



HPV DNA methylation at the early promoter and E1/E2 integrity: A comparison between HPV16, HPV18 and HPV45 in cervical cancer

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ABSTRACT

Objectives: To compare and describe type-specific characteristics of HPV16, HPV18 and HPV45 in cervical cancer with respect to 3'LCR methylation and disruption of E1/E2.

Methods: The methylation level of 137 cervical cancer samples (70 with HPV16, 37 with HPV18, and 30 with HPV45) of Brazilian patients was analyzed by pyrosequencing. PCR amplifications were performed to characterize E1 and E2 disruption as an episomal surrogate.

Results: The 3'LCR of HPV16 showed a higher methylation at all CpG sites (7%, 9%, 11%, 10% and 10%) than homologous HPV18 regions (4%, 5%, 6%, 9% and 5%) and HPV45 regions (7%, 7% and 5%). Presence of intact E1/E2 was associated with higher HPV16 and HPV18 methylation levels at all CpG sites ($p < 0.05$). Disruption of E1/E2 was more frequently found in HPV45 (97%) and HPV18 (84%) than in HPV16 DNA (30%). HPV16 disruption was more frequently found in E1 (48%) unlike HPV18, where it was found in E2 (61%). Concomitant disruption of E1/E2 was most frequent in HPV45 (72%).

Conclusions: The findings showed a higher methylation associated with intact E1/E2 for HPV16 and HPV18. The closely phylogenetic related HPV18 and HPV45 share a similar methylation level and the frequency of viral genome disruption.

1. Introduction

The biology of human papillomaviruses (HPV) has been extensively described in the literature [1] in view of its association with specific cancer types, mainly cervical cancer (CC). Twelve mucosal genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) have been characterized as high-risk HPV (HR-HPV) for cancer development [2] consequently to their high prevalence in CC and to the molecular evidence associated to carcinogenesis.

The HPV genome is a circular double-stranded DNA of approximately 8 Kilo-base pairs (kb) contained in an icosahedral capsid comprising three regions: (i) the E region, with genes coding for proteins predominantly expressed in early stages of infection (E1, E2, E4, E5, E6 and E7) and associated with viral DNA replication, regulation of HPV gene expression, control of cell cycle and oncogenesis, accounting for 4 kb of the viral genome; (ii) the L region, with genes encoding structural capsid proteins (L1 and L2) expressed in later stages of infection, comprising ~3Kb of the viral genome, and (iii) the Long Control Region

(LCR), a non-coding region of approximately 1 kb which controls viral replication and transcription through DNA motifs recognized by DNA-binding proteins [3].

The LCR consists of three functionally separate segments, the 5' segment (5'LCR), the central (or enhancer) and the 3' segment (3'LCR or early promoter) (Fig. 1A) [3]. The 5'LCR is approximately 300 bp long and is located between the terminal L1 codon and the E2 protein binding site (named E2BS#1). This segment contains a nuclear matrix attachment region, a transcription termination region and polyadenylation sites [4]. The central, or enhancer segment, of approximately 400 bp, is flanked by two E2 binding sites (E2BS#1 and E2BS#2). Several cell transcription factors (i.e.: AP-1, NFI, YY1, Oct-1, TF-1, TEF-2, glucocorticoid and progesterone receptor) are capable of binding to this segment and transactivate HPV gene expression [5,6]. The 3'LCR, of approximately 140 bp long and flanked by E2BS#2 and E6, is capable of controlling the expression of E6 and E7 viral oncogenes. This promoter has been well characterized in HPV16 and HPV18 (named P97 and P105, respectively), and is essential for

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that encodes a dose-dependent transcriptional repressor of the early promoter in 3'LCR [9–13]. E2 activity is associated with displacement of the Sp1 and TFIID promoter activators from their respective binding sites. Thus, the absence or low level of E2 proteins results in over-expression of the E6 and E7 oncogenes and cancer progression.

Furthermore, it has been suggested that methylation at CpG sites in the 3'LCR of HPV16 in CC could trigger cancer development by modulating E2 protein activity when episomal HPV DNA is present [14]. Methylation at this segment has shown to be complex, with conflicting findings among CC samples; most available data being restricted to the HPV16 genotype [15,16]. Moreover, clinical factors have also been found to affect methylation levels [17].

