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Screening methylation of DNA repair genes in the oral mucosa of chronic smokers

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ABSTRACT

Objective: The aim of this study was to evaluate the epigenetic changes in the process of oral carcinogenesis by screening the methylation of repair genes in chronic smokers.
Design: Two groups were formed: Group 1: 16 smokers with consumption of 20 cigarettes/day for at least 10 years; and Group 2: 10 non-smoking. Exfoliative cytology of the tongue was performed, and the extracted DNA was treated by enzymes. The PCR Array System performed methylation screening to evaluate 22 DNA repair genes, and the results were validated by RT-qPCR for each gene with methylation levels ≥10%.
Results: Highest percentages of methylation were observed for *MLH3* and *XRCC1* genes (11–20% methylation) and in one case for *MRE11A* and *PMS2* (> 50% methylation). Statistical analysis showed significant differences in the expression of the genes *MRE11A* (p = 0.0002), *PMS2*(p = 0.0068), *XRCC1* (p = 0.0080) and *MLH3* (0.0057) between the two groups.
Conclusion: The effects of chronic smoking on oral mucosa led to the methylation of genes *MRE11A PMS2*, *XRCC1* and *MLH3*, but resulted in a reduction of gene expression of *MRE11A* and *PMS2*, which showed ≥50% methylation. These results provide evidence that smoking cause methylation and reduced expression of repair genes.

1. Introduction

Epigenetics is a mechanism that leads to the modification of gene expression without altering the DNA sequence (Hitchins, 2010). Described as modifications in the spatial conformation of the DNA molecule and its transcriptional activity, they are involved in maintaining the stability and integrity of DNA, leading to changes only in chromatin structure (Arantes, de Carvalho, Melendez, Carvalho, & Goloni-Bertollo, 2014). These changes can be reversible and are not necessarily hereditary (Arantes et al., 2014; Breitling, 2013).

There are numerous epigenetic mechanisms such as DNA methylation, changes in the conformation of chromatin, histone modification and post-transcriptional modification (Arantes et al., 2014). All of these mechanisms lead to changes in gene expression. The most common epigenetic change is DNA methylation, which is the addition of a methyl group (−CH3) on the carbon 5 of a nitrogen base cytosine (C) in regions called CpG islands, becoming a 5-methylcytosine (Lee & Pausova, 2013; Zhu & Yao, 2009).

Hypermethylation functions as gene silencing and can be observed at a high frequency in squamous cell carcinoma (SCC) as well as in tissues adjacent to tumors and dysplastic tissues (Lingen et al., 2011). The most commonly methylated genes are tumor suppressors, metastasis-related, DNA repair genes, hormone receptors and angiogenesis inhibitors (Shaw, 2006).

The repair genes are responsible for identifying errors in DNA replication and its correction. DNA repair requires the recognition of DNA damage and the rapid activation of specific machinery to repair that damage to avoid a delay in the progression of the cell cycle while

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carrying out this repair (Lazzaro et al., 2009). The repair of DNA damage should be performed to prevent loss or incorrect transmission of genetic information because errors in this process initiate the development of abnormalities and oncogenesis (Branzei & Foiani, 2008). Repair genes can be grouped according to their performance category in DNA repair: repair of base excision, repair of nucleotide excision, mismatch base repair and repair of double-strand breaks. These functions are extremely important in maintaining the stability of the genetic material and cell cycle regulation (Woods et al., 2007). Repair gene silencing through various mechanisms, such as methylation, can also lead to susceptibility to genetic mutations (Fishel & Kolodner, 1995).

Changes in the methylation profile are described in several types of malignancies as an event related to initial stages of carcinogenesis (Arantes et al., 2014). Specifically in oral cancers, it is well known that tobacco represents a key factor in its carcinogenesis (Lima et al., 2015), and it has been described that DNA methylation caused by smoking can occur by different mechanisms (Lee & Pausova, 2013). In despite of this, no pattern of methylation levels among chronic smokers and nonsmokers has been established in the literature, especially when no invasive methods (e.g. exfoliative cytology) are applied for the diagnosis.

The objective of this study was to evaluate the methylation status of several DNA repair genes, using a PCR Array System, and relate to the chronic use of tobacco.

