Analysis of *p16^{CDKN2A}* Methylation and HPV-16 Infection in Oral Mucosal Dysplasia

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Key Words

DNMT3B • Polymorphism • *p16^{CDKN2A}* • Methylation • HPV •
Head and neck cancer • Oral premalignant lesions

Abstract

Objective: The purpose of this study was to investigate the relationship between *p16^{CDKN2A}* methylation and epithelial dysplasia (ED). We also evaluated the expressions of proteins related to methylation (DNMT3B and DNMT1). Finally, we tested whether HPV-16/18 or the *dmt3b* (C46359T) polymorphism is associated with *p16^{CDKN2A}* methylation status. **Methods:** To test the hypothesis, a case-control study with 72 (control, n = 24; ED, n = 48) tissue samples from subjects was performed. Methylation-specific PCR, RFLP, and immunohistochemical analyses were performed to evaluate *p16^{CDKN2A}* methylation status, *dmt3b* (C46359T) genotyping, and protein levels, respectively. **Results:** The methylation of *p16^{CDKN2A}* and HPV-16 was associated with ED gradation (p = 0.001 and 0.002, respectively). In addition, most HPV-16-positive samples (77.8%) exhibited *p16^{CDKN2A}* methylation; however, changes in DNMT3B and DNMT1 protein levels were

not observed in HPV-positive samples. Neither HPV-18 nor the *dmt3b* polymorphism was associated with *p16^{CDKN2A}* methylation. **Conclusions:** There is an association between the presence of HPV-16 in ED and the occurrence of *p16^{CDKN2A}* methylation. Both variables are also associated with ED development, but further studies are necessary to clarify if they operate independently and if they have any impact on OD malignization.

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Introduction

Oral squamous cell carcinoma (OSCC) is a disease associated with major morbidity and mortality and represents a major worldwide public health problem [1–4]. OSCC can be preceded by a group of lesions that are considered potentially malignant disorders [5, 6]. It is thought that most OSCCs are preceded by a period during which the affected epithelium shows histological evidence of epithelial dysplasia (ED), although this may not always be clinically apparent [5, 7].

The development of OSCC in humans involves progressive genetic alterations [8–11]. Recent evidence suggests that epigenetics is an important mechanism in oral carcinogenesis [12–16]. DNA methylation is the addition of methyl radicals to specific regions of DNA that predominantly contain cytosine nucleotides. It is catalyzed by a family of enzymes called DNA methyltransferases (DNMTs) [17]. DNMT1 is a maintenance enzyme, and both DNMT3a and DNMT3b are responsible for de novo methylation, which is the establishment of a new methylation pattern [17, 18]. It has been demonstrated that variant T of the *dmt3b* (C46359T) genetic polymorphism promotes increased protein expression, which could result in a predisposition to aberrant de novo methylation [19–21]. It has been demonstrated that high levels of DNMT3B protein contribute to *p16^{CDKN2A}* methylation in esophageal cancer [22, 23].

High-risk HPV infection has been shown to be associated with OSCC [24–26]. In contrast to cervical cancer, in which HPV has been established as a primary cause, oral cavity cancers are caused primarily by tobacco use, and high-risk HPV might be a cofactor for oral carcinogenesis [15, 27]. Studies using squamous cell carcinoma samples suggest that high-risk HPV infection could interfere in epigenetic regulation [28], specifically in the induction of the *p16^{CDKN2A}* gene methylation [12, 23, 29].

The aim of this study was to investigate the association between *p16^{CDKN2A}* methylation and ED. In addition, we also evaluated the expression of proteins related to methylation (DNMT3B and DNMT1). Finally, to our knowledge, as no study has attempted to evaluate *p16^{CDKN2A}* methylation and high-risk HPV simultaneously in this context, we tested whether HPV-16/18 or *dmt3b* (C46359T) polymorphism was associated with *p16^{CDKN2A}* methylation status.

