses or where HPV has not been detected at all may contain still unidentified HPV types, a search for new HPV types in such lesions is warranted. A similar line of reasoning applies to the occasionally encountered "HPV-negative" condyloma acuminata and to cervical lesions that are negative for known HPV types, but contain morphological changes typical of an HPV infection.

This report describes the characterization of three novel HPV types. HPV109 was found in a squamous cell carcinoma of the skin and HPV112 was cloned from a typical condyloma acuminata that was "HPV-negative" by general primer PCR. The third type, HPV114, was isolated from a low-grade cervical lesion. The prevalence and viral load of these types was then investigated in a panel of 3394 skin and genital samples.

Results

Characterization of the novel types

The index case from which HPV109 was isolated was a 59 year old male, participating in a case-control study of NMSC (VIRASKIN), that had tested positive with the general primer system FAP in 2 different laboratories (Forslund et al., 2007). The 450 bp PCR amplimer sequence was previously described and deposited in GenBank as FA137 (Forslund et al., 2004). Eightythree SCC samples from the VIRASKIN study were subjected to amplification of circular DNA using multiple displacement amplification (MDA), digested and run on a gel, and the sample from the index patient from whom we isolated HPV109 was the only sample where an HPV-type was found. The complete genome of HPV109 was 7346 bp with a GC-content of 38.3%. It was most closely related to the *gamma*-type HPV4 with 65% similarity in the L1 open reading frame (ORF), using the third ATG as the start of the ORF.

The index case from which HPV112 was isolated was a 25 year old male with a condyloma acuminata that tested negative with the general primer PCR system GP5+/6+ (de Roda Husman et al., 1995). By amplification with MDA and FAP a novel HPV-type, later denoted HPV112, was found. HPV112 had a genome of 7227 bp with a GC content of 37.5% and was most closely related to HPV65, also a *gamma*-type, with a similarity of 64% in the L1 ORF.

The index case from which HPV114 was cloned from was a cervical brush sample from a low-grade cervical lesion (27 year old woman with cytological diagnosis cervical intraepithelial neoplasia grade I (CIN I)). The HPV PCR detection and typing system used for typing of a study of 1581 women with low-grade cytological changes (atypical squamous cells of undetermined significance (ASCUS) or CIN I) from Stockholm, Sweden was a modified HPV general primer system (MGP (Soderlund-Strand et al., 2009)) that contains typing using type-specific probes attached to Luminex beads (Schmitt et al., 2006). A bead with a "universal probe" that is common to most HPV types is included and if the "universal

probe" is positive but the type-specific probes are negative, the amplimer is sequenced. When four such specimens (three with the cytological diagnosis CIN I and one with ASCUS) had been found to have the same sequence we decided to clone the virus. HPV114 contains 8069 bp, with a GC-content of 45.4%. The L1 ORF showed 84% sequence similarity to HPV84, belonging to genus *alpha* species 3. A fragment of the same sequence was previously described in a psoriatic lesion of the skin and denoted SW1 (Weissenborn et al., 1999).

All three types had a typical genomic organization for papillomaviruses, except that HPV114 was missing an E5 ORF. The E6 proteins from the three types contained two conserved zinc-binding domains separated by 36 amino acids and the E7 proteins contained one zinc-binding domain (Ullman et al., 1996). The E7 proteins also contained a pRb-binding domain, although this was not totally conserved in HPV112 (Radulescu et al., 1995). The ATP-binding site of the ATP-dependent helicase of E1 contained at least one proline residue (Raj and Stanley, 1995)(GPPNTGKS for HPV109, GPPDSGKS for HPV112 and GPSDTGKS for HPV114). Polyadenylation sites for processing of early mRNA transcripts were located at the 5'-end of L2 in HPV109 and 112, but for HPV114 the polyadenylation site was located in L1. The long coding region (LCR) of all three types contained a polyadenylation site for late transcripts. The LCR also contain putative binding sites for E2 [$ACCN_6GGT$](Hawley-Nelson et al., 1988) and for transcription regulatory factors such as NF-1(Apt et al., 1993), AP-1(Chan et al., 1990) and SP1(Gloss and Bernard, 1990).