2. Materials and methods

There were two groups of participants. One from the Outpatient Program for the Treatment of Smoking, Heart Institute, University Hospital, Medical School, São Paulo University (INCOR-HCFMUSP), and the other from the Oral Medicine of the São Paulo State University (Unesp), Institute of Science and Technology (ICT-UNESP).

The inclusion criteria for both groups were the following: no history of malignant neoplasia, absence of visible alterations in the normal oral mucosa, and a maximum weekly intake of 3 alcoholic drinks (Lima et al., 2010; Lima et al., 2015). All patients underwent an intra- and extra-oral clinical examination and answered a questionnaire when they were asked about the frequency and quantity of their cigarette consumption. The patients were grouped as follows:

Group 1 (chronic smokers): 16 chronic smokers, exclusively male, with tobacco consumption equal to or greater than 20 cigarettes/day for at least 10 years before anti-smoking treatment; and

Group 2 (control): 10 male nonsmokers, age-matched to the average age of group 1.

As an objective indicative of cigarette consumption, breath carbon monoxide (CO) was measured in parts per million, as a marker of smoking status, using a calibrated PiCO + Smokerlyzer® instrument (Bedfont Scientific Ltd, UK).

After being informed about the proposal and the conditions of this study, those who agreed to participate signed a consent form. The Ethics Committee Research in ICT/UNESP approved this study under protocol: CAAE 07386212.0.1001.0077.

2.1. Sample collection

Cells representative of many layers of tongue epithelium were collected by scraping two areas of the tongue border using a Rovers® Orcellex® Brush Soft Oral Cell Samplex (Rovers Medical Devices, NL, Netherlands). As such a procedure is only minimally painful, it is not necessary to use local anesthesia when performing it. Samples were collected from border of the tongue, which is one of the most affected by OSCC intra-oral sites (Lima et al., 2017; Pires et al., 2013).

Patients did not use mouthwash on the day of the procedure. Samples were stored in 2 mL of cell lysis solution (Qiagen, CA, USA) for DNA extraction and 2 mL of RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) for RNA extraction and then stored at − 80 °C.

Table 1
Repair genes evaluated.

Category Of DNA Repair	Genes
Base Excision Repair	*APEX1, LIG3, PARP1, POLB, UNG, XRCC1*
Nucleotide Excision Repair	*CCNH, RAD23A, RAD23B, XPC*
Mismatch Repair	*MLH1, MLH3, MSH2, PMS2, POLD3*
Double-Strand Break Repair	*BRCA1, BRCA2, FEN1, MRE11A, RAD50, AD51*
Genes Related to DNA Repair	*ATM*

2.2. Methylation screening

Methylation analysis of 22 repair genes (Table 1) was performed on all samples of Group 1 and Group 2 patients. Samples were centrifuged at 14,000 rpm for 5 min and DNA was extracted from pellets using the QIAAmp kit and DNA Mini Kit (Qiagen, CA, USA). Methylation was analyzed after digesting DNA with restriction enzymes sensitive to methylation (undigested methylated genes) and methylation-dependent enzymes (digested methylated genes); digestion with both types of enzymes (background control) was performed using the restriction system kit (Qiagen, CA, USA). Enzymatic treatment of 1 µg DNA was performed during 6 h at 37 °C, followed by enzymatic inactivation at 65 °C for 20 min. DNA was amplified by RT-qPCR using the EpiTect Methyl II PCR Array System (Qiagen, CA, USA) and primers flanking the region of interest, with the following cycling conditions: 1 cycle 10 min 95 °C, 3 cycles of 99 °C 30 s and 72 °C for 1 min and 40 cycles of 97 °C for 15 s and 72 °C for 1 min.

2.3. RT-qPCR

RT-qPCR was performed using the protocol described by Alves et al. (2017); on genes that showed a percentage of methylation ≥10. Trizol reagent (Ambion, Inc., Carlsbad, CA, USA) was used to extract total RNA from the cells of mucosa of the mouth. At first was performed an incubation of the collected cells with 1.0-mL of TRIzol at room temperature (RT) for 10 min. Following, was added 200 µL of chloroform (Sigma-Aldrich, St. Louis, MO, USA) which were centrifuged at 12,000*g* for 15 min at 4 °C, and 500 µL of isopropanol (Sigma-Aldrich, St. Louis, MO, USA) was added to the pellet. The pellet was washed with 70% ethanol (Sigma-Aldrich, St. Louis, MO, USA) and resuspended in 50 µL of RNA storage buffer (Ambion Inc., Carlsbad, CA, USA). The NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) was used to evaluate the concentration, purity and quality of the RNA.

