Gene expression and carbonic anhydrase IX promoter methylation in oral cancer and smokers oral mucosa: a pilot study

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Aim: To evaluate the methylation of carbonic anhydrase IX (CAIX) promoter and CAIX gene expression in oral cancer and smokers. Methods: The collected oral cells samples were classified in five groups, with each group containing 15 samples. Group 1, oral squamous cell carcinoma (OSCC); group 2, normal contralateral mucosa (NCM) of patients from group OSCC; group 3, normal mucosa in chronic smokers (CS); group 4, normal mucosa in ex-smokers (ES) in cessation for one year; and group 5, normal mucosa in never-smokers (NS). Samples were evaluated through methylation-specific polymerase chain reaction (PCR) and quantitative PCR. Results: The CAIX gene was methylated in only 20% of samples from group OSCC; in groups NCM, CS, ES and NS, all cases were unmethylated. CAIX expression of group OSCC was lower than in NS (P = 0.005). There was no statistically significant difference between CAIX expression in other groups, NCM and NS (P = 0.285), CS and NS (P = 0.530) and ES and NS (P = 0.068). Conclusion: CAIX methylation is higher in oral cancer and its expression is reduced in the same cases whose methylation is present. However, the methylation profile and the expression of this gene are not modified in chronic smokers and ex-smokers after one year of smoke cessation.

Key words: Carcinoma, squamous cell, methylation, carbonic anhydrase IX, gene expression, tobacco

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INTRODUCTION

Oral carcinogenesis is a multifactorial process with several stages, involving numerous genetic and epigenetic changes. It is modulated by hereditary predispositions and environmental influences\(^1\). Abnormalities of these processes can give rise to a phenotype with increased cell proliferation and loss of cellular cohesion, causing local infiltration and metastasis\(^2\).

The squamous cell carcinoma (SCC) is the most common oral malignancy. It is more common in men and the tongue is the most frequent location\(^3\). The diagnosis of oral SCC (OSCC) is based on clinical and histopathological findings. However, the analysis of exfoliative cytology can detect early-stage lesions, reducing morbidity and mortality\(^4\). This analysis is a noninvasive, painless, convenient and low-cost complementary test\(^5\), which can provide information about epithelial cells\(^6\).

The process of carcinogenesis is accompanied by metabolic disorders, such as hypoxia and acidification of the extracellular medium\(^7,8\). Carbonic anhydrase (CA) are transmembrane zinc metalloenzymes which catalyze the reversible hydration of carbon dioxide into carbonic acid, thereby regulating the pH\(^9\). Pérez-Sayáns et al.\(^{10}\) in 2012 concluded that CAIX was overexpressed in tumor cells and advanced stage tumors, these expression levels were statistically significantly higher than initial stage tumors. The authors emphasized the significant improvement in the survival of patients with moderate or negative CAIX expression in contrast to those with intense CAIX expression. Therefore, it appears to be a good prognostic marker for OSCC\(^10\). Choi et al.\(^{11}\) in 2008 and Brockton et al.\(^{12}\) in 2011 agreed that the higher expression might be associated with less favorable prognosis.

There are previous studies of CAIX expression in oral cancer\(^{10,11,13}\) and epithelial dysplasia\(^{14}\). To the best of our knowledge, there are no studies linking tobacco use to CAIX expression. And there are no studies that assess the methylation of CAIX promoter in OSCC. Thus, this study aimed to evaluate the CAIX promoter methylation and CAIX expression in oral cancer and chronically exposed oral mucosa to tobacco using exfoliative cytological samples.

METHODS

Patients and samples

This study was a multicenter study, and it was approved by the research ethics committee of Galicia, Spain and by the São Paulo State University (Unesp), Institute of Science and Technology. Informed consent was obtained from each participant.