As the L1 nucleotide sequence of HPV109 and 112 showed only 65% and 64% similarity to their closest relatives (HPV4 and -65, respectively) they should therefore constitute two new species in the genus *gamma*, but HPV109 also had an equal degree of similarity to a group of recently discovered and unclassified HPV types lacking an E6 gene (HPV101, 103 (Chen et al., 2007b) and 108 (Nobre et al., 2009)) (Table 1). In addition, HPV109 had more than 60% similarity in the L1 ORF to the *pi*- and *beta*- papillomaviruses (Table 1). Comparisons of the

HPV109 ORFs other than L1 (both nucleotide and amino acid sequences) show an equal level of similarity to the *gamma*-PVs and to the new unclassified group of HPV types lacking E6 (Table 1).

Phylogenetic analysis confirms that HPV112 constitutes a new species in the genus *gamma*, and that HPV114 belongs to species a*lpha* 3 (Fig 1). The bootstrap value for HPV109 was 55, well below 70, which indicates low reliability of the alignment (Bull, 1993). Therefore, HPV109 is difficult to classify. It appears to be a close-to-root type that is about equally related to the species g*amma* and *pi* and to the new group of HPVs that lack an E6 ORF.

Prevalence of HPV109, 112 and 114 in cutaneous and genital samples

The primers and probes for a triplex real-time PCR were designed at sites with low similarity to closely related types and putative types in order to avoid cross-reactivity. A sensitivity of five copies for each HPV type in the PCR was confirmed using dilution series of positive controls (purified plasmids) in every run.

HPV109 was found in three specimens, the index SCC, a seborrhoeic keratosis (SK) and as a coinfection with HPV114 in a CIN I lesion. The viral loads in the SK and CIN I specimens were very low ($4*10^{-2}$ and $5*10^{-6}$ copies per cell respectively), but for the index SCC the viral load was 11 copies per cell. HPV112 was only found in the index condyloma sample, with a viral load of 842 copies per cell. HPV114 was found in 48 of 2856 genital samples: 0.3% (1/312) of cases and 1.1% (3/271) of controls from a hospital-based cervical cancer case-control study from Mocambique (Naucler et al., 2004), 0% (0/431) of cervical cancers and 0.9% (2/234) controls from a population-based case-control study from Latvia (Silins et al., 2004) and in 2.7% (42/1581) of women with low-grade cytological changes (ASCUS or CIN I) from Stockholm, Sweden (L. Dillner, personal communication). Viral copy numbers for HPV114 varied from $5*10^{-4}$ to 240 copies per cell with a mean of 11.2 copies per cell (data

not shown). The total prevalence of HPV114 in the Stockholm study was 1.8% for the women with ASCUS and 3.1% for women with CIN I, diagnosed with cytology (Table 2). In five specimens (four with the cytological diagnosis CIN I and one with ASCUS) HPV114 appeared as a single infection. For the other 37 samples HPV114 coexisted with one or up to 5 different HPV types. For 1112 women, we had a histopathological diagnosis on a subsequently taken biopsy, and 2.5% (28/1112) women were HPV114 positive (Table 2). The highest prevalence of HPV114 was found in the samples from women with CIN I lesions (4.1%), but the small differences in HPV114 prevalence by histopathological diagnosis were not significant (Table 2). All samples in the Stockholm study were previously tested with Hybrid Capture II (HC II) (L. Dillner, personal communication) and 2.5% (11/440) of women with a negative HC II-result were positive for HPV114 (Table 2). For two of these HC II-negative samples we had histopathological diagnosis on a subsequently taken biopsy (one women with CINI in cytology that had a benign lesion and one woman with ASCUS in cytology that had a CIN II) (not shown).