The specificity of HPV types in the etiology of cervical cancer shows a phylogenetic imprinting, with some members of the Alpha-papillomavirus genus associated with all cervical cancers [18]. Within this genus, however, specific species (α 1–11, 13 and 15) and even HPV genotypes show different pathogenicity, with HPV16 (belonging to the α 9 species) accounting for more than 60% of all CC worldwide [19]. Nevertheless, distinct biological characteristics between HPV genotypes have been identified among CC samples. Presence of viral DNA in the episomal state has been observed in approximately 40% of HPV16 + tumors, while higher frequency of episomal DNA has been detected in tumors associated with HPV52 and HPV58 infection (75% and 88% respectively) [20,21]. Differently, most cervical cancers harboring HPV18 contained an integrated viral genome [21,22]. Moreover, in addition to their frequency of integration, HPV16 differs from HPV18 with respect to CC progression. Reviews and meta-analyses have reported a lower frequency of HPV18 in pre-neoplastic lesions than in CC, suggesting a more rapid progression of HPV18 lesions [23–25]. Moreover, HPV18 + or HPV45 + CC (two different α 7 species types) showed a higher proportion of adenocarcinomas than HPV16 + CC [23–25]. An additional difference between HPV genotypes accounts for the number of CpG sites along their genome, with 111 CpG sites in HPV16 vs 168 in HPV18 [26].

In this paper, we compare and describe type-specific characteristics of HPV16, HPV18 and HPV45 in CC samples with respect to 3'LCR methylation and disruption of E1/E2 open reading frames (ORFs). Moreover, we intent to verify whether methylation was associated with E1/E2 disruption in HPV18 and HPV45, as previously reported in HPV16 [17].

2. Material and methods

2.1. Samples

The study material consisted of 137 CC samples selected from a previously reported pool of 590 biopsies of invasive cervical cancer [27], 70 associated with HPV16, 37 with HPV18 and 30 with HPV45. Samples were collected at diagnosis from patients treated at the Instituto Nacional de Câncer (INCA - Rio de Janeiro, Brazil) referred for treatment between June 2011 and March 2014. The set of HPV16 + and HPV18 + samples was selected as previously reported [17] in according to their histopathological presentation while all HPV45 + tumors were included in view of their lower number in the pool with respect to HPV16 + and HPV18 + samples (Table 1). DNA isolation and HPV identification was as previously described [27]. Genomic DNA from HeLa, CaSki and SiHa cell lines was used as control for bisulfite treatment and pyrosequencing.

All procedures were approved by the Ethics Committee of Instituto Nacional de Câncer (protocol CAAE 53398416.0.0000.5274). All patients signed an informed consent and filled an epidemiological questionnaire.

2.2. Sodium bisulfite treatment and PCR amplification

Sodium bisulfite treatment was carried out with EpiTect Bisulfite Kit

Table 1
Characteristics of study group by HPV type.

Characteristics (N)	Total	HPV16	HPV18	HPV45
Overall population (N = 137)	137	70	37	30
Patient Age (yo)				
Mean; SD	46; 13	45; 13	47; 13	49; 12
Median	45	43	45	47
Tumor types				
ADN	39	23	10	6
SCC	96	47	25	24
No information	2	–	2	–
FIGO Stage				
I	31	23	4	4
II	54	23	15	16
III	47	21	17	9
IV	5	3	1	1
No information	–	–	–	–
Tumor Grade				
G1	12	6	3	3
G2	81	45	18	18
G3	25	11	10	4
No information	19	8	6	5

Note. ADC: adenocarcinoma, SCC: squamous cell carcinoma. N = number of samples. Yo: years old. FIGO: International Federation of Gynecology and Obstetrics.

(cat. no. 59104, Qiagen, Germany), with an input of 300–1500 ng of DNA and a final elution volume of 40 μ L. Following treatment, regions covering 178 bp, 245 bp and 149 bp of the 3'LCR of HPV16, HPV18 and HPV 45, respectively, were PCR-amplified (Table 2). PCR was carried out in 30 μ L mixtures containing 0.2 mM of each dNTP, 6 pmol of each primer, 1 U of Platinum Taq DNA Polymerase (Life Technologies, California, USA) and 1 \times PCR buffer (67 mM Tris pH 8.8, 6.7 mM MgSO₄, 16.6 mM (NH₄)₂SO₄, and 10 mM 2-mercaptoethanol) [28]. PCR conditions consisted of 95°C for 6 min followed by 45 cycles of 95°C for 1 min, annealing (temperatures listed in Table 2) for 1 min, 72°C for 1 min, and one final extension step at 72°C for 5 min. Presence of amplified products was verified in 2% ultrapure agarose gels (Life Technologies, California, USA).

