Phylogenetically related, clinically different: human papillomaviruses 6 and 11 variants distribution in genital warts and in laryngeal papillomatosis

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Abstract

Genital warts (GWs) and laryngeal papillomatosis (LP) are two usually benign pathologies related to infection with human papillomaviruses (HPVs), mainly HPV6 and HPV11. The aim of this work was to describe the genetic diversity of HPV6 and HPV11 isolates found in GWs and LPs, and to analyse the differential involvement of viral variants in either lesion. A total of 231 samples diagnosed as GWs (n = 198) or LP (n = 33) and caused by HPV6 or HPV11 monoinfections were analysed. The phylogenetic relationships of the retrieved viral sequences were explored. We have identified the long control region and the intergenic E2–L2 region as the two most variable regions in both HPV6 and HPV11 genomes. We have generated new HPV6 (n = 166) or HPV11 (n = 65) partial sequences from GWs and LPs lesions spanning both regions and studied them in the context of all available sequences of both types (final n = 412). Our results show a significant ($p < 0.01$) differential presence of HPV6 variants among both pathologies, with HPV6 B variants being preferentially found in GW versus LP samples. No differential involvement of HPV11 variants was observed. Our findings suggest that different HPV6 variants may either show differential tropism or have different potential to induce lesions in different epithelia.

Keywords: Genital warts, human papillomaviruses, laryngeal papillomatosis, phylogeny, recurrent respiratory papillomatosis, tissue tropism, variants

Original Submission: 25 June 2013; **Revised Submission:** 2 September 2013; **Accepted:** 30 September 2013

Editor: G. Antonelli

Article published online: 9 December 2013

Clin Microbiol Infect 2014; **20:** O406–O413

doi: 10.1111/1469-0691.12420

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Introduction

Papillomaviruses are small, non-enveloped viruses with a circular double-stranded DNA genome of around 8000 bp [1]. More than 250 complete papillomavirus genomes have been described, infecting human and non-human hosts (http://pave.niaid.nih.gov/#home). Human papillomaviruses (HPVs) are the causative agents of cancer of the cervix, and are also involved in cancers of the penis, anus, vagina, vulva, and head and neck, as well as in other benign, wart-like lesions [2]. Based on this association to cervical cancer, HPVs have been epidemiologically stratified into three risk groups: carcinogenic, probably and possibly carcinogenic, and not carcinogenic to humans [3]. Alphapapillomaviruses HPV6 and HPV11 belong to the non-carcinogenic group, being the most common non-oncogenic HPVs found in cervical specimens in the general population [4].

HPV6 and HPV11 are the causative agents in some conspicuous lesions, namely anogenital warts (GWs) and

laryngeal papillomatosis (LP). GWs are benign tumours of the epithelium caused by papillomavirus infection, mainly with HPV6 and HPV11 (85% of the cases) [5]. Co-infections by oncogenic and non-oncogenic types are commonly detected in a high proportion of anogenital warts (45%), which have been proposed as a partial explanation of the increased risk of cervical intraepithelial neoplasia and invasive cervical carcinoma in women with GWs [6]. GWs are closely associated with sexual behaviour, with number of sexual partners being the main risk factor [7]. The highest incidence rate for GWs in women is at 20–24 years, which correlates well with the peak of papillomavirus infection in the female genital tract [4]. In men, the incidence peak occurs at 20–29 years of age [2].

Laryngeal papillomatosis, or recurrent respiratory papillomatosis, is a neoplastic disease of the airways mainly caused by HPV6 and HPV11, although HPV16 has also been identified in a few cases [8]. It represents the most common benign tumour of the larynx in infants and children [9]. Some studies have identified infection with HPV11 as being associated with more aggressive disease and higher recurrence of lesions [8,10], and malignant transformation of lesions has been described in approximately 5% of cases [11]. The clinical complications of this pathology include dysphonia, dyspnoea and, in serious cases, complete obstruction of the airways [12].

Papillomavirus variants are defined as viral sequences sharing >98% identity in the nucleotide sequence in the L1 gene [13]. Based on this criterion, HPV6 and HPV11 variant lineages have been described [14]. Several studies have addressed the genetic diversity of HPV6 and HPV11 [15–17], and some of them have aimed to establish a link between genetic variation and differential outcome of the infection [8,18].

