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Research paper

Comparative analysis of human papillomavirus type 6 complete genomes originated from head and neck and anogenital disorders

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ABSTRACT

It is increasingly recognized that fundamental differences exist between high-risk and low-risk human papillomavirus (HPV) genotypes regarding interactions with the host. This study aims to join the recently emerging efforts to uncover these differences at the complete genome level and to study how they may influence the disease caused.

Sixteen samples of thirteen patients with various HPV6-mediated benign mucosal disorders (nine recurrent respiratory papillomatoses with 2–8 recurrences, one condyloma acuminatum and three premalignant lesions of the genital mucosa) were sampled to determine the complete virus genomes. We collected the 197 HPV6 complete genomes deposited in the GenBank for cluster analysis to determine (sub)lineages. Genome polymorphisms were determined against the reference sequences of the (sub)lineages. Genome polymorphisms of the long control region (LCR) were tested for putative transcription factor binding sites; their functional analysis was performed by transient transfection of cloned whole LCRs into HEp-2 cells using a luciferase reporter system.

Genomes from the same patients were always identical. Three, nine and one patients carried HPV6 lineage A, sublineage B1 and B2 variants, respectively. The three lineage A sequences were highly similar to each other, but distinct from the reference genome. A unique non-synonymous single nucleotide polymorphism (SNP) was found in the E5a open reading frame (ORF). Sublineage B1 genomes were more diverse, exhibited unique non-synonymous SNPs in the LCR and the E2/E4, L1, L2 ORFs. LCR activity of lineage A and sublineage B1 differed significantly; activity of one sublineage B1 LCR exhibiting two unique SNPs was significantly higher than that of other B1 LCR variants, close to the mean of LCR activities of lineage A variants.

Different HPV6 lineages showed marked differences in variability patterns of the different genome regions. This may be involved in the differences in their distribution in different diseases or patient populations.

1. Introduction

Though the bulk of our present knowledge on mucotropic HPV genotypes is concerned with genotypes associated with high oncogenic risk, recently low-risk genotypes HPV11 and HPV6 started to receive research attentions well. This has been enhanced by appearance of the quadrivalent vaccine, into which these two low-risk genotypes were also incorporated (Villa et al., 2006). Similarly to HPV16 and HPV18 (Yamada et al., 1997; Villa et al., 2000; Lizano et al., 2006; Lee et al., 2008), intratypic variants and subtypes were described for HPV6 and HPV11 (Heinzel et al., 1995; Burk et al., 2011; Burk et al., 2013; Jelen et al., 2014; Jelen et al., 2016), but uncovering the phylogenetic structure of these genotypes has only begun. HPV11 seems to show low diversity with two major lineages based mostly on European sequences (Maver et al., 2011; Jelen et al., 2016), and diseases with prominent severity seem to be linked to unique virus variants (Gáll et al., 2011; Yuan et al., 2012; Gáll et al., 2013). In case of HPV6, in contrast, existence of three major subtypes, HPV6a, HPV6b and HPV6vc is long known (de Villiers et al., 1981; Schwarz et al., 1983; Kovelman et al., 1999; Kocjan et al., 2011). A recent study based on a representative 2800 bp-long sequence fragment using the 48 most divergent published sequences suggested two major HPV6 lineages A and B. Lineage A corresponds to the former HPV6b, while lineage B contains five sublineages, sublineage B1 (formerly HPV6vc), sublineage B3 (formerly

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HPV6a) and three more novel sublineages (B2, B4 and B5) (Jelen et al., 2014).

These lineages and sublineages seem to exhibit differences in geographic distribution. In Europe, where most studied sequences originate from as well as in Caucasian populations in other continents HPV6 sublineage B1 (HPV6vc) and HPV6 lineage A (HPV6b) are the two most frequent variants (Kocjan et al., 2011; Burk et al., 2013; Danielewski et al., 2013; Jelen et al., 2014); dominance of HPV6 sublineage B3 (HPV6a) and B1 (HPV6vc) was reported from Brazil (de Matos et al., 2013; Measso do Bonfim et al., 2015; Flores-Díaz et al., 2017), while in Asia and in Africa dominance of lineage A and sublineage B3, respectively, was shown (Seedat et al., 2010; Jelen et al., 2014). Regarding the anatomical site of origin, lineage B, especially sublineages B1 and B3, was reported to be associated with the anogenital region (Jelen et al., 2014; Flores-Díaz et al., 2017).

Differences in pathogenicity may be inferred, e.g. the first HPV6 sublineage B1 was found in vulvar cancer (Kovelman et al., 1999), but in spite of these novel data, clinical characteristics are rarely reported together with sequence data, and the knowledge on differences in pathogenicity or on association with different diseases remain scant (DiLorenzo et al., 1992; Rübben et al., 1992; Gáll et al., 2011; Gáll et al., 2013; Seedat et al., 2016). In high-risk genotypes, oncoproteins and the differences in the transactivation activity of the long control region (LCR) defining oncoprotein levels are key points in pathogenesis (Xin et al., 2001; Lee et al., 2008; Doorbar et al., 2012; Roman and Munger, 2013; Vande Pol and Klingelhutz, 2013), however, variability in E6 and E7 was reported to be low in HPV11 (Kocjan et al., 2011; Gáll et al., 2013; de Matos et al., 2013). Instead, differences in the LCR sequence and consequently in transactivation activity corresponded to differences in the severity of the respiratory papillomatosis caused (Gáll et al., 2011; Yuan et al., 2012; Gáll et al., 2013). Similar link between LCR alterations and disease coursed were also reported in HPV6 (DiLorenzo et al., 1992; Ure and Forslund, 2012; Seedat et al., 2016).