From the RNA extracted 1 µg was treated with DNase I (Turbo DNase Treatment and Removal Reagents, Ambion Inc., Carlsbad, CA, USA) and transcribed into complementary DNA (cDNA) using the SuperScript® III First-Strand Synthesis SuperMix for RT-qPCR Kit (InvitrogenTM, Carlsbad, CA, USA).

The reference gene of choice was tubulin, *TUB*, after analysis of the profile of the application of three constituent genes: *GAPDH, TUB* and *ACTB*, in all experimental samples. Results were analyzed at http://www.leonxie.com/referencegene.phpe for selecting the best reference gene.

RT-qPCR analysis was applied to detect the amount of cDNA in the exponential phase of the amplification reaction. The detection system used was SYBR® Green fluorophore (Platinum® SYBR® Green qPCR SuperMix-UDG Applied Biosystems, Framingham, MA, USA) in the following reaction: 12.5 µL of Super mix Platinum SYBR Green, 1 µL of ROX (reference dye), 300 µM of the forward primer, 300 µM of the reverse primer, 2 µL of cDNA solution and 2.1 µL of Ultrapure water (InvitrogenTM, Carlsbad, CA, USA), to obtain a final volume of 20 µL in each well of a 96-well plate (InvitrogenTM, Carlsbad, CA, USA). All primers were selected from the reviewed literature, and sequences were confirmed using BLAST (basic local alignment search tool) (Table 2). As a negative control, all reagents were added to the last wells of the plate

Table 2
Sequence of the primers for RT-qPCR of the genes with increased methylation levels.

Primer	Sequence
MRE11A	CGGGTTCTCAGAGGCAAGTT
	CGACCCAAGGCTGTCTTCTT
PMS2	TGCCCCTGGACTTTTCTATG
	TCTGTTCCCCTTCACTTTGC
XRCC1	GTGCTGAGTGGCTTCCAGAAC
	TTGGCAAAGGCACAGATGAG
MLH3	CCTGGCATTCTCACCATCTT
	GGGCAAGTTCCTCAACACAT
TUB	CCGGGCAGTGTTTGTAGACT
	TTGCCTGTGATGAGTTGCTC

except cDNA. The instrument of analysis used was the StepOnePlus™ System (Applied Biosystems, Framingham, MA, USA). The cycling parameters used were: 50 °C for 2 min, followed by an initial denaturation at 95 °C for 2 min and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. All dissociation (melting) curve analysis were evaluate to the absence of any bimodal curve or abnormal amplification signal was observed and analyzed every 0.1 °C. The method of analysis of gene expression was $2^{-\Delta\Delta CT}$ described by Livak and Schmittgen (2001).

The results of the gene expression were presented graphically as means ± standard deviations.

3. Results

We evaluated 16 chronic male smokers, with a mean age of 58.44 ± 11.91 (minimum 38, maximum 80). The average consumption of cigarettes per day was 28.44 ± 9.25 cigarettes/day in 41.19 ± 13.29 years, with a mean tobacco history of 57.16 ± 23.22 pack/year. The CO average was 12.75 ± 7.17 ppm (minimum: 5, maximum:19). Two patients evaluated for DNA methylation in the control group were 66 and 35 years old and had never smoked.

After evaluating the methylation status of 22 repair genes for all samples, only four genes presented methylation ≥10% in smokers: *MRE11A, PMS2, XRCC1,* and *MLH3*. High percentage methylation was observed in three cases, showing*MLH3*and *XRCC1* with 11–20% methylation, and one case with *MRE11A* and *PMS2* above 50% methylation. Methylation levels of nonsmokers (controls) were lower than 10% for all evaluated genes. Data on the age of the patients, the consumption of tobacco and the methylation profiles for these genes are shown in Table 3.

The results of methylation analysis are represented by Fig. 1 which shows a heat map based on a hierarchical clustering method with Pearson correlation used to measure the similarity of methylation in intra- and inter-groups (smokers: s and controls: c).

To better assess the gene expression of genes highlighted by methylation, PCR Arrays were included with 8 more controls, totaling 10 cases. The control group consisted of 10 men, with mean ages of 57.8 ± 12.8 (minimum 35, maximum 74).

