



Differential presence of Papillomavirus variants in cervical cancer: An analysis for HPV33, HPV45 and HPV58

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ARTICLE INFO

Article history:

Received 12 June 2012

Received in revised form 5 September 2012

Accepted 12 September 2012

Available online 26 September 2012

Keywords:

Human papillomaviruses
Variants
Phylogeny
Squamous cell carcinoma
Infection
Cancer

ABSTRACT

Background: Certain human papillomaviruses (HPVs) are the causative agents of cervical carcinomas in humans. The identification of the link between infection and cancer has resulted in the successful establishment of clinical strategies such as screening or vaccination programs, aiming to prevent this pathology. More than 150 different HPVs have been described and classified and the large majority of them are not related to cancer. The genus *Alphapapillomavirus* encompasses many PVs, some of which are identified in humans as oncogenic, according to the epidemiological connection between infection and cervical cancer. Variants of some of these “high-risk” HPVs may have an increased involvement in cervical cancer, although definitive data are still wanting. The aim of the present work was to analyze the presence of HPV33, HPV45 and HPV58 variants in cases of cervical cancer.

Methods: Samples from cervical lesions in the context of different cervical cancer surveys were analyzed for presence of HPV DNA. Samples positive for HPV33, HPV45 or HPV58 DNA were selected and the E6/E7 genes were amplified and sequenced. The phylogenetic relationships of these sequences were inferred using an evolutionary placement algorithm and accordingly classified at the variant level.

Results: All viral E6/E7 sequences were successfully placed in the classification schemes of the corresponding viruses. For HPV33 ($n = 23$), 45 ($n = 61$) or 58 ($n = 29$), the distribution of variants found in cases of cervical cancer is not a random sample of the corresponding diversity. In all three HPVs, the respective A variants were more prevalent in the viral DNA-positive cases of cervical cancer analyzed. This is the first study trying to discern the phylogenetic connection between variants of the oncogenic HPV33, 45 and 58, and squamous cell carcinoma of the cervix.

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1. Introduction

Papillomaviridae are a family of epitheliotropic, non-enveloped viruses with a circular double stranded DNA genome of around 8000 bp. Papillomaviruses (PVs) were initially described in mammals, but they have also been found in birds (Terai et al., 2002), turtles (Herbst et al., 2009) and snakes (Lange et al., 2011), and probably infect all amniotes (Bravo et al., 2010). The association

between PVs and mammals is thus very ancient, and probably the ancestral mammals were already the hosts to a number of ancestral PVs (Gottschling et al., 2011b). Currently, more than 250 full-length PV genomes are available in the databases, and most of them have been isolated from humans (PAvE, Papillomavirus Episteme Database, <http://pave.niaid.nih.gov/>). The 2004 ICTV guidelines for PV classification defined clear-cut nucleotide identity values in the L1 gene for the definition of genera (more than 60% identity), species (between 60% and 70% identity), types (between 71% and 89% identity) and variants (more than 98% identity) (de Villiers et al., 2004). However, the distribution of genetic distances across taxonomic categories is not the same for all genera, e.g. distances between comparable categories are consistently larger for *Alpha*-PVs than for *Beta*-PVs (Bravo and Alonso, 2007). The revised 2010 ICTV guidelines for PV classification (Bernard et al., 2010) have thus refrained from establishing absolute limits for defining taxonomic categories and have explicitly included the

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genealogy-driven biological classification schemes advocated by Darwin (1872), as proposed elsewhere for PVs (Bravo and Alonso, 2007; Bravo et al., 2010, 2011).

Virtually all humans are infected since early childhood by a large number of different human PVs (HPVs) (Antonsson et al., 2003b; Michael et al., 2008). Most of these infections are asymptomatic and the skin of healthy individuals is colonized by dozens of different HPVs without apparent damage (Antonsson et al., 2000, 2003a; de Koning et al., 2007; Hsu et al., 2009). However, some infection generates conspicuous, hyperkeratotic, benign lesions, typically visible as warts in the hands, fingers, feet, lips or eyelids (Kashima et al., 1994; Keefe et al., 1994; Piecyk-Sidor et al., 2009; Rudlinger et al., 1989; Sun et al., 2009). A limited number of HPVs are associated with malignant proliferative anogenital lesions in humans (Munoz et al., 2003; zur Hausen, 2002). The link between infection and human cancer has historically been the main interest for the study of PVs, and this human medical focus largely dominates the field.

The vast majority of HPV types involved in anogenital infections in humans belong to *Alphapapillomavirus*. This genus belongs to the Alpha-Omicron-PV crown group (Gottschling et al., 2007, 2011b), which gathers eminently mucosotropic PVs that cause anogenital infections in different host species (Gottschling et al., 2011a; Rector et al., 2008; Rehtanz et al., 2006). Epidemiological studies analyzing the differential cancer risk associated to individual viral infections have identified a number of HPVs as necessary albeit not sufficient cause of cervical cancer (Walboomers et al., 1999). The most prominent among the so-called “high-risk” HPVs are HPV16 and HPV18, which together account for more than 70% of the cases of cervical cancer worldwide (de Sanjose et al., 2010). All “high-risk” HPVs are paraphyletic, i.e. they all share a common ancestor, but some of the descendants of this ancestor are not classified as “high-risk” (Bravo and Alonso, 2004; Schiffman et al., 2005). Thus, phylogenetic relationships among HPVs roughly match the phenotypic characteristics of the infection but many questions remain unclear (Bravo et al., 2010). We still do not understand what makes HPV16 and HPV18 different from the rest of the members of the *AlphaPVs* species (species 9 and 7, respectively), accounting for their increased carcinogenic potential; we do not know why HPV67 and HPV97, close relatives of HPV16 and HPV18 respectively, are only weakly associated to the development of cervical cancer (Schiffman et al., 2009); finally, we have little information about HPV54, a distant relative of both “high-risk” and “low-risk” that could help us understand the emergence of the transforming abilities of oncogenic HPVs.

The oncogenic potential is not evenly distributed among types within a PV species (Bravo and Alonso, 2004; Schiffman et al., 2005, 2009), and the same may hold true for the differential oncogenicity among variants within a PV type. A number of studies, mostly focusing on HPV16, have addressed the differential role of specific variants in the persistence of infection and the subsequent development of cervical lesions. Nevertheless, the potential differential carcinogenicity of variants in other HPVs is still largely unknown (Chan et al., 2002; Khouadri et al., 2006; Xin et al., 2001). The availability of increasing numbers of full-length genomes of many “high-risk” HPVs has allowed sampling the genotypic diversity around the genome sequences historically defined as “references” (Chen et al., 2009, 2011). This deep description of genetic variability is the prerequisite for more thorough analyses of the differential association between PV variants and cancer.

In the present study we have addressed the description of variants of HPV33, HPV45 and HPV58 present in cervical cancer samples. These three viruses are among the eight most prevalent types of HPV in cervical cancer, and account for 4%, 6% and 2%, respectively, of the cases of cervical cancer worldwide (de Sanjose et al., 2010). We have mapped the diversity of viral variants present

in cases of cervical cancer onto the respective global diversity, described in the latter data from literature (Chan et al., 2011; Chen et al., 2011) and have found that for all three analyzed PVs the repertoire of variants is not equally represented in cervical cancer.

2. Material and methods

2.1. Samples

Samples analyzed in the study were selected among those available from two different HPVs worldwide survey studies: the RIS HPV TT project, coordinated by the Catalan Institute of Oncology (ICO, Barcelona) (de Sanjose et al., 2010) and a case-control study performed at the International Agency for the Research on Cancer (IARC, Lyon) (Bosch et al., 1992; Hammouda et al., 2005). From the RIS HPV TT project formalin-fixed paraffin-embedded (FFPE) tissues were used, and from the IARC study both FFPE tissues and material from cervical swabs were used. The RIS HPV TT study included only cancer cases. Therefore no cancer-risk associated to viral variants could be explored because of the lack of proper control population.