2.3. Quantitation of DNA methylation by pyrosequencing

PCR products were submitted to pyrosequencing in a PyroMark Q24 platform (Qiagen, Hilden, Germany) following a standard protocol [17]. Briefly, streptavidin beads, PyroMark binding buffer (Qiagen, Hilden, Germany) and PCR products were mixed and incubated on a shaking platform. A Biotage Q24 Vacuum Prep Workstation was used for separating, denaturing and washing PCR products, which were subsequently added to a microtiter plate containing annealing buffer with sequencing primers (see Table 2). Primer annealing was carried out by incubation at 80°C for 2 min and cooling to room temperature before pyrosequencing. PyroGold reagents, including enzyme, substrate and nucleotides were used for the pyrosequencing reaction. Pyrograms were generated and analyzed with PyroMark Q24, v.2.0.6 (Qiagen, Hilden, Germany). Controls for hyper-methylation (with CaSki cells) and hypo-methylation (with SiHa and HeLa cells) were used as previously reported [29,30].

2.4. E1 and E2 disruption

HPV integration into the host DNA genome frequently occurs within E1 or E2 disruption [31] resulting in suppression of E2 transcription. The presence of intact E1/E2 was identified by PCR amplification of overlapping fragments encompassing the E1 and E2 coding regions of HPV16, HPV18 and HPV45 using primers pairs listed in Table 3, with some of them previously published [32,33]. PCR was carried out as described [17] with different annealing temperatures shown in Table 3.

Table 2
Primers for methylation analysis.

Assay	Primers (5'-3')	CpG (nt position)	Amplicon size	T.A.
HPV16_3LCR*	forward 3'LCR: TTGTAATAATTGTATATGGGTGTG reverse 3'LCR (biotin): AAATCCTAAAAACATTACAATTCTC sequencing (forward) 3'LCR: AATTTATGTATAAAAATTAAGGG	31, 37, 43, 52, 58	178 bp	61°C
HPV18_3LCR	forward: ATTTTAAATATGAATTATAATATGATTAAG reverse (biotin): CACAAATCAAATAACTTATAAAATC sequencing (forward): GTAGTATATAAAAAGGGAGTAA	44, 50, 54, 60, 66	245 bp	57°C
HPV45_3LCR	forward (biotin): GTTTATGTAATAGAAAAGGTTGGGTTTAA reverse: CCTATAATACACTTTTCCACAAAACCTT sequencing (reverse): ACTTTCCACAAAACCTT	41, 47, 63	149 bp	62°C

Note. *Primers reported by Rajeevan et al. (2006). CpG (nucleotide position) based on HPV reference: HPV16REF K02718.1, HPV18REF X05015.1, HPV45REF X74479.1. TA: Temperature of Annealing; bp: base pairs; nt: nucleotide.

Table 3
Primers for analysis of *E1* and *E2* gene disruption.

