Effect of age on the association between p16CDKN2A methylation and DNMT3B polymorphism in head and neck carcinoma and patient survival

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Abstract. *De novo* DNA methylation is a relevant epigenetic mechanism, which represses gene transcription and commonly inactivates tumor suppressor genes in carcinogenesis. A single nucleotide polymorphism of DNMT3B, C46359T (-149C→T) was reported to modulate individual's susceptibility to cancer. We investigated the role of this polymorphic variant regarding the methylation status of the p16CDKN2A gene in young and older patients with head and neck squamous cell carcinoma (HNCC) matched by the TNM staging system, together with its impact on patients survival. The results showed that the presence of the allele T of the polymorphism DNMT3B (-149C→T) was associated with advanced TNM staging and smoking habit, but no association was found between this polymorphisms and DNMT3B immunostaining. While *p16CDKN2A* methylation was significantly associated with smoking habit in older patients, this parameter was associated with family history of cancer in young patients. Moreover, in older patients the absence of *p16CDKN2A* promoter methylation had a negative impact on survival. In conclusion, nucleotide polymorphism of DNMT3B is not associated with methylation of *p16CDKN2A* gene in HNSCC. The association of *p16CDKN2A* gene methylation with smoking, family history of cancer and survival is dependent on age.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer and represents 350,000 cancer deaths worldwide every year (1,2). It includes malign epithelial neoplasms that arise in the paranasal sinuses, nasal cavity, oral cavity, pharynx, and larynx (3). In Brazil, considering only oral cavity, the estimate for 2009 is 10,300 new cases of squamous cell carcinoma according to the National Institute of Cancer (INCA) (4). HNSCC has been regarded as a disease that generally affects men between the sixth through the eighth decades of life following long-term exposure to smoking and alcohol intake (5). However, an increase in the incidence of head and neck cancer among younger patients under the age of 45 years has been reported worldwide (6-12). Evidence suggests that carcinogenesis in young adults have a distinct mechanism of disease and often is not associated with classic risk factors for HNSCC (6-10,12). Differences in prognosis were observed in HNSCC patients according to age (13). Classically, the development of cancer in human has been viewed as a disease related to progressive genetic alterations (14-16). Recently, evidence indicates that not only genetic factors but also epigenetic modifications are similarly relevant in carcinogenesis (17,18). In contrast to genetic alterations, epigenetic modifications are reversible (19,20). This feature makes them attractive targets for therapeutic intervention (21,22).

The DNA methylation is the addition of methyl radicals to specific regions of DNA containing, predominantly, cytosine nucleotides. It is catalyzed by a family of enzymes denominated DNA methyltransferase (DNMTs), including three catalytically active enzymes - DNMT1, DNMT3a and DNMT3b. Although these enzymes act cooperatively to establish a pattern of genomic methylation, specific functions are performed by DNMTs. DNMT1 is an enzyme of maintenance, while DNMT3a and DNMT3b are responsible for the establishment of a new methylation pattern, known as *de novo* methylation (23,24). In addition to an important role in controlling gene activity, embryonic development, genomic

imprinting (25), methylation has been associated with the development of cancer by transcriptional inactivation of tumor suppressor genes (17,25). The pattern of methylation has been linked to several cancer types, such as lung (17), oral (26) and head and neck cancer (27). Hypermethylation as well as hypomethylation can promote the development of the carcinogenesis (28). Genetic polymorphisms of the DNMT3b gene were described and it is associated with susceptibility of a variety of cancers (29-34), including head and neck squamous cell carcinoma (35-37). Genetic polymorphism of DNMT3b was described in the -149 position (C46359T). It was postulated that the variant T might regulate this gene, promoting an increase in its expression, and resulting in a predisposition to aberrant *de novo* methylation of tumor suppressor genes and repair genes (38-40).

The *p16CDKN2A* is a tumor suppressor gene that encodes a cyclin-dependent kinase inhibitor which plays an important role in the regulation of the G1/S phase cell cycle checkpoint. The inactivation of this gene was observed in many tumor types (41-45). P16 protein can be inactivated by point mutation, homozygous deletion and methylation of the promoter region (46,47). Although increased expression of DNMT3B gene is associated with P16 inactivation in esophageal and lung cancer (48,49), their role in HNSCC has not been established. We hypothesized that the polymorphism of DNMT3B (C46359T) could promote high levels of DNMT3B expression and induce consequently p16CDKN2A methylation. Furthermore, we attempted to verify whether this possible association is dependent on age and has impact on patient survival.