For HPV58, the samples were selected from the RIS HPV TT study according to the following criteria: cervical cancer cases with HPV58 single infections and enough material in terms of DNA available for the analyses. From the cases that met the criteria ($n = 192$), a random sample of 50 cases was taken, including 49 squamous cell carcinoma (SCCs) and one neuroendocrine (NEC) tumor. Samples with HPV33 and/or 45 were obtained from tumor biopsies or scrapes of the IARC case-control study. Twenty-three HPV33 and 87 HPV45 single infections were selected, as well as 82 multiple infections with HPV33 and/or HPV45 were identified. The quality of the material for processing was not sufficient for all these samples, so finally the number of invasive cervical cancer (ICC) samples of adequate quality was: ten samples with single infections and 13 with multiple infections of HPV33; 47 samples with single infections and 14 with multiple infections of HPV45.

A description of the samples and the geographical origin is given in [Supplementary material \(Table S1\)](#).

2.2. DNA amplification

Different protocols for DNA extraction and amplification were performed, according to the source and kind of the samples.

For HPV58 positive samples, paraffin tissue sections were treated with freshly prepared proteinase K solution to extract DNA as established in the working protocol used in the original project (de Sanjose et al., 2010). Briefly, proteinase K (10 mg/mL) was added to the digestion buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5% Tween 20). Each sample was mixed with 250 μ L of proteinase K solution and incubated in a heating block for 18 h at 56 °C.

Specific primers used for the amplification of 335 bp of the HPV58 E7 gene were designed (Table S3). All manipulations were carried out under conditions of pre- and post-PCR separation. The primers were tested and validated on cervical specimen positives for HPV58. Amplification was performed using HotStar Taq Polymerase (Qiagen, Hilden, Germany) according to the manufacturer's indications. The two strands of the PCR products were sequenced by the fluorescent dye dideoxy termination method using the amplification primers.

For HPV33 and/or HPV45, DNA was isolated by high-pure PCR template preparation kit (Roche, Mannheim, Germany) according to the manufacturers' instructions. Presence of HPV33 and/or HPV45 was verified by GP5 + /6 + PCR-EIA testing and subsequent reverse line blot genotyping as described earlier (Snijders et al., 2005).

The E6–E7 region of either HPV33 or HPV45 was amplified by PCR with specific primers generating overlapping amplicons with

total lengths between 140 and 851 bp (Table S4; Table S5). The two strands of the PCR products were sequenced by the fluorescent dye dideoxy termination method using the amplification primers.

Accession numbers for sequences generated in this study (GenBank JQ976760–JQ976877) are given in Table S1 (Supplementary material). Table S2 (Supplementary material) shows in detail the genetic differences in the analyzed sequences.

2.3. Sequence analysis

Phylogenetic inference was performed using the Evolutionary Placement Algorithm (EPA), originally designed for phylogenetic assignment of short DNA sequences onto a previously computed robust topology generated with a larger amount of information, under a maximum likelihood framework (Berger and Stamatakis, 2011; Stark et al., 2010). This algorithm has successfully been tested and applied to the phylogenetic placement of short PV sequences (Mengual-Chuliá et al., 2012).

The reference trees were computed using full-genome sequences from HPV33 (Chen et al., 2011), HPV45 (Chen et al., 2009) and HPV58 (Chan et al., 2011). Accession numbers and lineages for the reference sequences are given in Table 5 of the Supplementary material. The complete genomes were aligned at the nucleotide level with MUSCLE 3.7 (Edgar, 2004). The final sets contained 21 sequences, 7912 nucleotides and 137 alignment patterns for HPV33; 13 sequences, 7858 nucleotides and 116 alignment patterns for HPV45; and 37 sequences, 7838 nucleotides and 87 alignment patterns for HPV58. The phylogenetic relationships were inferred under a maximum likelihood framework using RAxML v7.2.8 (<http://www.exelixis-lab.org/>) (Stamatakis, 2006), with the GTR + Γ 4 model and the number of required bootstrap cycles was determined with the *-autoMRE* command (Pattengale et al., 2010). The results for the reconstruction of the reference trees are shown in Supplementary Text1.

Sequences generated in this study were added to the corresponding complete genome alignments using the *-add* command in MAFFT (Katoh et al., 2005). These alignments were subsequently chopped to the respective region common to all sequences. The final alignments contained 36 sequences, 1050 nucleotide positions and 34 alignment patterns for HPV33; 77 sequences, 1050 nucleotide positions and 35 alignment patterns for HPV45; and 66 sequences, 269 nucleotide positions and 34 alignment patterns for HPV58. The EPA algorithm was performed as implemented in RAxML v7.2.8 (option *-fv*) using the GTR + Γ 4 model. The query sequences (i.e. those generated in this work) were launched onto the corresponding reference tree (i.e. the one precalculated with the complete genomes) and their positions were inferred without modifying the scaffold. The algorithm calculates the node/s for insertion of new sequences in the reference trees together with the likelihood value/s of these insertions (Berger and Stamatakis, 2011; Stark et al., 2010). The algorithm does not compute the relationships among the query sequences, and they would yield an apparent polytomy if they were assigned to the same node. The likelihood weights for the assignment of the sequences to each clade or subclade are shown in Table S6 of the Supplementary material.

The results of the variants distribution in cancers obtained in the analyses of HPV58 variants were compared with the global distribution in the population based on available literature information (Chan et al., 2011; Chen et al., 2009, 2011) by means of the Pearson's Chi-squared Test for Count Data, as implemented in R.

3. Results and discussion

In the present study we have analyzed the diversity of HPV types 33, 45 and 58 in cervical cancer samples, obtained from

two international survey studies in cervical cancer (Bosch et al., 1992; de Sanjose et al., 2010; Hammouda et al., 2005), including cervical smears and FFPE samples. DNA from all samples was obtained and tested for HPV detection in the context of these studies. A total of 23, 61 and 29 samples positive for HPV33, HPV45 and HPV58, respectively were initially selected for the study, on the basis of the presence of sufficient left-over material for the proposed analyses.

The analyses for the genetic variability of each genotype were performed by sequencing targeted regions of the viral genome (Fig. 1). For HPV58, a 269 bp region of the E7 gene was analyzed in all samples (positions 602–870 bp in the HPV58 reference sequence, GenBank #D90400). For HPV33 and HPV45, the analyzed region comprised 1050 bp and spanned the complete E6 and E7 genes (positions 1–1050 in the HPV33 reference sequence, GenBank #M12732; positions 1–1050 in the HPV45 reference sequence, GenBank #EF202167). The two strands of all amplicons were sequenced and assembled, and those with enough quality were used for the subsequent phylogenetic analyses.

An Evolutionary Placement Algorithm was used for phylogenetic inference with the sequences obtained (Berger and Stamatakis, 2011; Stark et al., 2010; Mengual-Chuliá et al., 2012). For each of the three viruses, a reference tree was assembled with the full-length genomes of the respective genotypes (Figs. 2a, 3a and 5a; Table S1). Subsequently, the newly generated sequences were added onto this reference tree. For all three HPV types studied the phylogenetic relationships reconstructed matched the previous descriptions (Chan et al., 2011; Chen et al., 2009, 2011).

