Virus Research 232 (2017) 48–53

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Methylation at 3′LCR of HPV16 can be affected by patient age and disruption of *E1* or *E2* genes

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ARTICLE INFO

Article history:
Received 8 December 2016
Received in revised form 24 January 2017
Accepted 24 January 2017
Available online 29 January 2017

Keywords:
Human papillomavirus
HPV16
Invasive cervical cancer
Methylation
Episomal HPV genome
HPV integration

ABSTRACT

CpG methylation at early promoter of HPV16 DNA, in the 3′ end of the Long Control Region (3′LCR), has been associated to the presence of episomal forms of viral genome and, consequently, intact E1 and E2 ORFs. The DNA methylation would block the access of E2 viral protein to the E2 binding sites at early-promoter. However, is still unclear if methylation at 3′LCR of HPV16 DNA can also vary depending of other tumor characteristics in addition to viral DNA physical state. In this study, we evaluate whether the methylation level at the five CpG located at 3′LCR of HPV16 is associated to patient age and E1 and/or E2 ORFs integrity. DNA pyrosequencing was used to measure the methylation level in 69 invasive cervical cancer samples obtained from biopsies of patients attended at Brazilian National Institute of Cancer (INCA). PCR amplifications were performed to assess disruption status of *E1* and *E2* genes of HPV16. The methylation average per sample ranged widely, from <1 to 88.00%. Presence of intact *E1*|*E2* genes and patient age were positively associated with average methylation in both bivariate analyses ($p = 0.003$ and $p = 0.006$, respectively), and multivariate analysis ($p = 0.002$ and $p = 0.021$, respectively), adjusted for tumor type (squamous cell carcinomas or adenocarcinomas) and HPV16 lineage. These findings showed that presence of intact *E1*|*E2* open reading frames was associated with high levels of DNA methylation, and older patients showed higher levels of methylation than younger ones independently of viral genome disruption.

1. Introduction

Human papillomaviruses (HPV) are non-enveloped viruses (~55 nm) with a circular genome of double-stranded DNA of approximately 8 Kilobase-pairs (kb) inside an icosaedric capsid. More than 40 HPV genotypes have been found to infect the genital mucosa and 12 among them are well characterized as high-risk types (HR-HPV) for cervical cancer according to the International Agency for Research on Cancer IARC Working Group on the Evaluation of Carcinogenic Risks to Human (2009). The HPV genome contains three different regions: (1) the early region (E), with genes coding for proteins predominantly expressed in early stages of infection (*E1, E2, E4, E5, E6* and *E7*), comprising 4 kb of the viral genome and associated with viral DNA replication, regulation of HPV gene expression, and oncogenesis; (2) the late region (L) of approximately 3 kb, with genes encoding structural capsid proteins (*L1* and *L2*) expressed in later stages of infection; and (3) the Long Control Region (LCR), a non-coding region of approximately 1 kb, functionally divided into 5′, central and 3′ portions, with sequence motifs required for gene expression and initiation of viral DNA replication (O'Connor et al., 1995).

As in eukaryotic organisms, the HPV virion presents the genome arranged in a chromatin-like structure (Favre et al., 1977). These structure may be modified by host enzymes like histone acetyltransferases (HATs) and histone deacetylases (HDACs), which may allow or block access to the transcriptional machinery (reviewed by Kalantari et al., 2004). Additionally, host DNA methyltransferases

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http://dx.doi.org/10.1016/j.virusres.2017.01.022
0168-1702/

(DNMTs) are capable of adding methyl groups to cytosines at CpG sites of the viral genome which may alter HPV expression (Badal et al., 2004).

Although methylation of CpG sites has been found to be a major epigenetic modification frequently associated with transcriptional repression, the effect of HPV DNA methylation in the regulation of viral transcription is still unclear (Bird, 2002). Methylation of the *L1* and/or *L2* genes of HPV16 and HPV18 has been proposed as a marker for cervical neoplastic progression. This event probably results in transcriptional silencing of these late genes whose products are not required for neoplastic transformation. On the other hand, methylation at the 3′ segment of the LCR (3′LCR) in some cervical cancers could be associated with the episomal state of HPV DNA (Chaiwongkot et al., 2013).

