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# Methylation analysis of cancer-related genes in non-neoplastic cells from patients with oral squamous cell carcinoma

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**Abstract** Early detection of Oral Squamous Cell Carcinoma (OSCC) is important to reduce mortality rates and to help provide successful cancer treatment. Hypermethylation of CpG islands is a common epigenetic mechanism that leads to gene silencing in tumors and could be a useful biomarker in OSCC. Abnormal DNA hypermethylation can occur very early in cancer development and may be induced by exposure to environmental carcinogens. We set out to investigate the methylation status of cancer-related genes in normal oral exfoliated cells from OSCC patients and healthy volunteers, as well as possible associations with alcohol/tobacco exposure or specific tumor characteristics. The methylation status of *CDKN2A* (*cyclin-dependent kinase inhibitor 2A or p16*), *SFN* (*stratifin* or *14-3-3 σ*), *EDNRB* (*endothelin receptor B*) and *RUNX3* (*runt-related transcript factor-3*) was evaluated by MSP (Methylation-Specific Polymerase Chain Reaction) analysis in non-neoplastic oral epithelial cells from OSCC patients ($n = 70$) and cancer-free subjects ($n = 41$). Hypermethylation was observed in *CDKN2A*, *EDNRB* and *SFN* genes, whereas no methylation was found in the *RUNX3* gene. *CDKN2A* hypermethylation occurred only in the OSCC group (5.7%) while *SFN* and *EDNRB* hypermethylation occurred in both groups. There was no association between hypermethylation and smoking, drinking habits or specific tumor characteristics.

**Keywords** Oral squamous cell carcinoma · Methylation status · Cancer-related genes · Normal oral mucosa

## Introduction

Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer, accounting for over 90% of all mouth malignancies and 38% of head and neck tumors [1, 2]. Worldwide, OSCC is the eighth most common human cancer [3], with more than 300,000 new cases being diagnosed every year [4]. Oral carcinoma detection in early asymptomatic stages dramatically improves cure rates and quality of life by minimizing debilitating surgical treatments [5].

Oral squamous cell carcinoma arises from multiple genetic and epigenetic alterations, which are modulated by inherited predispositions and environmental influences [6]. Several risk factors have been associated with the development of oral cancer, most of them are related to diet, lifestyle and living habits. Because of the well known association between cancers of the oral cavity and tobacco smoking and/or alcohol consumption, it has been suggested that these risk factors could be potential inducers of

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genetic/epigenetic alterations in oral mucosa cells, and that these alterations would accumulate in the normal-appearing mucosa of heavy smokers and heavy drinkers before any tumor lesion is detected [7–9].

Tumor suppressor gene transcriptional inactivation by CpG island hypermethylation within gene promoter regions could be the initial event for the development of some forms of cancer [10]. Previous studies have shown that the largest number of cancer transcriptional changes happen during the transition from normal mucosa to the premalignant state, suggesting that many of the molecular changes accumulated in oral cancer occur preferentially in the early stages [6, 11]. Thus, developing biomarkers for epigenetically altered cells can be a valuable tool in the evaluation of cancer risk and prognostic [10].

Hypermethylation of some tumor suppressor genes has been observed in OSCC normal adjacent mucosa [12], dysplastic tissue [13] and leukoplakia [14], therefore being considered as an early event in oral carcinogenesis. Moreover, previous studies have identified aberrant DNA methylation in matched body fluid samples, such as OSCC patient salivary rinses, proposing potential noninvasive biomarkers for early detection of OSCC [15, 16].

Methylation profiling has been previously shown to be effective in distinguishing normal from tumor tissues [17, 18]. Although some variability was found in these studies, it has become clear that a better characterization of tumor suppressor gene promoter methylation in cancer development and in response to common risk factors, such as alcohol and tobacco, can be useful as diagnostic, prognostic or therapeutic biomarkers.

We chose to investigate the promoter methylation status of four cancer-related genes (*CDKN2A*, *EDNRB*, *RUNX3* and *SFN*) in normal appearing oral mucosa of OSCC patients and cancer-free/carcinogen-free controls. These genes have been previously reported as hypermethylated in OSCC and therefore were considered potential biomarkers for this cancer [17, 18]. Based on these data, we have determined the relationship between normal mucosa gene methylation status and OSCC presence, carcinogen exposure or tumor characteristics of malignancy.