Patients and methods

Patients. The present analysis was based on a case-control study design. The patients were recruited from databases of the head and neck surgery services in Montes Claros, Brazil from 1996 through 2007 (6). The study group included 75 patients with HNSCC consisting of a case group of 25 patients aged ≤45 years (young) and a control group of 50 patients aged >45 years (older patients), that were matched for TMN staging, smoking and alcohol intake. Young and older patients were from the same geographical area.

Clinical data. The mean age was 42.1 years (SD 3.17 years; range, 33-45 years) for young and 62.2 years (SD 8.0 years; range, 49-82 years) for older patients with HNSSC. Physical description of skin color was not used because, in Brazil, it is a poor predictor of genomic ancestry (50,51). The study was approved by the local Ethics Committee (process no. 1085). Information on age, sex, tobacco smoking, alcohol drinking, medical history, family cancer history, tumor site, TNM clinical staging, and survival were obtained from medical charts.

All patients were staged according to the UICC TNM Classification of Malignant Tumors (1997) (52). Lesions of HNSCC were classified according to the primary site as described in the International Classification of Diseases (ICD-10) for Oncology. The anatomical sites reviewed in this study included: i) 28 (37.3%) mouth and perioral region (C00, C01, C02, C04, C05, C06.0, C06.2); ii) 22 (29.3%) oropharynx (C09-C10) of the patients; and iii) hypopharynx-larynx 25 (23.4%) (C12, C13, C32). Lesions located in the oral cavity were considered as the anterior group and those located in the oropharynx-hypopharynx-larynx as the posterior group. Patients with diagnosis of carcinoma *in situ* or multiple head and neck carcinomas were excluded. All patients were asked about the occurrence of cancer in a first degree relative. The term cancer was defined using the WHO definition of 'an uncontrolled growth and spread of cells that may affect almost any tissue of the body'.

Histological gradation. Histological sections of tissues were stained with hematoxylin-eosin and evaluated under conventional light microscopy. All patients had histologically confirmed squamous cell carcinoma of head and neck. Histopathological classification of the tumors as moderate, or poorly differentiated was based on the World Health Organization criteria (WHO, 1997) (53) and invasive front area was also evaluated as described elsewhere (54).

DNA isolation and bisulfite conversion of DNA for methylation-specific PCR (MSP). DNA was isolated from ten 10-μm-thick tissue sections from each tissue block of HNSCC specimens, using the DNeasy Tissue Kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. The *p16CDKN2A* gene methylation profile was evaluated through methylation-specific PCR (MSP). DNA samples were bissulfite-treated for 3 h and MSP-PCR was performed as described (55) and posteriorly modified (56). Primer sequences, PCR product and polymerase chain reaction thermal conditions for defining methylation status are presented in Table I. The p16CDKN2A promoter methylation status for methylated or unmethylated reactions was identified by a fragment of 150 and 151 bp respectively (Fig. 1).

DNMT3B genotyping. DNMT3B (C46359T) polymorphism was assessed by RFLP (Table I). Polymerase chain reaction for DNMT3B was performed in a total volume of 25 μl containing ~100 ng genomic DNA as template, 0.5 μl of each primer (20 pmol/μl), 2.5 μl dNTP-mix (25 mM of each, Amresco, Ohio, CA, USA), 2.5 μl 10X PCR buffer, 1.25 μl magnesium chloride (50 mM), and 2.5 U of Platinum Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA).

The 230-bp PCR product from the DNMT3B gene was digested with Bln1 restriction endonuclease (Sigma-Aldrich, St. Louis, MO, USA), that recognizes a restriction site (C/CTAGG) in T allele; wild-type C allele lacks the Bln1 restriction site. The wild-type C allele has only one band (230-bp), while the polymorphic T allele has two bands (172 and 58-bp). Thus, 10 μl amplified DNA was digested with 2.5 U of Bln1 for 16 h at 37˚C. PCR and restriction reactions were performed into a termocycler (Eppendorf AG, Hamburg, Germany) (Fig. 1). DNA sequencing was realized to confirm the DNMT3B genotyping by PCR-RFLP.