The 3′LCR, where the *E6/E7* promoter is located, controls expression of *E6* and *E7* viral oncogenes of HPV16 and HPV18. This promoter has been well characterized for HPV16 and HPV18, named P97 and P105, respectively. This region is essential for immortalization of human keratinocytes by triggering massive production of E6 and E7 oncoproteins in high-risk HPV infections. The activity of P97 and P105 can be regulated by host transcription factors like AP-1, NF1, SP1, TFIID, TF1, Oct-1 and the viral factor E2 (Desaintes and Demeret, 1996; Lung et al., 2012; Thierry and Howley, 1991).

The E2 viral protein plays a key role during HPV infection and HPV-induced carcinogenesis, repressing transcription of *E6* and *E7* in a dose dependent process (McBride, 2013). Three conserved E2 DNA binding sites (E2BS) at the 3′LCR, with the consensus palindromic motif ACCG$(N)_4$CGGT, have been described (Steger and Corbach, 1997a). E2BS at the 3′LCR are low affinity biding sites and, in presence of high concentrations of E2 proteins, transcription of the *E6/E7* promoter is repressed. E2 activity is associated to displacement of P97 and P105 promoter activators, Sp1 and TFIID, from their respective binding sites. Thus, the absence or low level of E2 proteins results in overexpression of *E6* and *E7* oncogenes and cancer progression (Tan et al., 1994; Steger and Corbach, 1997b).

Two E2BS at the 3′LCR of HPV16 (E2BS#3 and E2BS#4, distal and proximal, respectively) overlapping four CpG sites at genomic positions 37, 43, 52 and 58 (in respect to GenBank accession K02718.1), are flanked by a close CpG site, at position 31 of an SP1 transcription factor binding site. In invasive cervical cancer (ICC) with HPV16 infection, these sites have shown a wide spectrum of methylation levels between samples rather than a similar level of methylation (Chaiwongkot et al., 2013; Hong et al., 2008).

In malignant lesions, E2 functions are frequently lost due to integration of the HPV genome to the host chromosome. Integration occurs by rupture of the viral genome downstream of *E6* and *E7*, preventing *E2* transcription and resulting in upregulation of *E6* and *E7*. Conversely, in approximately 40% of cervical tumors, presence of episomal HPV16 DNA has been reported (Cricca et al., 2007; Cullen et al., 1991; Liu et al., 2016), suggesting presence of a functional E2 protein. Additionally, intact *E2* ORFs are also present in tumors with integrated, multiple viral genome tandem copies. Chaiwongkot et al. reported these findings in squamous cell carcinomas associated with HPV16, suggesting that a higher methylation level of E2BS at 3′LCR probably contributed to upregulate *E6* and *E7* transcription by blocking E2 binding (Chaiwongkot et al., 2013). However, these studies did not evaluated the presence of E2 protein.

Methylation at CpG sites is a complex event in the human genome, displaying a tissue-specific pattern, and influenced by aging and life style (Christensen et al., 2009; Gama-Sosa et al., 1983; Lim and Song, 2012). Moreover, the E6 protein of HPV16 in cervical carcinoma cell lines SiHa and CaSki has been shown to increase the level of DNMT1, one of the enzymes involved in CpG methylation and epigenetic silencing of tumor suppressor genes (Au Yeung et al., 2010).

In addition to presence of a functional E2 ORF, no data are available on the influence of other tumor characteristics on the methylation pattern of HPV DNA. In this study, we evaluate whether the methylation level of the five CpGs sites at the SP1 binding site, E2BS#3, and E2BS#4, at the 3′LCR of HPV16, may also be associated with patient age and E1 and/or E2 ORFs integrity.

2. Material and methods

2.1. Samples

The study material was selected from a pool of 334 biopsies of invasive cervical cancer associated to HPV16. Patients were referred to Instituto Nacional de Câncer (Rio de Janeiro, Brazil) for cancer treatment between June 2011 and March 2014. They were requested to provide epidemiological data in a questionnaire after sign a consent form. All procedures were approved by the Institutional Ethics Committee (CAAE: 53398416.0.0000.5274). Biopsies were collected and stored in RNA-Later at −80 °C. DNA was isolated with QIAamp DNA mini Kit (Qiagen, Hilden, Germany). Presence of HPV DNA was detected by polymerase chain reaction (PCR) with PGMY 09/11 primers or by nested PCR with PGMY09/11 and GP5+/GP6+. HPV types were identified by DNA sequencing and BLASTn (http://blast.ncbi.nlm.nih.gov/). Samples with multiple infections were characterized by visual inspection of electropherograms and the detection of overlapping sequence peaks, these samples were not included in this work.