Patients, Samples, and Methods

Tissue Specimens and Patients

This case-control study was performed on archived tissue blocks from surgically resected specimens of the control group obtained during surgery for oral mucocles (n = 24; male-to-female ratio 1:1.4; mean age 32.83 years; SD 15.78 years; range 15–74 years) and ED (n = 48; male-to-female ratio 1.5:1; mean age 53.90 years; SD 12.45 years; range 33–93 years). Descriptive data of the population are presented in online supplementary table 1 (for all online supplementary material, see www.karger.com/doi/10.1159/000334926). Tobacco smoking was classified according to our previous studies [1, 30, 31]. Ethical approval for this study was obtained from relevant local ethics committees (process No. 1133/08).

Morphological Classification

For morphological analysis, samples were fixed in formalin, embedded in paraffin, serially sectioned at 5 μ m, stained with hematoxylin and eosin, and evaluated under a conventional light microscope. The samples were classified according to the International Classification of Diseases for Oncology (ICD-10th revision) and the World Health Organization (WHO) criteria [32]. Morphological analysis was performed by the same oral pathologist without prior knowledge of the demographic or clinical characteristics related to the samples.

DNA Isolation and Bisulfite Conversion of DNA for Methylation-Specific PCR

DNA was isolated from ten 10- μ m-thick tissue sections from each tissue block of the specimens using a DNeasy tissue kit (Qiagen, Chatsworth, Calif., USA) according to the manufacturer's protocol. The *p16^{CDKN2A}* gene methylation profile was evaluated by using methylation-specific PCR (MSP). DNA samples were treated with bisulfite for 3 h, and MSP-PCR was performed as described previously [33] and later modified [34]. The *p16^{CDKN2A}* promoter was defined as methylated or unmethylated on the basis of 2 distinct PCR reactions that yielded fragment sizes of 150 and 151 bp, respectively. Samples that exhibited methylation in at least one of the alleles were considered methylated (fig. 1).

dnmt3b Genotypes

The *dnmt3b* (C46359T) polymorphism was assessed by RFLP as described previously [30]. In brief, PCR for DNMT3B was performed with the reaction mixture containing approximately 100 ng genomic DNA as a template, 20 pmol/ μ l of each primer, 25 mM of each dNTP-mix (AMRESCO, Irvine, Calif., USA), 50 mM magnesium chloride, and 2.5 U Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, Calif., USA).

The 230-bp PCR product from the DNMT3B gene was digested with the *Bln1* restriction endonuclease (Sigma-Aldrich, St. Louis, Mo., USA). Because the wild-type C allele lacks the *Bln1* restriction site, it has only 1 band (230 bp), whereas the polymorphic T allele has 2 bands (172 and 58 bp). Amplified DNA (10 μ l) was digested with 2.5 U *Bln1* for 16 h at 37°C. PCR and restriction reactions were performed in a thermocycler (Eppendorf AG, Hamburg, Germany) (fig. 1). DNA sequencing was performed to confirm the DNMT3B genotyping by PCR-RFLP.

HPV Identification

The HPV-DNA sequences were first amplified by PCR by L1 and then by HPV-16 and HPV-18. Beta-globin gene primers were used as an internal control. All primer sequences have been described by Katiyar et al. [35]. PCR was performed with approximately 100 ng genomic DNA as a template, 20 pmol/ μ l of each primer, 25 mM of each dNTP-mix (AMRESCO), 50 mM magnesium chloride, and 2.5 U Platinum *Taq* DNA polymerase (Invitrogen Life Technologies) (fig. 1).

Electrophoresis

The PCR products were verified on a 6.5% polyacrylamide gel electrophoresed and stained with silver nitrate. The electrophoresis results were estimated with a 100-bp ladder.