The differential role of the diverse HPVs variants in the development of cervical cancer is a function of multiple factors, including geographical criteria (Bernard et al., 2006), and is still poorly understood. As in many other respects, the best documented HPVs in the context of viral variants are HPV16 and HPV18. Relative risk of cervical cancer for HPV16 African and Asian-American variants is threefold greater than for the European ones (Burk et al., 2003; Schlecht et al., 2005; Sichero et al., 2007). This risk is also higher for the European variant of HPV18 than for the African or Asian-American variants (Burk et al., 2003; Schlecht et al., 2005; Sichero et al., 2007). For other “high-risk” HPVs, the differential risk for cancer development associated to viral variants is not well documented, with scarce data on HPV33, HPV45 or HPV58 variants. Recently published studies have explored in depth the variability of these genotypes in different studies (Chan et al., 2011; Chen et al., 2011), but no precise information on the prevalence of the different variants either in lesions or in healthy tissue has been provided. In the present study we have aimed to provide a description of variants for these viruses in cervical cancer cases. Historically, the yardstick for PV inter-type comparison has been the L1 gene (de Villiers et al., 2004). Although still commonly used as a reference, the choice of this marker has been superseded by a global phylogenetic approach (Bernard et al., 2010). However, there is no standard marker defined for the description of PV intra-type variation. For the best studied HPV16 and HPV18, intra-type sequence variability has been assessed using either the LCR and/or the E6 and L1 genes. For our analyses the E6–E7 region was chosen to maximize the phylogenetic signal available for the downstream evolutionary analyses (Bravo and Alonso, 2004, #58; Garcia-Vallve et al., 2005).

3.1. Variant A predominates in HPV45-positive cases of cervical cancer

HPV45 is a close relative of HPV18 (Gottschling et al., 2011b), belongs to the *AlphaPVs species 7* (Bernard et al., 2010), and is epidemiologically considered as a “high-risk” type (Munoz et al., 2003). This virus is the third most common genotype involved in invasive cervical carcinoma, found in 6% of cervical cancers (de

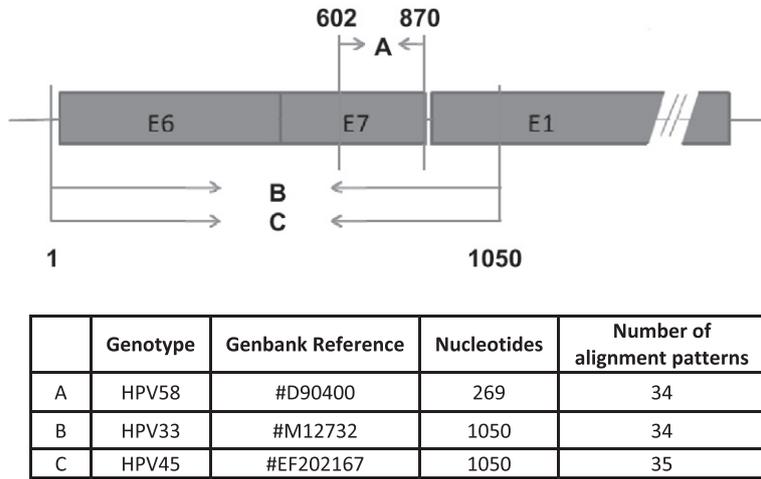


Fig. 1. Regions amplified for the analyses of each HPV genotype.

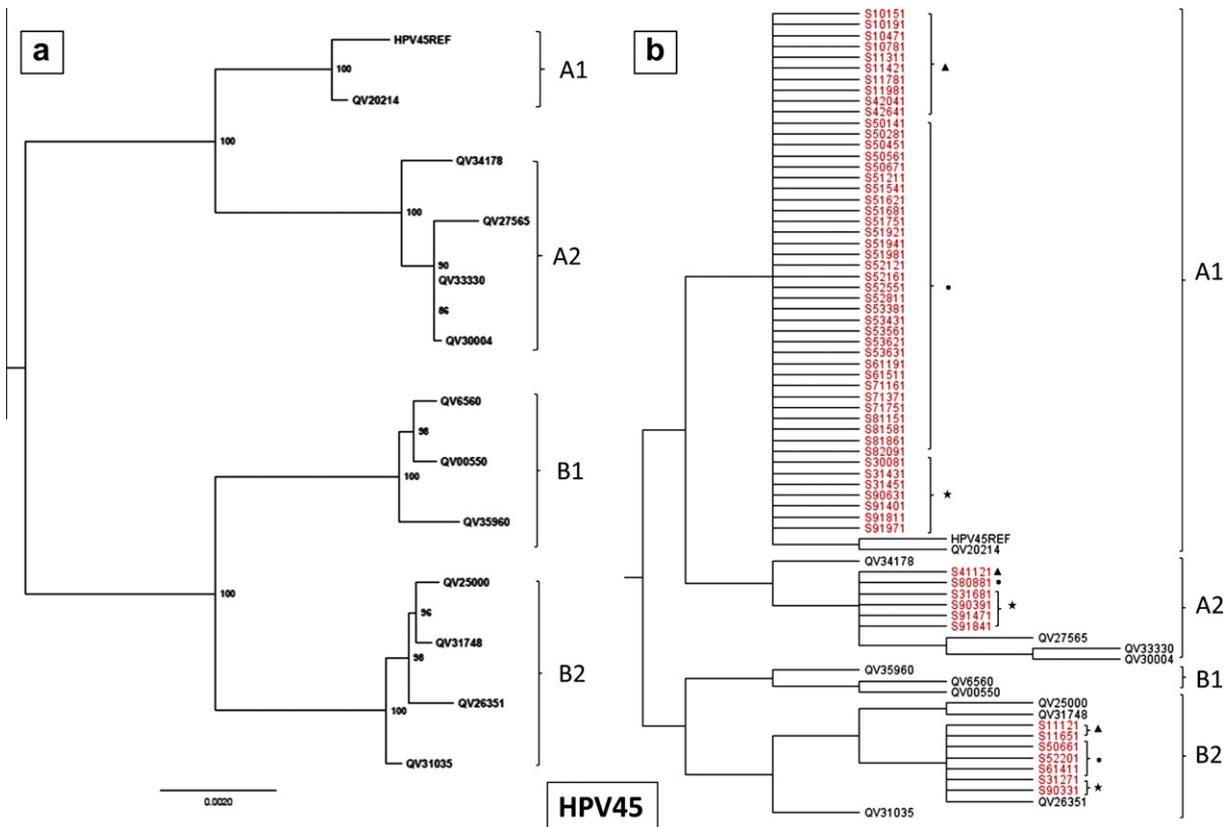


Fig. 2. (a) Best known ML phylogenetic tree of HPV45 variants using the full-length genome sequences from Chen et al. (2011). The tree has been midpoint rooted. The HPV45 variants are classified into two clades (A and B); (b) projection of the E6/E7 HPV45 sequences generated in this study (labeled in red) onto the scaffold of the best-known full-length ML tree. “Triangle”: South-American samples; “Circle”: Asian samples; “Star”: African samples; “Square”: European samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Sanjose et al., 2010), but in only 0.5% of women with normal cytology (Bruni et al., 2010).

For HPV45, most of the samples in our study (54 out of 61) fitted in clade A (48 samples in A1; six samples in A2) with only seven samples belonging to clade B, subclade B2. (Table 1 and Fig. 2). Thirty-two of the analyzed sequences (55.6%) were identical, most of them with Asian origin.

Phylogenetic diversity of HPV45 has recently been described (Chen et al., 2009) with the definition of two clades and four subclades. A later study of the same group has addressed the cancer risk associated to HPV45 variants, and found no differential connection

between infection with HPV45 variants A or B and risk of cancer, although the number of cervical cancer cases analyzed was small ($n = 7$) (Schiffman et al., 2010). In contrast, in the present study we have analyzed 61 cervical cancer cases containing HPV45 DNA, and our results show an excess of A variants over B variants in lesions (89% vs. 11%).

