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Research paper

Thioredoxin and metallothionein: Homeostasis-related proteins in lip carcinogenesis

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ABSTRACT

Objective: Thioredoxin (Trx) and metallothionein (MT) are involved in the development of some carcinomas; however, the role of these proteins in labial carcinogenesis has not yet been tested. The aims of the study were to evaluate and to correlate the immunoexpression of Trx and MT in actinic cheilitis, lip squamous cell carcinoma, and normal vermillion lip mucosa.
Design: Immunohistochemistry was undertaken for Trx and MT in samples of actinic cheilitis, lip squamous cell carcinoma, and normal lip mucosa. Qualitative and semi-quantitative evaluations were conducted. The proportion of stained cells, intensity of staining, and the cell compartment labeled were evaluated. A *quickscore* index was also calculated by multiplying the values of extension and intensity of nuclear and cytoplasmic staining, respectively, giving a maximum value of 9. Statistics were performed.
Results: A remarkable nuclear Trx staining was seen in normal lip mucosa and cheilitis, not in carcinoma ($p < 0.05$). Cytoplasmic Trx expression was widely detected in all lesions ($p > 0.05$). MT was broadly expressed in nuclei and cytoplasm of carcinoma, but not in normal lip mucosa and cheilitis ($p < 0.05$). *Quickscores* were in accordance with the qualitative results.
Conclusions: The current study showed a different immunopattern of Trx and MT between normal lip mucosa, actinic cheilitis and lip squamous cell carcinoma. The cellular compartment-based analyses evidenced differences that can be related to the proteins function. Considering the relevant roles of these proteins in cellular homeostasis, they seem to have an important role in lip carcinogenesis.

1. Introduction

Oral cancer is a public health problem worldwide, and there is an estimative of 11,140 new cases in men and 4350 in women in 2016 in Brazil (INCA, 2015). Lip cancer accounts for 25%–30% of all oral cancers (Vieira, Minicucci, Marques, & Marques, 2012), and it can be preceded by actinic cheilitis (AC), a potentially malignant disorder. Whereas intra-oral squamous cell carcinoma (SCC) is mainly associated with tobacco and alcohol intake, lip cancer has a different pathogenesis, being clearly associated with long-lasting unprotected sun exposure. Brazil is situated near the Equator line, and most of its territory presents a tropical climate with high incidence of solar radiation (Corrêa, 2015; INCA, 2010). At this scenario, the faired-skinned Brazilians with occupational exposure to solar radiation are at high risk of lip SCC development. There is an expectative of 80,850 new cases of sun-related skin cancer (except melanoma) in men and 94,910 in women in 2016 in Brazil (INCA, 2015).

AC is an inflammatory process that affects the inferior lip in almost all cases, and it is associated with chronic exposure to ultraviolet (UV) radiation (Vieira et al., 2012). The lesion is more prevalent in middle-aged white-skinned males (de Santana Sarmento, da Costa Miguel, Queiroz, Godoy, & da Silveira, 2014; Kaugars et al., 1999).

The human thioredoxin (Trx) system plays a major role in regulation of oxi-reduction cellular homeostasis, which is involved in several cellular functions, as DNA replication and repair (Holmgren & Lu, 2010; Lu & Holmgren, 2012). Trx1 is a cytosolic and extracellular enzyme whereas Trx2 exists in mitochondria (Arnér & Holmgren, 2006; Holmgren & Lu, 2010). Immunoex-

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pression of Trx can be found in cellular nucleus and cytoplasm. Due to its association with cell growth and anti-apoptotic function, the Trx system has been investigated in many cancer types, including oral lesions (Zhu, Huang, & Peng, 2011).

Metallothionein (MT) comprises a group of ubiquitously occurring proteins, which have specific capacities to bind heavy metals such as zinc, copper, cadmium, and platinum. MTs are involved in many cellular processes, including metal homeostasis and detoxification, protection against oxidative damage, maintenance of intracellular redox balance, cell proliferation and apoptosis, drug and radiotherapy resistance, defense against tissue injury and remodeling, among others (Miles, Hawksworth, Beattie, & Rodilla, 2000; Pedersen, Larsen, Stoltenberg, & Penkowa, 2009; Tapiero & Tew, 2003). A number of studies have shown MT overexpression in different human cancers (Pedersen et al., 2009). Like Trx, MT is detected by immunohistochemistry in cellular nucleus and cytoplasm.

Despite their role in cellular mechanisms important to carcinogenesis, especially in UV-induced damage, Trx and MT were never investigated in lip carcinogenesis. Thus, the aims of the present study were to evaluate and to compare the immunoexpression of Trx and MT in AC, lip SCC, and normal vermillion lip mucosa.