Each group was composed of 15 samples of oral smears from males, with the diagnosis of OSCC. OSCC is more common in men than in women. In the OSCC’s group, all patients were chronic smokers’ men, consuming, at least, 20 cigarettes/day and had drinking habits; the alcohol consumption was frequent and weekly. Group 2 equivalent normal contralateral mucosa (NCM) in patients from group OSCC; group 3, chronic smokers (CS) with consumption of at least ten packs of cigarettes/year; group 4 by 15 ex-smokers (ES) in cessation for one year and group 5 by never-smokers (NS). These samples were collected at the Oral Medicine, Oral Surgery, and Implantology Unit of the Faculty of Medicine and Dentistry, University, of Santiago de Compostela; at the Smoking Cessation Program of Heart Institute, University of São Paulo School and at the outpatient clinic of Oral Medicine of São Paulo State University (Unesp), Institute of Science and Technology. The exfoliative cytology from groups CS, ES and NE were performed in the oral mucosa with no visible clinical changes in equivalent locations of the lesions of group OSCC.

Inclusion criteria for CS, ES and NS groups were: (1) male individuals with no history of oral malignancy and (2) no visible clinical signs of any change at the evaluated location and less than 20 g of alcohol consumption per week\(^{15}\). Individuals with chronic diseases were excluded from the sample.

The following information was collected in group OSCC: age, gender, tumor site and tumor stage (according to the edition of the American Joint Committee on Cancer’s, Cancer Staging Manual)\(^{16}\).

Additionally, these groups were formed by individuals with gender and age (± three years) equivalent to individuals in group OSCC. Smears were collected from the same site of the lesion in cases from group OSCC. All patients were submitted to extraoral and intraoral clinical examination.

Exfoliative cytology

Smears were made using Orcellex Rovers Brush\(^\circledR\) (Rovers Medical Devices, NL, Holland), without previous use of mouthwashes\(^{17}\). The samples were transported in DNase, RNase and pyrogenic free tubes, protected from light, containing 2 mL of RPMI (Microvet, Madrid, Spain) and stored at 2-10 °C, also protected from light\(^{18}\).
Methylation-specific polymerase chain reaction
DNA extraction was performed by Trizol technique, and bisulfite conversion was made using EpiTect Bisulfite Kit, according to manufacturer’s information (Qiagen, Valencia, Spain).

Methylation-specific polymerase chain reaction (MSP-PCR) was used, under the following conditions: 30 s at 94 °C, 1 min at 53 °C, 1 min at 72 °C for 35 cycles, 7 min at 72 °C. The amplification products are run on 2% agarose gel staining solution, with 5 mg/mL of REALSAFE nucleic acid staining Solution (Real, Valencia, Spain) and then visualized under ultraviolet light and photographed with a digital camera. In vitro methylated DNA was the positive control for methylated sequences, water was used as a negative PCR control, and DNA from tonsils was used as a control for the unmethylated reaction.

Real-time reverse transcriptase reaction
Samples were processed at the Molecular Biology Laboratory, University Hospital and School of Medicine of Santiago de Compostela Compostela, Spain. RNA extraction was performed by Trizol technique.

One microliter of RNA was used to measure the absorbance at 260 (A260) and 280 (A280) nm in a NanoDrop 1000 Spectrophotometer for each sample (Thermo Scientific, Wilmington, Delaware). The estimated concentration of RNA was obtained by multiplying by 40 the value of A260 (ng/mL). The purity, which indicates the quality of RNA, was assessed by the A260/A280 and A260/A230 ratios, where A260/A280 ratio values between 1.8 and 2.0 and A260/A230 ratio values close to 1.7 suggest RNA free of contamination. Integrity was checked by electrophoresis, performed through 1% agarose gel. Only samples with intact RNA were used. Total RNA was used for reverse transcription with MuLV reverse transcriptase PCR buffer and RNase inhibitor random hexamers (Applied Biosystems, Foster City, USA). This association was incubated at 42 °C for 45 min, then for transcriptase denaturation at 95 °C for 5 min, and after at 10 °C. The cDNA was stored at -80 °C.

The primer sequences were confirmed in the NCBI/GenBank website, which was specific for the species Homo sapiens, and homology. The selected reference gene was glyceraldehyde-3-phosphate dehydrogenase (GAPDH), among other reference genes[18]. Primers information is described in Table 1.