3.2. Variant A predominates in HPV33-positive cases of cervical cancer

HPV33 is a relative of HPV16 (Gottschling et al., 2011b) and is classified into *AlphaPVs species 9* (Bernard et al., 2010). It is found

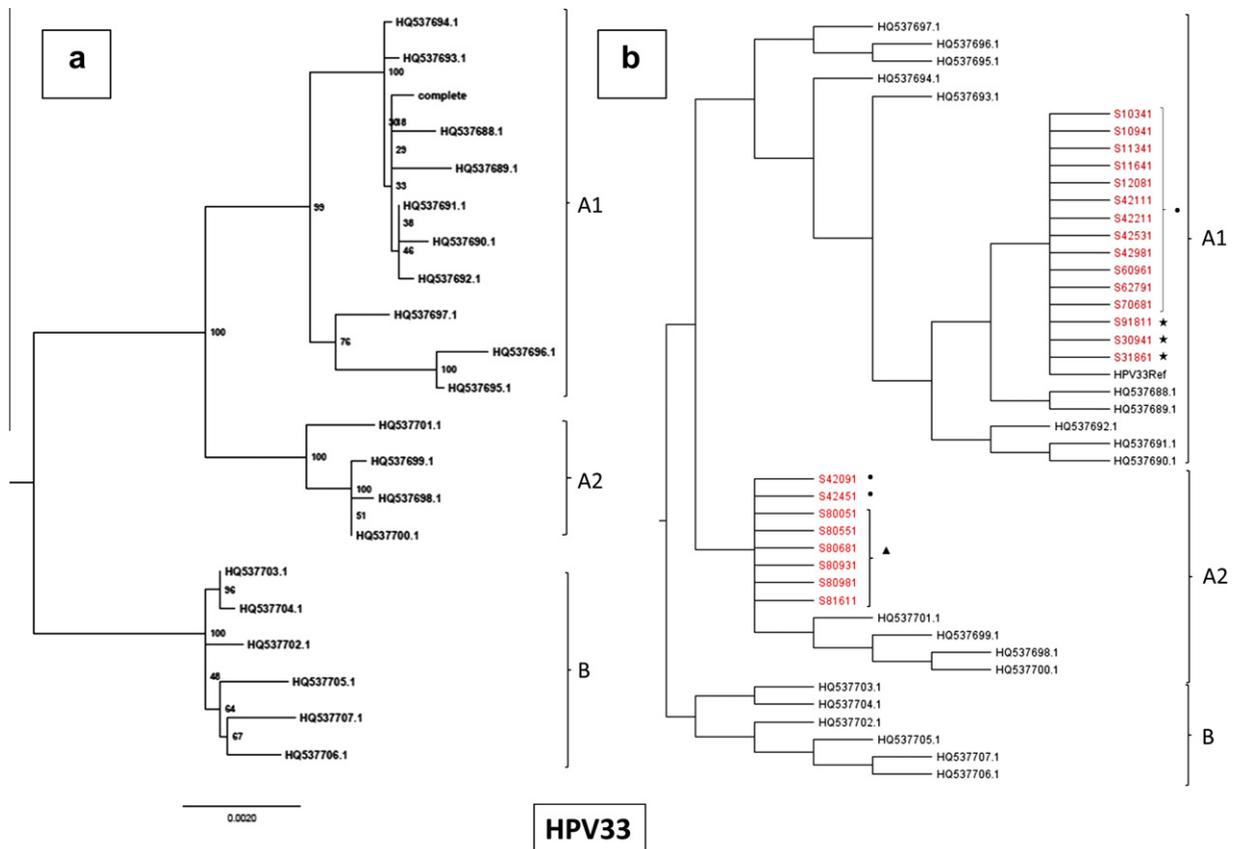


Fig. 3. (a) Best known ML phylogenetic tree of HPV33 variants using the full-length genome sequences from Chen et al. (2011). The tree has been midpoint rooted. The HPV33 variants are classified into two clades (A and B); (b) projection of the E6/E7 HPV33 sequences generated in this study (labeled in red) onto the scaffold of the best-known full-length ML tree. “Triangle”: South-American samples; “Circle”: Asian samples; “Star”: African samples; “Square”: European samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in 0.5% of women with normal cytological findings (Bruni et al., 2010) and accounts for approximately 4% of cervical cancer cases worldwide (de Sanjose et al., 2010). All sequences generated from HPV33-positive cervical cancer samples fitted in the clusters that describe the known diversity within HPV33 (Chen et al., 2011) and belonged to clade A (15 samples in A1; 8 samples in A2) (Table 2 and Fig. 3). For the geographical distribution of the variants, the only remarkable point is the appearance of all the three HPV33 African samples in clade A1.

A number of studies have addressed the description of HPV33 variants, analyzing the sequence in specific regions of the genome and their relationship to cancer development (Khouadri et al., 2006, 2007; Xin et al., 2001). A differential relation between cervical cancer and HPV variants is described in the work by Xin and colleagues (Xin et al., 2001), where the authors report that non-prototype variants were associated more frequently with cervical intraepithelial neoplasia (CIN) grade I/II diagnoses, while the prototype sequences were associated with CIN III/ICCs diagnoses. Unfortunately the sequences generated and analyzed by these authors were not deposited and are not available for phylogenetic inference.

Finally, one further description of the differential involvement of HPV33 variants in cervical cancer was reported from the Guanacaste Project (Schiffman et al., 2010). The authors referred variants in clades A and B to show a higher risk for cancer development compared with C variants, based on the frequency of finding the different variants in advanced CIN (Schiffman et al., 2010). Unfortunately, there was no description of the definition of such three clades in the report, while in the full description of the HPV33 diversity only two clades were defined (Chen et al., 2011). This

unfortunate issue highlights the need of standardizing nomenclature using neutral naming, as has been put forward in the last descriptions of HPVs variants diversity (Burk et al., 2011; Chan et al., 2011; Chen et al., 2011).

3.3. Variant A predominates in HPV58-positive cases of cervical cancer

HPV58 is a close relative of HPV33 (Gottschling et al., 2011b) and belongs to *AlphaPVs species 9* (Bernard et al., 2010). It is considered as “high-risk” (Munoz et al., 2003), is found in 0.7% of women with normal cervical cytology (Bruni et al., 2010) and accounts for ca. 2% cases of cervical cancer worldwide (de Sanjose et al., 2010). Concerning the viral diversity of HPV58, the 29 analyzed samples fitted in the clusters that describe the known diversity within the type (Chen et al., 2011). Twenty-five out of 29 sequences belonged to clade A (three samples in A1; 13 samples in A2; nine samples in A3), while three sequences belonged to clade C and one sequence belonged to clade D2 (Fig. 4b and Table 3).

The best description of the global diversity of HPV58 is that of Chen and colleagues (Chen et al., 2011). We have reconstructed the relationships among the variants that these authors communicated and have obtained a similar phylogenetic reconstruction (Fig. 4a). However, there is an unfortunate nomenclature assignment in this reference work (Chen et al., 2011), because the authors have described variant B as a lineage obviating that it is clearly paraphyletic, as variants C and D are nested within variant B (Fig. 4a and 6 in (Chen et al., 2011)). The ICTV does not provide recommendations for classification at shallow levels, beyond type. The study group of the ICTV for *Papillomaviridae* has put forward the need for explicitly introducing phylogeny in the definition of

Table 1
Distribution of the new samples in the phylogenetic clades HPV45.