The aim of this study was to analyse first the genetic diversity of HPV6 and HPV11 sequences retrieved from two different but related pathologies, namely GWs and LP. Further, the phylogenetic relationship of all HPV6 and HPV11 sequences and tissue-dependent distribution of the variants were analysed.

Methods

Samples

Samples analysed in this project originate from two different formalin-fixed paraffin-embedded (FFPE) sample repositories. GWs were obtained from the Surgical Genital Wart Biobank established in 1995 at the Sexual Health Clinic at Royal Perth Hospital, Perth, Australia. These samples include FFPE surgery specimens excised from patients who required surgical resection of anal and/or perianal GWs [19]. One hundred and forty-three HPV6 and sixty-four HPV11 single infected samples from the first surgical event of each patient were included.

Laryngeal papillomatosis samples originated from a multicentre study of cases diagnosed between 1985 and 2009, in the cities of Cali and Medellin, Colombia [10]. Forty-one HPV6 and eleven HPV11 single-infected samples, each from a different patient, were included. Detailed information about the samples included is shown in the Supplementary material, Table S1.

Presence of HPV DNA in the samples was assessed by using the SPF_{10}-DEiA-LiPA protocol (version 1; Laboratory Biomedical Products, Rijswijk, the Netherlands). The SPF_{10} system targets a 65-base pair region of L1 gene of a broad spectrum of Alphapapillomaviruses. HPV-positive samples were identified and genotyped by amplicon hybridization (DEiA) and reverse hybridization line probe assay, LiPA25. The detected viruses were HPV6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 58, 59, 56, 66, 68, 70 and 74.

Selection of the most informative genomic regions

Fragmentation of genetic material in the FFPE samples prevented us from obtaining full-genome sequences. To select the most informative regions for the study, the variability of different regions of the viral genomes was assessed.

HPV6 and HPV11 complete genome unique sequences were obtained via GenBank. The different open reading frames (ORFs: E6, E7, E1, E2, L1 and L2), the long control region (LCR), and the intergenic E2–L2 region (IntE2L2) were extracted and aligned. This intergenic region spans the E5a and E5b ORFs of HPV6 and HPV11 [20]. All sequences were aligned at amino acid level (except the non-coding LCR), back-translated and concatenated to obtain full-genome reference alignments. For each of the alignments, phylogenetic relationships were inferred under a maximum likelihood framework using RAxML v7.2.8 (http://www.exelixis-lab.org/) [21], using the GTR+Γ4 model, and the number of required bootstrap cycles was determined with the *–autoMRE* command [22]. The well-resolved phylogenetic trees obtained were further employed to compute tree-guided, model-based pairwise genetic distances between taxons (*–f x* command in RAxML).

PCR and sequencing

DNA was extracted by incubation of the material with 250 μL of proteinase K buffer (10 mg/mL proteinase K in 50 mM Tris–HCl, pH 8.0) overnight at 56°C. The samples were later incubated at 95°C for 8 min to inactivate proteinase K and were stored at −20°C until use.

Based on the pairwise distance results, the LCR and the IntE2L2 were chosen as amplification targets. Different type-specific PCR systems were designed for the amplification

of the samples. One primer set per region per genome was initially designed. For HPV6 samples not amplifiable by the primer set because of amplicon length, targeted regions were obtained by amplification of overlapping fragments. Primer sequences and amplified regions are shown in the Supplementary material, Table S2.

PCR products were sequenced at the Genoscreen facilities (Lille, France) in both strands.

Phylogenetic analyses

We have applied an Evolutionary Placement Algorithm (EPA) [23] to the inference of the phylogenetic relationships of the short fragments generated, in the context of the whole HPV6 or HPV11 variability. This methodology had been successfully applied for the analyses of short papillomavirus DNA sequences [24]. The reference tree described above, inferred using the genomic information of all full-length HPV6 or HPV11 variants, was used as scaffold.

The final set of reference sequences contained: for HPV6, 38 sequences, 8047 nucleotides and 172 alignment patterns; for HPV11, 26 sequences, 7878 nucleotides and 77 alignment patterns. These sets included sequences obtained from different pathologies (GWs, LP, cervical and lung samples) in different regions (Slovenia, Sweden and Thailand). Detailed information on the sequences is shown in Table S1.

