Archives of Oral Biology 92 (2018) 83–87

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

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

Celina Faig Lima Carta[a,b,*], Mônica Ghislaine Oliveira Alves[a,b,c], Patrícia Pimentel de Barros[a], Márcia Sampaio Campos[d], Jaqueline Scholz[e], Antonio Olavo Cardoso Jorge[a], Fábio Daumas Nunes[f], Janete Dias Almeida[a]

[a] Department of Biosciences and Oral Diagnosis, São Paulo State University (Unesp), Institute of Science and Technology, São José dos Campos, Brazil
[b] University Braz Cubas, Mogi das Cruzes, Brazil
[c] Universidade de Mogi das Cruzes, Mogi das Cruzes, Brazil
[d] Angiogenesis Research Laboratory, Department of Cariology, Restorative Sciences and Endodontics, University of Michigan School of Dentistry, Ann Arbor, MI, United States
[e] Heart Institute, University Hospital, Medical School, Universidade de São Paulo, São Paulo, Brazil
[f] Department of Oral Pathology, School of Dentistry, University of Sao Paulo, SP, Brazil

ARTICLE INFO

Keywords:
Methylation
Repair gene
Smoking
Oral mucosa
Carcinogenesis

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

* Corresponding author at: University Braz Cubas, Av Francisco Rodrigues Filho, 1233, Mogilar, Mogi das Cruzes, São Paulo, 08773-380, Brazil.
E-mail address: celina.carta@brazcubas.br (C.F.L. Carta).

https://doi.org/10.1016/j.archoralbio.2018.04.017
Received 22 October 2017; Received in revised form 29 April 2018; Accepted 30 April 2018

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