Electrophoresis. The PCR products for methylation and digested fragments were verified on 6.5% polyacrylamide gel electrophoresis at 120 V of constant voltage for 1.5 h and stained with silver nitrate. Electrophoresis results were estimated regarding a 100-bp ladder.

Table I. Primer sequences, PCR product and polymerase chain reaction thermal conditions.

	Primer sequences	PCR product (bp)	Refs.	PCR thermal conditions
DNMT3B C46358T	F 5'-TGGCTACCAGGTCTCCTTGGCC-3' R 5'-GGTAGCCGGGAACTCCACGG-3'	230	Own design[a]	1x95˚C-5' 35x95˚C-1' 68.4˚C-1' 72˚C-1' 1x72˚C-10
Methylated *p16*	F 5'-TTATTAGAGGGTGGGGCGGATCGC-3' R 5'-GACCCCGAACCGCGACCGTA-3'	150	(56)	1x95˚C-5' 35x95˚C-1' 64˚C-1' 72˚C-1' 1x72˚C-10'
Unmethylated *p16*	F 5'-TTATTAGAGGGTGGGGTGGATTGT-3' R 5'-CAACCCCAAACCACAACCATAA-3'	151	(56)	1x95˚C-5' 35x95˚C-1' 64˚C-1' 72˚C-1' 1x72˚C-10'

[a]Designed based on the GenBank reference sequence (accession no. NG_007290), using the software 'Annhyb' (http://annhyb.free.fr) and Blast (www.ncbi.nlm.nih.gov/blast).

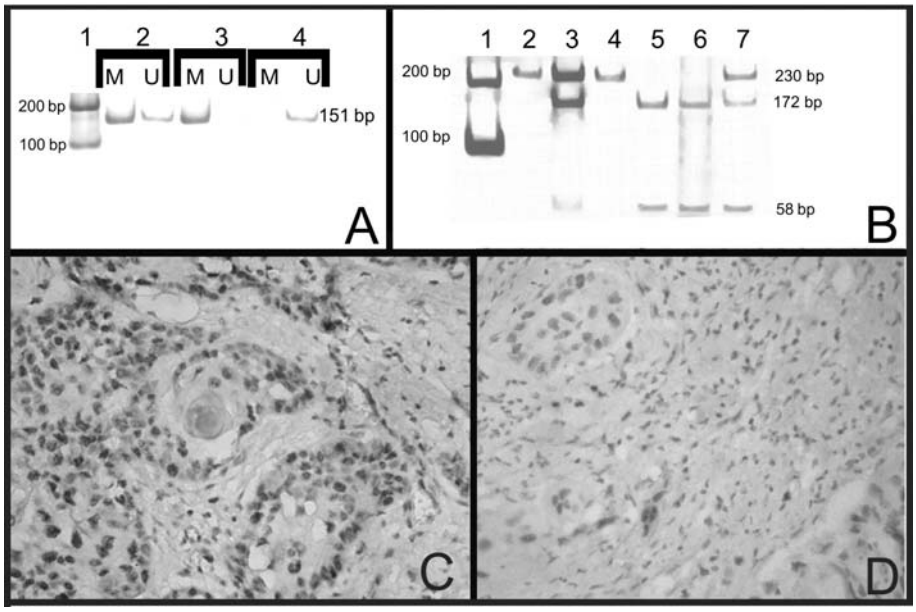

Figure 1. (A) Methylation-specific PCR of p16 gene. 'M' (150 bp) and 'U' (151 bp) represent primer sets specific to methylated and unmethylated DNA, respectively. Samples 2 and 3 contain methylated DNA (M) indicative of the presence *p16* methylation. Sample 4 shows the unmethylated status of *p16* gene because of absence of methylated reaction (M). Lane 1, 100-bp molecular marker. (B) PCR-RFLP for genotyping of DNMT3B (C46359T) polymorphism. Lane 1, 100-bp molecular marker; lanes 2 and 4, CC genotype; lanes 3 and 7, CT genotype; lanes 5 and 6, TT genotype. (C) Positive immunostaining of DNMT3B, magnification x400. (D) Negative immunostaining of DNMT3B, magnification x400.