Discussion

The three novel HPV types described in this paper contribute to our knowledge of HPV genomic diversity. HPV109 clusters with the group of recently identified HPV types HPV101, 103 and 108 in-between the *gamma* and *pi* genera, but the bootstrap value in the phylogenetic tree is too low (below 70) to define it as a true branch. HPV109 should probably not be classified as a member of the HPV101/103/108 group, because HPV109 contains all the ORFs typical for an HPV type whereas HPV101, 103 and 108 all lack the E6 gene. HPV101, 103 and 108 were also all found in genital lesions, but HPV109 came from an SCC of the skin. Only one out of 2856 genital lesions was HPV109-positive and at a very low copy number. For the index SCC, the viral load of HPV109 was 11 copies per cell. Although most cutaneous samples contain only low amounts (<1 copy/cell) of known viruses (Hazard et al., 2006; Vasiljevic et al., 2008; Weissenborn et al., 2005), there are many examples of skin lesions that do contain high amounts of virus per cell, e.g. HPV1 and 2 (Gissmann et al., 1977; Orth et al., 1977) in skin warts, HPV5 and 8 in skin cancers from epidermodysplasia verucciformis patients (Ostrow et al., 1982; Pfister et al., 1981), HPV92 in a BCC (Forslund et al., 2003) and HPV88 (Kullander et al., 2008) in an SCC.

HPV112 appears to be a very rare virus. In spite of the fact that we tested >3000 samples from both skin and mucosa, HPV112 was only found in the index condyloma sample. To our knowledge, *gamma* HPV types have so far only been in cutaneous lesions. The high viral load of HPV112 in the condyloma (842 copies per cell) indicates that its presence in this lesion is not merely a contamination.

The third HPV type, HPV114, was relatively common in a study of women with ASCUS/CINI, with 42 positive women. The MGP/Luminex typing system used missed only one of the HPV114-positive samples and MGP/Luminex seems sensitive for detection also of unknown HPVs as long as the HPV-types don't differ too much in their sequence compared

to the MGP primers and the "universal" probe in the luminex. HPV114 differs from the MGP primers in two nucleotides per primer and in none in the probe sequence.

In conclusion, we have characterized the complete genome of three new HPV types; HPV109, 112 and 114. HPV114 represents a new type in genus a*lpha*, species 3, and is not uncommon in a large material of genital lesions. The characterization of HPV112 from a condylomata acuminata expands the genus g*amma* with a new species and it also implies that *gamma* HPV types may occasionally associate with genital condyloma. HPV109 was found at a high copy number in an SCC of the skin and is not closely related to any other HPV type. Our findings expand the genomic diversity of HPVs and suggests that a continued search for unknown HPVs in skin SCCs and in typically HPV-associated lesions that appear HPV-negative is warranted.

Material and methods

Cloning of novel types

HPV109 (FA137) was isolated from an SCC of the skin using MDA with primers generic to any HPV(Kullander et al., 2008). After digestion with 20U of HincII (New England Biolabs, Ipswich, MA) and electrophoresis, we cloned an approximately 4 kb fragment. The Zero Blunt TOPO PCR Cloning kit (Invitrogen, Carlsbad, CA) used a gel-excised band purified with QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). The remainder of the genome was amplified by Expand High Fidelity PCR (Roche, Mannheim, Germany) with 2.5 µl of DNA from the patient, following the manufacturer's instructions with some modifications; 0.5 µM of each primer, annealing at 55°C, elongation at 68°C for 4 min plus 5 s/ cycle in cycle 11 to 40. The PCR mixture was prepared in a clean room, separated from the PCR. The product was electrophoresed and a band of approximately 3 kb was excised and purified as described above and cloned using TOPO TA Cloning kit (Invitrogen).

HPV112 (FA164) was isolated from a condyloma acuminate brush sample using the same modified MDA protocol as above followed by FAP-PCR (Forslund et al., 1999). The amplified DNA was separated by electrophoresis, excised and purified as described and cloned using TOPO TA Cloning kit (Invitrogen). This 446 bp fragment was sequenced and a BLAST search against GenBank revealed that the sequence was <80% similar to the most closely related type. This sequence was used as a basis for design of primers for amplifying the complete genome in an Expand Long Template PCR (Roche) following the manufacturers instructions for System 1 (0.5 to 9 kb) with an annealing temperature of 56°C, elongation at 10 minutes and with an additional 20s/cycle for cycle 11 to 40.

The third HPV type, HPV114 (SW1), was isolated from a CIN I lesion using the same PCR protocol as described for HPV112 with an anneling temperature of 55°C. The PCR products

from HPV112 and 114 were separated on E-Gel iBase Power System (Invitrogen) and cloned using TOPO TA Cloning kit (Invitrogen).