## Materials and methods

### Patients

Cytobrush samples were collected from seventy untreated OSCC patients at the Santa Rita de Cassia Hospital, Espirito Santo, Brazil. The cancer-free group was composed by 41 individuals who had a negative cancer familial history, without exposure to risk factors (tobacco and alcohol) and did not show pre-malignant or malignant oral lesions at the time of sample collection. All subjects provided signed informed consents approved by institutional review boards. This work was approved by the Research Ethics Committee of the Espirito Santo Federal University (CEP-UFES Protocol n° 120/2006).

### Sample collection and DNA extraction

Samples consisted of exfoliated oral cells, collected by scraping the oral mucosa and tongue posterior edge for 10 s using a sterile cytological brush. Immediately after collection, the brush was rinsed in TE (10 mM Tris–HCl, pH 7.6, 1 M EDTA) and samples were stored at 4–8°C. For DNA extraction, cells were treated by 1% SDS/proteinase K (0.5 mg/ml) at 48°C for 16 h. High molecular weight DNA was isolated by standard phenol/chloroform extraction and ethanol precipitation.

### Methylation-specific polymerase chain reaction (MSP)

Genomic DNA (270 ng) was subjected to bisulfite modification with the *methylSEQr*$^{TM}$ *Bisulfite Conversion Kit* (Applied Biosystems, Foster City, CA, USA). All primers used in this study have been previously described and were specific to methylated and unmethylated sequences [19–22]. DNA from the urothelial carcinoma derived T24 cell line was used as the methylation positive control for *CDKN2A* and *EDNRB* genes [23, 24] and DNA from the urothelial carcinoma derived 5,637 cell line was used as the methylation positive control for *RUNX3* and *SFN* genes [25, 26]. Normal lymphocyte DNA was used as unmethylated control for *CDNK2A*, *EDNRB* and *RUNX3* genes, and normal epithelial cell DNA was used for the *SFN* gene [22].

MSP was performed using specific primers for methylated and unmethylated sequences, in distinct 25 μl reactions, according to Platinum *Taq* DNA Polymerase enzyme manufacturer's recommendations (Invitrogen Life Technologies, Carlsbad, CA, USA). Amplified fragments were visualized by electrophoresis in 8% polyacrylamide gels and silver nitrate staining. Water blanks and unmodified DNA controls were used. Oligonucleotide sequences, annealing temperatures and MSP fragment sizes are shown in Table 1. Figure 1 shows a representative MSP acrylamide gel for all analyzed genes.

### DNA sequencing

To validate the assay methylation specificity, PCR products were purified using ExoSAP-IT PCR Clean-up Kit (GE Healthcare Life Sciences, Uppsala, Sweden) and

**Table 1** Oligonucleotide sequences, annealing temperatures, and fragment sizes for MSP analysis

| Gene | Primer 5′–3′ | Position of interrogated CpGs[a] | AT (°C) | Fragment size (bp) | Ref. |
|---|---|---|---|---|---|
| *CDKN2A* | | | | | |
| Methylated allele | F: TTA TTA GAG GGT GGG GCG GAT CGC | 149, 154, 156, 265, 269, 271, 276, 282 | 63 | 150 | [19] |
| | R: GAC CCC GAA CCG CGA CCG TAA | | | | |
| Unmethylated allele | F: TTA TTA GAG GGT GGG GTG GAT TGT | | 58 | 151 | |
| | R: CAA CCC CAA ACC ACA ACC ATA A | | | | |
| *EDNRB* | | | | | |
| Methylated allele | F: CGAAGAGGTTGCGGGCGGTATTAGCG | 116, 127, 131, 140, 225, 232, 236 | 62 | 130 | [20] |
| | R: TACTCCAAAAACGTCCGATAACCG | | | | |
| Unmethylated allele | F: TGGTGAAGAGGTTGTGGGTGGTATTAGTG | | | 134 | |
| | R: ACCTACTCCAAAAACATCCAATAACCA | | | | |
| *RUNX3* | | | | | |
| Methylated allele | F: TATTCGTTAGGGTTCGTTCGT | 144, 151, 235, 239, 249 | 61 | 119 | [21] |
| | R: AAACAACCACGAAAAACGAC | | | | |
| Unmethylated allele | F: AAGTGGGAAAGTAGAAGTGGTG | | | 125 | |
| | R: CCAAACAAACTACAAACAACCA | | | | |
| *SFN* | | | | | |
| Methylated allele | F: TGGTAGTTTTTATGAAAGGCGTC | 153, 156, 219, 228 | 61 | 104 | [22] |
| | R: CCTCTAACCGCCCACCACG | | | | |
| Unmethylated allele | F: ATGGTAGTTTTTATGAAAGGTGTT | | 56 | 106 | |
| | R: CCCTCTAACCACCCACCACA | | | | |