Fig. 1. Molecular features. **a** Positive and negative immunostaining samples of the different groups. **b** MSP of the *p16^{CDKN2A}* gene. M (150 bp) and U (151 bp) represent primer sets specific to methylated and unmethylated DNA, respectively. Samples 1 and 2 contain methylated DNA (M) indicative of the presence of *p16^{CDKN2A}* methylation. In contrast, sample 3 shows only positive amplifica-

tion for unmethylated DNA. **c** PCR-RFLP for genotyping of the *dnmt3b* (C46359T) polymorphism. Lane 1: 100-bp molecular marker; lane 2: CT genotype; lane 3: CC genotype; lane 4: TT genotype. **d** 100-bp molecular marker. Lanes 1 and 3: HPV-16 positive; lanes 2 and 4: HPV-18 positive (100 bp).

Immunohistochemical Analyses

Paraffin sections (3 μ m) were mounted on glass and dried overnight at 37 °C. The sections were incubated with anti-DNMT1, anti-DNMT3B (diluted 1:250; Imgenex, San Diego, Calif., USA), or anti-P16CDKN2A clone JC8 (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA) monoclonal antibodies at 4 °C for 18 h. Next, we followed a previously described method [31]. Normal mucosa was used as a positive control, and the primary antibody was replaced with phosphate-buffered saline as a negative control. After staining, the tissue sections were scored according to the percentage of positive cells among all cells.

Statistical Analysis

The χ^2 test or Fisher's exact test was used to test the relationships between categorical variables [i.e. *p16^{CDKN2A}* methylation status, HPV-16 or HPV-18 infection, ED, and the *dnmt3b* (C46359T) polymorphism]. The relationships between categorical variables (i.e. HPV infection, methylation, and genotype status) and immunostaining were evaluated using the Mann-Whitney test. The Pearson correlation test was used to evaluate correlations between the *dnmt3b* polymorphism, HPV positivity, *p16^{CDKN2A}* methylation, and the protein expressions of

P16CDKN2A, DNMT1, and DNMT3B. Binary logistic regression models were fitted to assess the relationships between the variables and the risk of advanced histological staging. The relationships are expressed as odds ratios (ORs) with their corresponding 95% confidence intervals (CIs). All analyses were conducted with SPSS 17.0 (SPSS, Inc., Chicago, Ill., USA), and $p < 0.05$ was considered statistically significant.

Results

Descriptive Statistics

Of the total study population, 18.1% presented with moderate or severe ED; 54.2% of the patients were male. The most common anatomical site was the buccal mucosa ($n = 17$) followed by the tongue border ($n = 14$) (online suppl. table 1).

Methylation of *p16^{CDKN2A}* was present in 61.1% of the general samples (online suppl. table 1). Samples that ex-

Table 1. Factors associated with ED

	Grading of oral dysplasia			p
	normal epithelium	mild	moderate/severe	
Gender				
Male	10 (41.7)	20 (57.1)	9 (69.2)	0.244
Female	14 (58.3)	15 (42.9)	4 (30.8)	
Age				
≤45 years	19 (79.2)	12 (34.3)	5 (38.5)	0.002
≥46 years	5 (20.8)	23 (65.7)	8 (61.5)	
Lesion site				
BM/RTM N/LM	18 (75.0)	18 (51.4)	5 (38.5)	0.066
LBT/FM/SP	6 (25.0)	17 (48.6)	8 (61.5)	
Tobacco habit				
Ever	4 (16.7)	25 (71.4)	12 (92.3)	0.000
Never	20 (83.3)	10 (28.6)	1 (7.7)	
Ethylism habit				
Ever	10 (41.7)	24 (68.6)	10 (76.9)	0.050
Never	14 (58.3)	11 (31.4)	3 (23.1)	
DNMT3B polymorphism				
CC	13 (54.2)	12 (34.3)	3 (23.1)	0.133
CT/TT	11 (45.8)	23 (65.7)	10 (76.9)	
HPV-16				
Negative	19 (79.2)	13 (37.1)	4 (30.8)	0.002
Positive	5 (20.8)	22 (62.9)	9 (69.2)	
HPV-18				
Negative	22 (91.7)	32 (91.4)	13 (100.0)	0.553
Positive	2 (8.3)	3 (8.6)	0	
<i>p16^{CDKN2A}</i> methylation				
Negative	22 (91.7)	4 (11.4)	2 (15.4)	0.000
Positive	2 (8.3)	31 (88.6)	11 (84.6)	
Protein expression				
Mean P16	40.79	35.60	31.00	0.361
Mean DNMT3B	42.33	33.13	34.81	0.239
Mean DNMT1	37.76	34.62	30.79	0.610