Immunohistochemical analyses. Paraffin sections (3-μm) were mounted on glass, and dried overnight at 37˚C. All sections were then deparaffinized in xylene, rehydrated through a series of alcohol, and washed in phosphate-buffered saline. Anti-DNMT3b monoclonal antibody (diluted 1:250, IMGENEX, CA, USA) was used as the primary antibody and the incubation time was 18 h at 4˚C. Endogenous peroxidase was blocked by incubation with 0.03% H_2O_2 in ethanol for 30 min. For antigen retrieval, sections were heated in a steam cooker filled for 5 min at 125˚C in Tris-EDTA buffer (1 mM Tris base, 1 mM EDTA solution, 0.05% Tween-20, pH 9.0). Signals were developed with 3'3-diaminobenzidine-tetrahydrocloridre for 5 min and counter-stained with Harris hematoxylin for 30 sec. Normal mucosa was used as positive control and, for negative control, the primary antibody was replaced with phosphate-buffered saline. After staining, tissue sections were scored according to the percentage of positive cells among the neoplastic cells.

Table II. *p16* methylation and their association with molecular and clinicopathological features in case and control HNSCC.

	All patients (%) *p16* methylation status		Younger patients (%) *p16* methylation status		Older patients (%) *p16* methylation status	
Variables	Positive	Negative	Positive	Negative	Positive	Negative
Age						
Younger	17 (68)	8 (32)				
Older	42 (84)	8 (16)				
p-value	0.099					
Gender						
Male	53 (89.8)	11 (68.7)	16 (94.1)	05 (62.5)	37 (88.1)	06 (75.0)
Female	06 (10.2)	05 (31.3)	01 (5.9)	03 (37.5)	05 (11.9)	02 (25.0)
p-value	**0.035**		**0.044**		0.328	
Family history of any cancer						
Absent	29 (49.2)	10 (62.5)	05 (29.4)	06 (75.0)	24 (57.1)	04 (50.0)
Present	30 (50.8)	06 (37.5)	12 (70.6)	02 (25.0)	18 (42.9)	05 (50.0)
p-value	0.343		**0.032**		0.709	
Smoking status						
Smokers	47 (79.7)	13 (81.3)	14 (82.4)	07 (87.5)	33 (78.6)	06 (75.0)
Non-smokers	0 (0.0)	03 (18.7)	0 (0.0)	01 (12.5)	0 (0.0)	02 (25.0)
Ex-smokers	12 (20.3)	0 (0.0)	03 (17.6)	0 (0.0)	09 (21.4)	0 (0.0)
p-value	**0.001**		0.169		**0.002**	
Alcohol consumption						
Drinkers	37 (62.7)	07 (43.8)	12 (70.6)	03 (37.5)	25 (59.5)	04 (50.0)
Non-drinkers	03 (5.1)	02 (12.4)	01 (5.9)	01 (12.5)	02 (4.8)	01 (12.5)
Ex-drinkers	19 (32.2)	07 (43.8)	04 (23.5)	04 (50.0)	15 (35.7)	03 (37.5)
p-value	0.318		0.289		0.676	
Anatomic sites						
Anterior	19 (32.2)	09 (56.2)	04 (23.5)	05 (62.5)	15 (35.7)	04 (50.0)
Posterior	19 (32.2)	03 (18.8)	13 (81.3)	03 (18.8)	27 (87.1)	04 (12.9)
p-value	0.176		0.075		0.351	
TNM clinical stage						
I/II	06 (10.2)	03 (18.8)	02 (11.8)	01 (12.5)	04 (9.5)	02 (25.0)
III/IV	53 (89.8)	13 (81.2)	15 (88.2)	07 (87.5)	38 (90.5)	06 (75.0)
p-value	0.349		0.958		0.217	
Tumor size						
T1/T2	13 (22.0)	07 (43.8)	04 (23.5)	04 (50.0)	09 (21.4)	03 (37.5)
T3/T4	46 (78.0)	09 (56.2)	13 (76.5)	04 (50.0)	33 (78.6)	05 (62.5)
p-value	0.081		0.186		0.329	
Locoregional metastasis						
Absent	21 (35.6)	06 (37.5)	05 (29.4)	03 (37.5)	16 (38.1)	03 (37.5)
Present	38 (60.4)	10 (62.5)	12 (70.6)	05 (62.5)	26 (61.9)	05 (62.5)
p-value	0.888		0.686		0.975	
WHO grade						
I	17 (28.8)	02 (12.5)	02 (11.8)	02 (25.0)	15 (35.7)	0 (0.0)
II	18 (30.5)	06 (37.5)	09 (52.9)	03 (37.5)	09 (21.4)	03 (37.5)
III	24 (40.7)	08 (50.0)	06 (35.3)	03 (37.5)	18 (42.9)	05 (62.5)

