Arch Virol (2017) 162:2855–2860
DOI 10.1007/s00705-017-3439-5

BRIEF REPORT

Genetic diversity of human papillomavirus types 35, 45 and 58 in cervical cancer in Brazil

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Received: 6 March 2017 / Accepted: 31 May 2017 / Published online: 9 June 2017

Abstract In Brazil, most studies of intra-type variants of human papillomavirus (HPV) have focused on HPV16 and HPV18, but other high-risk HPV types have not been studied. Here, we report the prevalence of lineages and variants of HPV35, HPV45 and HPV58 in cervical cancers from the Amazonian and Southeast Brazilian regions. The most frequent sublineages were A1 for HPV35, B2 for HPV45, and A2 for HPV58. The Southeast region had a higher frequency of the B2 sublineage of HPV45, and for HPV35, the genetic and nucleotide sequence diversity were higher in the Southeast region, suggesting that regional factors are influencing the diversity and lineage prevalence.

Keywords Cervical cancer · Papillomavirus · HPV35 · HPV45 · HPV58 · HPV diversity

Electronic supplementary material The online version of this article (doi:10.1007/s00705-017-3439-5) contains supplementary material, which is available to authorized users.

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Cervical cancer is the fourth most common cancer in women, with 527,000 cases and 265,000 deaths in 2012 [1]. In Brazil, this cancer accounted for 5,430 deaths in 2013 (https://mortalidade.inca.gov.br/MortalidadeWeb/) and approximately 16,300 new cases in 2016 [2]. Human papillomavirus (HPV) infection is considered an indispensable factor, albeit not sufficient, for the development of cervical cancer [3]. Fifteen HPV types have been associated with a high risk for development of cervical cancer (HR-HPV) [4], and among them, HPV16 and HPV18 are associated with approximately 75% of cervical cancer cases worldwide. Two commercially available vaccines for cervical cancer prevention have been developed against these two types. Recently, a new vaccine was approved, protecting against seven high-risk HPV types and two low-risk HPV types [5].

HPV taxonomy is based on the sequence variation of the *L1* gene, with differences greater than 10% defining HPV types [6]. Burk et al. [7] proposed a criterion for classifying intra-type HPV diversity based on overall genomic divergence, ranging from 1% to 10%, for defining different lineages within HPV types and differences between 0.5% to 1% for defining sublineages. They suggested an alphanumeric nomenclature for all HPVs lineages and sublineages, which we have used in this study, where lineages are identified by letters (A, B, C, D,…) and sublineages by letters and numbers (A1, A2, A3, B1, B2, B3,…). HPV lineages have been associated with different risks for cervical cancer. This is the case for specific HPV16 lineages and variants [8–11] as well as for HPV45 lineages [12]. Moreover, specific sequence variants of HPV58 are associated with the risk of developing CIN3 (cervical intraepithelial neoplasia grade 3) and invasive cancer [13].

In Brazil, most studies of intra-type variants of HPV have focused on HPV16 and HPV18 [9, 14–18], but other

relevant, high-risk types with different prevalence in different countries and continents have not been studied. Brazil has an admixed population, with Amerindian, African and European ethnic components with regional disparities, and Amazonian populations show a higher Amerindian genetic contribution than is observed in the southern regions [19]. This scenario makes the intra-type genetic diversity of high-risk HPV types relevant for analyzing the prevalence of HPV lineages and variants in different Brazilian regions. In this study, our objective was to describe and to compare the prevalence and the genetic diversity of lineages and variants, *sensu* Burk et al. [7], of the high-risk viruses HPV35, HPV45 and HPV58 in cervical cancer in women diagnosed in the Amazonian Region (Belém do Pará, Pará State, Brazil) and the Southeast Region (City of Rio de Janeiro, Rio de Janeiro State, Brazil). The genetic diversity of these HPV types was assessed by sequencing a contiguous HPV genomic region encompassing the LCR (long control region), *E6* and *E7*, which have been considered adequate for lineage and sublineage identification in previous work [20–22].

The study was carried out with a cohort of 1,275 women with cervical cancer, 634 from Instituto Nacional de Câncer (Rio de Janeiro, Southeast region) and 641 from Hospital Ophir Loyola in Belém (State of Pará, Amazonian region) following approval by the Ethics Committee of each institution. The identification and frequency of HPV types present in these tumor biopsies were carried out by PCR amplification of the L1 gene using the primers PGMY09/11 and GP05+/06+ [23, 24]. Samples from patients (n = 96) infected with HPV35 (n = 26), HPV45 (n = 53) and HPV58 (n = 17) in single infections were selected for this study (Supplementary Table 1).