This work endeavours to collect new data on virological factors affecting pathogenicity by analysis of whole genomes of HPV6s from different diseases.

2. Materials and methods

2.1. Patients and viruses

Since 2000, 34 patients with histologically confirmed recurrent respiratory papillomatosis (RRP) were tested for presence of HPV DNA using the MY/GP consensus nested polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis of amplimers (Kónya et al., 2000). In this population, we detected 29 HPV positive patients, of which thirteen proved to carry HPV6. Presence of HPV6 genome was confirmed using type-specific PCR (Evander and Wadell, 1991). The present study is concerned with nine of the thirteen detected HPV6 positive RRP patients (Table 1; sample amount did not allow for sequence analysis for the other four HPV6 positive patients).

Besides viruses of RRP patients, HPV6s of four patients originated from genital samples (one patient with low-grade squamous intraepithelial lesion [LSIL1/6]; one patient with atypical squamous cells of undetermined significance [ASCUS1/6]), with condyloma acuminatum (CAC1/6) of the vulva and with leukoplakia penis (LP1/6) (Table 1). In case of patients AO-RRP1/6, AO-RRP3/6 and AO-RRP5/6 multiple episodes were sampled, of which the complete genomes of HPV6s were determined from temporally distant samples. Written informed consent was collected from each patient. All samples were collected in Hungary in University of Debrecen (Clinic of Otorhinolaryngology and Head & Neck Surgery, Clinic of Obstetrics and Gynaecology) and originated from the EU regions Northern Great Plain and Northern Hungary. The study received ethical committee approval (approval number: 4169–2014).

2.2. Amplification and sequencing of the genomic regions

Sequences of primers used for complete genome amplification are shown in Supplementary Table 1. PCRs were performed using Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, USA); amplimers were purified by QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and were sequenced in duplicates from both directions using the dideoxy chain termination method (Macrogen Europe, Amsterdam, the Netherlands). Sequences were assembled and analysed using CLC Main Workbench 7.9.1 (Qiagen, Aarhus, Denmark). Genomes were deposited in the GenBank under accession numbers MK313769-MK313781.

2.3. Analysis of sequences

The 197 HPV6 complete genomes available in GenBank were collected (Supplementary Table 2) and aligned together with our sequences. Identical sequences were used only once. Dendrograms were reconstructed using the neighbour-joining method with bootstrapping 1000 times (CLC Main Workbench 7.9.1; Qiagen, Aarhus, Denmark) and were used to determine the (sub) lineage of the newly collected sequences. The HPV11 (M14119) reference sequence was included as outgroup (Supplementary Table 2).

As the reference sequence is generally the first sequence reported for the (sub)lineage and may differ substantially from the most frequent sequence variant, a consensus sequence was determined for all (sub) lineages in an endeavour to represent this most frequent sequence variant. The consensus was determined using (sub)lineage alignments prepared by realigning all sequences of the sublineage using the CLC Main Workbench 7.9.1 (Qiagen, Aarhus, Denmark).

New sequences determined in this study were analysed against the respective reference HPV6 genomes sequenced from condyloma acuminatum (NC_001355 for lineage A [HPV6b], FR751328 [CAC301] for sublineage B2 and L41216 for sublineage B3 [HPV6a]) and a vulvar squamous cell cancer (AF092932 for sublineage B1 [HPV6vc]) (Schwarz et al., 1983; Hofmann et al., 1995; Kovelman et al., 1999; Kocjan et al., 2011) as well as against the consensus sequences determined.

2.4. Functional analysis of the long control region of different HPV6 variants

The plasmid containing the reference lineage A (HPV6b; NC_01355) sequence was kindly provided by Dr. H. zur Hausen. For functional analysis of long control regions (LCRs), reference and unique LCR sequence variants were cloned to pALuc luciferase reporter vector using primers 6LCR_BamHI 5′-CCGGGATCCGCAAGTTTTTGTTACAAAGT-3′ (nucleotide 7171–7191; recognition site for BamHI is underlined), and 6LCR_KpnI 5′-GCCGGTACCATAGATTAAACGTCTTGCAC-3′ (nucleotide 148–168; recognition site for KpnI is underlined). Amplifications were performed with Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, USA). Initial denaturation (98 °C for 3 min) was followed by 35 cycles of PCR: denaturation at 98 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s. The final extension was 72 °C for 5 min.

The amplification products were analysed by electrophoresis on 0.8% low-melting agarose gels (Lonza, Basel, Switzerland), were excised from the gel, purified using Qiaquick Gel Extraction Kit (Qiagen, Hilden, Germany) and digested by BamHI and KpnI (Promega, Madison, USA), then purified again and ligated into precut luciferase reporter vector using T4 DNA ligase (Promega, Madison, USA). The ligated plasmids were transformed into *Escherichia coli XL-1* strain using TransformAid Bacterial Transformation Kit (Thermo Scientific, Waltham, USA) according to the manufacturer's recommendation. The purified (PureLink HiPure Plasmid Midiprep Kit, Thermo Scientific, Waltham, USA) plasmids were verified by sequencing and used in

Table 1
Clinical characteristics of patients diagnosed with diseases associated with human papillomavirus type 6.