Table II. Continued.

Variables	All patients (%) *p16* methylation status		Younger patients (%) *p16* methylation status		Older patients (%) *p16* methylation status	
	Positive	Negative	Positive	Negative	Positive	Negative
Invasive front grade						
Score 4-8	05 (8.5)	0 (0.0)	01 (5.9)	0 (0.0)	04 (9.5)	0 (0.0)
Score >8	54 (91.5)	16 (100.0)	16 (94.1)	08 (100.0)	38 (90.5)	08 (100.0)
p-value	0.288		0.489		0.363	
DNMT3B genotype						
CC	10 (16.9)	03 (18.8)	03 (17.6)	02 (25.0)	07 (16.7)	01 (12.5)
CT	41 (69.5)	11 (68.8)	11 (64.8)	06 (75.0)	30 (71.4)	05 (62.5)
TT	08 (13.6)	02 (12.5)	03 (17.6)	0 (0.0)	05 (11.9)	02 (25.0)
p-value	0.983		0.440		0.615	
Allele frequency						
C allele	51 (86.4)	14 (87.5)	14 (82.4)	08 (100.0)	37 (88.1)	06 (75.0)
T allele	08 (13.6)	02 (12.5)	03 (17.6)	0 (0.0)	05 (11.9)	02 (25.0)
p-value	0.912		0.296		0.310	
DMNT3B immunohistochemistry[a]						
Mean rank of positivity	37.90	28.9	12.81	10.14	25.31	20.44
p-value	0.133		0.384		0.368	

In bold, significant p-value <0.05. [a]Analyzed by Kruskal-Wallis test, the other analyzes were done using the χ^2 test.

Statistical analysis. Statistical significance of differences between case and control group distributions for alleles, genotypes, methylation status, immunohistochemical analysis and clinicopathological features was determined using Fisher or χ^2 tests. The possible association between genotypes and DNMT3B immunostaining was evaluated by Kruskal-Wallis test.

Time to survival was calculated from date of diagnosis to time of last follow-up visit or to time of death. The records of each patient were reviewed, considering the same parameters, for 0-2500 days. All deaths were caused by locoregional and/or metastatic disease. For the purposes of analysis, patients who died without evidence of recurrence were excluded. Time survival was displayed by means of the Kaplan-Meier method for the variables. The results of Kaplan-Meier were compared by the log-rank test. Variables with p≤0.25 additional to age, p16CDKN2A methylation status, DNMT3B genotypes and immunostaining were included in the Cox proportional hazards multivariate model. Categorical variables considered as referents were those associated with less risk of death in accordance with the literature. All analyses were assessed using SPSS 17.0 (SPSS Inc., Chicago) and statistical significance was set at p<0.05.

Results

Association of p16CDKN2A promoter methylation, DNMT3b polymorphism and clinicopathological parameters of HNSSC patients. The frequency distributions of p16CDKN2A promoter methylation according to age, molecular features and clinicopathological parameters are summarized in Table II. No association between p16CDKN2A methylation and age was observed. In young patients we identified a significant association between p16CDKN2A methylation with the presence of family history of cancer and male gender. On the other hand, in the older patients, p16 methylation was significantly increased regarding the presence of smoking habit. Considering all patients, p16 methylation was increased in male and smoker subjects (Table II). Differences in gender habits were observed according to age. In young patients, no differences of tobacco addiction and gender were observed (p=0.072). However, considering all patients together or only older patients, the male gender was associated with the presence of tobacco habit when compared with women (p=0.001) (data not shown). No relation between DNMT3B immunostaining and p16CDKN2A methylation was observed.