Assay		Primers (5'-3')	TA	Nucleotide position	Amplicon size (bp)	
HPV16	E1a	forward: CCATGGCTGATCCTGCAG reverse: TCTCCTTTTTGCAGCTCT	61 °C	863–1219	356 bp	
	E1b	forward: GACAGCGGTATGGCAAT reverse: CATTCCCATGAACATGC	65 °C	1254–1663	409 bp	
	E1c	forward: AATAAATCAACGTGTTGCGATTGG reverse: GTTTATAATGTCTACACATTGTTG	65 °C	1548–2084	536 bp	
	E1d	forward: GGATTGTGCAACAATGTG reverse: TGGAGGGCATTTTAGTTG	65 °C	2072–2527	455 bp	
	E1e	forward: CAACTAAAATGCCCTCCA reverse: CGCATGTGTTTCCAATAG	61 °C	2529–2845	316 bp	
	E2a	forward: CGAGGACAAGGAAAACGA reverse: CTTGACCTCTACACAG	65 °C	2738–3189	451 bp	
	E2b	forward: GGTTTATATTATGTTTCATGAAGG reverse: TATGGGTGAGTGTACTATTACA	56 °C	3220–3599	379 bp	
	E2c	forward: GTAATAGTAACACTACACCCATA reverse: GGATGCAGTATCAAGATTG	56 °C	3596–3853	257 bp	
	HPV18	E1P1	forward: GGTGTGCATCCAGCAGTAA reverse: GCCGCCACTACATACATTGC	59 °C	888–1403	515 bp
		E1P2	forward: GCGGCAATGTATGTAGTGGC reverse: GCTGCAACACTACTTCGCAA	59 °C	1400–1908	508 bp
E1P3		forward: TCAACCACCAAATGCGAAGT reverse: TCGTTTTTGGGCTCGCCTAT	59 °C	1877–2211	334 bp	
E1P4		forward: GCAAACATTATAGGCGAGCCC reverse: TGTCCAACACGTGGTCGTT	59 °C	2181–2546	365 bp	
E1P5		forward: GGTGGCCATGTTAGATGATGC reverse: GATTTTGTCTGCAACGCACT	59 °C	2506–2895	389 bp	
E2P1		forward: TCCAGATTAGATTTGCACGA reverse: CAATTGTCCTTTGTTGCCATC	61 °C	2786–3192	407 bp	
E2P2		forward: ATACAAAACCGAGGATTGGA reverse: ACTTCCCACGTACCTGTGTT	61 °C	3086–3388	303 bp	
E2P3		forward: AACACAGGTACGTGGGAAGT reverse: TTTCCGAATCTGTACCGTAA	61 °C	3369–3739	371 bp	
E2P4		forward: GACCTGTCAACCCACTTCT reverse: ACATGGCAGCACATACAT	61 °C	3598–3994	397 bp	
HPV45		E1a	forward: GGTGTAATGGCTGTTCTTTGT reverse: AATGGACTGTTTTCTTGTCTGC	55 °C	881–1139	259 bp
	E1b	forward: CAGTCCATTAGGGGAGCAGC reverse: GCTGCAACACTACTTCGCAA	57.5 °C	1131–1775	645 bp	
	E1c	forward: AGCACATTGTTGCACGTACC reverse: GGTCTCCAATCCCACCTTC	57.5 °C	1705–2144	440 bp	
	E1d	forward: AAGGTGGGGATTGGAGACCC reverse: AGGGATTCTTCGGTGTCTG	62 °C	2126–2727	602 bp	
	E1e	forward: TTTGCACGAGGACGATGAAGA reverse: CACCTGGTGGTTAGTTGGTAA	55 °C	2685–2890	206 bp	
	E2a	forward: GGACATGGTCCAGATTAGATTGC reverse: GCACGGTTTTACCGCCTTTT	55 °C	2666–3068	403 bp	
	E2b	forward: TACAGAACCGTCGAGTGT reverse: TCTGGATGTGGGGTTTTGGG	62 °C	3025–3431	407 bp	
	E2c	forward: AGACAGCTACAACACGCCTC reverse: TGCAGCACATAAAGGCAC	62 °C	3359–3893	535 bp	

Note. Bp, base pairs; primers covering *E1* and *E2* of HPV16 were designed by Vermont et al. (2007), and primers of *E2* of HPV18 by Collins Constandinou-Williams et al. (2009).

Negative reactions were repeated to confirm lack of amplification of target regions.

2.5. Statistical analysis

The level of methylation at each CpG site per sample was estimated as the proportion of methylated cytosines, ranging from 0 (without methylation) to 100% (totally methylated). Comparisons of methylation levels were carried out with the Mann-Whitney test between (i) homologous CpG sites (sites binding similar proteins) and (ii) individual CpG sites with and without *E1/E2* disruption. All statistical analyses and graphs were conducted with GraphPad Prism 7.

3. Results

3.1. Characteristics of study group

The clinical and pathology profiles of the 137 patients herein studied are summarized in Table 1. Age at diagnosis ranged from 19 to 80 years, with a mean of 46 (SD \pm 13) and a median of 45. A higher proportion of squamous cell carcinomas than adenocarcinomas (approximately 2:1) were selected among HPV16 + and HPV18 + tumor samples while, among HPV45 + tumors, the proportion of SCC was higher (4:1) due to the limited number of available samples. In 77% of all tumors, FIGO staging was equal or above IIA, with 90% tumor grade G2 or G3 (Table 1).

3.2. Methylation at the 3'LCR of HPV DNA

Following bisulfite treatment, the methylation level of each CpG site per sample was estimated by pyrosequencing in the 3'LCR of HPV16 (nt 31, 37, 43, 52 and 58; GenBank: K02718.1), HPV18 (nt 44, 50, 54, 60 and 66; GenBank: X05015.1) and HPV45 (nt 41, 47 and 63; GenBank: X774479.1) (Fig. 1B to 1D, respectively). Two samples (one HPV16 + and one HPV45 +) were excluded from analysis due to low quality of pyrosequencing data. The methylation levels of CpG sites per sample are listed in Supplemental Table 1.

The CpG sites in the 3'LCR of HPV16 showed a higher median methylation (7%, 9%, 11%, 10% and 10%) than the homologous segment of HPV18 (4%, 5%, 6%, 9% and 5%) and HPV45 (7%, 7% and 5%). Moreover, HPV16 showed a wider range of methylation per sample at E2 binding sites (0–90%, in nt 37) than HPV18 (1–72%, in nt 60) and HPV45 (1–85%, in nt 63) (Fig. 2).