Following the nomenclature proposed by Burk et al. (2013), HPV16 lineages were identified in a set of 334 samples based on *LCR* and *E6* sequencing. These comprised 217 of lineage A, 10 of B, 10 of C, and 97 of D (see Vidal et al., 2016). For the current study, seventy samples were selected taking into account the following criteria: (1) inclusion of samples representing all the four HPV16 lineages, and (2) samples representing the histological types adenocarcinomas (ADN) or squamous cell carcinoma (SCC). Thus, the select samples comprised: the 10 samples infected by HPV16 of lineage B (all SCC), the 10 infected by HPV16 of lineage C (1 ADN and 9 SCC), 26 infected by lineage A (12 ADN and 14 SCC), and 24 infected by lineage D (12 ADN and 12 SCC). The different histological types were randomly selected among the ADNs and SCCs of lineages A and D. One sample of lineage B was excluded because PCR products could not be amplified following sodium bisulfite treatment. Cell lines CaSki and SiHa were used as controls for bisulfite treatment and pyrosequencing.

2.2. Sodium bisulfite treatment and PCR amplification

Sodium bisulfite treatment was carried out with EpiTect-Bisulfite Kit (Qiagen, Germany), with an input of 300–1500 ng of DNA for cytosine to uracil conversion. Following treatment, a region covering 178 bp, containing five CpG sites (with cytosine positions 31, 37, 43, 52, and 58) at the 3′LCR of HPV16 was PCR-amplified by nested PCR following Rajeevan et al. (2006).

PCR was carried out in 30 μL mixtures containing 0.2 mM of each dNTP, 6 pmol of each primer, 1U of Platinum *Taq* DNA Polymerase (Life Technologies) and 1X PCR buffer (67 mM Tris pH 8.8, 6.7 mM $MgSO_4$, 16.6 mM $(NH_4)_2SO_4$, and 10 mM 2-mercaptoethanol (Kocher et al., 1989). PCR conditions were: 95 °C for 6 min, followed by 45 cycles of 95 °C for 1 min, 61 °C for 1 min, 72 °C for 1 min, and one step of final extension at 72 °C for 5 min. Presence of PCR products was verified in agarose gels.

2.3. Quantitation of DNA methylation by pyrosequencing

PCR products were submitted to pyrosequencing in a PyroMark Q24 platform (Qiagen, Germany) following a standard

protocol. Briefly, 1 μL of streptavidin beads (GE Healthcare, Buckinghamshire, UK), 40 μL PyroMark binding buffer (Qiagen), 20 μL PCR product and 19 μL water were mixed and incubated for 10 min on a shaking platform at 1400 rpm. A Biotage Q24 Vacuum Prep Workstation was used for separating, denaturing and washing PCR products which were subsequently added to a microtiter plate containing 25 μL of annealing buffer with 0.3 μM of sequencing primer (5′-AATTTATGTATAAAATTAAGGG-3′). 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 the PyroMark Q24, v.2.0.6 (Qiagen; Hilden; Germany). Dispensation order was 5′-YGTAATYGAAATYGGTTGAATYGAAATYG-3′. Based on the findings of Badal et al. (2003), and Fernandez et al. (2009), the genomic DNA of the cell lines CaSki and SiHa cell lines were used as controls for hypermethylation or hypomethylation pattern of the CpG at 3′ LCR, respectively.

2.4. *Disruption of* E1 *or* E2 *gene*

HPV integration to the host DNA genome frequently occurs with *E1* or *E2* disruption (Xu et al., 2013) resulting in suppression of *E2* transcription. The presence of a non-disrupted *E2* gene was identified by PCR amplification of overlapping fragments encompassing the *E1* and *E2* coding regions using the 8 primer pairs described by Collins et al. (2009) and Vernon et al. (1997).

PCR was carried out in 25 μL mixtures containing 0.2 mM of each dNTP, 25 pmol of each primer (Supplemental Table 1), 1 U of Platinum *Taq* DNA Polymerase (Life Technologies) and 1X buffer, 2 mM $MgCl_2$. PCR conditions were: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, annealing temperature for 30 s (see Supplemental Table 1), extension at 72 °C for 40 s, and final extension at 72 °C for 5 min. Presence of PCR products (Supplemental Fig. 1) was verified in 1.5% ultrapure agarose gels (Life Technologies). Absence of an amplicon, which indicates disruption of *E1* or *E2*, was confirmed by an independent PCR for the same region.