Table III presents the distribution of molecular and clinicopathological parameters grouped by age and DNMT3B variants. No association between clinicopathological parameters and the polymorphic variables were observed in young patients. The T allele of DNMT3B genotype was significantly associated with advanced TNM staging and tumor size in the older patients. Considering all samples, the distribution of allele T was increased in the T3/T4 tumors. No relation between polymorphic variants of DNMT3B gene and p16CDKN2A promoter methylation was observed.

Table III. DNMT3B genotype and their association with p16 methylation and clinicopathological features in case and control HNSCCs.

	All patients (%)			Younger patients (%)			Older patients (%)		
Variables	CC	CT	TT	CC	CT	TT	CC	CT	TT
Age									
Young	05 (20.0)	17 (68.0)	03 (12.0)						
Older	08 (16.0)	35 (70.0)	07 (14.0)						
p-value		0.899							
Gender									
Male	11 (84.6)	45 (86.5)	08 (80.0)	05 (100.0)	13 (76.5)	03 (100.0)	06 (75.0)	32 (91.4)	05 (71.4)
Female	02 (15.4)	07 (13.5)	02 (20.0)	0 (0.0)	04 (23.5)	0 (0.0)	02 (25.0)	03 (8.6)	02 (28.6)
p-value		0.864			0.326			0.235	
Family history of any cancer									
Absent	05 (38.5)	28 (53.8)	06 (60.0)	01 (20.0)	08 (47.1)	02 (66.7)	04 (50.0)	20 (57.1)	04 (57.1)
Present	08 (61.5)	24 (46.2)	04 (40.0)	04 (80.0)	09 (52.9)	01 (33.3)	04 (50.0)	15 (42.9)	03 (42.9)
p-value		0.527			0.395			0.933	
Smoking status									
Smokers	08 (61.5)	43 (82.7)	09 (90.0)	05 (100.0)	13 (76.5)	03 (100.0)	03 (37.5)	30 (85.7)	06 (85.7)
Non-smokers	01 (7.7)	02 (3.8)	0 (0.0)	0 (0.0)	01 (5.9)	0 (0.0)	01 (12.5)	01 (2.9)	0 (0.0)
Ex-smokers	04 (30.8)	07 (13.5)	01 (10.0)	0 (0.0)	03 (17.6)	0 (0.0)	04 (15.0)	04 (11.4)	01 (14.3)
p-value		0.437			0.692			0.054	
Alcohol consumption									
Drinkers	05 (38.5)	30 (57.7)	09 (90.0)	02 (40.0)	10 (58.8)	03 (100.0)	03 (37.5)	20 (57.1)	06 (85.7)
Non-drinkers	01 (7.7)	04 (7.7)	0 (0.0)	0 (0.0)	02 (11.8)	0 (0.0)	01 (12.5)	02 (5.7)	0 (0.0)
Ex-drinkers	07 (53.8)	18 (34.6)	01 (10.0)	03 (60.0)	05 (29.4)	0 (0.0)	04 (50.0)	13 (37.1)	01 (14.3)
p-value		0.165			0.367			0.425	
Anatomic sites									
Anterior	06 (21.4)	20 (71.4)	02 (7.1)	02 (40.0)	06 (35.2)	01 (33.3)	04 (50.0)	14 (40.0)	01 (14.3)
Posterior	07 (14.9)	32 (68.1)	08 (17.0)	03 (18.8)	11 (68.8)	02 (12.5)	04 (12.9)	21 (67.7)	06 (19.4)
p-value		0.418			0.976			0.330	
TNM clinical stage									
I/II	03 (23.1)	05 (9.6)	01 (10.0)	0 (0.0)	03 (17.6)	0 (0.0)	03 (37.5)	02 (5.7)	01 (14.3)
III/IV	10 (76.9)	47 (90.4)	09 (90.0)	05 (100.0)	14 (82.4)	03 (100.0)	05 (62.5)	33 (94.3)	06 (85.7)
p-value		0.401			0.448			**0.043**	
Tumor size									
T1/T2	07 (53.8)	12 (23.1)	01 (10.0)	01 (20.0)	07 (41.2)	0 (0.0)	06 (75.0)	05 (14.3)	01 (14.3)
T3/T4	06 (46.2)	40 (76.0)	09 (90.0)	04 (80.0)	10 (58.8)	03 (100.0)	02 (25.0)	30 (85.7)	06 (85.7)
p-value		**0.036**			0.301			**0.001**	
Locoregional metastasis									
Absent	06 (46.2)	18 (34.6)	03 (30.0)	01 (20.0)	07 (41.2)	0 (0.0)	05 (62.5)	11 (31.4)	03 (42.9)
Present	07 (53.8)	34 (65.4)	07 (70.0)	04 (80.0)	10 (58.8)	03 (100.0)	03 (37.5)	24 (68.6)	04 (57.1)
p-value		0.677			0.301			0.253	