The LCR and E6/E7 regions of HPV35, HPV45 and HPV58 were amplified using type-specific primers (Supplementary Table 2). PCR reactions were carried out with 1X PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM each DNTP, 10 pmol of forward and reverse primers per reaction, 50–100 ng of genomic DNA and 2.5 U of Platinum Taq DNA Polymerase (Life Technologies) in a final volume of 50 µl. The PCR conditions were 94 °C for initial DNA denaturation (5 min) followed by 35 cycles of 94 °C (1 min), 58 °C (1 min), and 72 °C (1 min), with final extension at 72 °C (15 min). PCR products were purified and sequenced as described above.

A contig encompassing the LCR, *E6*, and *E7* genomic regions was built for each sample (GenBank accession numbers KY565580 to KY565668). Sequences of each HPV type were aligned with reference sequences according to Burk et al. [7], and sequence variants (haplotypes) were identified with DNASP [25]. Maximum-likelihood (ML) phylogenetic reconstructions were carried out for each haplotype set with a reference sequence for each HPV type according to the GTR+I+G evolutionary model [26, 27]. ML was carried out with PHYML v.3.0 [28] with node support based on 1,000 bootstrap pseudoreplicates. Phylogenetic trees were rooted by the mid-point method. Median-joining analysis was carried out using Network v.4.6.1.1 [29]. Genetic and nucleotide diversity indices were estimated with Arlequin v.3.5.2.2 [30].

Analysis of HPV35, encompassing 1,587 nucleotides, showed 13 haplotypes in 24 samples (Table 1 and Supplementary Table 3A and B), two samples were excluded because the PCR for the LCR did not work. ML analysis (Fig. 1A) grouped reference sequence A1 with eight haplotypes (H2, H3, H5, H7, H9, H11, H12 and H13) in one group and with two others (H1 and H10) in a sister group. The eight haplotypes showed an exclusive insertion of 16 bp between positions 229 and 244. Three other haplotypes (H4, H6 and H8) grouped with sublineage reference sequence A2. The network arrangement (Supplementary Figure 1) was coincident with the ML topology. All haplotypes with frequency > 1 were present in both localities. Haplotypes grouped with sublineage A1 were more frequent (in 20 women), with haplotype H2 in 11 patients, eight from Belém do Pará and three from Rio de Janeiro (Table 1). HPV35 from Belém do Pará showed less genetic and nucleotide sequence diversity with respect to samples from Rio de Janeiro. HPV35 showed the least genetic and nucleotide sequence diversity when compared with HPV45 and HPV58 (Table 2).

Although some nucleotide substitutions could have resulted from mutations that occurred during cancer progression, the phylogenetic analysis supported haplotype identification at the sublineage level with bootstrap values >80%. HPV35 analysis showed less intra-type genetic diversity than the other types, in agreement with previous evidence that this type shows less intra-type genetic diversity than other HR-HPV types [7]. In this study, A1 was the most frequent HPV35 sublineage, in 20 of 24 isolates, as has been seen in different regions of the world [21, 31–33]. This sublineage was reported to have a higher persistence and oncogenicity than sublineage A2 [21, 34]. The ML topology showed a distinct group of eight haplotypes (H2, H3, H5, H7, H9, H11, H12 and H13) of sublineage A1 comprising the largest number of HPV35 isolates reported herein (18/24). These haplotypes, associated with invasive cancer, are common in other regions of the world, sharing a characteristic 16 bp insertion in their LCR [29, 33, 35]. The high frequency of these haplotypes, however, must be evaluated in women with normal cervix and pre-neoplastic lesions to determine whether this group is associated with higher oncogenicity.

HPV45 was detected in 53 women, 33 from Rio de Janeiro and 20 from Belém do Pará. Analysis of 51 of these samples, covering 1,569 nucleotide positions

Table 1 Number of samples (n) with respect to lineages, sublineages and haplotypes per locality for each HPV type

HPV type	Lineage/sublineage (n)	Haplotype	n - Rio de Janeiro	n - Belem do Pará
HPV35	A1(20)	H1	1	-
		H2	3	8
		H3	1	-
		H5	1	-
		H7	1	-
		H9	-	1
		H10	-	1
		H11	-	1
		H12	-	1
		H13	-	1
	A2(4)	H4	1	-
		H6	1	1
		H8	1	-
HPV45	A1(12)	H4	1	-
		H5	1	-
		H7	5	-
		H10	1	-
		H16	1	-
		H20	1	-
		H21	-	1
		H23	-	1
	A2(6)	H15	2	1
		H27	-	3
	A3(1)	H9	1	-
	B1(7)	H14	1	-
		H18	1	3
		H24	-	1
		H26	-	1
	B2(25)	H1	2	-
		H2	4	5
		H3	1	-
		H6	1	-
		H8	1	-
		H11	1	-
		H12	2	-
		H13	2	-
		H17	1	-
		H19	1	-
		H22	-	3
		H25	-	1
HPV58	A2(10)	H1	1	1
		H2	1	-
		H3	2	-
		H4	1	2
		H6	1	-
		H9	-	1
	A3(2)	H5	1	-
		H7	1	-
	C(2)	H8	1	1