2.5. *Statistical analysis*

Exploratory analysis was carried out to describe sample characteristics. The level of methylation of each CpG site per sample was estimated as the proportion of methylated cytosines, ranging from 0 (without methylation) to 1 (totally methylated). To evaluate the influence of other factors on the methylation level of this region, we considered the average methylation of the five CpG sites per sample as outcome, because the binding of E2 proteins to E2BS#3 and E2BS#4 physically encompass the CpG sites at genomic positions 31, 37, 43, 52, and 58. Bivariate models were elaborated to evaluate the association between the level of average methylation with presence of intact or disrupted *E2*/*E1* open reading frames (ORFs), and patient age at diagnosis, analyzed as a continuous variable. As our outcome variable (average methylation level) was a continuous proportion with non-normality assumption, a Quasi-likelihood regression with logit link was used. In multivariate models, we considered tumor type (SCC/ADN) and lineages as control variables because samples were not randomly selected. A p value < 0.05 was considered significant. Statistical analyzes were conducted with R v.3.2.4.

3. Results

Clinical and pathological characteristics of the 69 patients herein studied are summarized in Table 1. Age at diagnosis ranged from 19 to 79 years, with a mean of 44.61 (SD = 13.45) and a median of 43.00. In 21 tumors (31.34%) disruptions were detected contrary to 46 (68.66%) in which these regions were intact. *E1* disruption occurred in 13 tumors, while E2 was disrupted in two, and both genes were disrupted in six (Supplemental Table 2).

Table 1
Characteristics of Study Population.

Characteristics	N
Overall population	69
Patient Age (year)	
Mean (SD): 44.61 (13.45)	
Median: 43	
Tumor types	
ADN	25
SCC	44
No information	–
HPV16 lineage	
A	26
B/C	19
D	24
No information	–
FIGO Stage	
I	22
II-IV	47
No information	–
Disruption of E1/E2	
Yes	21
No	46
No information	2

ADN: adenocarcinomas, SCC: squamous cell carcinomas.
All CpGs: average methylation of all five CpG.
N = number of samples.

Following bisulfite treatment, the methylation level of each CpG site per sample was estimated by pyrosequencing for viral genomic positions 31, 37, 43, 52 and 58 (in respect to GenBank K02718.1). The methylation level (%) of each CpG site, and the average of methylation of these sites per sample are listed in Supplemental Table 2. The lowest methylation level was found at nt 31 (mean = 18%), overlapping with an Sp1 binding site. The other CpG sites showed a similar methylation level with a mean per site ranging from 21.50% to 23.00% (Fig. 1a). No statistical difference between the CpG sites were observed. The average methylation of the five CpG sites per sample ranged from <1 to 88.00% (Fig. 1b). Similar as previously reported (Badal et al., 2003), a higher average methylation was found for the CaSki cell line (96.00%) and a lower one for SiHa (3.20%).

Bivariate analysis showed that presence of disruption and patient age were positively associated with average methylation at the five CpG sites per tumor sample (p = 0.003 and p = 0.006, respectively; Table 2). With multivariate analysis, both variables (E1/E2 integrity and patient age) were also positively associated with average methylation level (p = 0.002 and p = 0.021, respectively; Table 2) following adjusting for tumor type (SSC or AND) and HPV16 lineage. These findings showed that presence of intact *E1*/*E2* open reading frames was associated with higher levels of methylation. Additionally, older patients showed higher levels of methylation than younger ones independently of HPV DNA disruption.