Clade	No. samples	%
A	54	88.52
A1	48	78.69
A2	6	9.83
B	7	11.48
B1	0	0.00
B2	7	11.48
Total	61	100.00

Table 2
Distribution of the new samples in the phylogenetic clades HPV33.

Clade	No. samples	%
A	23	100.00
A1	15	65.22
A2	8	34.78
B	0	0.00
TOTAL	23	100.00

taxonomic categories, refraining from the use of absolute boundaries of sequence identity for defining genera, species, types and variants (Bernard et al., 2010). This switch reflects the need to account for the differential pace of evolution in different PV crown-groups and the difficulty of dealing with overlapping distances between categories (Bravo and Alonso, 2007; Gottschling et al., 2011b; Bernard et al., 2010). The systematic nomenclature of HPV58 variants may require amendment.

Table 3
Distribution of the new samples in the phylogenetic clades HPV58.

Clade	No. samples	%
A	25	86.21
A1	3	10.34
A2	13	44.84
A3	9	31.03
B	0	0.00
C	3	10.34
D	1	3.45
D1	0	0.00
D2	1	3.45
TOTAL	29	100.00

A number of studies regarding HPV58 variants have been previously published, but most of them analyze the results in terms of a signature of single nucleotide polymorphisms that could differentiate between variant lineages (Cento et al., 2011; Wu et al., 2009). Indeed, some of these sequence patterns can be related to progression or appearance of a cervical pathology (Cento et al., 2011; Xin et al., 2001). However, the absence of a phylogenetic framework for interpreting the results prevents the comparison with our results.

Total variability of the genotype is shown in the work we used as a reference for the trees reconstruction (Chen et al., 2011), but not their frequencies in the population. Because of this, the comparison of our data can only offer us results on how our variants are clustered in the known clades. We observed a high proportion of A variants in our population when compared with the data published in (Chen et al., 2011) (86.2% of the lesions in our study presented these variants). Further, no precise information about the

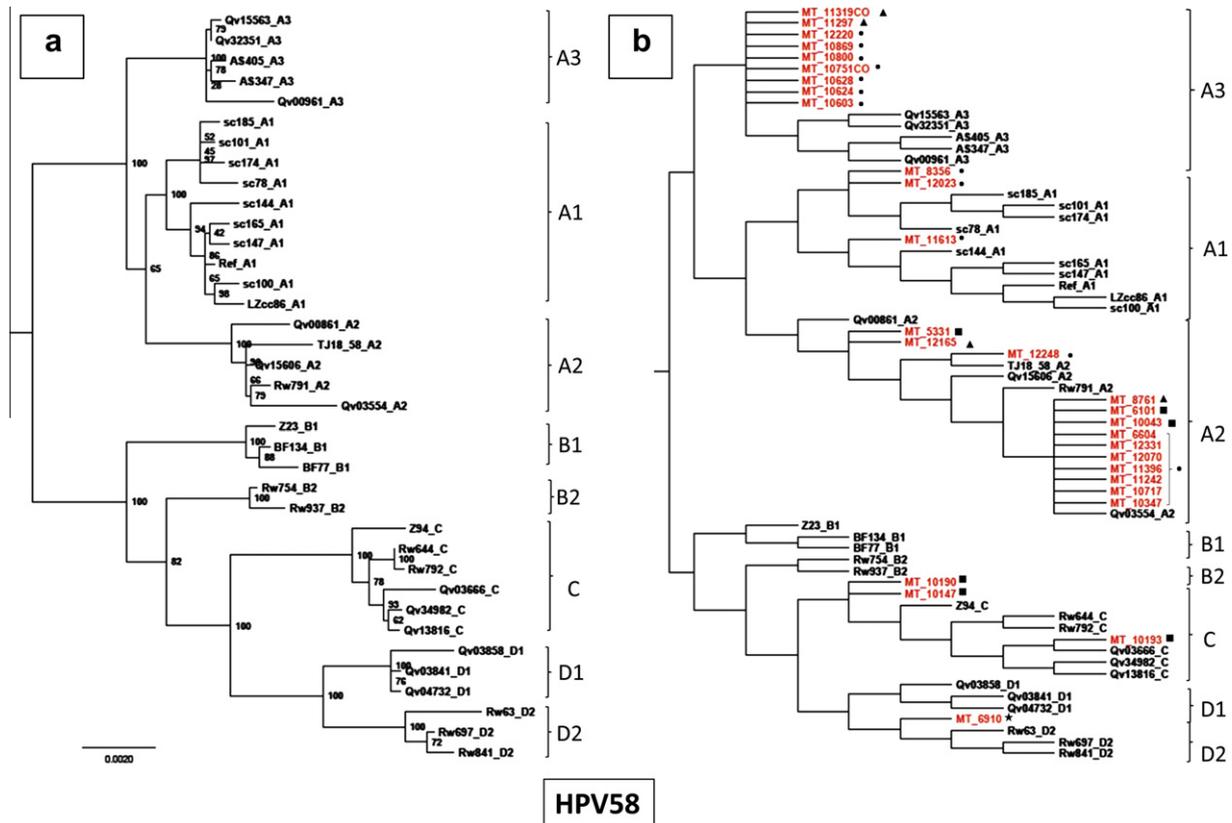


Fig. 4. (a) Best known ML phylogenetic tree of HPV58 variants using the full-length genome sequences from Chen et al. (2011). The tree has been midpoint rooted. The HPV58 variants are classified into four clades (A–D); (b) projection of the new HPV58 sequences generated in this study (labeled in red) onto the scaffold of the best-known full-length ML tree. Most of new sequences cluster with confidence in the A clade. “Triangle”: South-American samples; “Circle”: Asian samples; “Square”: African samples; “Star”: European samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

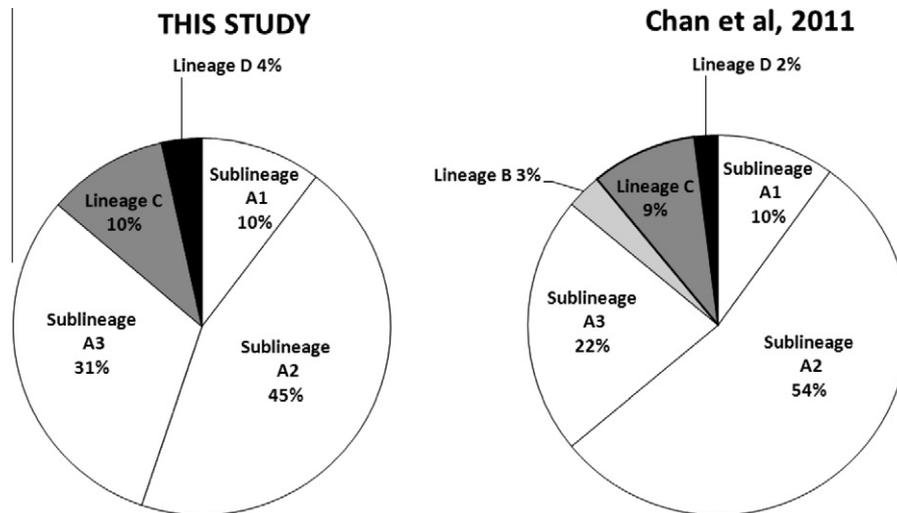


Fig. 5. Comparison of the HPV 58 variants distribution in this study (left) and in [43], (right). There are no statistically significant differences between both distributions ($\chi^2 p = 0.24$).

composition of the study population is presented in the mentioned work (Chen et al., 2011). The samples analyzed were generated from the Guanacaste cohort and comprise both samples with lesions and HPV positive controls (Chen et al., 2011). Another HPV variant study has previously been performed using this Guanacaste cohort (Schiffman et al., 2010) and the authors presented data supporting a different relative risk for specific HPV58 variants, with the variants in clade A being the only ones found in CIN2+ cases. Our results show that not only A variants but also C and D variants are found in cervical cancer. The sample size in the Guanacaste study is larger than ours (57 CIN2+ vs 29 SCCs). It is therefore unlikely that the differences in variants distribution appear as an artifact due to sample size. We interpret that such differences reflect rather the differences in genetic background of the two study populations: the Guanacaste cohort includes samples from patients residing in Central-South America while our study population includes women from Asia, Europe, Central-South America and Africa (Fig. 4b). It is remarkable that all three Italian samples fit in clade C, and all Asian samples in clade A. These results suggest a geographical component in the differential distribution of the HPV58 variants.