2. Material and methods

2.1. Ethical statement

The study protocol was approved by the Ethics Committee of Universidade Federal de Minas Gerais (19345313.8.0000.5149).

2.2. Samples

Samples of AC, lip SCC, and normal vermillion lip mucosa were retrieved from archived formalin-fixed paraffin-embedded tissues of the laboratories of the School of Dentistry of Universidade Federal de Minas Gerais and School of Dentistry of Universidade Federal de Goiás. Normal lip mucosa specimens were from esthetical corrective surgery or normal tissue excised along with pigmented lesions in the lower lip.

2.3. Epithelial dysplasia and tumor grading

The presence and degree of epithelial dysplasia in AC samples and the histological grade of malignancy of lip SCC samples were established according to the WHO criteria (WHO, 2005). Two oral pathologists (P.C.C. and M.C.F.A.) independently reviewed the hematoxylin and eosin-stained slides. Discrepancies were resolved via discussing the cases.

2.4. Immunohistochemistry

Three μm sections were dewaxed in xylene and hydrated with graded ethanol. After antigen retrieval, hydrogen peroxide block solution (Spring BioScience, code: DHP-125) and protein block solution were applied (Spring BioScience, code DPB-125), respectively. Slides were incubated with the primary antibody diluted 1:100, for 1 h at room temperature (Anti-Trx polyclonal, Santa Cruz Biotechnology, Inc., code TRX (FL-105): sc- 20146. Anti-MT, clone E9, Dako North America, Inc., code M0639). Detection was undertaken with ready-to-use reagents (Spring BioScience, codes DCMT-999 and DHRR-999). Reactions were revealed with DAB chromogenic solution (Spring BioScience, code DAB-999). Mayer's hematoxylin was used for counterstaining. Negative controls were obtained by omission of primary antibody and samples of intra-oral SCC with known positive reactivity were included as positive controls, as Trx and MT immunoexpression have previously been demonstrated in intra-oral SCC (Theocharis et al., 2011; Zhu et al., 2011).

2.5. Immunohistochemistry evaluation

The evaluation of immunostaining was based on previously published methods (Brazão-Silva et al., 2013; Miranda Viana et al., 2013; Zhu et al., 2011). One observer (P.C.C.) evaluated the slides under light microscopy, with a counting grid. The sections were scanned at low power to select the area with the highest degree of staining ("hotspot"), in which quantification was performed in 10 high-power fields. This evaluation was done in a unique axis (horizontal or vertical), and both the intensity and proportion of brown-stained cells were evaluated (Detre, Saclani Jotti, & Dowsett, 1995). For the intensity classification, the following definition was applied: no staining: blue staining, with no brown coloration; weak: faint brown staining; moderate: an intermediate brown coloration between weak and strong; and strong: dark-brown staining. Additionally, the subcellular distribution was taken into account since it might be related to the protein function (Cherian, Jayasurya, & Bay, 2003; Coyle, Philcox, Carey, & Rofe, 2002; Holmgren & Lu, 2010; Lu & Holmgren, 2012; Yoshioka, Schreiter, & Lee, 2006; Zhu et al., 2011). The five parameters were numerically graded as follows:

1. Predominant subcellular localization (0: no staining, 1: cytoplasmic, 2: nuclear, 3: cytoplasmic and nuclear)
2. Extension of nuclear staining (0: no staining, 1: 1%-25% of cells stained, 2: 26%-75%, 3: >75%);

Table 1
Clinical and histological characteristics of actinic cheilitis and lip squamous cell carcinoma.

		Number	Percentage (%)
Actinic Cheilitis			
	Total	**32**	**100.0**
Gender	Male	25	78.1
	Female	7	21.9
Age	Younger than 50	13	41.9
	Older than 50	18	58.1
	Data not available	1	–
Epithelial dysplasia	Absent	9	28.1
	Mild	10	31.3
	Moderate	9	28.1
	Severe	4	12.5
Lip squamous cell carcinoma			
	Total	**20**	**100.0**
Gender	Male	13	68.4
	Female	6	31.6
	Data not available	1	–
Age	Younger than 50	4	21.1
	Older than 50	15	78.9
	Data not available	1	–
"T" stage	T1	11	57.9
	T2	6	31.6
	T3	0	0
	T4	2	10.5
	Data not available	1	–
Histological classification	Well differentiated	20	100.0
	Moderately differentiated	0	0
	Poorly differentiated	0	0