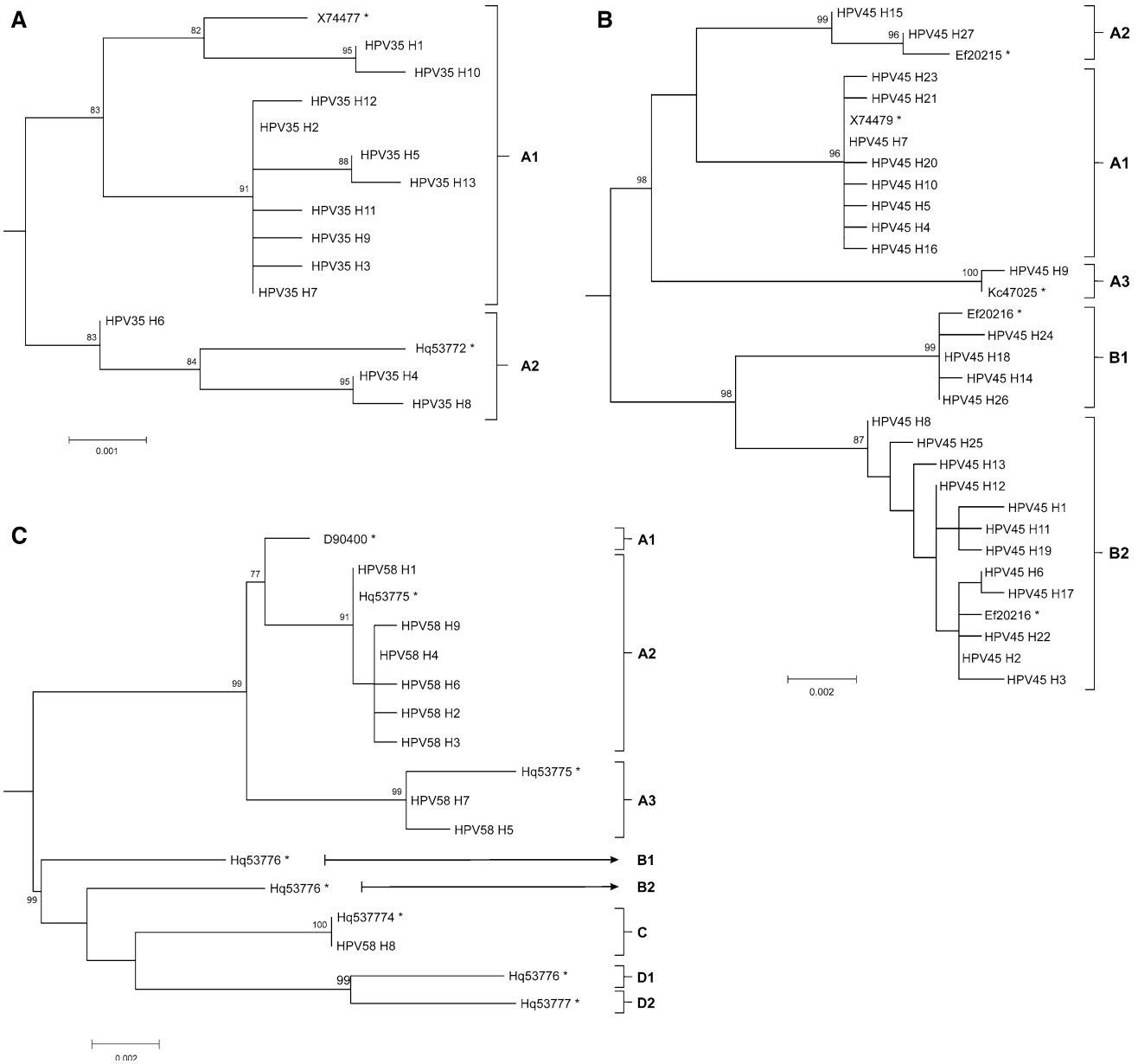

Fig. 1 Maximum-likelihood topologies for the haplotypes of each HPV type analyzed. The topologies were generated using the concatenated nucleotide sequences of the LCR, *E6* and *E7* regions for each haplotype. Numbers at the nodes are bootstrap values $\geq 70\%$, based on 1000 replicates. (A) HPV35. (B) HPV45. (C) HPV58. *Reference sequences [7] for each HPV lineage/sublineage are identified with the respective GenBank accession number