A more closely related population to the one in our work is the study population by Chan and colleagues (Chan et al., 2011). Here, the authors present the results of HPV58 variants distribution worldwide, including both lesions and normal samples. In this case, total and comparable data on HPV58 variants prevalence are included (Fig. 5). As in our work, Chan and colleagues analyzed a worldwide population with an important contribution of HPV58-positive samples of Asian origin, namely 62.0% and 59.4% of the total cases, respectively. This overrepresentation of Asian samples reflects the increased prevalence of HPV58 in ICC cases in Asia, where it is found to be involved in 4% of the cases, in contrast with the described worldwide prevalence of 2% (de Sanjose et al., 2010; Zuna et al., 2009). There is no statistically significant difference between the variant distributions found by Chan and colleagues (Chan et al., 2011) and in the present work ($\chi^2 p$ value = 0.24).

Finally, scarce information is available concerning the distribution of these HPV variants in women without cervical lesions. The biggest control group is the one described in the work by Schiffman et al. (2010). In this study, nested in the Guanacaste cohort, women without lesions but positive for HPV infection are used as control population. There, the authors grouped the different HPV variants according to a “theoretical cancer-risk”, and compared intra-type variants. For HPV33 ($n = 69$), 89.8% of the variants

were type A or B and 10.2% were C; for HPV45 ($n = 114$), 47.4% A vs. 52.6% B; for HPV58 ($n = 194$), 93.8% A vs. 6.2% B/C/D.

No comparisons for cancer-risk associated to variants in case-control samples could be performed between these data and ours. The different classification of variants (3 clades for HPV33 in Schiffman’s work, 2 clades in HPV33 phylogenetic description; the grouping of variants, which do not allow knowing the exact number of each one) and our small sample size prevent any additional analyses in this way.

3.4. Conclusion

Our data suggest that the different variants of HPV 58, HPV33 and HPV45 are not equally present in cervical cancer samples. These findings are important for our basic knowledge of PVs, as they provide us with evidence on the different ways the PV infection can be involved in cancer development and suggest differential interaction between variability at the level of the host and at the level of the viruses. The data we have presented here arise from studies based on cancer samples and do not incorporate results from control samples. Therefore, no data on the differential prevalence of viral variants in cervical cancer compared to normal tissue can be established here. For the statement of a real risk associated to variants, epidemiologic studies with proper case-control design are needed. However, the low prevalence of these HPV genotypes in normal population may limit the possibilities to obtain the necessary sample size for these studies. Finally, our results for HPV58 suggest that there may be an important interaction component between the geographical origin of the samples (which may be a proxy for genetic background of the population) and the differential distribution of viral variants in cancer cases. Further research is thus required for the study of such differential presence in control samples from various geographical locations.

Author’s contribution

JMG analyzed the data and drafted the manuscript. MT and TG generated data on HPV58. DAMH, PJFS and CJLMM generated data on HPV33 and HPV45. LA coordinated the study. FXB and SdS designed and coordinated the study. IGB conceived data analysis, analyzed data and drafted the manuscript. All authors contributed to the final draft and have read and approved the final manuscript.

Acknowledgements

We thank Dr. Robert D. Burk for providing us with HPV58 full genome sequences before public release. We thank the contribution to the project of the HPV Variants Study Group. We thank Debby Boon for expert technical assistance. JMG is funded by a Red Temática de Investigación Cooperativa en Cáncer, RTICC Grant (RD06/0020/0095). IGB is funded by the disappeared Spanish Ministry for Science and Innovation, MICINN (CGL2010-16713). The study has been partly supported by Spanish public grants from the Instituto de Salud Carlos III (Grants numbers FIS PI030240, FIS PI061246, RCEP C03/09, RTICESP C03/10, RTIC RD06/0020/0095, and CIBERESP), Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR2005SGR 00695 and 2009SGR126), Marató de TV3 Foundation (051530), and GlaxoSmithKline Biologicals, Sanofi Pasteur MSD, and Merck who had no role in the interpretation of the results or in the preparation of this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.meegid.2012.09.011>.