Homologous CpG sites in E2 binding site motifs of each HPV genotype comprised: (i) nt 37, 44 and 41 of HPV16, HPV18 and HPV45, respectively, at the 5' end of E2BS#3; (ii) nt 43, 50 and 47 of HPV16, HPV18 and HPV45, respectively, at the 3' end of E2BS#3; (iii) nt 52 and 60 of HPV16 and HPV18, respectively, at the 5' end of E2BS#4; and (iv) nt 58, 66 and 63 of HPV16, HPV18 and HPV45, respectively, at the 3' end of E2BS#4 (Fig. 2). Significant differences were found in the methylation level of E2BS#3 CpG sites between HPV16 and HPV18 ($p = 0.0018$ and $p = 0.0490$), and in one E2BS#4 CpG site between HPV16 and HPV18 ($p = 0.0109$) (Fig. 2). Significant differences were also found between E2BS#3 CpG sites of HPV45 and HPV18 ($p = 0.0029$ and $p = 0.0039$) (Fig. 2).

A hypermethylation pattern was found at CpG sites in the 3'LCR of HPV16 in CaSki cells (90%, 86%, 97%, 92%, 98%) as well as a hypomethylation pattern in SiHa cells (2%, 3%, 2%, 4% and 5%). HPV18 + HeLa cells showed a pattern of hypomethylation (1%, 1%, 1%, 2% and 1%).

3.3. E1 and E2 gene integrity

PCR amplification covering *E1* and *E2* was used for detecting HPV disruption, a finding suggesting HPV integration into the host genome and lack of *E2* expression. Disrupted *E1* and *E2* were found in 30% (21/

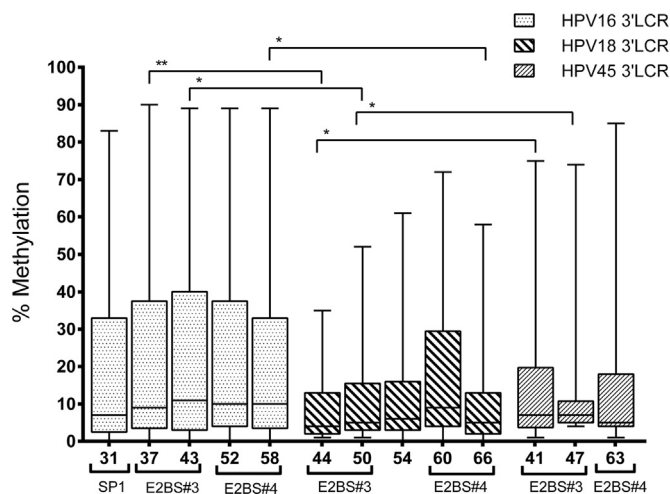


Fig. 2. Methylation status of homologous CpG sites in the 3'LCR of HPV16, HPV18 and HPV45. The methylation level of each CpG site is represented by a box displaying upper and lower quartiles separated by the median line, and whisker plots. HPV16 displayed a higher level of methylation than HPV18 and HPV45, mainly at E2 binding sites (E2BS#3 and #4). This was statistically different when comparing the homologous CpG sites of HPV16 vs HPV18 (CpG 37 vs 44, $p = 0.0018$; CpG 43 vs 50, $p = 0.0490$; and 58 vs 66, $p = 0.0109$), and HPV18 vs HPV45 (CpG 44 vs CpG41). Analyses were performed with the Mann-Whitney *U* test.

69) of HPV16 + samples, 84% (31/37) of HPV18 + samples, and 97% (28/29) of HPV45 + samples.

In HPV16, the most frequent disruption occurred between nt 2529–3189 (67% - 14/21) encompassing the 3' end of *E1* and the 5' end of *E2* (HPV16REF; K02718.1). Forty-eight percent (10/21) of disruptions were exclusively found in *E1* and 14% (3/21) in *E2*. Disruption in both *E1* and *E2* were found in 38% (8/21) of samples (Fig. 3A). In HPV18, the most frequent disruption occurred between nt 3369 and 3739 (90% - 28/31), a region inside *E2* and overlapping the *E4* ORF; nt 3418–3684 (HPV18REF; X05015.1) (Fig. 3B). *E2* was more frequently disrupted (61% - 19/31), followed by disruption of both genes (35% - 11/31), and a single disruption in *E1* (3% - 1/31) (Fig. 3B). In HPV45, the concomitant *E1* and *E2* disruption was the most frequent pattern (72% - 20/28) resulting in lack of amplification of a large extension of these genes; 46% of samples (13/28) showing loss of more than 1.7 Kb (between nt 2126 and 3893) (HPV45REF; GenBank accession number X74479.1) (Fig. 3C). Similarly, to HPV18, the most frequently missing amplicon (*E2c*) overlapped the *E4* ORF (nt 3359–3893) (HPV45REF; X74479.1). Additionally, in 14% (4/28) of samples, losses were exclusively found in *E1* and *E2*.