Patient ID	Gender	Age at the time of diagnosis (years)	Localization	No. of surgical interventions since diagnosis	Dysplasia	Follow up time (years)	Adjuvant therapy
JO-RRP1/6	M	12	Larynx	5	No	62	No
JO-RRP2/6	F	8	Larynx	5	Moderate	9	No
AO-RRP1/6[a]	M	28	Larynx	8	Mild	16	CDV
AO-RRP2/6	M	25	Pharynx, larynx	1	Moderate	3	No
AO-RRP3/6[a]	M	36	Nasal cavity	6	No	10	CDV
AO-RRP4/6	M	71	Larynx	2	Moderate	6	No
AO-RRP5/6[a]	M	22	Pharynx	8	No	19	CDV
AO-RRP6/6	M	30	Larynx	2	No	5	No
AO-RRP7/6	M	27	Larynx	2	No	7	No
CAC1/6	F	41	Vulva	3	No	6	Podophyllin
LSIL1/6	F	23	Cervix	N.A.	N.A.	N.A.	N.A.
ASCUS1/6	F	51	Cervix	N.A.	N.A.	N.A.	N.A.
LP1/6	M	28	Penis	N.A.	N.A.	N.A.	N.A.

JO-RRP juvenile-onset recurrent respiratory papillomatosis; AO-RRP adult-onset recurrent respiratory papillomatosis; CAC condyloma acuminatum; LSIL low-grade squamous intraepithelial lesion; ASCUS atypical squamous cells of undetermined significance; LP leukoplakia penis; M male; F female; CDV cidofovir; N.A. not applicable.
None of the patients had tracheostomy, spreading to lower airways or malignant transformation.
[a] Multiple samples from different recurrences were analysed.

transient transfection experiments as described earlier (Gáll et al., 2013). Briefly, HEp-2 (ATCC Number CCL-23) laryngeal cancer cells were transfected with 2 μg of the reporter vector pALuc containing the unique LCR sequence variants and 1 μg of RSV-β-Gal plasmid as an internal control for transfection efficiency. Cells were transfected in 6-cm-diameter dishes (10^6 cells/dish) with Lipofectamine 2000 (Invitrogen, Gaithersburg, USA), according to the manufacturer's recommendation. Cells were harvested 48 h posttransfection by the addition of 500 μL Reporter Lysis buffer (Promega, Madison, USA) and lysed by a freeze–thaw cycle; the luciferase activities of the cell extracts were measured using the Luciferase Assay System (Promega, Madison, USA) according to the manufacturer's recommendation. Transfection was standardized using β-galactosidase assay by adding 1 mg/mL ortho-nitrophenyl-β-galactoside chromogenic substrate and normalized to the protein concentration as measured by the Bradford method. LCR activities of all sequences were tested simultaneously in three independent experiments, means of luciferase activities from the three experiments were compared using ANOVA with Tukey's post-tests with Bonferroni correction as appropriate (PaSt2.17c; Hammer et al., 2001).

In case of HPV6 sublineage B1, the reference LCR was not available for functional characterization; for this reason, three of the seven LCRs with sequences identical to the reference sequence (AF092932) derived from our clinical samples were chosen randomly and tested. The mean of all nine luciferase activities measured in the triplicate experiments with these three LCRs identical to the reference sequence was regarded as the reference LCR activity of sublineage B1 as well as of sublineage B2. Relative luciferase activities were calculated as the ratio of the mean measured activity of a sequence variant divided by the reference luciferase activity.

Putative transcription factor binding sites were predicted by the PROMO tool on the ALGGEN server (Farré et al., 2003) using a maximum dissimilarity of 10%. Loss or gain of putative transcription factor binding sites in the LCRs were determined compared to the reference sequence of the (sub)lineage.

3. Results

3.1. Sequences and lineage determination

Sequences from different samples of the same patients (AO-RRP1, AO-RRP3 and AO-RRP5) were always identical. Among the thirteen complete genomes three clustered together with the HPV6 lineage A reference (Table 2), nine with the sublineage B1 reference (Table 3) and one sequence clustered with sequences of sublineage B2 (Table 4) (Fig. 1). Sublineage B3 (HPV6a), B4 and B5 did not occur. In case of HPV6 lineage A and sublineage B1, size of all genomes was identical to the size of the reference genomes (7902 and 8012 bps, respectively); the sequence from sublineage B2 showed a 19 bps deletion absent in the 19 available sublineage B2 genomes. ORFs E2, E6 and E7 were highly conserved, while ORFs E5a, L1, L2 and the LCR showed higher variability. Dendrograms built using individual ORF or LCR sequences yielded topologies largely identical to the complete genome dendrograms (data not shown).

3.2. Genome analysis

In case of HPV6 lineage A, thirteen nucleotide positions were different between the reference sequence and the consensus; in addition, a 94-bp insertion was present in the LCR of the consensus sequence (between nucleotide positions 7250 and 7251), which is absent from the reference sequence or the sequences of this study (Table 2). In case of sublineage B1 in contrast, the reference and the consensus differed only at a single nucleotide position (Table 3). In case of sublineage B2 six nucleotide positions were polymorphic between the reference sequence and the consensus (Table 4).

At DNA level, 25, 26 and six single nucleotide polymorphisms (SNPs) were identified in case of HPV6 lineage A, sublineage B1 and B2 (Tables 2, 3 and 4), respectively, compared to the reference genomes. Lineage A genomes carried 17–21 SNPs/genome, sublineage B1 genomes exhibited 2–8 SNPs per genome, while the single sublineage B2 genome had six SNPs; corresponding to 2.15–2.66, 0.25–1.00 and 0.75 SNPs per 1000 bps, respectively. In HPV6 sublineage B1, the variability of genomes was higher in case of sequences from juvenile-onset RRPs and from condyloma than in case of adult-onset RRPs (0.87–1.00 *vs.* 0.25–0.62 SNPs per 1000 bps).

In case of HPV6 lineage A, SNPs were found in ORFs E1, E5a, E6, L1 and L2 as well as in the noncoding region between E5b and L2 and the LCR (Table 2). At the deduced protein level, nevertheless, these did not cause an amino acid alteration, except for two polymorphisms found in a single lineage A sequence in the E5a ORF.

