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

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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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(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