4. Discussion

Our results showed that the range of average methylation of the CpG sites located in the 3′ LCR of HPV16, per invasive cervical cancer sample, was similar to previously reported (from 2.00% to 84.00%) in 17 squamous cell carcinomas analyzed by bisulfite treatment and pyrosequencing (Jacquin et al., 2013). Conflicting reports on the 3′LCR methylation pattern of HPV16 in invasive cervical cancer are likely to have resulted from using different methodologies and sample size. This is because methylation of this HPV16 region

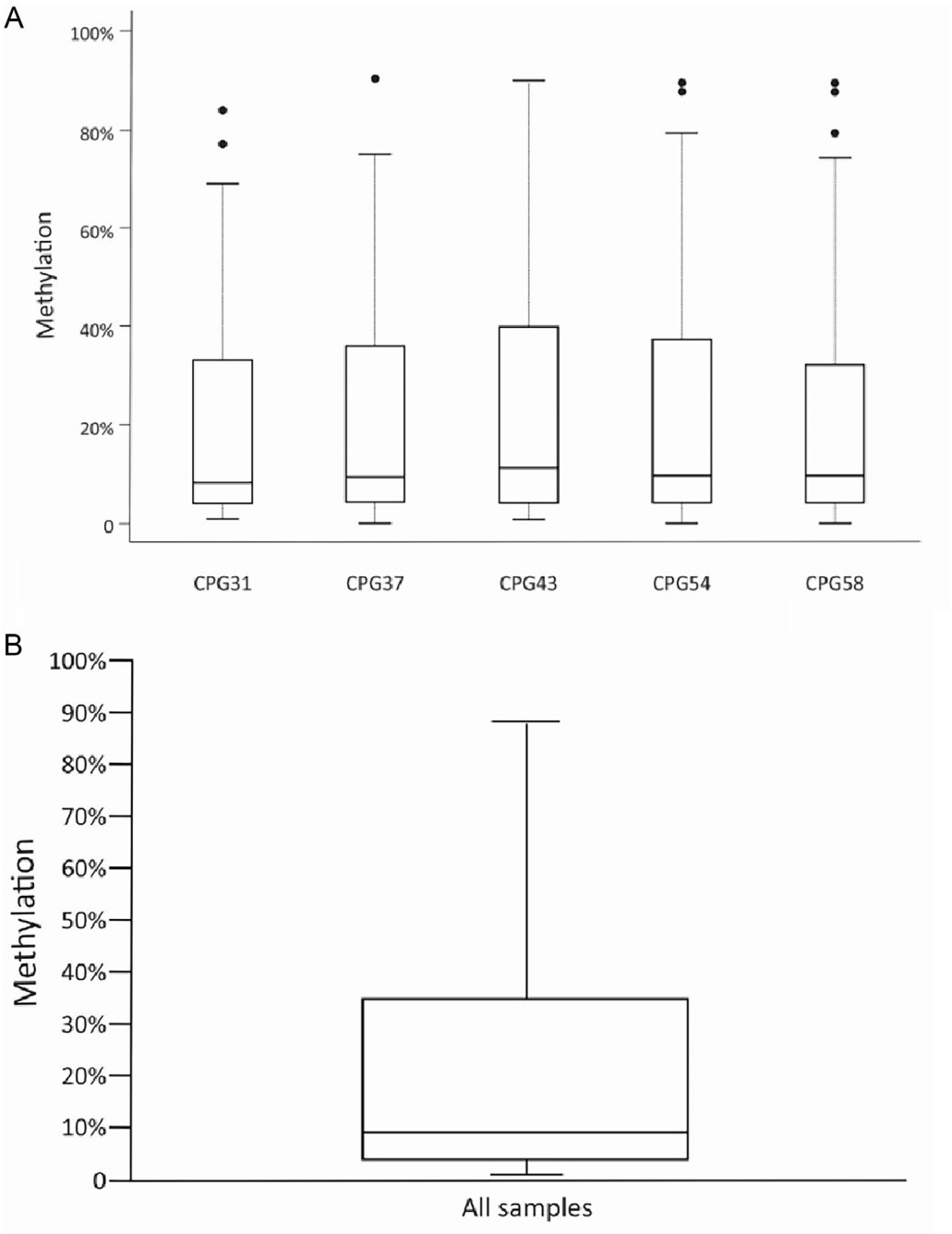

Fig. 1. Average methylation at 3′LCR of HPV16. In A, the average methylation is represented by individual CpG sites, and in B is shown the overall average methylation for all five CpG sites. Methylation levels are represented in boxes displaying upper and lower quartiles separated by the median line and whisker plots; black circles indicate outliers.

Table 2
Bivariate and multivariate analyzes of the average methylation of the five CpGsites at 3′ LCR.