The quantitative real-time polymerase chain reaction (qRT-PCR) was carried out in 96-well plates (Applied Biosystems, Madrid, Spain) in an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Madrid, Spain), with a final volume of 20 μL. These tests were carried out with triplicates. As a negative control, all reagents were added to the last wells of the plates, except for cDNA, and the wells were sealed with optical adhesive (InvitrogenTM, Carlsbad, CA, USA). Thermal cycling conditions were: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. A dissociation curve analysis was added after the final PCR cycle to evaluate the presence of nonspecific PCR products and primer dimers. During initial optimization runs, 10-fold serial dilutions were employed to demonstrate a linear amplification range for each gene-specific primers.

The relative levels of gene expression were determined using the method of a quantitative curve. Normalization of quantitative cDNA in each sample was performed using the expression of the GAPDH gene as a reference gene.

Statistical analysis
The statistical analysis of the data obtained by qRT-PCR was performed using GraphPad Prism software 5.03 and SPSS 20.0. Data were presented as median, mean and standard deviation. Significance between two groups was determined by Student’s t test. A level of significance of 5% was adopted.
RESULTS

The average age of group OSCC was 58 ± 12.65 years (range 45 to 80), all patients in this group had primary OSCC and the most common locations were tongue (46%) and the floor of the mouth (40%). At diagnosis, 26.66% of patients had stage I disease, 20% had stage II disease, 0% had stage III disease, and 53.33% had stage IV disease. Well-differentiated cases were 20%, 40% moderately differentiated and poorly differentiated cases were 20% of the total.

The CAIX gene was methylated in 20% of group OSCC cases and no case in groups NCM, CS, ES, and NS. The results of the methylation analysis in different cases can be seen in Table 2. There was no statistically significant difference related the methylation status of histological grade (P = 0.43), stage (P = 0.11) or location of the lesion (P = 0.28).

The gene expression can be visualized in Figure 1. There was a statistically significant difference between CAIX expression of group OSCC and group NS patients (P = 0.005), with lower expression in group OSCC. There was no statistically significant difference between CAIX expression of the group NCM and NS patients (P = 0.285), group CS and group NS patients (P = 0.530) and between group ES and NS patients (P = 0.068).

DISCUSSION

CAIX methylation was higher in OSCC and its expression was reduced in the same cases with methylation. However, the profile of gene methylation and expression was not modified in chronic smokers that used tobacco for 20 years or in ex-smokers after one year of smoke cessation. This study aimed to connect methylation CAIX promoter with the low expression of CAIX in oral carcinogenesis process, using exfoliative cytological samples. Therefore, the tumor contralateral normal mucosa was assessed to study the cancerization field, the normal mucosal cells of chronic smokers to study damage caused by chronic use of tobacco, and the normal mucosal cells of ex-smokers after one year of smoke cessation to study the changes in the damage associated to smoke cessation.

CAIX promoter methylation was observed in 20% of OSCC. No correlation between histological grade, stage or location of the disease was observed. This may be due to a limited number of samples. Studies have demonstrated that CAIX expression is predominantly regulated by methylation of a single CpG site, at the 74 bp position in some kinds of cancers, like renal cancer [19,20] or in diffuse-type gastric cancer [21,22]. These results suggest the hypoxia-independent regulation of CAIX in this type of cancer [21,22]. There are no comparable studies concerning the methylation of CAIX promoter in OSCC. Therefore, it is not possible to compare the preliminary results found. Nevertheless, Beasley et al. [23] (2001) previously suggested that CAIX is regulated by hypoxia in vivo.