Table III. Continued.

Variables	All patients (%)			Younger patients (%)			Older patients (%)		
	CC	CT	TT	CC	CT	TT	CC	CT	TT
WHO grade									
I	02 (15.4)	14 (26.9)	03 (30.0)	02 (40.0)	02 (11.8)	0 (0.0)	0 (0.0)	12 (34.3)	03 (42.9)
II	07 (53.8)	14 (26.9)	13 (30.0)	03 (60.0)	07 (41.1)	02 (66.7)	05 (40.0)	07 (20.0)	01 (14.2)
III	04 (30.8)	24 (46.2)	04 (40.0)	0 (0.0)	08 (47.1)	01 (33.3)	05 (40.0)	16 (45.7)	03 (42.9)
p-value		0.461			0.250			0.214	
Invasive front grade									
Score 4-8	01 (7.7)	04 (7.7)	0 (0.0)	0 (0.0)	01 (5.9)	0 (0.0)	01 (12.5)	03 (8.6)	0 (0.0)
Score >8	12 (92.3)	48 (92.3)	10 (100.0)	05 (100.0)	16 (94.1)	03 (100.0)	07 (87.5)	32 (91.4)	07 (100.0)
p-value		0.662			0.783			0.656	
p16 methylation status									
Positive	10 (76.9)	41 (78.8)	08 (80.0)	03 (60.0)	11 (64.7)	03 (100.0)	07 (87.5)	30 (85.7)	05 (71.4)
Negative	03 (23.1)	11 (21.2)	02 (20.0)	02 (40.0)	06 (35.3)	0 (0.0)	01 (12.5)	05 (14.3)	02 (28.6)
p-value		0.983			0.440			0.615	
DMNT3B immuno-histochemistry[a]									
Mean rank of positivity	29.14	37.54	35.85	14.5	11.91	9.7	13.64	26.25	26.86
p-value		0.472			0.584			0.084	

In bold, significant p-value <0.05. [a]Analyzed by Kruskal-Wallis test, the other analyzes were done using the χ^2 test.

Moreover, neither polymorphic variables nor clinicopathological parameters showed association with immunoexpression of DNMT3B.

Molecular results and survival of HNSSC patients. The mean overall survival of patients was 1021.5 days after the diagnosis. Factors which impacted in survival were differently distributed among the groups. In young patients no factor was associated with death. Considering both groups together, only TNM staging impacted on survival. However, in older patients, not only TNM staging but also the absence of p16CDKN2A promoter methylation showed a negative impact on survival (Table IV).

Discussion

The determinants of DNMT expression in human tissues have not been clearly defined. Recently, the role of ageing in DNA methylation was reported in liver cells (57). On the other hand, methylation of tumor suppressor genes, such as p16, has been associated with HNSCC development (48,58,59). In the present study we hypothesized that DNMT3B C46359T polymorphism may be associated with DNMT3B immuno-expression and p16CDKN2A methylation. In addition, we tested if these parameters are related to the clinical stage, family history or the prognosis of HNSCC in young and older patients matched by the TNM staging, smoking and alcohol intake.

We observed that the frequency of p16 methylation in young patients was not different from older patients with HNSCC and we noted that this epigenetic alteration was present in most of the samples of both groups (76%). Taken together, the data indicate that p16 methylation is a common event in HNSCC development. Our data showed also that p16 methylation in young patients was associated with family history of cancer. However, there are some limitations that should be regarded. The high presence of subjects with family history of cancer in the young patients group could be a confounding factor. Therefore, a larger scale study is needed to confirm these data. Although we found association between p16 methylation in young patients and male gender, the limited number of patients of female gender indicates that the data must be analyzed cautiously.