Table 2 Genetic and nucleotide sequence diversity for each HPV type per locality

HPV type/ locality	Number of samples	Gene diversity	Nucleotide sequence diversity
HPV35 RJ	10	0.9333 ± (0.0773)	0.004979 ± (0.002866)
HPV35 PA	14	0.6923 ± (0.1366)	0.002293 ± (0.001390)
HPV45 RJ	31	0.9570 ± (0.0212)	0.009324 ± (0.004773)
HPV45 PA	20	0.8842 ± (0.0416)	0.009788 ± (0.005099)
HPV58 RJ	9	0.9722 ± (0.0640)	0.007416 ± (0.004217)
HPV58 PA	5	0.9000 ± (0.1610)	0.007350 ± (0.004709)

(encompassing the LCR, *E6*, and a portion of the *E7* region), identified 27 haplotypes (Table 1 and Supplementary Table 3C and D), two samples were excluded because PCR for the LCR did not work. The ML topology (Fig. 1B) showed haplotypes grouping with one reference sequence of each sublineage: A1 with eight haplotypes, A2 with two, A3 with one, B1 with four, and B2 with 12. In this last group, a larger number of haplotypes was present in samples from Rio de Janeiro than in those from Belém do Pará (10 *vs.* 3). The results of median-joining network analysis (Supplementary Figure 2) coincided with the ML topology. Sublineages were present in both localities except for A3 (H9), which occurred in Rio de Janeiro. A large number of haplotypes (20 in 31 samples) was found in samples from Rio de Janeiro. HPV45 showed the highest nucleotide sequence diversity with respective to the other HPV types analyzed (Table 2). Previous reports showed that the HPV45 B2 sublineage was associated with a larger risk for cervical cancer than other sublineages [12, 21]. Our findings showed that B2 was the most frequent HPV45 sublineage, with a higher number of haplotypes in Rio de Janeiro than in Belém do Pará (Table 1).

HPV58 was detected in 17 patients. Analysis of 14 samples (nine from Rio de Janeiro and five from Belém do Pará), covering 1,567 nucleotide positions encompassing the LCR, *E6*, and *E7* showed nine haplotypes (Table 1 and Supplementary Table 3E and F), three samples were excluded because the PCR for the LCR,*E6* and *E7* did not work. ML topology (Fig. 1C) showed six haplotypes grouping with the reference sequence of sublineage A2, two with A3, and one with C. None of these haplotypes grouped with representative sequences of sublineages A1, B1, B2, D1, and D2. The results of median joining network analysis (Supplementary Figure 3) showed was coincident with the ML topology, with three groups respective to sublineages A2 and A3 and lineage C. A larger number of haplotypes was found in Rio de Janeiro than in Belém do Pará (Table 1), but these localities showed similar genetic and nucleotide sequence diversity (Table 2).

HPV58 is the third most frequent papillomavirus infecting women with cervical cancer in Eastern Asia [36]. The frequency of HPV58 varies around the globe and is more frequent in East Asia, where lineage A is the most frequent one, followed by lineage C [37]. Our findings showed the presence of two sublineages (A2 and A3) and one lineage (C), with A2 showing the highest frequent in both regions (58.8%). These findings are in agreement with those of Chan et al. [13, 37], who reported the highest frequency of A2 in cervical samples worldwide.

Our findings show differences in the frequency and presence of specific HPV variants between the Southeast and Amazonian regions, and this may be associated with differences in ethnic genetic background of the patients, as reported by Lopera et al. [38] for the Colombian population and HPV16 lineages. The higher proportion of Amerindian ethnic component in the Brazilian Amazonian population in comparison to the Southeast population has been reported by many authors [19, 39, 40]. However, to access the possible association between the ethnic component with specific lineages/sublineages of HPV35, HPV45, or HPV58 a larger sample size will be required, taking into account the prevalence of these three HPV types in the populations studied: only in 96 of the 1275 patients included (8.1%), was the presence of HPV35 or HPV45 or HPV58 detected.

Acknowledgements We want to thank the physicians who collected the tumor biopsies. This work was supported by the National Institute for Cancer Control (INCT do Cancer: http://www.inct-cancer-control.com.br/); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) Grants 573806/2008-0, 484005/2013-8 and 305873/2014-8; Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) Grant E26/170.026/2008; Ministry of Health – Brazil; and Pan-American Health Organization (PAHO).

Compliance with ethical standards

Ethical approval All procedures were approved by the Research Ethics Committees of the Instituto Nacional de Câncer (CAAE 53398416.0.0000.5274) and the Hospital Ophir Loyola (CAAE 03288212.0.1001.0018).

Informed consent All patients signed an informed consent form.

Animal rights This article does not contain any studies with animals.

Conflict of interest The authors declare no conflict of interest.

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