Table 2: Results of the methylation of carbonic anhydrase IX analysis in all cases

<table>
<thead>
<tr>
<th>Cases</th>
<th>Oral cell squamous carcinoma</th>
<th>Normal contralateral mucosa</th>
<th>Smokers</th>
<th>Ex-smokers</th>
<th>No smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>M 0.006</td>
<td>Case 1 U 0.331</td>
<td>Case 1 U 0.628</td>
<td>Case 1 U 1.101</td>
<td>Case 1 U 1</td>
</tr>
<tr>
<td>Case 2</td>
<td>U 0.411</td>
<td>Case 2 U 0.872</td>
<td>Case 2 U 0.815</td>
<td>Case 2 U 0.129</td>
<td>Case 2 U 1</td>
</tr>
<tr>
<td>Case 3</td>
<td>U 0.240</td>
<td>Case 3 U 0.755</td>
<td>Case 3 U 1.259</td>
<td>Case 3 U 1.390</td>
<td>Case 3 U 1</td>
</tr>
<tr>
<td>Case 4</td>
<td>M 0.007</td>
<td>Case 4 U 0.455</td>
<td>Case 4 U 0.727</td>
<td>Case 4 U 1.259</td>
<td>Case 4 U 1</td>
</tr>
<tr>
<td>Case 5</td>
<td>U 0.369</td>
<td>Case 5 U 0.441</td>
<td>Case 5 U 1.014</td>
<td>Case 5 U 0.512</td>
<td>Case 5 U 1</td>
</tr>
<tr>
<td>Case 6</td>
<td>U 0.344</td>
<td>Case 6 U 0.405</td>
<td>Case 6 U 1.106</td>
<td>Case 6 U 1.419</td>
<td>Case 6 U 1</td>
</tr>
<tr>
<td>Case 7</td>
<td>M 0.034</td>
<td>Case 7 U 0.475</td>
<td>Case 7 U 1.224</td>
<td>Case 7 U 0.455</td>
<td>Case 7 U 1</td>
</tr>
<tr>
<td>Case 8</td>
<td>U 0.363</td>
<td>Case 8 U 0.702</td>
<td>Case 8 U 0.982</td>
<td>Case 8 U 1.356</td>
<td>Case 8 U 1</td>
</tr>
<tr>
<td>Case 9</td>
<td>U 0.372</td>
<td>Case 9 U 0.621</td>
<td>Case 9 U 2.562</td>
<td>Case 9 U 0.535</td>
<td>Case 9 U 1</td>
</tr>
<tr>
<td>Case 10</td>
<td>M 0.055</td>
<td>Case 10 U 0.468</td>
<td>Case 10 U 0.987</td>
<td>Case 10 U 0.492</td>
<td>Case 10 U 1</td>
</tr>
<tr>
<td>Case 11</td>
<td>M 0.094</td>
<td>Case 11 U 0.416</td>
<td>Case 11 U 0.691</td>
<td>Case 11 U 1.419</td>
<td>Case 11 U 1</td>
</tr>
<tr>
<td>Case 12</td>
<td>U 0.440</td>
<td>Case 12 U 0.464</td>
<td>Case 12 U 0.511</td>
<td>Case 12 U 0.109</td>
<td>Case 12 U 1</td>
</tr>
<tr>
<td>Case 13</td>
<td>U 1.109</td>
<td>Case 13 U 0.841</td>
<td>Case 13 U 0.585</td>
<td>Case 13 U 0.455</td>
<td>Case 13 U 1</td>
</tr>
<tr>
<td>Case 14</td>
<td>U 1.129</td>
<td>Case 14 U 0.543</td>
<td>Case 14 U 0.664</td>
<td>Case 14 U 1.510</td>
<td>Case 14 U 1</td>
</tr>
<tr>
<td>Case 15</td>
<td>U 1.257</td>
<td>Case 15 U 0.505</td>
<td>Case 15 U 0.504</td>
<td>Case 15 U 1.390</td>
<td>Case 15 U 1</td>
</tr>
</tbody>
</table>

M: methylated; U: unmethylated
OSCC cases presented lower expression than never-smoker patients’ mucosa. To best of our knowledge, no other studies addressing CAIX methylation in OSCC, as well as related to CAIX gene expression in oral mucosa chronically exposed to tobacco. There are many studies evaluating CAIX protein expression in OSCC using different immunohistochemical evaluation methods. Pérez-Sayáns et al. (2012) concluded that CAIX was overexpressed in OSCC. In contrast, other authors demonstrated protein expression in a limited number of cells. Kondo et al. (2011) reported that 98% of OSCC cases showed more than 10% positive cells in the sample. Kim et al. (2007) found that 63.3% cases showed more than 10% positive cells in the sample. Eckert et al. (2012) found CAIX positivity in 42.5% of OSCC samples. Choi et al. (2008) reported that 58.1% OSCC cases showed more than 5% positive cells in the sample. Roh et al. (2009) found 40.47% OSCC cases with more than 1% positive cells in the sample.