*MSP* methylation-specific polymerase chain reaction, *AT* annealing temperature, *bp* base pair, *F* forward primer, *R* reverse primer

[a] Position in relation to transcription start site

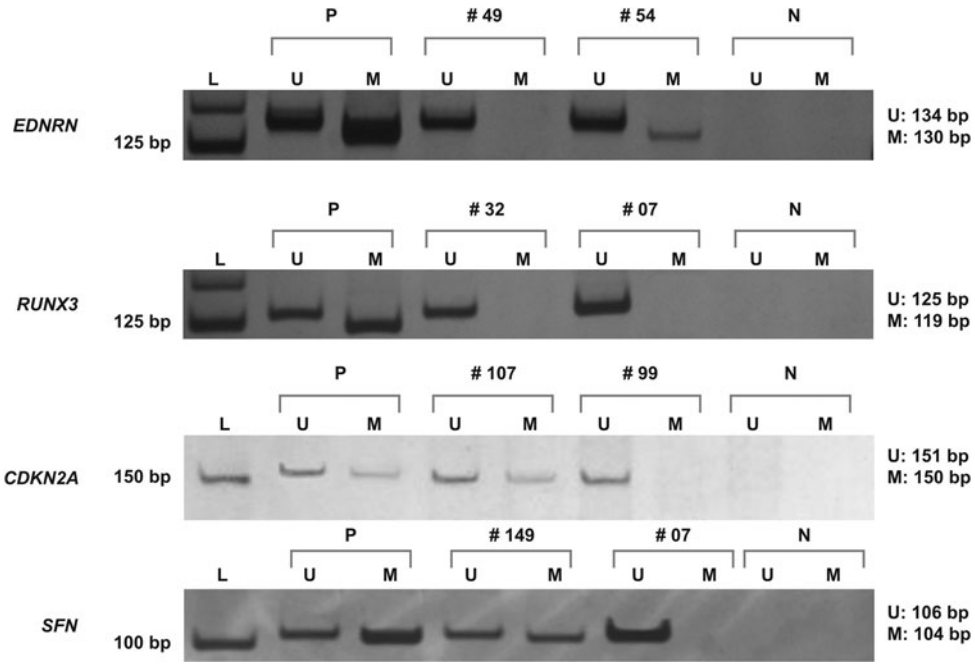


**Fig. 1** Representative MSP acrylamide gel. *EDNRB, RUNX3, CDKN2A and SFN* genes promoter methylation assay. *U* unmethylated control; *M* methylated control; *P* positive control; *N* negative control. *L* size ladder. # Patient case number

sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Capillary electrophoresis was performed in the ABI Prism® 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Results were blasted in the MethBLAST site (http://medgen.ugent.be/methBLAST/).

### Statistical analysis

Statistical analysis was performed by the chi-square test or Fisher's exact test ($P < 0.05$) using the GRAPHPAD PRISM V5 statistical software. Age comparison was performed using the nonparametric Mann–Whitney test.

## Results

### Population description

Of the seventy OSCC patients, 53 (75%) were smokers, 17 (25%) were nonsmokers, 44 (63%) were drinkers, 26 (37%) were non-drinkers and 40 (57%) were smokers and drinkers simultaneously. Subjects age ranged from 24 to 87 years (mean = 59 years, 53 males and 17 females). The cancer-free group was formed by 41 volunteers (31 females and 10 males). The mean age of the control group was 47 years old. Age and gender of OSCC cases were significantly different from controls ($P < 0.05$).

### Methylation status of OSCC patients vs. cancer-free controls

Methylation patterns were not significantly different between non-neoplastic oral cells from OSCC patients and from healthy control individuals. Nonetheless, the *CDKN2A* gene showed a hypermethylation pattern in mucosa of OSCC patients (5.7%), whereas no hypermethylation was found in cancer-free controls. In contrast, *SNF* gene methylation pattern was lower in non-adjacent, non-neoplastic cells of OSCC patients (54.3%) when compared to OSCC-free controls (73.2%). Methylation analysis results are shown in Table 2. Random results were selected and validated by sequencing. A statistically significant relationship between methylation status and age was not found in this study.