Sequences obtained from our samples, and those partial sequences retrieved from GenBank were included and aligned with the reference sequences. Genome alignments were chopped to the length of the larger partial sequence in the alignments. Final alignment included 253 sequences, 1432 nucleotides and 343 alignments patterns for HPV6; 159 sequences, 1452 nucleotides and 157 alignment patterns for HPV11. The EPA algorithm was performed as implemented in RAxML v7.2.6, using the GTR+Γ4 model.

The results of the variants distribution in different pathologies were compared by means of the Pearson's chi-squared test for count data as implemented in R.

Results

LCR and IntE2L2 are the most informative regions

We studied the distribution of pairwise genetic distances for the different genomic regions among full-length genomes of HPV6 and HPV11. The most variable regions in both HPVs were IntE2L2, E2 and LCR; and E6 for HPV11 only (see Supplementary material, Table S3). The accumulated frequencies of the pairwise distances for each ORF and the values of the 95th centile are depicted in Fig. 1. The study of E6 was discarded because we chose to use the same regions for the study of both HPV6 and HPV11, and the E6 gene was not informative for HPV6 variability [25]. E2 was discarded because we aimed to maximize the number of sequences from other studies for our combined analyses, and the only E2 sequences available in the GenBank were those of the full-length genomes. Hence, the most informative regions, IntE2L2 and LCR, were chosen for further analyses.

HPV6 variants analysis

One hundred and sixty-eight GW and LP samples were successfully amplified and sequenced. All newly generated sequences fitted into the previously described clades [14]

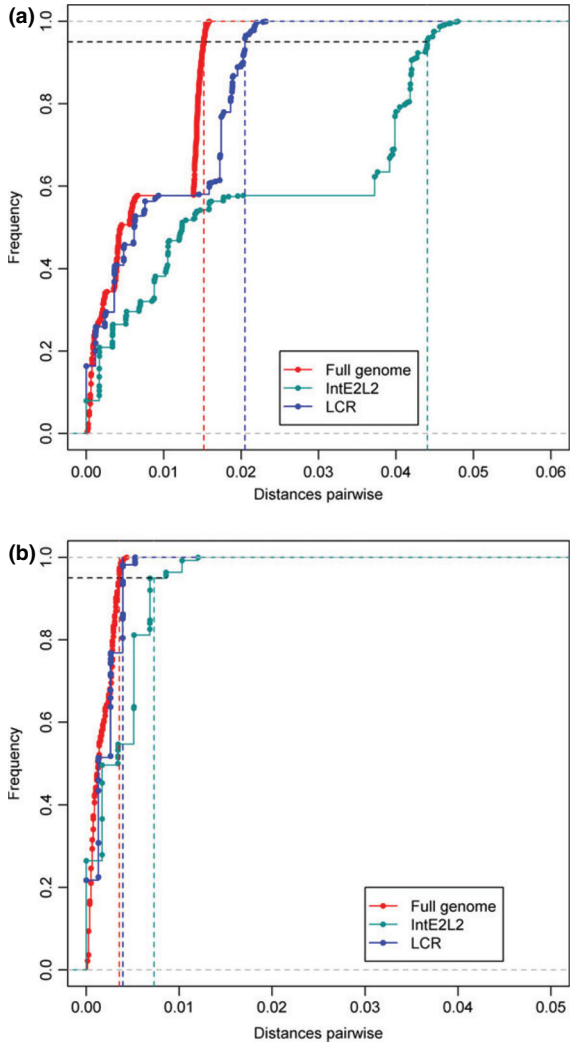

FIG. 1. (a) Human papillomavirus strain 6 (HPV6) pairwise distances calculated for full-genome, LCR and IntE2L2 of reference sequences; (b) HPV11 pairwise distances calculated for full-genome, LCR and IntE2L2 of reference sequences. Note that both plots are represented at the same scale. Dashed lines represent the distances pairwise values for 95th centile.

(Fig. 2). Likelihood weights for the ascription of each individual sequence to each clade/subclade are shown in the Supplementary material, Table S4. Detailed information on the distribution of the sequences in the different clades is presented in Table 1.