SNPs were found in the LCR as well as in all ORFs except for E7 in case of sublineage B1 (Table 3); in contrast to lineage A, four, one and three SNPs causing amino acid changes were detected in the E2/E4, L2 and L1 ORFs, respectively (Table 3). All of these were unique except for Y219D in the L1 ORF, which was present in all sublineage B1 sequences from this study.

Thus, at the deduced protein level only one of the three lineage A sequences was different from the reference, while all sublineage B1

Table 2
Nucleotide changes in human papillomavirus type 6 lineage A (HPV6b) complete genomes.

ORF	Nucleotide position	Reference sequence NC_001355	Consensus[1]	AO-RRP2/6 MK313770	AO-RRP3/6[2] MK313771	AO-RRP4/6 MK313772	Amino acid change/comment
E6	221	A	T	T	T	T	no
(nt 102..554)	473	G	A	A	A	A	no
E1	1554	G	A	A	A	A	no
(nt 832..2781)	1926	G	A	A	A	A	no
	1938	C	C	T			no
	2518	C	C			T	no
	2709	T	A	A	A	A	no
E5A	4003	G	G			C	E39D
(nt 3887..4162)	4118	C	C			T	P78S
Non coding sequence	4308	A	A		G		
L2	4731	T	C	C	C	C	no
(nt 4423..5802)	4740	T	T			C	no
	4755	G	G	T	A	T	no
	5406	G	A	A	A	A	no
	5637	T	T		G		no
L1	6073	A	A			G	no
(nt 5789..7291)	6166	T	T		G		no
	6598	A	T	T	T	T	no
	7099	G	A	A	A	A	no
LCR[3]	7373	G	T	T	T	T	loss of putative binding site for FOXP3; new putative binding site for GR-β
(nt 7292..7902, 1..101)	7585	A	C	C	C	C	loss of putative binding site for C/EBP-β, GR-β; new putative binding site for LEF-1, SRY
	7653	G	G	C	C	C	loss of putative binding site for
							C/EBP-β; new putative binding sites for GR-β, C/EBP-α
	7654	C	A	A	A	A	loss of putative binding site for C/EBP-β, NF-1; new putative binding sites for GR-β
	7815	G	G	C		C	putative binding site for C/EBP-β
	7860	G	A	A	A	A	localized immediately next to an E2 binding site; new putative binding sites for C/EBP-β

Nucleotide changes were not found in ORFs E2/E4, E5b and E7, therefore these are not shown. Empty grey cells represent the same nucleotide as in the reference sequence. ORF open reading frame; E early; L late; LCR long control region; nt nucleotide; AO-RRP adult-onset recurrent respiratory papillomatosis.
[1]The consensus sequence was determined from alignments of the lineage A genomes available in the GeneBank as described in the Materials and Methods.
[2]Multiple samples were analysed.
[3]The consensus carries a 94-base-pair long insertion between nucleotide positions 7250 and 7251.

sequence variants exhibited at least one SNP leading to amino acid alteration compared to the reference genome.

The sublineage B2 sequence differed from the B2 reference sequence in the E1, E5a, L1 and L2 ORFs as well as in the LCR (Table 4); of which only a single SNP in the L2 (H157N) resulted in amino acid change compared to the reference genome.

3.3. Sequence and functional analysis of LCRs

In HPV6 lineage A sequences, LCRs exhibited six SNPs compared to the reference sequence, all of which except for G7815C were present in all three genomes (Table 2). Putative transcription factor binding sites disrupted or created by SNPs are shown in Table 2. Transcription activation potential of all three sequences was statistically comparable to each other and to the reference LCR ($p = .47–0.99$) (Fig. 2).

In case of HPV6 sublineage B1 genomes, sequences of seven of the nine LCRs were identical to the reference LCR, the remaining two exhibited SNPs; in JO-RRP2/6 an A7332C and in CAC1/6 A7342G and T7909G SNPs were found (Table 3). These SNPs do not alter any putative transcription factor binding sites. The mean luciferase activities of the three tested LCRs identical to the reference sequence were closely similar ($p = .83–0.96$); the reference luciferase activity was defined as the mean of the luciferase activities measured for these LCRs. Activity of the JO-RRP2/6 was comparable to the reference activity (relative activity 0.88, $p = .20$). Activity of CAC1/6, in contrast, was significantly higher than either the reference activity (relative activity 1.74, $p < .001$) or the activity of JO-RRP2/6 ($p < .001$). The LCR of the single sublineage B2 genome carried a 19-bps deletion (between nucleotid positions 7361 and 7379) and two SNPs G7623T and T7645C compared to the B2 reference sequence. Curiously, with this 19-bps deletion, the size of its LCR is identical to that of sublineage B1 (compared to sublineage B1 reference four SNPs were found in the LCR, i.e. C7613G, T7626C, C7669G, C7900A). The deletion and the T7645C SNP led to loss of four and two putative transcription factor binding sites, respectively (Table 4). LCR activity (Fig. 2) was comparable to that of sublineage B1 sequences ($p = .20–0.81$), except for CAC1/6 ($p < .001$). Lineage A LCRs showed significantly higher activity than sublineage B1 or B2 LCRs when comparing all individual sequence variants as well as when comparing means of HPV6 lineage A to means of HPV6 lineage B1 sequences ($p < .001$ in both comparisons).

4. Discussion

Lack of sublineage B3 (HPV6a) and relative abundance of sublineage B1 (HPV6vc) among these new whole genomes is in line with the presently available data regarding the European distribution of HPV6 lineages (Jelen et al., 2014).