### Methylation status vs. tumor characteristics

The following tumor characteristics were analyzed: tumor stage (0, I, II, III and IV), histological grade (low, moderate and high differentiation grade and undifferentiated), anatomical site (upper/lower lip, lateral/ventral/base tongue, mouth floor, buccal mucosa, hard/soft palate, retromolar area, superior/inferior alveolar ridge). No statistically significant relationship between stage, anatomical site or histological grade variables and DNA methylation patterns was found.

### Methylation status vs. tobacco/alcohol consumption

No statistically significant relationship was found between gene methylation status and tobacco smoking/alcohol intake.

## Discussion

Cancer initiation and progression are accompanied by changes in DNA methylation, which can be used as potential biomarkers for cancer diagnostic, prognostic or treatment [27, 28]. Various studies indicate that abnormal DNA methylation may occur early in cancer development, including OSCC, contributing to cancer initiation [10, 29]. Hypermethylation favors cancer progression by silencing tumor suppressor genes or DNA repair genes [10, 30].

In addition, DNA methylation is influenced by age [31–33], dietary intake [34, 35] and environmental factors [33, 36–38]. Analysis of healthy control individuals is important in the characterization of promoter methylation variation according to age, and in response to common environmental exposures such as alcohol and tobacco smoking [33, 39].

In this study we have analyzed the methylation status of four cancer-related genes in non-neoplastic oral cells from OSCC patients and OSCC-free controls that were not exposed to tobacco smoking or alcohol consumption. Our aim was to investigate whether normal cells from oral cancer patients would be more committed to epigenetic

**Table 2** Univariate analysis of gene methylation status in OSCC patients vs. normal controls

| Gene/methylation status | OSCC oral mucosa DNA (%) | Normal control DNA (%) | *P* value |
|---|---|---|---|
| *CDKN2A* gene | | | |
| Negative | 66 (94.3) | 41 (100) | |
| Positive | 4 (5.7) | 0 | 0.2946 |
| *EDNRB* gene | | | |
| Negative | 55 (78.6) | 31 (75.6) | |
| Positive | 15 (21.4) | 10 (24.4) | 0.8148 |
| *RUNX3* gene | | | |
| Negative | 70 (100) | 41 (100) | |
| Positive | 0 | 0 | – |
| *SFN* gene | | | |
| Negative | 32 (45.7) | 11 (26.8) | |
| Positive | 38 (54.3) | 30 (73.2) | 0.0688 |
| ≥2 methylated genes | | | |
| Negative | 59 (84.3) | 32 (78) | |
| Positive | 11 (15.7) | 9 (22) | 0.4491 |

early events than similar cells from individuals without oral neoplasia, and if such changes would be associated with exposure to alcohol, tobacco or specific tumor characteristics. Our findings demonstrate a remarkable difference between the *CDKN2A* methylation pattern in non-neoplastic cells from OSCC patients (5.7%) and cancer-free controls (0%). Despite not been statistically significant, our data show an increased rate of *CDKN2A* methylation in normal mucosa of OSCC patients. *CDKN2A* encodes the cell cycle regulatory protein *p16* (*INK4A*) which binds cyclin-dependent kinases and inhibits their activities, resulting in hypophosphorylation of the retinoblastoma protein (pRb), an inhibitor of cell cycle progression. *CDKN2A* promoter methylation has been widely investigated and reported in the literature, varying from 31 to 67% [12, 13]. In addition, *CDKN2A* gene methylation was also found in dysplastic tissue (33%) [13], leukoplakia (44%) [14] and tumor adjacent normal cells (27–50%) [12, 40], reasons because it has been previously described as an early change in oral carcinogenesis. Although some studies have reported a high methylation frequency of normal tissue surrounding tumors, there is a lack of information regarding tumor-distant cells. In this study, 5.7% of OSCC patients showed *CDKN2A* methylation in non-neoplastic cells distant from the tumor site, whereas oral cancer free controls showed 0% methylation in such cells.