Primer pairs (Cybergene, Huddinge, Sweden) used for PCR are listed in Table 3.

Sequencing

Sequencing of HPV109 used primer walking with ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems, Foster City, CA) and a 3730 sequencer (Applied Biosystems).

The complete sequences of HPV112 and 114 were obtained using EZ-Tn5 <TET-1> Insertion Kit (Epicentre Biotechnologies, Madison, WI) as previously described (Vasiljevic et al., 2008) and sequencing using a 3730 sequencer.

The complete sequences were deposited with the International Reference Center for Papillomaviruses at the German Cancer Research Center, Heidelberg, Germany, where they were labelled: HPV109 (GenBank accession nr **EU541441**), HPV112 (GenBank accession nr **EU541442**) and HPV114 (GenBank accession nr **GQ244463**).

Phylogenetic analysis

Phylogenetic analysis was based on an alignment of the L1 ORFs performed by ClustalW Multiple Alignment in BioEdit version 7.0.5.3. MEGA 3.1 was used for phylogenetic analysis, with neighbour joining and bootstrap with 1000 replications and Kimura 2-parameter as the model.

Patient panel

All samples were analyzed for human DNA content using β-*globin* PCR(Saiki et al., 1985) and had to be positive to be included.

Skin biopsies were collected from 269 immunocompetent patients, attending Swedish hospitals. The sample series included actinic keratosis (AK) (n=52), SK (n=47), BCC (n=118) or SCC (n=52). After tape stripping (Forslund et al., 2004), a biopsy was taken from the lesion and from adjacent healthy skin of the same patient. Hence, 538 cutaneous samples were included. The DNA was extracted using a phenol-free method (Forslund et al., 1999). Genital samples from four different studies were also included. From Mocambique, 320 cervical cancer biopsies and 272 brush samples were obtained (Naucler et al., 2004)(Naucler,personal communication). Eight cancer biopsies and 1 swab sample were excluded as they were β-*globin* negative. These samples were stored in GITS (4 M Guanidium Thiocyanate, 22 mM NaCitrate and 5 % Sarcosyl (N-Lauroylsarcosine sodium salt)). To use the DNA in the biopsies 400 µl of the sample was transferred to a tube and 150 µl saturated ammonium acetate was added followed by a centrifugation step at 16 000 for 15 minutes. After addition of 900 µl ice cold 99.5% EtOH to the supernatant the DNA was precipitated for 30 min at -20ºC. The tube was thereafter centrifuged at 16 000g for 10 minutes. The supernatant was discarded and the DNA was washed once with 500µl of 70% EtOH and centrifugation at 16 000g for 5 min. The dried pellet was dissolved in 200 µl TE-buffer (10 mM Tris-HCl, 0.1 mM EDTA pH8). The DNA from the swab samples was retrieved in a similar way; 35 µl saturated ammonium acetate was added to 100 µl sample and the DNA was precipitated with 200 µl ice cold 99.5% EtOH and centrifuged at 16 000g for 15 minutes. The supernatant was discarded and the DNA was washed twice first with 250µl of 70% EtOH and centrifugation at 16 000g for 10 min and then 500µl 70% EtOH and a centrifugation step at 16 000 g for 5 min. The dried pellet was dissolved in 100 µl TE-buffer. If β-*globin* results were negative the samples were re-extracted using SDS/Proteinase K (Sambrook and Russell, 2001)

From Latvia, 431 brush samples from cervical cancer and 234 brush samples from population-based controls were included(Silins et al., 2004) after excluding 3 controls as they were β-*globin* negative. The samples were extracted using a freeze/thaw method as previously described (Forslund et al., 2002). β-*globin* negative samples were re-extracted using SDS/Proteinase K (Sambrook and Russell, 2001) and thereafter with QIAmp MinElute Media Kit (Qiagen) if still negative. A third series of 1581 genital samples were from Stockholm, from women attending the Swedish cervical screening program who had had ASCUS or CIN I in cytology (L. Dillner, personal communication).The ages varied from 23 to 61 years old, and 584 women were 35 years or older. DNA from the samples was extracted using SDS/proteinase K (Sambrook and Russell, 2001). All women in this group were previously tested for HPV by HC II and the positive patients were referred for colposcopy and biopsy. Of these 1141 HC II-positive women the histopathological report is missing for 103 patients. Conversely, 74 of the 440 HCII-negative patients (for whom a biopsy was not receommended) still had a biopsy and a histopathological report. In total 1112 of the women included in the current study had a histopathological report.