HPV6 genome was stable in consecutive samples; a finding also reported unequivocally by earlier studies (Kocjan et al., 2013; Measso do Bonfim et al., 2015). Low variability in a given host was also observed in case of HPV11 (Gáll et al., 2011; Gáll et al., 2013; Kocjan et al., 2013), but the opposite was reported for HPV16 by some authors (Mendes de Oliveira et al., 2015; Dube Mandishora et al., 2018). Thus, low-risk types seem to be markedly conserved between temporally distant virus sequences, while in high-risk types within-host variability was observed. This genome stability, at the same time, confirms that the symptomatic episodes are linked to reactivation of the virus in the patient rather than reinfections.

Major genome rearrangements are rarely reported in low-risk HPVs and are related to unfavourable outcome; malignant transformation (DiLorenzo et al., 1992; Yuan et al., 2012) or extreme high number of recurrences (Seedat et al., 2016).

Notably, such genome rearrangements are not needed for progression of the malignancies caused by high-risk types, where tumour progression was repeatedly found to be associated with accumulation of point mutation in the viral genome, especially in E6 ORF (Zehbe et al., 1998; Villa et al., 2000; Zehbe et al., 2009; Tamegao-Lopes et al., 2014; Cao et al., 2016). In contrast, SNPs causing amino acid change were never found in E6 in the sequences reported in this study. This is in line with the findings in Hungarian HPV11 genomes as well (Gáll et al., 2011; Gáll et al., 2013).

These suggest that E6 may be more conserved in low-risk types than in high-risk HPVs (Gáll et al., 2011; Danielewski et al., 2013; Kocjan et al., 2013; de Matos et al., 2013). The conserved nature of E7, in contrast, seems to be a shared characteristic between the two groups of HPVs (Zehbe et al., 1998;. Van Doorslaer, 2013).

Other early and late ORFs were more variable among the HPV6 sequences reported in this study; moreover, the variability of these ORFs and the LCR showed a notable difference between (sub)lineages. In our three HPV6 lineage A genomes almost all SNPs were synonymous, and the sequences were highly similar. Differences between the reference sequence and the consensus, especially in the LCR, suggests that the lineage A reference sequence may be a unique variant and does not represent the wild type similarly to the HPV11 reference sequence (M14119, Gáll et al., 2013). In contrast, our HPV6 sublineage B1 sequences were diverse and were positioned at various distances to the reference sequence, which was highly similar to the consensus. Most SNPs in sublineage B1 sequences were unique, many of which result in amino acid changes. Sequences with these unique SNPs originated from RRPs with several episodes of recurrence (5–8) and/or mild to moderate dysplasia in the papilloma/condyloma tissue, while the two sequences with only the ubiquitous L1 polymorphism showed only a single recurrence after removal of the initial papilloma.

ORFs showing the highest diversity were E2/E4, L1 and L2, suggesting that individual viruses may exhibit differences in the function of these proteins, which, in turn, may explain, at least partly, the higher number of recurrences; similarly to findings reported for HPV11 (Gáll et al., 2013).

Only a single lineage A sequence (AO-RRP4/6) showed SNPs leading to amino acid change, both in the E5a. SNP E39D (notably, the amino acid in this position of HPV11 is D) corresponded to the loop between the putative first and the second transmembrane domains of HPV16 E5, which is localized probably on the extracellular part of the protein, while the other (P78S) created a potential phosphorylation site mapping to the putative third transmembrane domain of HPV16 E5 (DiMaio and Petti, 2013). Hypothetically, at least the probably extracellularly localized E39D may influence growth factor binding, thus may contribute to the moderate dysplasia found in the papilloma of this patient.

In HPV6 sublineage B1 sequence variants, E2/E4, L1 and L2 ORF showed notable variability at the deduced protein level. Two polymorphisms in the E2/E4 ORFs affected the transactivation domain (T116N in AO-RRP1/6 and S144T in JO-RRP2/6) of the E2 regulatory protein; one polymorphism was in the hinge region (S246A in JO-RRP1/6), eliminating a potential phosphorylation site in this phosphorylation hot-spot of the protein; and one (E340D in AO-RRP5/6) was in the DNA-binding and dimerization domain. As inferred from their position, these polymorphisms may influence E1 binding and consequently initiation of DNA replication as well as transcriptional activation; intracellular localization, chromatin binding and self-

Table 3
Nucleotide changes in human papillomavirus type 6 sublineage B1 (HPV6vc) complete genomes.

ORF	Nucleotide position	Reference sequence AF092932	Consensus[1]	JO-RRP1/6 MK313776	JO-RRP2/6 MK313777	AO-RRP1/6[2] MK313769	AO-RRP5/6[2] MK313773	AO-RRP6/6 MK313774	AO-RRP7/6 MK313775	CAC1/6 MK313778	LSIL1/6 MK313779	LP1/6 MK313780	Amino acid change/ comment
E6 (nt 103..555)	399	C	C	T									no
	507	T	T				C						no
E1 (nt 832..2781)	1831	T	T		G								no
	2146	A	A						C				no
	2170	T	T			C	C	C			C	C	no
	2278	T	T		G								no
E2 (nt 2724..3830) E4 (nt 3256..3585)	2814	C	C	T									no
	3070	C	C			A							T116N
	3153	T	T		A								S144T in E2
	3459	T	T	G									S246A in E2 S68R in E4 (S50R in E1^E4)
	3743	G	G				C						E340D in E2
E5A (nt 3887..4162)	4100	T	T	C									no
	4101	T	T	C	C				C	C			no
Non coding sequence	4385	A	A				C						
L2 (nt 4424..5803)	4441	C	C					T					no
	4786	T	T							C			no
	5546	A	A							G			T375A
L1 (nt 5790..7292)	5930	T	T		G								no
	6065	A	A							G			no
	6323	T	T	C									no
	6444	T	G	G	G	G	G	G	G	G	G	G	Y219D
	7112	T	T							G			F441L
	7134	A	A	G									K449E
LCR[3] (nt 7293..80121..102)	7332	A	A		C								
	7342	A	A							G			
	7909	T	T							G			putative binding sites for C/EBP-β, GR