References

- Antonsson, A., Forslund, O., Ekberg, H., Sterner, G., Hansson, B.G., 2000. The ubiquity and impressive genomic diversity of human skin papillomaviruses suggest a commensal nature of these viruses. *J. Virol.* 74, 11636–11641.
- Antonsson, A., Erfurt, C., Hazard, K., Holmgren, V., Simon, M., Kataoka, A., Hossain, S., Hakangard, C., Hansson, B.G., 2003a. Prevalence and type spectrum of human papillomaviruses in healthy skin samples collected in three continents. *J. Gen. Virol.* 84, 1881–1886.
- Antonsson, A., Karanfilovska, S., Lindqvist, P.G., Hansson, B.G., 2003b. General acquisition of human papillomavirus infections of skin occurs in early infancy. *J. Clin. Microbiol.* 41, 2509–2514.
- Berger, S.A., Stamatakis, A., 2011. Aligning short reads to reference alignments and trees. *Bioinformatics* 27, 2068–2075.
- Bernard, H.U., Calleja-Macias, I.E., Dunn, S.T., 2006. Genome variation of human papillomavirus types: phylogenetic and medical implications. *Int. J. Cancer* 118, 1071–1076.
- Bernard, H.U., Burk, R.D., Chen, Z., van Doorslaer, K., Hausen, H.Z., de Villiers, E.M., 2010. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. *Virology* 401, 70–79.
- Bosch, F.X., Munoz, N., de Sanjose, S., Izarzugaza, I., Gili, M., Viladiu, P., Tormo, M.J., Moreo, P., Ascunze, N., Gonzalez, L.C., et al., 1992. Risk factors for cervical cancer in Colombia and Spain. *Int. J. Cancer* 52, 750–758.
- Bravo, I.G., Alonso, A., 2004. Mucosal human papillomaviruses encode four different E5 proteins whose chemistry and phylogeny correlate with malignant or benign growth. *J. Virol.* 78, 13613–13626.
- Bravo, I.G., Alonso, A., 2007. Phylogeny and evolution of papillomaviruses based on the E1 and E2 proteins. *Virus Genes* 34, 249–262.
- Bravo, I.G., de Sanjose, S., Gottschling, M., 2010. The clinical importance of understanding the evolution of papillomaviruses. *Trends Microbiol.* 18, 432–438.
- Bravo, I.G., de Sanjose, S., Gottschling, M., 2011. Papillomaviruses and Darwinian classification: response to van Doorslaer et al. *Trends Microbiol.* 19, 49–50.
- Bruni, L., Diaz, M., Castellsague, X., Ferrer, E., Bosch, F.X., de Sanjose, S., 2010. Cervical human papillomavirus prevalence in 5 continents: meta-analysis of 1 million women with normal cytological findings. *J. Infect. Dis.* 202, 1789–1799.
- Burk, R.D., Terai, M., Gravitt, P.E., Brinton, L.A., Kurman, R.J., Barnes, W.A., Greenberg, M.D., Hadjimichael, O.C., Fu, L., McGowan, L., Mortel, R., Schwartz, P.E., Hildesheim, A., 2003. Distribution of human papillomavirus types 16 and 18 variants in squamous cell carcinomas and adenocarcinomas of the cervix. *Cancer Res.* 63, 7215–7220.
- Burk, R.D., Chen, Z., Harari, A., Smith, B.C., Kocjan, B.J., Maver, P.J., Poljak, M., 2011. Classification and nomenclature system for human alphapapillomavirus variants: general features, nucleotide landmarks and assignment of HPV6 and HPV11 isolates to variant lineages. *Acta Dermatovenerol. Alp. Panonica Adriat.* 20, 113–123.
- Cento, V., Rahmatalla, N., Ciccozzi, M., Perno, C.F., Ciotti, M., 2011. Intra-type variations of HPV 31 and 58 in Italian women with abnormal cervical cytology. *J. Med. Virol.* 83, 1752–1761.
- Chan, P.K., Lam, C.W., Cheung, T.H., Li, W.W., Lo, K.W., Chan, M.Y., Cheung, J.L., Cheng, A.F., 2002. Association of human papillomavirus type 58 variant with the risk of cervical cancer. *J. Natl. Cancer Inst.* 94, 1249–1253.
- Chan, P.K., Luk, A.C., Park, J.S., Smith-McCune, K.K., Palefsky, J.M., Konno, R., Giovannelli, L., Coutlee, F., Hibbitts, S., Chu, T.Y., Settheetham-Ishida, W., Picconi, M.A., Ferrera, A., De Marco, F., Woo, Y.L., Raiol, T., Pina-Sanchez, P., Cheung, J.L., Bae, J.H., Chirenje, M.Z., Magure, T., Moscicki, A.B., Fiander, A.N., Di Stefano, R., Cheung, T.H., Yu, M.M., Tsui, S.K., Pim, D., Banks, L., 2011. Identification of human papillomavirus type 58 lineages and the distribution worldwide. *J. Infect. Dis.* 203, 1565–1573.
- Chen, Z., DeSalle, R., Schiffman, M., Herrero, R., Burk, R.D., 2009. Evolutionary dynamics of variant genomes of human papillomavirus types 18, 45, and 97. *J. Virol.* 83, 1443–1455.
- Chen, Z., Schiffman, M., Herrero, R., Desalle, R., Anastos, K., Segondy, M., Sahasrabudhe, V.V., Gravitt, P.E., Hsing, A.W., Burk, R.D., 2011. Evolution and taxonomic classification of human papillomavirus 16 (HPV16)-related variant genomes: HPV31, HPV33, HPV35, HPV52, HPV58 and HPV67. *PLoS ONE* 6, e20183.
- Darwin, C., 1872. *The Origin of Species by Means of Natural Selection*, sixth ed. John Murray, London, pp. 363–403.
- de Koning, M.N., Struijk, L., Bavinck, J.N., Kleter, B., ter Schegget, J., Quint, W.G., Feltkamp, M.C., 2007. Betapapillomaviruses frequently persist in the skin of healthy individuals. *J. Gen. Virol.* 88, 1489–1495.
- de Sanjose, S., Quint, W.G., Alemany, L., Geraets, D.T., Klaustermeier, J.E., Lloveras, B., Tous, S., Felix, A., Bravo, L.E., Shin, H.R., Vallejos, C.S., de Ruiz, P.A., Lima, M.A., Guimera, N., Clavero, O., Alejo, M., Lombart-Bosch, A., Cheng-Yang, C., Tatti, S.A., Kasamatsu, E., Iljazovic, E., Odiida, M., Prado, R., Seoud, M., Grce, M., Usubutun, A., Jain, A., Suarez, G.A., Lombardi, L.E., Banjo, A., Menendez, C., Domingo, E.J., Velasco, J., Nessa, A., Chichareon, S.C., Qiao, Y.L., Lerma, E., Garland, S.M., Sasagawa, T., Ferrera, A., Hammouda, D., Mariani, L., Pelayo, A., Steiner, I., Oliva, E., Meijer, C.J., Al-Jassar, W.F., Cruz, E., Wright, T.C., Puras, A., Llave, C.L., Tzardi, M., Agorastos, T., Garcia-Barrion, V., Clavel, C., Ordi, J., Andujar, M., Castellsague, X., Sanchez, G.L., Nowakowski, A.M., Bornstein, J., Munoz, N., Bosch, F.X., 2010. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. *Lancet Oncol.* 11, 1048–1056.
- de Villiers, E.M., Fauquet, C., Broker, T.R., Bernard, H.U., zur Hausen, H., 2004. Classification of papillomaviruses. *Virology* 324, 17–27.
- Edgar, R.C., 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 5, 113.
- García-Valve, S., Alonso, A., Bravo, I.G., 2005. Papillomaviruses: different genes have different histories. *Trends Microbiol.* 13, 514–521.
- Gottschling, M., Stamatakis, A., Nindl, I., Stockfleth, E., Alonso, A., Bravo, I.G., 2007. Multiple evolutionary mechanisms drive papillomavirus diversification. *Mol. Biol. Evol.* 24, 1242–1258.
- Gottschling, M., Bravo, I.G., Schulz, E., Bracho, M.A., Deaville, R., Jepson, P.D., Bressen, M.F., Stockfleth, E., Nindl, I., 2011a. Modular organizations of novel cetacean papillomaviruses. *Mol. Phylogenet. Evol.* 59, 34–42.
- Gottschling, M., Goker, M., Stamatakis, A., Bininda-Emonds, O.R., Nindl, I., Bravo, I.G., 2011b. Quantifying the phylogenetic forces driving papillomavirus evolution. *Mol. Biol. Evol.* 28, 2101–2113.
- Hammouda, D., Munoz, N., Herrero, R., Arslan, A., Bouhade, A., Oubli, M., Djedat, B., Fontaniere, B., Snijders, P., Meijer, C., Franceschi, S., 2005. Cervical carcinoma in Algiers, Algeria: human papillomavirus and lifestyle risk factors. *Int. J. Cancer* 113, 483–489.
- Herbst, L.H., Lenz, J., Van Doorslaer, K., Chen, Z., Stacy, B.A., Wellehan Jr., J.F., Manire, C.A., Burk, R.D., 2009. Genomic characterization of two novel reptilian papillomaviruses, *Chelonia mydas* papillomavirus 1 and *Caretta caretta* papillomavirus 1. *Virology* 383, 131–135.
- Hsu, J.Y., Chen, A.C., Keleher, A., McMillan, N.A., Antonsson, A., 2009. Shared and persistent asymptomatic cutaneous human papillomavirus infections in healthy skin. *J. Med. Virol.* 81, 1444–1449.
- Kashima, M., Adachi, M., Honda, M., Niimura, M., Nakabayashi, Y., 1994. A case of peculiar plantar warts. Human papillomavirus type 60 infection. *Arch. Dermatol.* 130, 1418–1420.
- Katoh, K., Kuma, K., Toh, H., Miyata, T., 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Res.* 33, 511–518.
- Keefe, M., al-Ghamdi, A., Coggon, D., Maitland, N.J., Egger, P., Keefe, C.J., Carey, A., Sanders, C.M., 1994. Cutaneous warts in butchers. *Br. J. Dermatol.* 130, 9–14.
- Khoudri, S., Villa, L.L., Gagnon, S., Koushik, A., Richardson, H., Ferreira, S., Tellier, P., Simao, J., Matlashewski, G., Roger, M., Franco, E.L., Coutlee, F., 2006. Human papillomavirus type 33 polymorphisms and high-grade squamous intraepithelial lesions of the uterine cervix. *J. Infect. Dis.* 194, 886–894.
- Khoudri, S., Villa, L.L., Gagnon, S., Koushik, A., Richardson, H., Matlashewski, G., Roger, M., Ferenczy, A.S., Franco, E.L., Coutlee, F., 2007. Viral load of episomal and integrated forms of human papillomavirus type 33 in high-grade squamous intraepithelial lesions of the uterine cervix. *Int. J. Cancer* 121, 2674–2681.
- Lange, C.E., Favrot, C., Ackermann, M., Gull, J., Vetsch, E., Tobler, K., 2011. Novel snake papillomavirus does not cluster with other non-mammalian papillomaviruses. *Virol. J.* 8, 436.
- Mengual-Chuliá, B., García-Pérez, R., Gottschling, M., Nindl, I., Bravo, I.G., 2012. Novel animal papillomavirus sequences and accurate phylogenetic placement. *Mol. Phylogenet. Evol.* in press, <http://dx.doi.org/10.1016/j.ympev.2012.08.011>.
- Michael, K.M., Waterboer, T., Sehr, P., Rother, A., Reidel, U., Boeing, H., Bravo, I.G., Schlehofer, J., Gartner, B.C., Pawlita, M., 2008. Seroprevalence of 34 human papillomavirus types in the German general population. *PLoS Pathog.* 4, e1000091.
- Munoz, N., Bosch, F.X., de Sanjose, S., Herrero, R., Castellsague, X., Shah, K.V., Snijders, P.J., Meijer, C.J., 2003. Epidemiologic classification of human