Table 3
Smokers' age, tobacco data, and highest methyl profile.

Patients' Data	S1	S6	S8	S11	S15
Age	46	39	80	62	71
Daily consumption tobacco	40	40	30	40	20
Years of consumption tobacco	40	25	62	45	68
CO2 (ppm[a])	13	16	11	14	19
MRE11A	51.43%	10%	10%	10%	10%
PMS2	10%	10%	54.4%	10%	10%
XRCC1	11.68%	11.98%	10%	15.48%	10%
MLH3	16.32%	16.47%	10%	10%	13.58%

[a] Parts per million.

The results of the gene expression can be seen in Fig. 2.

Statistical analysis using Student's *t*-test showed significant differences in expression of *MRE11A* (p = 0.0002), *PMS2* (p = 0.0068), *XRCC1* (p = 0.0080) and *MLH3* (0.0057) among groups 1 and 2. Pearson correlation test (α = 5%) did not show statistical significance when relating *MRE11A* and *PMS2* methylation to age (p = 0.6125, p = 0.9464), smoking history (p = 0, 6846; p = 0.6125), and CO level (p = 0.1675, p = 0.1873). The same was found for *XRCC1* and *MLH3* to age (p = 0.7827, p = 0.8028), smoking history (p = 0.2954, p = 0.9071) and CO level (p = 0.3674; p = 0.1901).

4. Discussion

The effects of chronic smoking on oral mucosa leads to the methylation of some repair genes (*MRE11A, PMS2, XRCC1* and *MLH3*), but the reduction of gene expression occurred only to genes which showed ≥50% methylation (*MRE11A* and *PMS2*). These results provide evidence that smoking causes methylation and reduced expression of repair genes.

The function of DNA methylation comprises, other than the regulation of gene expression, the protection of the integrity of the genome (Lee & Pausova, 2013). These changes are also related to those in the gene expression of important genes in the development of various types of cancer (Arantes et al., 2014).

The studies of methylation profile have used different types of samples, such as tissue, serum/plasma and exfoliated cells (Arantes et al., 2014; Longo et al., 2014). Therefore, cytology is the method of choice, as it is minimally invasive, practical and easy to perform (Almeida, Cabral, & Brandão, 1992). For ethical reasons a clinically normal mucosa cannot be biopsied. The use of Rovers® Orcellex® Brush as an instrument for collection of cells makes it possible to reach the deeper layers of the epithelium. The changes occurring in the basal layer are perpetuated in cells that detach from the epithelium in the maturation process, including DNA methylation.

Exposure to tobacco and alcohol promote not clinically evident epithelial changes in the oral mucosa, and the entire area is at increased risk for the development of malignant lesions, also named field cancerization (Arantes et al., 2014; Slaughter, Southwick, & Smejkal, 1953). Tobacco-related genetics and epigenetics have aroused the interest of many studies prior to the universal acceptance of tobacco as a triggering factor for many diseases (Breitling, 2013). The effects of tobacco-related methylation can be variable. In embryonic development, tobacco can cause a global hypomethylation (Lee & Pausova, 2013; Wan et al., 2012). In adulthood, tobacco-related epigenetic events can cause hypermethylation in the promoter regions of genes related to DNA repair, cell cycle regulation, tumor suppressors and apoptosis (Arantes et al., 2014; Talikka et al., 2012). Although the status of hypermethylation persists for years after smoking cessation, the methylation levels are always higher in active smokers compared to former smokers, as this process is reversible (Talikka et al., 2012; Yanagawa et al., 2011).

DNA methylation caused by smoking occurs by different mechanisms (Lee & Pausova, 2013). This effect can occur by damage to the DNA itself that leads to the recruitment of DNMTs (DNA methyl transferases). DNA methylation is catalyzed by three types of methyltransferases (MT): DNMT1, DNMT2, and DNMT3. DNTM1 functions maintaining methylation during cell division, and for this reason, it is expressed in proliferating cells (Lee & Pausova, 2013). Hypermethylation in smokers can also be explained by the high concentrations of DNMT1. This is because it causes the inhibition of degradation by the NKK (nicotine-derived nitrosamine ketone) of cigarettes (Lin et al., 2010; Talikka et al., 2012).