Significant associations are in bold. Figures in parentheses are percentages.

hibited methylation in at least one of the alleles were considered methylated (fig. 1b). HPV-16 was present in 50% of the samples; however, only 6.1% of the samples had HPV-18 (online suppl. table 1). Figure 1d shows the gel band of HPV positivity. The CT genotype of the *dmt3b* (C46359T) polymorphism was the most frequent (55.6%), followed by CC (38.9%) and TT (5.6%) (online suppl. table 1). Figure 1c shows the *dmt3b* (C46359T) genotypes. Immunohistochemical analysis showed that P16 was nuclear and cytoplasmic in positive cases in both the case and control groups (fig. 1a). DNMT3B manifested as nuclear staining distributed in epithelial tissue in both groups; a similar profile was observed for DNMT1 (fig. 1a).

Table 2. Molecular findings associated with the risk of present ED evaluated by binary logistic regression

Variables	p	OR	95% CI	
			lower	upper
HPV-16				
Negative		Referent		
Positive	0.026	8.137	1.291	51.287
HPV-18				
Negative		Referent		
Positive	0.160	0.150	0.011	2.113
<i>p16^{CDKN2A}</i> methylation				
Negative		Referent		
Positive	0.000	79.691	11.496	552.423
DNMT3B polymorphism				
CC		Referent		
CT/TT	0.084	0.179	0.025	1.259

Significant p values <0.05 are in bold. The term referent is associated with categorical variables with a lower risk of developing the disease.

Clinical and Molecular Parameters Associated with ED

To determine the associations between parameters and ED, the χ^2 test was performed (table 1). The methylation of *p16^{CDKN2A}* was associated with ED gradation in both univariate (table 1) and multivariate analyses (table 2). Methylated samples were associated with an increased risk (79.6 times) of high-grade ED. Similarly, HPV-16 was related to high-grade ED (tables 1, 2). On the other hand, HPV-18, *dmt3b* (C46359T) polymorphism, and DNMT1 and DNMT3B levels were not associated with ED grading. In the current study, classical clinical parameters such as smoking, age, and ethylism were associated with ED gradation.

Factors Associated with *p16^{CDKN2A}* Methylation

Considering the anatomical sites, the lateral border of the tongue, the floor of the mouth, and the soft palate were associated with *p16^{CDKN2A}* methylation ($p = 0.012$) (online suppl. table 2). Smoking was also associated with *p16^{CDKN2A}* methylation ($p = 0.001$). There were no associations among HPV-18, *dmt3b* (C46359T) polymorphism, and clinical data (online suppl. table 2).

To test whether *p16* methylation is associated with the presence of HPV and DNMT3B polymorphisms, we performed analyses using the χ^2 test and Fisher's exact test (online suppl. table 3). HPV-18 and the *dmt3b* (C46359T)

genetic polymorphism were not significantly associated with *p16^{CDKN2A}* methylation. Moreover, the *dmt3b* (C46359T) polymorphism did not affect DNMT3B levels (online suppl. table 2). On the other hand, our data show a positive association between the presence of HPV-16 and *p16^{CDKN2A}* methylation. It is important to highlight that HPV positivity was not related to P16, DNMT1, or DNMT3B protein levels (online suppl. table 2). We also performed correlation analyses to check if the expressions of DNMT1 or DNMT3B influence P16; however, no correlations were observed (online suppl. table 4).