Ten partial sequences were excluded for the analyses of the global dataset because no information regarding anatomical site of the lesion was available. Finally, the analysis conducted with 243 sequences showed that 32 out of 85 LP samples belonged to clade A (37.7%), and the remaining sequences belonged to clade B (n = 53, 62.3%) [B1: 30 (35.3%); B2: 6 (7.1%); B3: 17 (20.0%)]. Among all HPV sequences identified in GWs, ten (6.3%) belonged to clade A, and 148 (93.7%) to clade B [B1: 108 (68.4%); B2: 34 (21.5%); B3: 6 (3.8%)] (Table 1). Fig. 2(b) displays the generated tree, including the whole set of sequences.

The differential presence of the different variants in GWs and in LP was further analysed. In both GWs and LP HPV6 B variants were the most common, 93.6% and 62.4%, respectively.

TABLE 1. Distribution of the analysed human papillomavirus 6 (HPV6) sequences into phylogenic clades

	GWs		LP	
Clade	**No. samples**	**%**	**No. samples**	**%**
A	10 (7)	6.33	32 (14)	37.65
B	148 (135)	93.67	53 (10)	62.35
B1	108 (97)	68.35	30 (–)	35.30
B2	34 (33)	21.52	6 (1)	7.05
B3	6 (5)	3.80	17 (9)	20.00
Total	158 (142)	100.00	85 (24)	100.00

The comparison of the distribution of HPV6 variants among both pathologies, genital warts (GWs) and laryngeal papillomatosis (LP), shows a statistically significant difference (chi-squared test $p < 0.01$).
Numbers in brackets correspond to new sequences generated from the samples collections described.

However, a significant difference (chi-squared test; $p < 0.01$) was observed between the distribution of variants between the two types of lesions. While in GWs almost two-thirds of sequences belonged to subclade B1, in LP we found an increased contribution of A (38%) and B3 variants (20%).