- papillomavirus types associated with cervical cancer. *N. Engl. J. Med.* 348, 518–527.
- Pattengale, N.D., Alipour, Masoud, Bininda-Emonds, Olaf R.P., Moret, Bernard M.E., Stamatakis, Alexandros, 2010. How many bootstrap replicates are necessary? *J. Comput. Biol.* 17, 337–354.
- Pieczk-Sidor, M., Polz-Dacewicz, M., Zagorski, Z., Zarnowski, T., 2009. Occurrence of human papillomavirus in pterygia. *Acta Ophthalmol.* 87, 890–895.
- Rector, A., Stevens, H., Lacave, G., Lemey, P., Mostmans, S., Salbany, A., Vos, M., Van Doorslaer, K., Ghim, S.J., Rehtanz, M., Bossart, G.D., Jenson, A.B., Van Ranst, M., 2008. Genomic characterization of novel dolphin papillomaviruses provides indications for recombination within the Papillomaviridae. *Virology* 378, 151–161.
- Rehtanz, M., Ghim, S.-J., Rector, A., Van Ranst, M., Fair, P.A., Bossart, G.D., Jenson, A.B., 2006. Isolation and characterization of the first American bottlenose dolphin papillomavirus: *Tursiops truncatus* papillomavirus type 2. *J. Gen. Virol.* 87, 3559–3565.
- Rudlinger, R., Bunney, M.H., Grob, R., Hunter, J.A., 1989. Warts in fish handlers. *Br. J. Dermatol.* 120, 375–381.
- Schiffman, M., Herrero, R., Desalle, R., Hildesheim, A., Wacholder, S., Cecilia Rodriguez, A., Bratti, M.C., Sherman, M.E., Morales, J., Guillen, D., Alfaro, M., Hutchinson, M., Wright, T.C., Solomon, D., Chen, Z., Schussler, J., Castle, P.E., Burk, R.D., 2005. The carcinogenicity of human papillomavirus types reflects viral evolution. *Virology* 337, 76–84.
- Schiffman, M., Clifford, G., Buonaguro, F.M., 2009. Classification of weakly carcinogenic human papillomavirus types: addressing the limits of epidemiology at the borderline. *Infect. Agent Cancer* 4, 8.
- Schiffman, M., Rodriguez, A.C., Chen, Z., Wacholder, S., Herrero, R., Hildesheim, A., Desalle, R., Befano, B., Yu, K., Safaeian, M., Sherman, M.E., Morales, J., Guillen, D., Alfaro, M., Hutchinson, M., Solomon, D., Castle, P.E., Burk, R.D., 2010. A population-based prospective study of carcinogenic human papillomavirus variant lineages, viral persistence, and cervical neoplasia. *Cancer Res.* 70, 3159–3169.
- Schlecht, N.F., Burk, R.D., Palefsky, J.M., Minkoff, H., Xue, X., Massad, L.S., Bacon, M., Levine, A.M., Anastos, K., Gange, S.J., Watts, D.H., Da Costa, M.M., Chen, Z., Bang, J.Y., Fazzari, M., Hall, C., Strickler, H.D., 2005. Variants of human papillomaviruses 16 and 18 and their natural history in human immunodeficiency virus-positive women. *J. Gen. Virol.* 86, 2709–2720.
- Sichero, L., Ferreira, S., Trottier, H., Duarte-Franco, E., Ferenczy, A., Franco, E.L., Villa, L.L., 2007. High grade cervical lesions are caused preferentially by non-European variants of HPVs 16 and 18. *Int. J. Cancer* 120, 1763–1768.
- Snijders, P.J., van den Brule, A.J., Jacobs, M.V., Pol, R.P., Meijer, C.J., 2005. HPV DNA detection and typing in cervical scrapes. *Methods Mol. Med.* 119, 101–114.
- Stamatakis, A., 2006. RAXML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22, 2688–2690.
- Stark, M., Berger, S.A., Stamatakis, A., von Mering, C., 2010. MLTreeMap-accurate Maximum Likelihood placement of environmental DNA sequences into taxonomic and functional reference phylogenies. *BMC Genomics* 11, 461.
- Sun, C., Chen, K., Gu, H., Chang, B., Jiang, M., 2009. Association of human papillomavirus 7 with warts in toe webs. *Br. J. Dermatol.* 162, 579–586.
- Terai, M., DeSalle, R., Burk, R.D., 2002. Lack of canonical E6 and E7 open reading frames in bird papillomaviruses: *Fringilla coelebs* papillomavirus and *Psittacus erithacus* timneh papillomavirus. *J. Virol.* 76, 10020–10023.
- Walboomers, J.M., Jacobs, M.V., Manos, M.M., Bosch, F.X., Kummer, J.A., Shah, K.V., Snijders, P.J., Peto, J., Meijer, C.J., Munoz, N., 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* 189, 12–19.
- Wu, E.Q., Zha, X., Yu, X.H., Zhang, G.N., Wu, Y.G., Fan, Y., Ren, Y., Kong, L.Q., Kong, W., 2009. Profile of physical status and gene variation of human papillomavirus 58 genome in cervical cancer. *J. Gen. Virol.* 90, 1229–1237.
- Xin, C.Y., Matsumoto, K., Yoshikawa, H., Yasugi, T., Onda, T., Nakagawa, S., Yamada, M., Nozawa, S., Sekiya, S., Hirai, Y., Shiromizu, K., Fujii, T., Taketani, Y., 2001. Analysis of E6 variants of human papillomavirus type 33, 52 and 58 in Japanese women with cervical intraepithelial neoplasia/cervical cancer in relation to their oncogenic potential. *Cancer Lett.* 170, 19–24.
- Zuna, R.E., Moore, W.E., Shanesmith, R.P., Dunn, S.T., Wang, S.S., Schiffman, M., Blakey, G.L., Teel, T., 2009. Association of HPV16 E6 variants with diagnostic severity in cervical cytology samples of 354 women in a US population. *Int. J. Cancer* 125, 2609–2613.
- zur Hausen, H., 2002. Papillomaviruses and cancer: from basic studies to clinical application. *Nat. Rev. Cancer* 2, 342–350.