We observed that p16CDKN2A methylation in older patients was significantly associated with smoking habit. Smoking is an important factor associated with the methylation of genes related to cancer (59,60). 7-methylguanine (m7Gua) is a biomarker of methylating agents present in tobacco (61).

Table IV. Cox regression analyses in the HNSCC patients with a follow-up of 0-2500 days.

	All patients 95.0% CI				Young patients 95.0% CI				Older patients 95.0% CI			
Variables	p-value	OR	Lower	Upper	p-value	OR	Lower	Upper	p-value	OR	Lower	Upper
Age												
>45 years	Referent				-	-	-	-	-	-	-	-
≤45 years	0.115	1.822	0.864	3.844	-	-	-	-	-	-	-	-
P16 methylation												
Positive	Referent				Referent				Referent			
Negative	0.274	1.482	0.733	2.996	0.088	0.268	0.059	1.217	**0.001**	7.832	2.796	21.940
DNMT3B genotype												
CC	Referent				Referent				Referent			
CT	0.822	1.110	0.449	2.744	0.438	1.991	0.350	11.336	0.973	0.980	0.297	3.233
TT	0.871	1.096	0.362	3.318	0.327	0.286	0.023	3.496	0.824	1.168	0.297	4.595
TMN												
Early	Referent				Referent				Referent			
Late	**0.029**	5.022	1.175	21.470	0.985	NA	0.011	8.449	**0.011**	8.449	1.622	44.015
DNMT3B immuno-histochemistry	0.753	1.141	0.501	2.597	0.583	0.666	0.156	2.848	0.079	2.623	0.894	7.696

In bold, significant p-value <0.05. The term referent is associated with categorical variables with a lower risk of developing the disease. NA, not applicable.

Recent reviews observed that age and cigarette smoking were positively correlated with the urinary m7Gua level (62). The urinary excretion of m7Gua was shown to be higher in smokers than in non-smokers (63). Furthermore, the m7Gua level in human urine decreased after smoking cessation (64). It is important to highlight that, in the present study, no p16CDKN2A methylation was observed in non-smokers. Therefore, our study gives additional support that both smoking and aging are important factors involved in DNA methylation.

In recent years, several studies have demonstrated that genetic polymorphisms are associated with susceptibility to, or protection from, cancer development (36,65,66). Until now, 13 polymorphisms were described in the coding region of gene DNMT3B according NCBI database, but the association between these genetic variations and cancer prognosis remains unclear. Polymorphisms may alter gene transcription and/or protein synthesis and function. Recently, an increased risk of HNSCC development was demonstrated with the T allele of DNMT3B -149 polymorphism in oral cavity, pharynx and larynx but not in nasopharynx (36). In the present study we attempted to verify the possible association between this polymorphism and early HNSCC development. We did not observe association between DNMT3B polymorphic variant and age, but in the older patients group we found association between the higher DNMT3B genotype (allele T) with an advanced TNM staging. Although this finding suggests that polymorphisms might influence cancer progression, it will take a long time to prove this association. Here, the immunoexpression of DNMT3B protein was not related to the genetic polymorphisms in HNSCC samples studied. Moreover, this polymorphism did not show association with p16 methylation. This may be explained by the fact that other factors, such as HPV high-risk infection, may induce p16 methylation (47,67). Furthermore, other DNMT polymorphisms may be more relevant to this epigenetic alteration.

Evidence suggests that there are differences in molecular mechanisms between younger and older HNSCC patients (6,9). We found that absence of p16 methylation was associated with low survival in older patients. Therefore, it can be speculated that different epigenetic and genetic pathways may affect cancer evolution and prognosis. Although we are only just starting to understand the impact of molecular findings in patient prognosis, previous reports have already suggested that HPV infection has an impact on the prognosis of HNSCC (68,69).

In conclusion, our data suggest that while genetic factors are more importantly involved with p16 methylation in younger individuals with HNSCC, environmental factors are more relevant for this epigenetic change in older patients. Finally, the present study shows that p16 methylation status has impact on the prognosis of patients with HNSCC.

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