Nucleotide changes were not found in ORFs E5b and E7, therefore these are not shown. Empty grey cells represent the same nucleotide as in the reference sequence. ORF open reading frame; E early; L late; LCR long control region; nt nucleotide; JO-RRP juvenile-onset recurrent respiratory papillomatosis, AO-RRP adult-onset recurrent respiratory papillomatosis; CAC condyloma acuminatum; LSIL low-grade squamous intraepithelial lesion; LP leukoplakia penis.
[1]The consensus sequence was determined from alignments of the sublineage B1 genomes available in the GeneBank as described in the Materials and Methods.
[2]Multiple samples were analysed.
[3]In HPV6 sublineage B1, SNPs did not influence the binding sites for transcription factors.

regulation; or L1 binding (Siddiqa et al., 2015) and regulating oncoprotein expression through LCR binding of the E2, respectively (McBride, 2013). The polymorphism S246A also affected the E1/E4 fusion protein as S50R (corresponding to S68R when directly translating the E4 ORF alone) localized in the negatively charged proline-rich region (Doorbar, 2013).

The unique polymorphisms F441 L in patient CAC1/6 and K449E in patient JO-RRP1/6 are localized in the C-terminus invading arms linking L1 capsomers, thus in the region involved in capsid assembly and encapsidation as well as in heparan sulfate binding, which also contains several epitopes. Remarkably, heparan sulfate binding is linked to lysine residues in HPV16, thus the loss of lysine (K449E) may impair this binding (Bishop et al., 2007; Buck et al., 2013).

The localization and the inferred functional characteristics of these polymorphisms suggest that variations may exist between individual viruses in regulation of viral transcription and/or replication (E2), virus release (E4), capsid assembly (L1, L2) or interaction with the host (E5a, L1, L2).

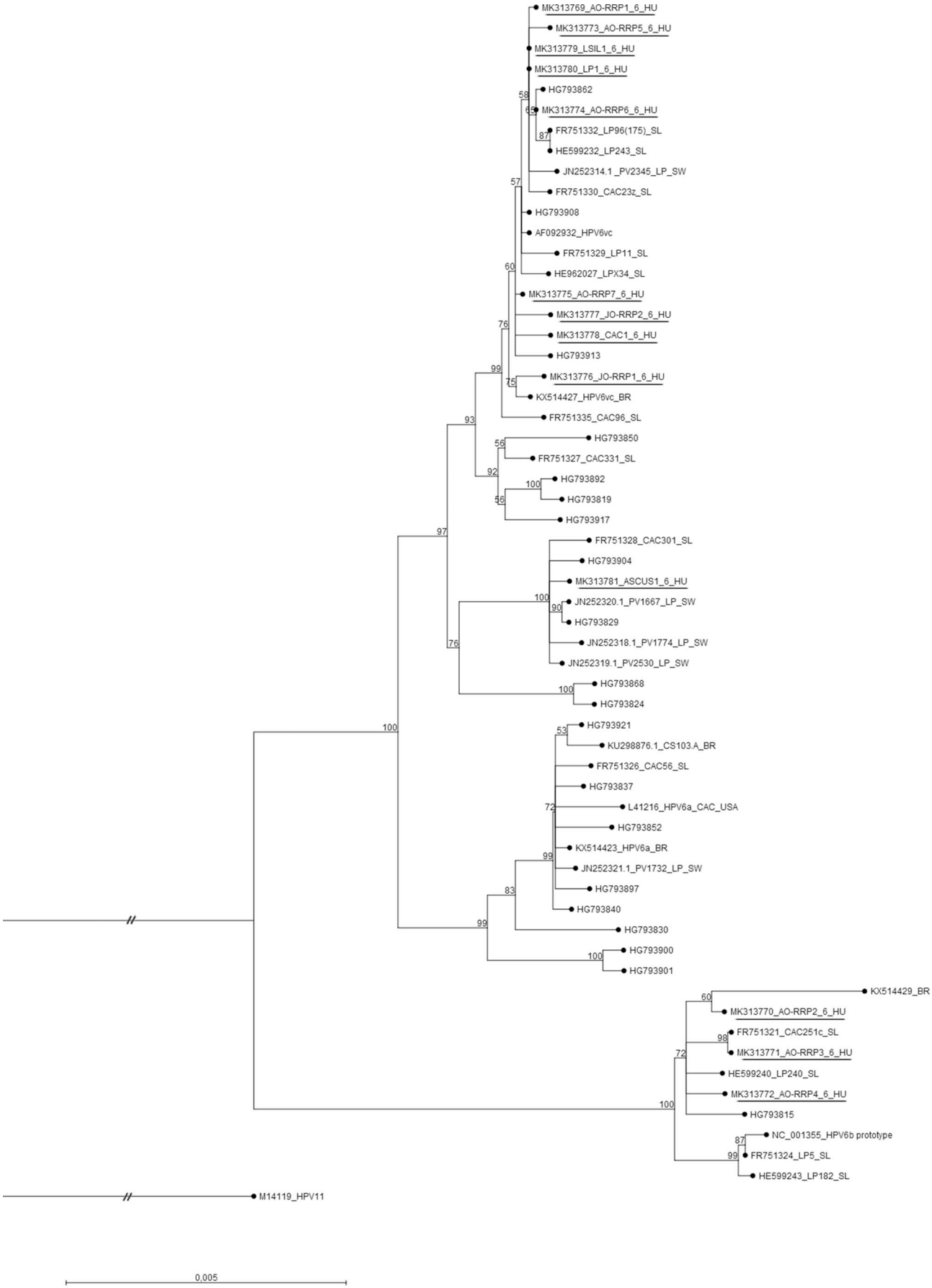
The oncoproteins E6 and E7, in contrast, were highly conserved in case of HPV 11 (Danielewski et al., 2013; Gáll et al., 2013) as well as in case of HPV6 (Grassmann et al., 1996; this study), even across genotypes, suggesting that their role is very tightly regulated, and their function can tolerate no or minimal structural alterations. This may also apply for the E5b ORF of as yet unknown function. This is in line with the widely accepted fact that the mechanism of induction of cell proliferation by low-risk HPVs shows important differences compared to high-risk HPVs (Doorbar et al., 2012). Key oncoproteins E6 and E7 play a fundamental role in cell transformation and proliferation caused by high-risk genotypes and the differences in oncogenic risk between genotypes and subtypes is predominantly determined by E6 and E7 variability (Xin et al., 2001; Lizano et al., 2006; Lee et al., 2008; Mirabello et al., 2017). In contrast, in case of low-risk genotypes differences in pathogenesis between individual viruses seems hardly affected by these oncoproteins, the differences are more likely to be linked to proteins encoded by the more variable ORFs E1, E2/E4 and E5a, to capsid proteins or to differences in LCR activity.