HPV16 + and HPV18 + samples with intact *E1* and/or *E2* showed higher median methylation levels in all 3'LCR sites ($p < 0.05$; Mann-Whitney *U* Test) than samples with disruptions (Fig. 4A and B). This analysis could not be performed with HPV45 + samples because only a single sample lacking *E1/E2* disruption was detected. A comparison of the methylation level between homologous CpG sites in samples with *E1/E2* disruption showed a higher methylation at E2BS#3 (nt 41) and E2BS#4 (nt 63) in HPV45 than in HPV18 (nt 44 and 66, respectively; $p = 0.0163$ and $p = 0.0246$, respectively; Supplemental Fig. 1).

4. Discussion

The present study compared HPV16, HPV18, and HPV45 (three HPV associated with high risk for cervical cancer development) with respect to 3'LCR methylation and *E1/E2* integrity, a comparison not carried out to present. Methylation at CpG sites in the LCR of HPV16 has been extensively studied [34] and, in high-grade lesions and cervical tumors, a lower methylation level has been found in the LCR than

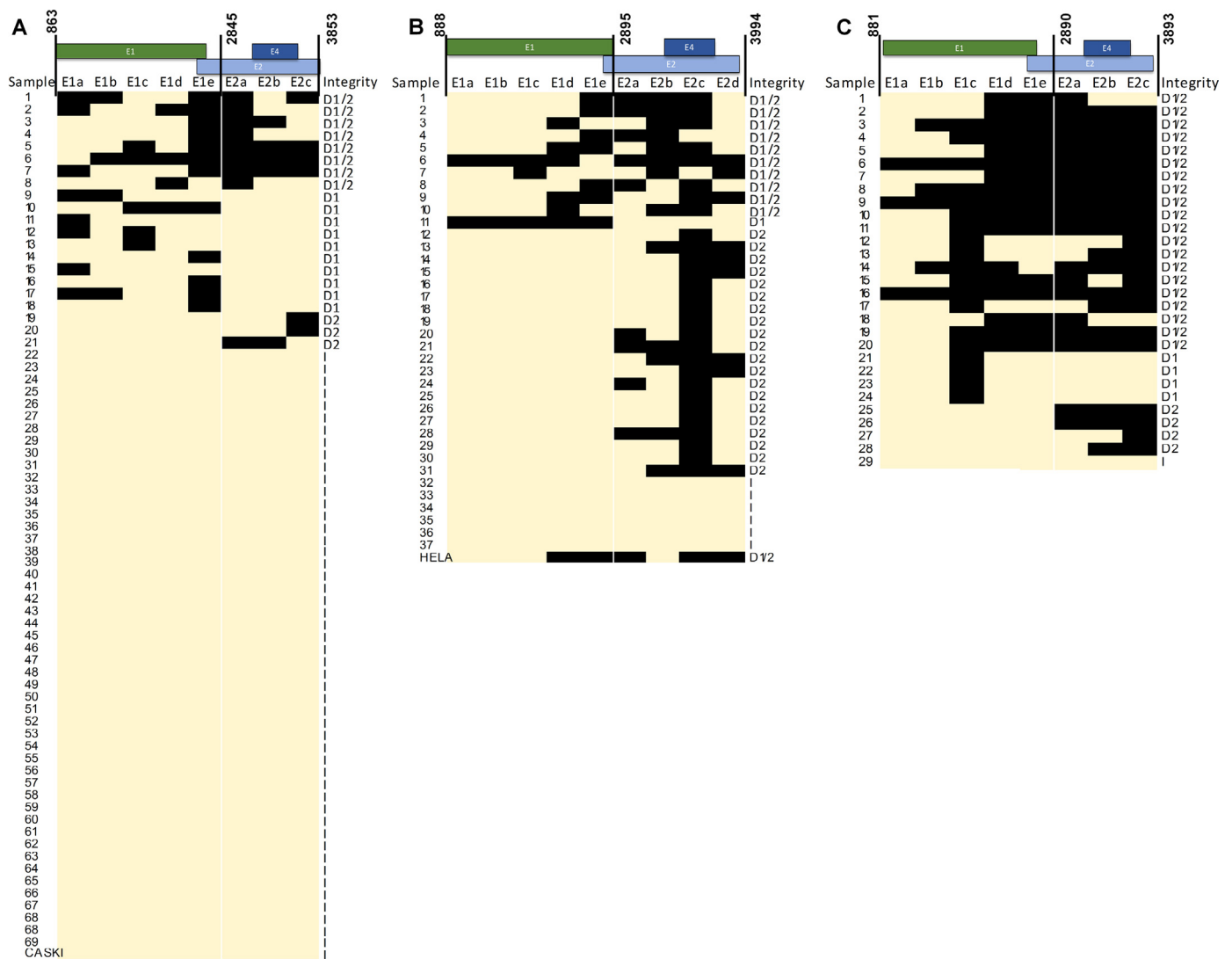


Fig. 3. Map of disruptions sites in *E1* and *E2* of the 3'LCR of HPV per sample. A, *E1* and *E2* of HPV16 predominately showed intact genes (light orange color - I), with high disruption of *E1*. B, integrity of HPV18 *E1* and *E2*, with most samples displaying disrupted DNA, predominately of *E2*. C displays *E1* and *E2* integrity of HPV45 showing significant loss of both genes and larger deletions; light orange indicate presence of amplification. D1/2 indicates *E1* and *E2* disruption; D1: exclusive *E1* disruption; D2: exclusive *E2* disruption; I: intact *E1* and *E2*. E1a, E1b, E1c, E1d and E1e represent amplicons covering the *E1* gene. E2a, E2b, E2c and E2d represent amplicons covering the *E2* gene. The genome position of each amplification is described in Table 2.