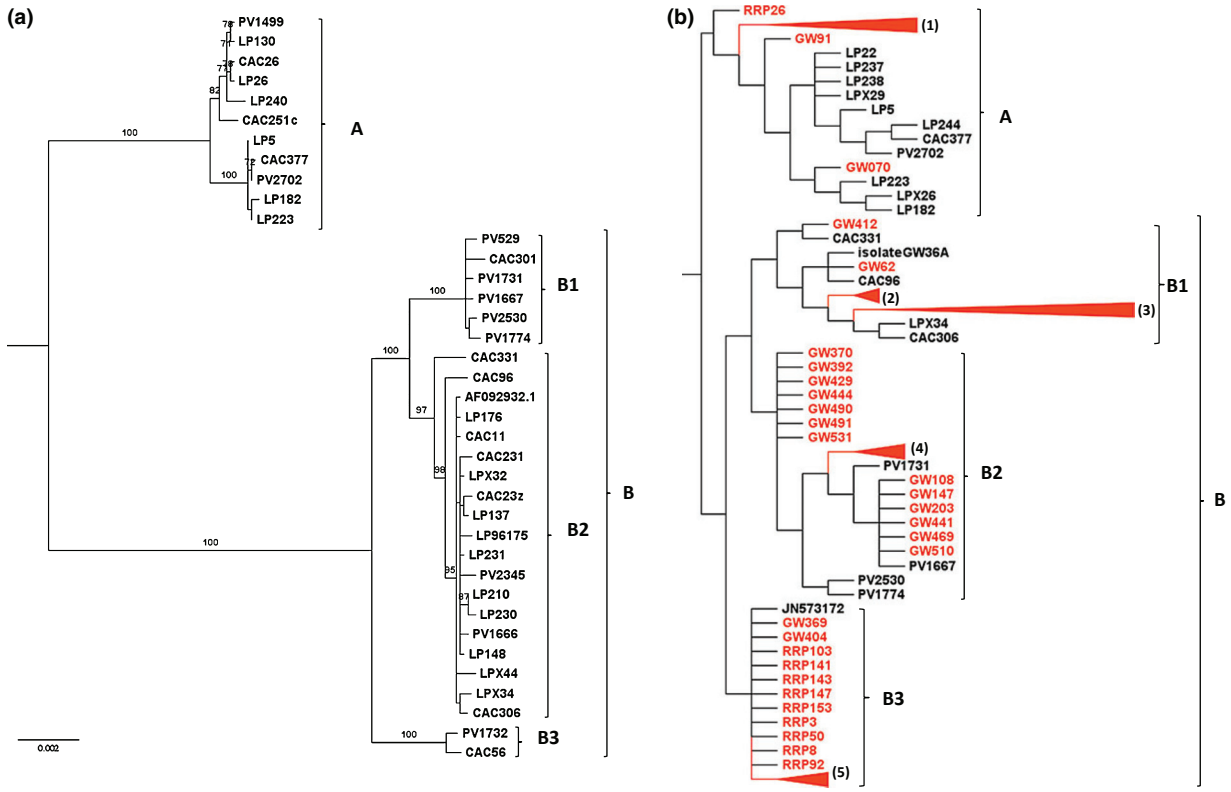

FIG. 2. (a) Midpoint rooted best-known maximum likelihood (ML) phylogenetic tree of human papillomavirus strain 6 (HPV6) isolates using 38 unique full-length genome sequences retrieved from GenBank. HPV6 variants are classified into two clades, A and B. Only bootstrap values over 70 supporting each branch of the generated tree are represented; (b) projection of the LCR/IntE2L2 HPV6 sequences analysed in this study onto the scaffold of the best-known full-length ML tree using the Evolutionary Placement Algorithm approach. Some branches have been collapsed for better presentation, the collapsed branches include: (1) 6 GWs/20 LPs/2 Cervix; (2) 32GWs; (3) 66 GWs/45 LP/2 Cervix; (4) 23 GWs/4 LPs; (5) 4 GWs/12 LP. Sequences newly generated in this study are shown in red. An uncollapsed version of the tree is available from the authors under request.

HPV11 variants analysis

Sixty-five samples were successfully amplified and sequenced. All HPV11 sequences fitted into the previously described clades (Fig. 3). Likelihood weights for the assignment of each individual sequence to each clade/subclade are shown in Table S4.

Detailed information on the distribution of the sequences in the different clades is presented in Table 2.

Eleven partial HPV11 sequences retrieved from GenBank were not included in the final analyses because of unknown origin of the material. Fig. 3(b) shows the generated tree, including the complete set of sequences. In both GWs and LP the vast majority of sequences belonged to the HPV11 A2 clade, with no difference between variant distributions depending on the different types of lesion (chi-squared test p 0.493).

TABLE 2. Distribution of the analysed human papillomavirus 11 (HPV11) sequences into phylogenic clades

	GWs		LP	
Clade	**No. samples**	**%**	**No. samples**	**%**
A1	6 (2)	5.50	3 (1)	7.5
A2	103 (54)	94.50	37 (8)	93.5
Total	109 (56)	100.00	40 (9)	100.00

The comparison of the distribution of HPV11 variants among both pathologies, genital warts (GWs) and laryngeal papillomatosis (LP), shows no statistically significant difference (chi-squared test p 0.493).
Numbers in brackets correspond to new sequences generated from the sample collections described.

Discussion

Both GWs and LP are benign proliferative lesions caused mainly by HPV6 and HPV11 [2]. Both types of lesions present similar clinical features, such as high recurrence and need of long-term treatments, and have been linked to the development, in a small proportion of patients, of malignant neoplasms [11]. Previous studies have addressed differential HPV6 and HPV11 genotype distributions in GWs and LP, but mainly at a national/regional level [18,26,27]. Here we provide the first study analysing HPV6 and HPV11 variant distribution in two different pathologies, with a large number of samples, GWs (n = 198) and LP (n = 33). The combination with all available