Sequence variation of the LCR across the HPV6 lineages showed differences markedly similar to those found in the variability of ORFs. While two HPV6 sublineage B1 LCRs contained altogether three SNPs, in case of lineage A the differences are shared by all three sequences. In line with this, transactivating potential of our lineage A LCRs was highly similar to each other as well as to the reference. In case of sublineage B1, LCRs of seven of nine viruses were identical, a LCR with the unique SNPs A7342G and T7909G (Patient CAC1/6 with six episodes) showed a significantly increased activity compared to other sublineage B1 LCRs. This pattern reflects what was reported by Measso do Bonfim et al. (2015) concerned with the link between LCR sequence differences and transactivating potential. Existence of HPV6 (Combrinck et al., 2012; Seedat et al., 2016) and HPV11 (Yuan et al., 2012) variants with partially duplicated LCRs and consequent higher

Table 4
Nucleotide changes in human papillomavirus type 6 sublineage B2 complete genome.

ORF	Nucleotide position	Reference sequence FR751328	Consensus[1]	ASCUS1/6 MK313781	Amino acid change/comment
E1 (nt 833..2782)	1072	A	G	A	no
	2407	C	A	A	no
E5A (nt 3888..4163)	4159	C	A	A	no
L2 (nt 4424..5803)	4892	C	A	A	H157N
L1 (nt 5790..7292)	5978	A	A	T	no
LCR (nt 7293..8031, 1..102)	7361-7379			19 bp-long deletion[2]	loss of putative binding sites for C/EBP-β, FOXP3, PR A, PR B
	7623	G	T	T	putative binding site for GR
	7645	T	T	C	putative binding sites for HOXD9, HOXD10 loss of putative binding sites for GR-β, TFIID,

Nucleotide changes were not found in ORFs E2/E4, E5b, E6 and E7, therefore these are not shown. ORF open reading frame; E early; L late; LCR long control region; nt nucleotide; ASCUS atypical squamous cells of undetermined significance.
[1]The consensus sequence was determined from alignments of the sublineage B2 genomes available in the GeneBank as described in the Materials and Methods.
[2]TATATGTATGTGTGTTGTA.
Empty grey cells represent that the 19 bp-long deletion is not present in the reference and consensus sequences.



(caption on next page)

Fig. 1. Dendrogram of representative archive and presently reported HPV6 genomes with the HPV11 reference sequence (M14119) used as an outgroup. Of the 197 genomes available in the GenBank, selection of representative archive sequences followed Jelen et al. (2014), i.e. included the most diverse sequences. In addition, we used all sequences which could be identified in the dendrogram reported by Jelen et al. (2014), all reference sequences for the (sub)lineages and all genomes determined in the present study. The dendrogram was constructed using the neighbour-joining method with 1000 bootstrapping and rooted to the outgroup. Bootstrap percentages higher than 50% are shown on the branches. New sequences reported in this study are underlined.