Drinking and smoking habits were expected to induce widespread field change in the upper aero-digestive tract [41–43], and some studies have demonstrated a synergistic effect between alcohol and tobacco [44]. Moreover, the presence of *CDKN2A* methylation in oral cavity cells from smokers without clinical evidence of oral cancer has been demonstrated [15, 36, 43, 45], suggesting that smoking increases epigenetic changes in normal cells. Nonetheless, in our study, *CDKN2A* hypermethylation was not associated with smoking or drinking habits, either separately or concurrently, corroborating other studies who found a low *CDKN2A* methylation rate in normal cells from the resection margin of smoker patients with OSCC or oral dysplasia [46, 47]. In addition, *CDKN2A* methylation status was not altered by tumor histological grade or anatomical site. An overall methylation trend has been associated with age in normal human prostate and colon tissues for several genes [32, 48], suggesting that the aging process may influence methylation changes, which could account for some of the methylation found in our study.

*RUNX3* gene hypermethylation was absent in both sample groups. Runt-related transcription factor 3 (*RUNX3*) is a tumor suppressor gene that appears to be an important component of the transforming growth factor-beta (TGF-$\beta$)-induced tumor suppression pathway. The lack *RUNX3* gene expression stimulates cell proliferation and suppression of apoptosis in epithelial cells [49]. *RUNX3* reduced expression was observed in various cancers and was frequently caused by CpG island hypermethylation [50–56]. Gao et al. [56] determined the *RUNX3* methylation status in OSCC tumor adjacent tissue and mucosa from healthy volunteers. Whereas no methylation was found in the latter group, a high rate was found in OSCC (70%) and intermediate rates were reported in tumor adjacent normal cells (53.3%). The absence of *RUNX3* methylation in normal mucosa distant from tumor reveals that abnormal *RUNX3* hypermethylation is not a widespread event in the oral cavity prior to or contributing with cancer initiation, therefore it should not be used as a marker for early oral cancer detection. It has been previously reported in bladder cancer that *RUNX3* methylation is increased as a function of age at diagnosis and smoking history [57]. Unlike bladder cancer, *RUNX3* methylation was not found in normal oral cells of individuals exposed to tobacco or alcohol, neither an age association was detected.

Interestingly, hypermethylation was observed in both samples groups for *EDNRB* and *SFN* genes, but was not associated with alcohol or tobacco use, age or gender. *EDNRB* encodes the endothelin B receptor that mediates vasoconstriction. Although its role in carcinogenesis is still largely unknown, *EDNRB* has been reported to be silenced in some cancers through CpG island methylation [16, 58] and was considered as a potential molecular marker for patients at risk of developing precancerous lesions and oral cancer [16]. Nonetheless, our results suggest that *EDNRB* methylation is not an effective biomarker for early diagnosis of OSCC.

*SFN* is a major G2/M checkpoint control gene and its inactivation in various cancers has been shown to occur mostly by epigenetic mechanisms instead of gene mutation. Gasco et al. [59] reported a *SFN* methylation frequency of 34.8% in OSCC lesions, 50% in oral dysplasias and no methylation was found in normal tissue. Bhawal et al. [60] reported a methylation rate of 13% in OSCC but not in adjacent non-malignant cells and normal gingival tissues. Our results showed a hypermethylation pattern in 54.3% of non-adjacent, non-neoplastic cells of OSCC patients and 73.2% in OSCC-free controls. Despite not been statistically significant, $P$ value was borderline significant ($P = 0.068$) indicating that a lower *SFN* methylation rate is found in OSCC patients. Because *SFN* methylation has been reported in normal lymphocytes [61] these findings may indicate that lymphocytes could be less prevalent in predisposed mucosa. Moreover, *SFN* hypermethylation was not associated with tobacco or alcohol consumption.

Due to the fact that aberrant DNA methylation of cancer-related genes has been associated with cancer initiation and progression, as well as with carcinogen exposure, we have set out to investigate a possible role of *CDKN2A*, *EDNRB*, *RUNX3* and *SFN* in unaffected mucosa of OSCC patients, in

an attempt to identify useful biomarkers for non-invasive early cancer detection or prognosis. We have shown that these genes are not epigenetically silenced in normal mucosa of carcinogen-exposed individuals, and do not correlate with tumor features of malignancy. Nonetheless, we have found that *CDKN2A* promoter methylation occurs only in the normal mucosa of OSCC patients, when compared with oral cancer-free controls. Therefore, *CDKN2A* methylation could be useful as a non-invasive assay for early detection of OSCC, suggesting that further studies are needed to fully characterize its potential as a biomarker.

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