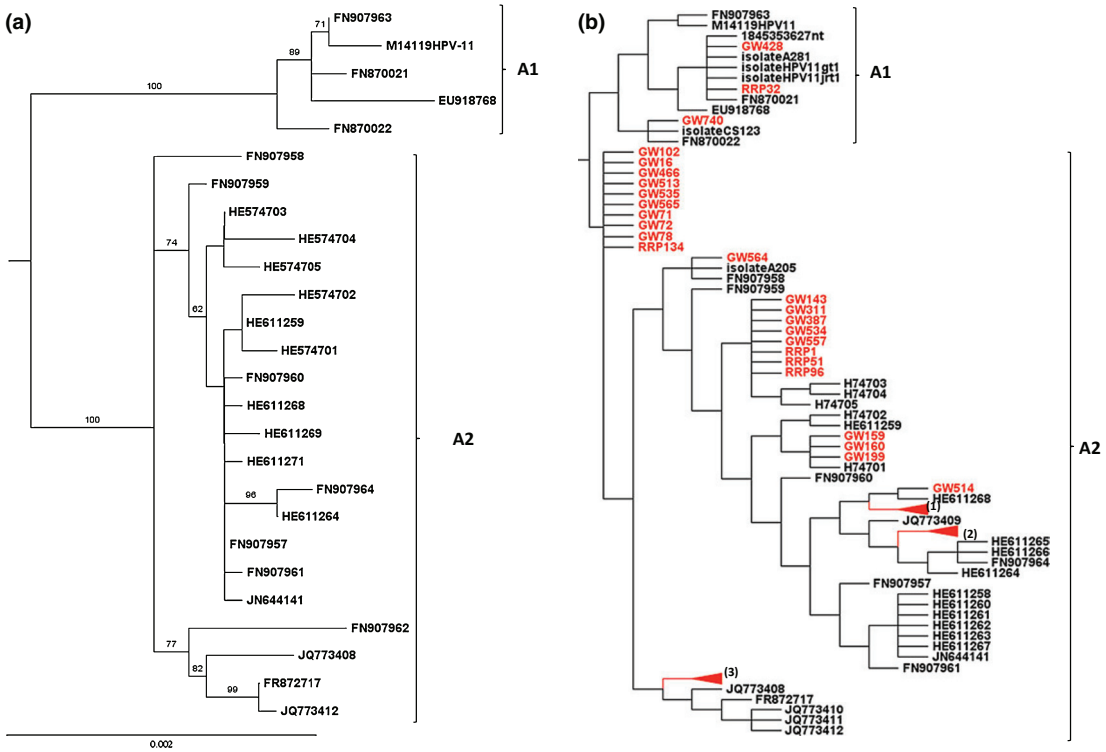

FIG. 3. (a) Midpoint rooted best known maximum likelihood (ML) phylogenetic tree of human papillomavirus strain 11 (HPV11) variants using 26 unique full-length genome sequences retrieved from GenBank. The HPV11 variants are classified into one clade (A) and two sub-clades (A1, A2). Only bootstrap values over 70 supporting each branch of the generated tree are represented; (b) projection of the LCR/IntE2L2 HPV11 sequences analysed in this study onto the scaffold of the best-known full-length ML tree. Some branches have been collapsed for better presentation, the collapsed branches include: (1) 31 GWs/6 LPs; (2) 26 GWs/4 LP/2 Cervix; (3) 18 GWs/1 LPs. Sequences newly generated in this study are shown in red. An uncollapsed version of the tree is available from the authors under request.

sequences in the GenBank generates a final dataset of 253 samples for HPV6, and 159 samples for HVP11 sequences, encompassing ten countries (Australia, China, Colombia, Germany, Hungary, Slovenia, South Africa, Sweden, Thailand, USA) and five continents.

We have identified the most suitable genomic regions for assessing intratype genetic diversity, which for HPV6 and HPV11 are the LCR and the IntE2L2 regions (Table S3). Similar data on the heterogeneous rate of variation throughout the papillomavirus genomes had been previously described. Among coding regions, the E5 genes are the fastest evolving ORFs, and E1 and L1 are the more slowly evolving genes [20,28]. The LCR itself, devoid of the selective pressures for protein encoding, accumulates changes more than twice as fast as the L1 or the E1 genes [28]. Our results confirm therefore that the general trend of variation accumulation is conserved also at shallower levels within Papillomaviridae.