Additionally, it is important to note that unlike the studies cited, this study used exfoliative cytology, and smears in OSCC were collected in an ulcerated lesion. In other groups, the epithelium was intact.

A lower expression was observed in OSCC if compared to the control. This lower expression may be due to the fact that there are three cases in this group with methylation in CAIX promoter, with lower expression in these cases. As it was observed in the evaluation of methylation, the CAIX profile expression is not modified in tumors in the contralateral normal mucosa, in chronic smokers, and in ex-smokers after a year of smoke cessation. Notably, the exfoliated cells are removed from the surface layers of the epithelium. In this case, it was performed in mucosa without any alteration, as already mentioned. Taking this into consideration, in this study, there was no observed change of the epithelium in the expression of CAIX caused by tobacco use, since CAIX is usually absent in normal epithelium.

Pérez-Sayáns et al. (2014) studied epithelial dysplasia adjacent to surgical resection margins of OSCC and found no statistically significant relationship between the expression of CAIX with any other variables, including the degree of dysplasia and the intensity of expression in the tumor. Furthermore, CAIX expression was significantly associated with postoperative disease recurrence and with worse overall survival in patients with oral cancer. Kim et al. (2007) showed that the correlation of CAIX expression with prognosis might become indirect evidence that hypoxia is a poor prognostic sign. Pérez-Sayáns et al. (2012) concluded that survival in patients with moderate or negative expression improves significantly in contrast to those patients with intense CAIX expression. However, Kondo et al. (2011) found that CAIX expression had no significant impact on the survival rate of the patients.

Eckert et al. (2010) studied the expression of CAIX and HIF-1α, finding that low expression of CAIX is associated with increased HIF-1α expression and it was correlated to worse prognosis, but also that a low expression of both CAIX and HIF-1α was associated with the best prognosis. The authors discussed that there are two possibilities to explain this phenomenon: CAIX plays no important role in progression of OSCC, or this enzyme possesses different roles in OSCC. Additionally, Eriksen et al. (2007) also concluded that there is no correlation between patient or tumor characteristics with CAIX expression and, apparently, no prognostic value of CAIX was demonstrated. Their results indicate that CAIX alone is not a specific marker for head and neck SCC with known modifiable hypoxia.

On the other hand, Eckert et al. (2012) found that elevated stromal CAIX expression

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**Figure 1:** Carbonic anhydrase IX expression, relative quantification (Log) using quantitative real-time polymerase chain reaction. Values are expressed as mean and standard deviation. Student’s $t$-test was used to compare gene expression ($^{*}P < 0.05$).
is associated with reduced 5-year disease-specific survival in patients with OSCC.

In conclusion, within the limitations of this study, since it is just a pilot study, it was demonstrated that CAIX's methylation is higher in OSCC and its expression is reduced in cases with methylation. Therefore, authors may consider that CAIX promoter methylation appears in more favorable prognosis cases and not in all cases. The methylation and expression's gene profile was not modified in chronic smokers and ex-smokers with one year of smoking cessation. Further studies, with larger samples, are necessary to better determine the CAIX's role in carcinogenesis stages better.

DECLARATIONS

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Conflicts of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

Patient consent
Informed consent was obtained from each participant.

Ethics approval
It was approved by the research ethics committee of Galicia, Spain and by the São Paulo State University (Unesp), Institute of Science and Technology.

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stomach complementary DNA sequence and expression in human and rat alimentary tracts. *Gastroenterology* 1997;112:398-408.