Twentyseven brush samples from condyloma acuminate patients, previousy found to be "HPV negative" with GP5/6+ PCR were also included (Sturegard et al., 2008). These samples were extracted with MagNA Pure LC using the kit Total Nucleic acid (Roche). In total, 2856 samples from the genital area were screened.

All patients provided informed consent and the studies were approved by the appropriate Ethical Review Boards.

Real-time PCR

Primers and minor groove binder (MGB) probes (Applied Biosystems) (Table 3), for triplex real-time PCR, specific for HPV109, 112 and 114 were designed using Primer Express 2.0 software program (Applied Biosystems) in L1.

Standard curves used serial dilutions from 100 000 copies to one copy, and also five copies, of purified viral DNA from plasmids containing the genomes of HPV109, 112 and 114. The PCR-mixtures were prepared in a clean room. The 6 µl PCR mix contained 2 µl of sample, 1x QuantiTect Multiplex PCR Master Mix (Qiagen) and 0.2 µM of each primer and probe. Real time PCR was carried out on a 7900HT (Applied Biosystems) using the following parameters: 2 min at 50°C and 10 min at 95°C followed by 50 cycles of 15 s at 94°C and 1.5 min at 60°C. All samples except the condyloma samples were diluted 1:2 in TE buffer before analysis. The condyloma samples were diluted 1:5 as the extraction buffer from MagNAPure inhibited the PCR at higher concentrations. All positive samples were verified and had to be positive in at least two out of three times to be considered positive. All samples except the condyloma samples were verified undiluted.

To determine the viral load/cell, the number of copies of cellular DNA was determined by realtime-PCR for the β-globin gene as described previously (Hazard et al., 2006).

Statistical analysis

LogXact (version 8; Cytel Software Corporation) was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) using multivariate logistic regression.

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Table 1: Similarity between HPV109 ORFs and other representative PVs.

	Gamma-PV									Pi-PV		Beta-PV				Alpha-PV	
HPV109	HPV4	HPV50	HPV48	HPV60	HPV88	HPV112	HPV101	HPV103	HPV108	McPV2	HaOPV	HPV49	HPV96	HPV5	HPV38	HPV114	HPV16
E6aa*	33.1	34.5	33.1	37.1	32.1	37.2	-	-	-	34.5	33.6	27.5	26.3	27.0	29.1	27.3	25.0
E6 nt**	48.8	51.9	50.9	51.4	46.4	52.4	-	-	-	45.3	44.3	46.2	46.8	44.6	45.7	44.3	44.7
E7aa	36.3	36.5	42.7	36.1	35.7	30.9	38.8	42.4	39.8	29.0	20.6	34.9	40.0	39.4	38.5	28.7	26.5
E7 nt	48.9	50.3	58.1	52.6	57.5	47.9	50.5	51.3	52.5	44.3	43.7	51.6	49.8	51.3	52.8	46.1	49.8
E1aa	51.2	45.6	45.2	50.2	49.5	45.2	47.6	47.7	50.5	45.8	51.0	42.3	43.2	45.4	44.2	39.9	41.1
E1 nt	62.5	60.3	59.6	62.0	61.1	60.4	61.4	61.5	62.2	57.9	58.3	58.3	57.3	57.2	57.6	53.8	55.6
E2aa	44.3	43.5	42.0	40.4	43.1	41.0	47.8	46.2	47.0	38.6	37.8	35.6	38.0	37.3	37.0	28.8	27.5
E2 nt	56.5	57.8	55.1	55.9	56.3	56.3	57.0	57.3	57.1	51.1	53.0	50.8	51.0	49.1	50.6	47.6	49.5
L2aa	46.4	36.2	38.0	43.0	42.1	37.2	45.6	49.2	46.6	40.3	39.3	40.1	38.5	39.7	35.3	29.5	31.2
L2 nt	55.7	51.0	52.8	54.6	54.2	51.4	54.2	54.9	54.2	49.8	50.5	52.0	52.6	52.2	49.4	45.6	49.4
L1aa	60.8	55.4	55.7	55.1	54.5	58.0	61.7	60.8	61.6	63.1	59.2	57.3	55.3	56.6	56.7	51.2	50.7
L1 nt	64.9	61.1	60.0	62.1	60.6	62.2	63.0	62.5	63.4	61.8	60.8	62.9	62.2	61.3	62.4	58.1	59.0