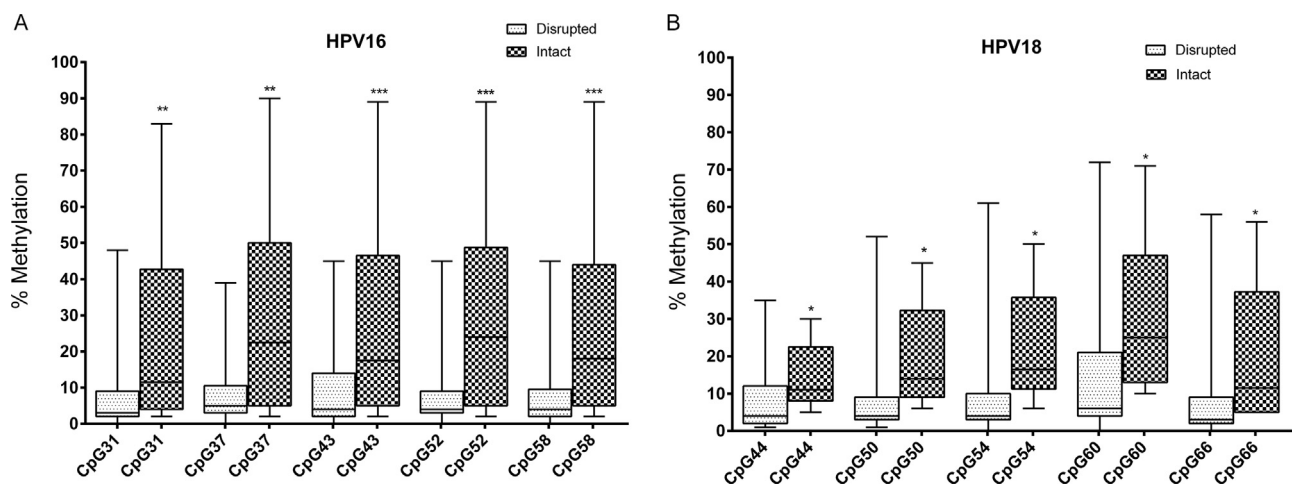


Fig. 4. 3'LCR of HPV16 and HPV18 relative to *E1* and *E2* integrity. The methylation level of each CpG site is represented by a box displaying upper and lower quartiles separated by the median line, and whisker plots. A higher methylation at all CpG sites was found in samples with intact *E1* and *E2* than with disrupted genes (* $p < 0.05$; ** $p < 0.005$ and *** $p < 0.0005$, Mann-Whitney Test).

in L1 or L2 [35,36]. Other studies have pointed a wide range of methylation in the 3'LCR of HPV16 in clinical samples of CC [37,38], suggesting a different regulation of HPV expression and a likely application for prognosis [38,39]. Moreover, our previous study [17] suggested that patient aging may also contribute, independently, to increase methylation in the 3'LCR of HPV16, pointing to the complexity of HPV DNA methylation.

High methylation in the 3'LCR of HPV16 in cervical tumors has been associated with presence of episomal DNA [14] resulting in loss of negative control of transcription of *E6* and *E7* oncogenes by the *E2* protein [40,41]. To present, there is no evidence that this association (methylation vs integration) might occur in other HPV genotypes. The increased methylation level at the 3'LCR of HPV16 + and HPV18 + samples were herein associated with presence of intact *E1/E2* genes (Fig. 4A and B), a finding suggestive for the presence of episomal viral DNA in these tumors. This association could not be assessed in HPV45 + samples due to their small number with intact *E1/E2*.