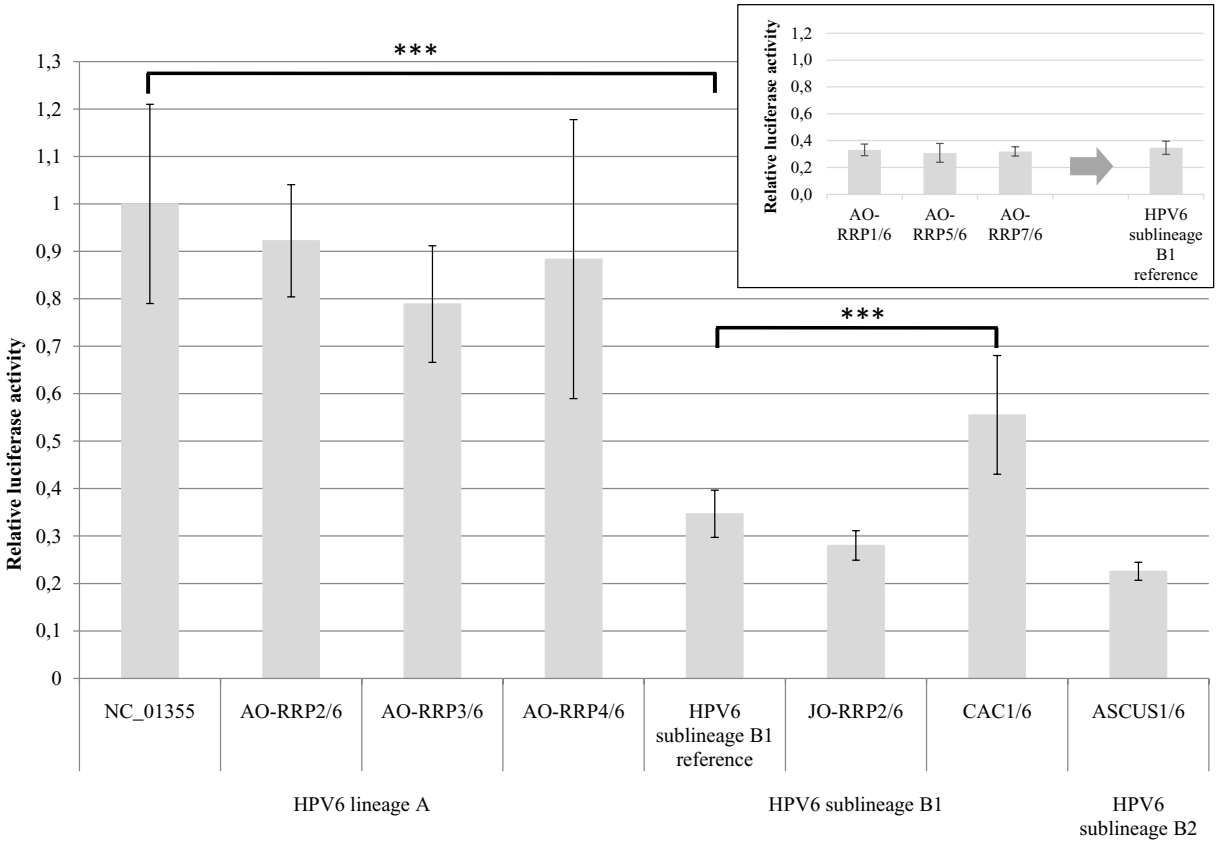


Fig. 2. Transcriptional activity of the LCRs of HPV6s derived from recurrent respiratory papillomatoses with different severity and anogenital disorders, as determined by luciferase tests in HEp-2 cells. Data presented represent the averages of three independent experiments, with error bars indicating the standard deviations. The inset figure shows the determination of the reference LCR activity for sublineage B1 (see text for details). Statistically significant comparisons are shown by linking. ***: $p < .001$. AO-RRP adult-onset recurrent respiratory papillomatosis; JO-RRP juvenile-onset recurrent respiratory papillomatosis; CAC condyloma acuminatum; ASCUS atypical squamous cells of undetermined significance.

transactivating potential, which caused more aggressively spreading disease lend further support to this hypothesis. However, Grassmann et al. (1996) did not report differences in LCR activities of LCR sequence variants, as also seen with the polymorphic LCR in JO-RRP2/6 with an activity comparable to the reference activity. Thus, as also reported in HPV11 (Gáll et al., 2013), polymorphic sites may or may not cause significant alterations in LCR activity.

Measso do Bonfim et al. (2015) also reported a major difference between the LCR activity of HPV6 sublineages; HPV6 sublineage B3 (HPV6a) was found to exhibit lower activity than HPV6 sublineage B1. Notably, transactivating potential of HPV6 lineage A in our study was significantly higher than that of sublineage B1 sequence variants and of the lineage B2 sequence, except for the mutant LCR of Patient CAC1/6, which has statistically similar, though somewhat lower, transactivating potential. This, together with our data suggests that the LCR activity shows a gradient from the highest HPV6 lineage A through sublineage B1 to sublineage B3 showing the lowest activity.

These taken together suggests that there are differences in the ways to achieve cell proliferation between the HPV6 lineage A and sublineage B1, possibly between the two main HPV6 lineages A (represented by HPV6b) and B (represented by HPV6 sublineage B1 and the single B2 sequence in this study). HPV6 lineage A seems to rely more on transactivating potential than sublineage B1; the possible greater importance of LCR activity is also supported by the frequent alteration of transcription binding sites by the SNPs in lineage A but not in sublineage B1 LCR. Similarly, such differences between clusters were also suggested for HPV11 (Gáll et al., 2013).

HPV6 sublineage B1 also shares some similarities with HPV11 generally accepted to be more aggressive (Rabah et al., 2001; Wiatrak et al., 2004). Both JO-RRPs were caused by HPV6 sublineage B1, while HPV6 lineage A genomes were found exclusively in AO-RRPs. E2/E4, L2 and L1 ORFs contained unique SNPs. Some genomes had unique LCRs, which showed increased transactivating potential as compared to that of the reference LCR. Nevertheless, differences in the disease course or severity among the patients infected with either detected subtypes of HPV6 were much less prominent than those reported among HPV11s in a similarly small patient population (Gáll et al., 2013).

5. Conclusion

Similarly to high-risk types, lineages of HPV6 show marked differences in variability patterns of the different genome regions. This may be involved in differences in the distribution of the variants in different

diseases or in different patient populations. Thus, differences in pathogenicity reported for high-risk genotypes as well as for HPV11 are likely to exist also in HPV6, and may, at least partly, be a lineage-specific characteristic.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.03.019.

Conflict of interest

The authors declare that they have no conflict of interest.

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