	Bivariate Analysis			Multivariate analysis*		
	Coefficient	Std. Error	p value	Coefficient	Std. Error	p value
Disruption of E1/E2						
Yes	1	–		1	–	
No	1.373	0.438	0.003	1.422	0.443	0.002
Age	0.034	0.012	0.006	0.028	0.012	0.021

*Adjusted for tumor type (SCC *vs* ADN) and HPV16 lineage (A vs. B/C/D).

has been investigated with non-quantitative methods like Bisulfite Restriction Analysis (COBRA), methylation-specific PCR (MS-PCR) and PCR-based methylation sensitive restriction analysis (MSRA) (Ding et al., 2009; Leung et al., 2015; Mazumder Indra et al., 2011; Vinokurova and von Knebel Doeberitz, 2011), a reason why these findings cannot be compared with the quantitative data herein reported.

Our study showed association between methylation patterns and presence of disruption. Viral DNA integration with disruption of *E1* or *E2* and loss of the repressive activity of E2 protein over the earlier promoter has been considered an essential step during malignant transformation. However, in 20% of cervical cancers, HPV16 DNA integration was not detected, potentially retaining *E2* function (Cricca et al., 2007; Cullen et al., 1991). In addition, experimental data showed that methylation of CpG sites at the 3′LCR E2BS blocked E2 binding in association with loss of E2 repressive activity (Tan et al., 1994; Thain et al., 1996). Our findings were coincident with this proposition, showing that a higher methylation at the 3′LCR in invasive cervical carcinomas was associated with a potentially functional *E2* gene. However, is important to highlight these findings do not allow to establish a direct association between the presence of E2 protein and the higher methylation of CpG sites at 3′LCR. This is because the presence of an intact *E2* ORF does not imply in the presence of E2 protein, as reported in samples from SCC and the CaSki cell line (Xue et al., 2012).

A positive correlation between methylation level at the 3′LCR of HPV16 and patient age was found in this study. In humans, aging has been associated with global hypomethylation and site-specific hypermethylation, mainly in CpG islands (Christensen et al., 2009). To date, our findings represent the first report showing the likely influence of aging on HPV16 DNA methylation in cancer samples, independently of gene disruptions. Previous reports on the association between HPV DNA methylation and patient age did not consider the presence of an episomal or integrated viral genome, and showed contradictory findings for CpGs placed in LCRs and in other HPV genomic regions. Leung et al. reported association between patient age and increase of methylation level for HPV18 E2BS4 (in 5′LCR), but not for HPV16 in samples of SCC (Leung et al., 2015). Mirabello et al. reported increase in methylation in CpGs along the HPV16 genome associated with patient age in CIN2-3 (Cervical Intraepithelial Neoplasia 2 and 3) but not in control samples (women with HPV16 clearance in the period of <2 years) (Mirabello et al., 2013).

The positive correlation between patient age and 3′LCR methylation indicates that aging affects tumor epigenetics. In normal tissues was observed a global decline of CpG methylation with human age (Zampieri et al., 2015) and an inverse correlation between the amount of transcripts of DNA Methyltransferases (DNMT1 and DNMT3b) with age (Ciccarone et al., 2016). In cervical cancer and CIN1-3 a higher expression of DNMT1 protein was observed when compared with normal squamous epithelium (Sawada et al., 2007), and in cervical cancer cell lines was shown that E6 and E7 increased the expression of DNA Methyltransferases and the methylation of tumor suppressor genes (Au Yeung et al., 2010; Li et al., 2015). The correlation between methylation and age are probably associated with the neoplastic process and the over expression of the DNMTs, our data in cervical cancer and those from Mirabello et al. (2013) (for CIN2-3 and women with HPV16 clearance) showed that this correlation is observed only in high grade lesions and cancer but not in women with HPV16 clearance.

Although this study might be restricted to a relatively small sample of different HPV16 lineages and histological tumor types, our findings highlighted the importance of considering patient age as a factor that might affect methylation status in HPV DNA. This is because methylation has been used as a potential marker for presence of high grade lesions or invasive cancer. Further studies should be carried out for testing the association of patient age with methylation in other HPV types and HPV genomic regions.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We want to thank Dr. Héctor N. Seuanez for a critical revision of the manuscript. This work was supported by National Institute for Cancer Control (INCT do Cancer: http://www.inct-cancer-control.com.br/), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Ministry of Health – Brazil, Pan-American Health Organization (PAHO).

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.virusres.2017.01.022.

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