In this study, a higher percentage of DNA methylation ≥10% was observed in four repair genes out of a total of 22 evaluated genes: *MRE11A* (*meiotic recombination 11 homolog A*), *PMS2* (*postmeiotic segregation increased 2*), *XRCC1* (*X-ray cross complementing group 1*), and

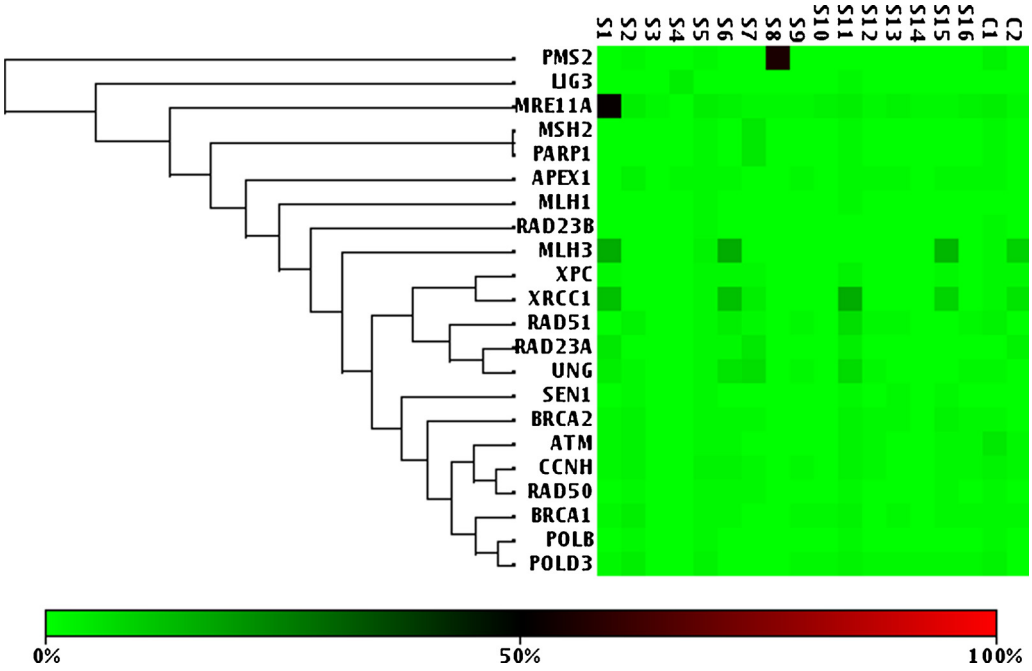

Fig. 1. Samples with similar methylation profiles for smokers and controls. The black pixels represents the highest methylation levels founded in the evaluated samples. The green pixels represents the absence ou lower levels of methylation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

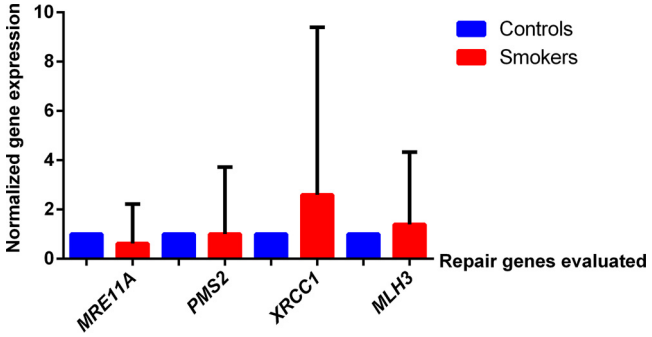

Fig. 2. Graph of gene expression (mean ± standart deviation) of methylated genes in all participants in the smoking group (n = 16). The genes *XRCC1* and *MLH3* exhibited an increase in their expression.

MLH3 (*mutL homolog 3*). Although significant methylation percentages (≥10%) have been identified in only four genes, the study was performed by combining a panel of 22 genes to increase the sensitivity of the assessment of methylation rate (Arantes et al., 2015) and increase the reliability of the results. When expression in these genes were evaluated in the same patients, only *MRE11A* and *PMS2* showed a decrease in expression by RT-qPCR, while *XRCC1* and *MLH3* showed an increase in expression. This can be attributed to a methylation percentage ≥50% in *MRE11A* and *PMS2* genes.