*aa = aminoacid

**nt = nucleotide

Table 2: HPV114 positivity and odds ratios (ORs) for women included in a Stockholm study at baseline cytology, for HC II positivity and for the histopathological report.

Diagnosis	HPV114 status no (%) of samples		OR (95% CI*)	Adjusted OR** (95% CI)
Baseline cytology (n=1581)	Positive samples (n=42)	Negative samples (n=1539)		
ASCUS (n=554)	10 (1.8)	544 (98.2)	1.0 (ref)	1.0 (ref)
CIN I (n=1027)	32 (3.1)	995 (96.9)	1.8 (0.8-4.0)	1.8 (0.9-4.3)
HC II positivity (n=1581)	Positive samples (n=42)	Negative samples (n=1539)		
HC II neg (n=440)	11 (2.5)	429 (97.5)	1.0 (ref)	1.0 (ref)
HC II pos (n=1141)	31 (2.7)	1110 (97.3)	1.1 (0.5-2.4)	1.2 (0.5-2.6)
Histopathological report (n=1112)***	Positive samples (n=28)	Negative samples or no histopathological report (n=1084)		
Benign (n=244)	4 (1.6)	240 (98.4)	1.0 (ref)	1.0 (ref)
Condyloma (n=150)	3 (2.0)	147 (98.0)	1.2 (0.2-7.3)	1.3 (0.2-7.8)
Atypia (n=105)	3 (2.9)	102 (97.1)	1.8 (0.3-	1.8 (0.3-11.0)

			10.6)	
Adenocarcinoma/AIS**** (n=7)	0 (-)	7 (100)	-	-
CIN I (n=221)	9 (4.1)	212 (95.9)	2.5 (0.7-11.5)	2.7 (0.8-12.2)
CIN II (n=200)	7 (3.5)	193 (96.5)	2.2 (0.5-10.3)	2.3 (0.6-10.9)
CIN NOS (n=18)	0 (-)	18 (100)	-	-
CIN III (n=140)	2 (1.4)	138 (98.6)	0.9 (0.1-6.2)	0.9 (0.1-6.3)
Cancer (n=8)	0 (-)	8 (100)	-	-

*CI=confidence interval

**Adjusted for age (<35 or ≥35)

***The number of patients with a histopathological report exceeds the total number in the subgroups since patients with histopathological diagnoses “insufficient” or “irrelevant” are not included in the analysis.

****AIS=adenocarcinoma in situ

Table 3: Primers and probes for sequencing of complete genomes and real-time PCR

HPV type	Forward (5´-3´)	Reverse (5´-3´)	Probe (Reporter dye-5´-3´-MGBNFQ*)
Primers to receive whole genome			
HPV109	GTGGAAGAAGCTGAATGCGAGGAC	CTGCGAGAGCCAGTATTAAGGAC	
HPV112	GAGCATTAGCTGTACCGAAAGTTTC	CTGGTAAAGCCGGATTTGGAATTTC	
HPV114	CCAGGCCTCCACGGAATATAAG	GTAGCGGAGGCCTGGGTAG	
Primers for triplex			
HPV109	GGGCAGCAGCAAAACACTGT	TTGTTACTAAAGACCCGCTTGGA	6-FAM-AAGCCCAGTGTATTTTGCTA-MGBNFQ
HPV112	GCCCTCTGGGACTTGGTACAT	GGATTTTCTGTATCGGCCACTT	VIC-AGGCCATCCGTTGTTTAAT-MGBNFQ
HPV114	CTGGGTATGGCGCTATAGACTTC	GGTCCAAAGGCACCTCAGATT	NED-TGGCGCTACAGGAAA-MGBNFQ