Furthermore, we observed a higher methylation level of HPV16 than in HPV18 and HPV45, particularly at CpG sites in E2BS#3 (Fig. 2), a finding that might be associated to different patterns of DNA disruption. A higher frequency (70%) of intact *E1/E2* was observed in HPV16 + samples than in HPV18 + and HPV45 + samples (16% and 3%, respectively), where only few tumors showed intact *E1/E2*. The similar methylation pattern and *E1/E2* disruption frequency of HPV18 and HPV45 might be associated with their close phylogenetic relationship; both belonging to species α 7, differently from HPV16 that belongs to species α 9 [42].

Differences in *E1/E2* disruption patterns were also observed because *E1* disruption was very frequent in HPV16 while HPV18 disruption occurred most frequently in *E2*. In HPV45, a different pattern was observed, with concomitant disruption frequently affecting both genes and with deletions of large viral genomic segments suggesting total loss of *E1* and *E2*. A higher frequency of *E2* disruption has been observed in HPV18 than in HPV16, although *E1* disruptions were not assessed [32]. Differently, a high frequency of *E1/E2* deletions have been reported in HPV16 + CC samples, with low-grade cervical intraepithelial neoplasia associated to exclusive *E1* deletions [43]. In this study, we did not observe any association between disruption patterns and cancer staging (Supplemental Table 2).

The *E4* ORF overlapping with the *E2* ORF was the most frequently disrupted region in HPV18 and HPV45 DNA which also contains the highest numbers of CpG sites ($N = 19$ and $N = 18$, respectively). These findings might indicate that the methylation patterns of these CpG sites during early events of infection might affect the instability of HPV DNA.

Our findings highlight the importance of considering the frequency of specific disruptions when evaluating integration status. It is important to highlight that assessment of *E1/E2* integrity by PCR has some limitations. Lack of *E1/E2* amplicons confirms samples with disrupted *E1/E2* viral genes, suggesting integration into host DNA in single copy HPV integration events. On the other hand, presence of intact *E1/E2* did not exclude integration of multiple viral genomes (in tandem or multiple independent integrations) or the presence of concomitant forms (episomal and integrated). This approach, however, includes tumor samples in two groups: (i) unable to translate *E2* (with only disrupted *E1/E2* copies), and (ii) capable of translating *E2*, negatively regulating LCR promoter activity (intact *E1/E2*).

Different methods for detecting HPV DNA integration have been used; among which the most frequently ones were based on the integrity of a small *E2* region by Real-Time PCR, usually estimated by the ratio between *E2: E6* or *E2: E7* copy number [44,45]. More recently, an alternative approach carried out by HPV DNA capture with specific probes and NGS (next-generation sequencing) allowed a more precise identification of integration sites through detection of chimeric DNA (HPV DNA + host DNA). The few studies that used this approach reported a large diversity of host and HPV DNA breakpoints [22,46] but, at present, it is still unclear whether breakpoints identified by NGS were

relevant for cervical carcinogenesis.

The role of CpG methylation at upstream LCR *E2* binding sites (encompassing the CpG sites (nt) 7453, 7459 and 7860 in HPV16; accession number AF125673) is still unclear, but there is evidence that CpG methylation might also affect progression from pre-cancer lesions to invasive cancer. In the proposed model for HPV16 [12], *E2* binds with higher affinity to the upstream *E2* binding sites (E2BS1 and E2BS2), activating transcription of *p97* [12]. Additionally, cell lines transfected with plasmid constructs containing full LCR and methylated CpGs at E2BS1 (nt 7453 and 7459) presented a higher *p97* promoter activity than with unmethylated constructs [48]. On the other side, significant differences in methylation level in invasive cancer were restricted to E2BS3 and E2BS4 (the targets of this work) between samples with episomal vs. integrated HPV16 viral genome, but not for the upstream E2BS1 and E2BS2 [14].

5. Conclusion

Our study showed a similar association between the methylation pattern at 3'LCR of HPV16 and HPV18 with the disruption of viral genome at *E1/E2*, reinforcing the importance of DNA methylation for *E2* function. Additionally, we showed differences in the frequency of *E1/E2* disruptions among the three HPV types herein studied, with the closely related HPV18 and HPV45 sharing a higher frequency of *E1/E2* disruptions and the methylation level of the 3'LCR.

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Conflict of interest

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.pvr.2018.04.002>.

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