Those studying methylation should consider that the pattern of methylation may vary in the same individual over time (Bjornsson et al., 2008; Dobrovic & Kristensen, 2009). In the present study, the age of subjects was not correlated with an increase in methylation. This association is often described as weak or non-existent (Dobrovic & Kristensen, 2009).

Another factor that is not determined by researchers to be involved in methylation variation is the genetic polymorphism of the evaluated genes. The literature discloses some studies on polymorphism of the evaluated genes, including *XRCC1*. The protein encoded by this gene acts interactively with other proteins responsible for base excision repair (BER). The description of other polymorphisms that are related to mismatch repair (MMR), *MLH1, MSH2* and *MSH3*, contribute to the SCC head and neck prognosis, especially in smokers (Nogueira et al., 2015).

The loss of any DNA repair mechanism increases the risk of cancer development in humans (Nunn et al., 2003). The effect of gene expression on the *PMS2* and *MLH3* genes can be understood according to the overall performance in its class, MMR. MMR defects in the DNA repair mechanism may manifest as microsatellite instability (Nunn et al., 2003).

The impact of *MLH3* methylation may be linked to their function as a heterodimeric molecule with *MLH1*. This heterodimer, known as MutLγ, helps with base–base mismatch repair and extra-helical single nucleotides, but does not function in the repair of insertion and deletion errors in loops of 2–4 nucleotides (Flores-Rozas & Kolodner, 1998; Korhonen, Vuorenma, & Nyström, 2008). The *MLH1-MLH3* heterodimer is also an active participant in meiotic recombination, due to its endonuclease activity (Larrea, Lujan, & Kunkel, 2010; Muro, Sugiura, & Mimori, 2015).

Similarly decreased expression of the *PMS2* gene affects the performance of the heterodimer *MLH1-PMS2* (MutLα). This heterodimer is very active and responsible for repairs initiated by the MutS complex, due to its endonuclease activity that cleaves the defective chain near the error location in base mismatch (Larrea et al., 2010; Muro et al., 2015).

The effects of changes in the expression of the *PMS2* gene were reported by Gibson et al. (2006) as a cause of changes in the MMR pathway, resulting in hypermutability and tolerance to DNA replication errors. However, the authors observed that overexpression increased the frequency of spontaneous mutation. The *PMS2* gene also participates in the signaling pathway of apoptosis by interacting with the *p73* pro-apoptotic factor, so the inhibition of its expression can reduce apoptotic events. The expression levels of MMR heterodimers have been described by Jessri, Dalley, and Farah (2015) as being related to an increase in the severity of tissue changes in the dysplastic tissues of the oral mucosa and SCC, whereas a decrease in the expression of *hMLH1* and *hPMS2* was observed.

The effect on the repair of double-strand breaks (DBS) has been described by Mondal et al. (2013). The researchers suggest that the *MRE11A* gene is one of the most important genes that can modify the risk to the development of SCC and leukoplakia. Thus, studies in SCC of the head and neck are scarce. *MRE11A*expression changes can affect performance in the MRN complex (*MRE11A-RAD50-NBS1*) in its maintenance activity of telomeres, meiotic recombination and

detection of DNA damage (Denchi, 2009; Rein & Stracker, 2014; Zhong et al., 2007). This likewise affects the activation of kinases *ATM* and *ATM/RAD3* (*ATR*) regulated by this complex.

The increase of gene expression of *XRCC1*, related to single-strand break repair (SSB), is reflected in radio resistance (Lattanzio et al., 2015) to future SCC development in patients with overexpression of this gene, as observed in the smoker group assessed for methylation.

With regards to gene expression and tobacco consumption, correlations between smoking history and CO concentrations were observed. These results contrast with studies from Leoncini et al. (2015) which describe the duration of smoking as a risk factor for SCC of the head and neck. This finding may be related to small smoking history variation, which provided a homogeneous sample for correlation evaluations.

Based on these results, we conclude that the effects of chronic smoking on the oral mucosa lead to methylation of the genes *MRE11A, PMS2, XRCC1 and MLH3*. The decrease in gene expression was observed only in *MRE11A* and *PMS2*, which showed higher percentages of methylation $\geq 50\%$. These results show the reduction in the activity of DNA repair genes due to smoking.

Conflict of interest

The authors have no potential conflict of interest. All authors have read and approved the final article.

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Ethical approval

The Ethics Committee Research in ICT/UNESP approved this study under protocol: CAAE 07386212.0.1001.0077.

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