Oliveira Alves *et al. Stomatological Dis Sci* 2017;1:109-15
DOI: 10.20517/2573-0002.2017.05

Stomatological Disease and Science
www.sdsjournal.com

Original Article **Open Access**

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

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How to cite this article: Oliveira Alves MG, Lima Carta CF, Padín-Iruegas ME, Pérez-Sayáns M, López-López R, Lago NM, Suárez-Peñaranda JM, Issa JS, García-García A, Almeida JD. Gene expression and carbonic anhydrase IX promoter methylation in oral cancer and smokers oral mucosa: a pilot study. *Stomatological Dis Sci* 2017;1:109-15.

Article history:
Received: 25 May 2017
First Decision: 10 Oct 2017
Revised: 23 Oct 2017
Accepted: 21 Nov 2017
Published: 25 Dec 2017

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

ABSTRACT

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 to 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.

Quick Response Code:

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® (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[6].

Table 1: Primers information

		Primers sequences
qRT-PCR	*CAIX*	Forward 5'-GCCCAGCACTTTATGATGGT-3'
		Reverse 5'-TACAGGGCTTTGGAGCAACT-3'
	GAPDH	Forward 5'-AGGTCATCCATGACAACTTTG-3'
		Reverse 5'-TTCAGCTCAGGGATGACCTT-3'
MSP-PCR	*CAIX*	Methylate forward 5'AGTGTAATGGCGCGATTTC
		Methylate reverse 5'AACGAAACCCCGTCTCTACTAA
		Unmethylate forward 5'TGGAGTGTAATGGTGTGATTTT
		Unmethylate reverse 5'AACAAAACCCCATCTCTACTAAAAA

qRT-PCR: quantitative real time polymerase chain reaction; MSP-PCR: methylation specific polymerase chain reaction; *CAIX*: carbonic anhydrase IX; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase

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/Gene Bank 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.

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

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

M: methylated; U: unmethylated

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 40%, 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*.

In the cases studied in the present research, *CAIX* methylation was higher in OSCC. Cases with *CAIX* methylation presented reduced expression of *CAIX*.

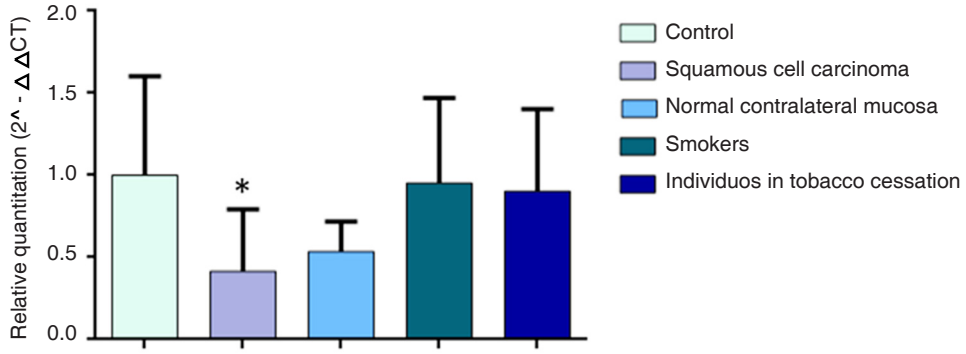

Figure 1: Carbonic anhydrase IX expression, relative quantification (Log) using quantitative real-time polymerase chain reaction. Values are expressed as mean and standard deviation. Student's *t*-test was used to compare gene expression (*$P \leq 0.05$)

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[10,11,13,24-26]. Pérez-Sayáns *et al.*[10] (2012) concluded that *CAIX* was overexpressed in OSCC. In contrast, other authors demonstrated protein expression in a limited number of cells[11,13,24-26]. Kondo *et al.*[24] (2011) reported that 98% of OSCC cases showed more than 10% positive cells in the sample. Kim *et al.*[13] (2007) found that 63.3% cases showed more than 10% positive cells in the sample. Eckert *et al.*[25] (2012) found *CAIX* positivity in 42.5% of OSCC samples. Choi *et al.*[11] (2008) reported that 58.1% OSCC cases showed more than 5% positive cells in the sample. Roh *et al.*[26] (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[27].

Pérez-Sayáns *et al.*[27] (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[11]. Kim *et al.*[13] (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.*[10] (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.*[24] in 2011 found that *CAIX* expression had no significant impact on the survival rate of the patients.

Eckert *et al.*[28] (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[25]. Additionally, Eriksen *et al.*[29] (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[29].

On the other hand, Eckert *et al.*[28] and Brockton *et al.*[30] (2012) found that elevated stromal *CAIX* expression

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

Acknowledgments
The authors thank Rovers Medical Devices for donating a Rovers® Orcellex® Brush, and the Pathology Department of the University Hospital and School of Medicine of Santiago de Compostela, Spain, for their collaboration. The authors also acknowledge the efforts of Dr. Jon Wanger in refining the final manuscript.

Authors' contributions
Conception and design of the study: M.G. Oliveira Alves, C.F. Lima Carta, M.E. Padín-Iruegas, M. Pérez-Sayáns
Acquisition of data: M.G. Oliveira Alves, C.F. Lima Carta, M.E. Padín-Iruegas, N.M. Lago, J.S. Issa, A. García-García
Analysis and interpretation of data: M.G. Oliveira Alves, M.E. Padín-Iruegas, M. Pérez-Sayáns, J.M. Suárez-Peñaranda
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Financial support and sponsorship
The study was supported by the Coordination for the Improvement of Higher Education Personnel (CAPES), grants #2012/05371-4 and 2013/06251-5, São Paulo Research Foundation (FAPESP).

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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