*MGBNFQ = minor groove binder, non fluorescent quencher

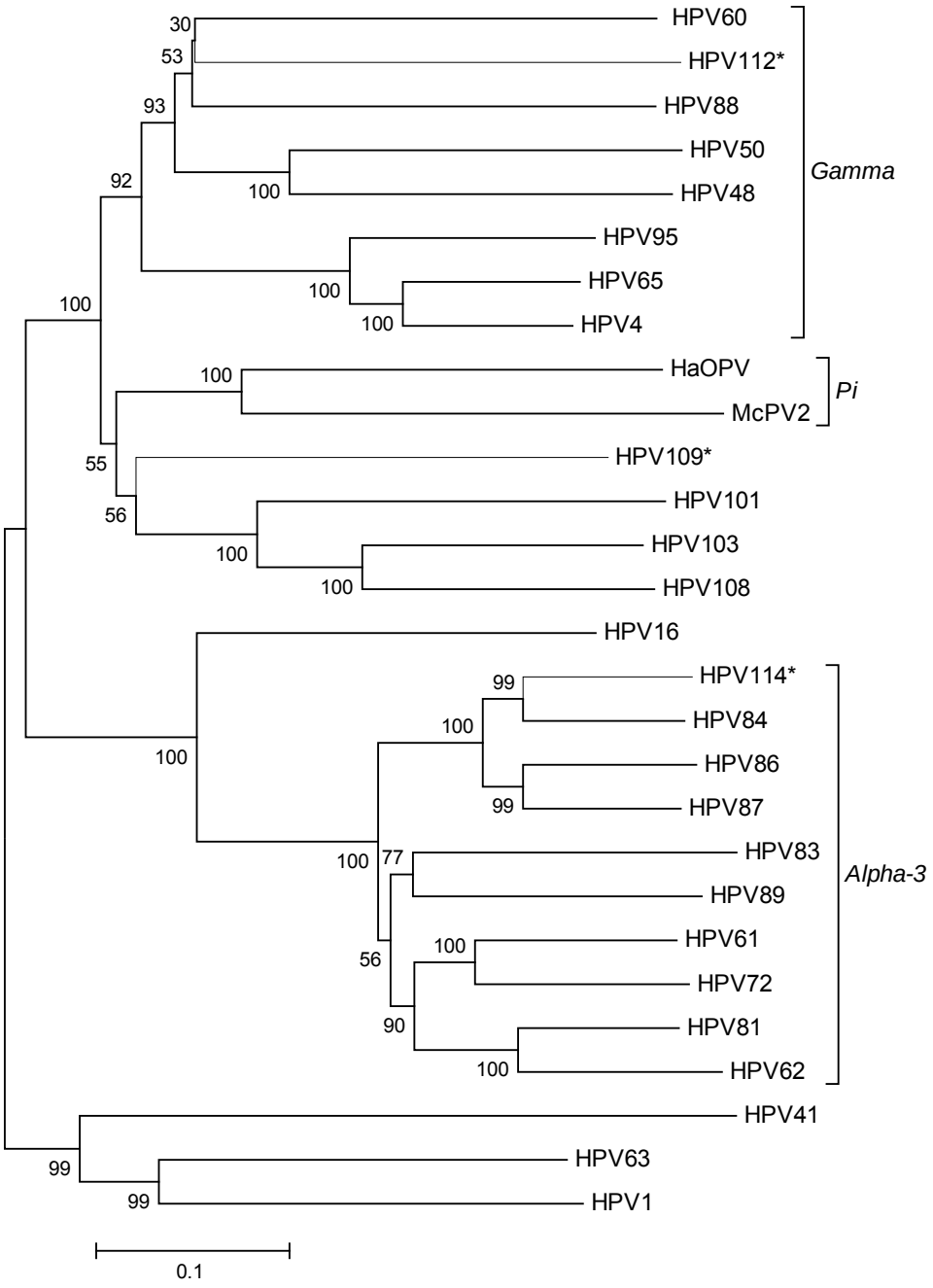

Fig 1: A neighbour joining tree based on the alignment of the L1-sequences of HPV109, 112 and 114 with representative types. The new types are indicated by an asterisk.