HPV6 and HPV11 are close relatives, and belong together in Alphapapillomaviruses, species 10. Genetic diversity is about four times greater among HPV6 isolates than among HPV11 isolates, as concluded after the analyses of all available full-length genomes for both viruses (Wilcoxon's test, $p < 0.01$) (Fig. 1). These diversity values fit well the described taxonomic definition: for HPV6 variants based on nucleotide similarities in the L1 gene, intravariant differences are around 0.7% and intervariant differences are around 1.5%; for HPV11, no different variants are described and nucleotide differences are below 0.5% [14]. The EPA approach [23] allowed us to assign all partial sequences into the different clades defined using the full-length genome sequences (Fig. 2, Fig. 3). The most important finding of our study is that HPV6 A and B variants are not equally distributed in GWs and LP ($p < 0.01$). Specifically, HPV6 B variant isolates are preferentially found in GWs compared with LP. Furthermore, the contribution of subclades within HPV6 B variants is also different in GWs and in LP ($p < 0.01$). No distribution difference could be observed in our data for HPV11 variants. It could be argued that the observed differences arise from a geographical bias for the origin of the samples analysed. However, previous research did not identify geographical origin as an important component of viral diversity for HPV6 and HPV11. Heinzel and co-workers communicated a global study of these two HPVs [15], including 19 samples containing HPV6 and ten samples containing HPV11. More recently, de Matos *et al.* presented data on the phylogenetic relations of HPV6 variants using 117 sequences from South America, Europe and South Africa [29], suggesting no evidence of a geographical distribution of HPV variants in these lesions. Further, more local studies have reported that the whole repertoire of HPV6 variants can be found in isolates originating from a single country [25].

It was not the aim of this study to assign a differential risk for certain variants, as this is not a case–control study. The low prevalence of both studied HPVs in a healthy population worldwide (0.5% for HPV6; 0.2% for HPV11) [4] makes it difficult to obtain a sample size with enough statistical power for a meaningful comparative study.

In summary, in this study we demonstrate the differential presence of papillomavirus variants in different pathologies, with variants B1 of HPV6 being more prevalent in GWs than in LP. The current state of knowledge therefore supports our finding of a preferential involvement of HPV6 B1 variants in GWs, which may reflect a biological difference in the interplay between viruses and the different mucosal epithelia. Such differences may arise from either a preferential tropism or a differential viral fitness and potential to induce lesions between anogenital and laryngeal mucosa. Similar scenarios have been proposed for HPV16 variants differentially enriched in vulvar cancer compared with cervical cancer [30]. Further research on the prevalence of the different variants within HPV6 in healthy tissue from both locations will be necessary to solve this question.

Acknowledgements

The authors want to thank Dr Gustavo Cuello, MD, otorhinolaryngologist in Medellín, Colombia, for his help with initial sample collection and data processing. The authors thank Catalina Bonet for her assistance in statistical analysis and Stéphanie Bedhomme for critical review of the results and analyses. This work was supported by grants from the Spanish Ministry for Science and Innovation (MICINN Grant CGL2010-16713); Agencia de Gestió d'Ajuts Universitaris i de Recerca (Catalan Government, AGAUR Grant 2009 SGR 1026); Red Temática de Investigación Cooperativa en Cáncer (RTICC Grant RD06/0020/0095); Spanish Ministry for Economy and Competitivity (PFIS Grant FI12/0142 and FIS Grant PI11/02096); the Lilly Foundation (Premio de Investigación Biomédica Preclínica 2012 to FXB); and the Institut d'Investigació Biomédica de Bellvitge – IDIBELL. None of the aforementioned agencies had any role in the interpretation of the results or in the preparation of this manuscript.

Authors' Contribution

JMG conceived and coordinated the study, analysed the data and drafted the manuscript. SN performed experiments and analysed the data. VP performed initial HPV detection. BM analysed the data. GIS and NM obtained and characterized LP

samples. JMc obtained and characterized GW samples. FXB performed epidemiological analysis. IGB designed the analyses and supervised the study. All authors contributed to, read and approved the final manuscript.

Transparency Declaration

NM is member of the HPV Global Board of Merck. FXB is member of the speakers' bureau from GlaxoSmithKline and Sanofi Pasteur MSD.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Descriptive table with the information concerning the analysed sequences with HPV6 and HPV11 infection.

Table S2. Primers for HPV6 and HPV11 amplification and sequencing.

Table S3. 95th centile, 10th centile and Median Absolute Deviation (MAD) values of the pairwise distances for each viral open reading frame.

Table S4. Likelihood weights for assignment of human papillomavirus variants to the different clades.

Data S1. Human papillomavirus (HPV) reference trees generated for the analyses.

Data S2. Sequences not submitted to GenBank, because total length is below 200 bp.

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