Discussion

Variables Associated with ED

A large number of studies have attempted to identify biological markers associated with oral carcinogenesis [6, 9, 24, 25, 30, 36, 37]. In this field, molecular biology has emerged as an important tool for identifying groups of people susceptible to particular diseases [6, 30, 33, 34, 38, 39]. For example, the loss of heterozygosity could be responsible for a 33-fold increased risk of leukoplakia malignant transformation [9]. Recently, epigenetic imbalance has emerged as an important cause of carcinogenesis [40]. Given the reversible nature of epigenetic modifications [41, 42], they are attractive targets for therapeutic intervention, especially for premalignant lesions [43, 44]. In the current study, we demonstrated that ED was associated with *p16^{CDKN2A}* methylation. A recent study suggested that *p16^{CDKN2A}* methylation is associated with oral carcinogenesis [36, 45]. Moreover, evidence suggests that *p16^{CDKN2A}* methylation may serve as a useful molecular marker for predicting local recurrence in tongue carcinoma [46]. Based on these facts, *p16^{CDKN2A}* methylation may be relevant for oral carcinogenesis.

High-risk HPV infection has emerged as a possible etiological factor for head and neck squamous cell carcinoma at a young age [47]. High-risk HPV is most commonly found in the oropharynx [26] as well as other head and neck sites such as the oral cavity [48], larynx [49], and hypopharynx [50]. In addition, recent studies have suggested that there is an association between high-risk HPV and potential malignant lesions in the oral cavity [51]. Interestingly enough, in the current study, we observed HPV-16 in 20.8% of normal mucosa and 69.2% of moderate/severe ED. These results are concordant with those of a previous study [52]. No associations were found between HPV-18 and ED. Our data suggest that HPV-16 could be involved in ED development.

It is well known that exposure to smoking and ethylism for long periods are the principal causes of OSCC [1, 30, 31]. In the current study, age, smoking, and ethylism were associated with worse ED gradation.

*Molecular and Clinical Factors and *p16^{CDKN2A}* Methylation*

Although epigenetic imbalance seems to be important in oral carcinogenesis, little is known about the interaction between epigenetics and the other cofactors involved in oral carcinogenesis. In the current study, smoking and anatomical site were associated with *p16^{CDKN2A}* methylation, which corroborates the results of previous studies [45, 53].

The importance of functional genetic polymorphisms has been demonstrated in the vast majority of oral diseases [54–56]. In the current study, the T allele of the *dnmt3b* (C46359T) polymorphism was not associated with ED or *p16^{CDKN2A}* methylation status. No associations were observed between the *dmt3b* (C46359T) polymorphism and the expressions of DNMT1, DNMT3B, or P16 protein. To our knowledge, the current study is the first to attempt to evaluate the *dnmt3b* (C46359T) polymorphism in the context of ED. Our study does not support the notion that the T allele of the *dnmt3b* (C46359T) polymorphism regulates protein expression, at least in the context of ED lesions.

In theory, there is no advantage for high-risk HPV to downregulate *p16^{CDKN2A}* [15, 57–60]. In addition, aberrant *p16^{CDKN2A}* gene methylation is not observed in E6/E7 immortalized cells [61]. Moreover, an absence of *p16^{CDKN2A}* mutations and the expression of P16 is observed in HPV tumors [62]. On the other hand, studies of lung SCC samples suggest a possible role of high-risk HPV infection in epigenetic regulation, specifically in *p16^{CDKN2A}* methylation [12, 29]. Because the presence of HPV-16 was associated with *p16^{CDKN2A}* methylation in the current study, we can speculate that the former might be responsible for P16 silencing and cancer progression in some HPV-16-positive cases. Given our previously published data [30] and the lack of association between the expressions of P16, DNMT1, and DNMT3B and HPV positivity observed in the current study, we conclude that *p16^{CDKN2A}* methylation and HPV-16 infection are important factors involved in oral carcinogenesis, although more studies are necessary to clarify whether they operate independently.

Conclusion

The presence of HPV-16 in ED and the occurrence of *p16^{CDKN2A}* methylation are associated. Both variables are also associated with ED development, although further studies are necessary to clarify if they operate independently and if they have any impact on OD malignization.

Acknowledgements

This study was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG). Dr. Guimarães and Dr. Gomez are research fellows at CNPq. Dr. De-Paula is a research fellow at FAPEMIG.

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