Archives of Oral Biology 77 (2017) 75–81

Contents lists available at ScienceDirect

Archives of Oral Biology

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

Research paper

Thioredoxin and metallothionein: Homeostasis-related proteins in lip carcinogenesis

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ARTICLE INFO

Article history:
Received 6 January 2016
Received in revised form 27 January 2017
Accepted 28 January 2017

Keywords:
Actinic cheilitis
Lip neoplasms
Squamous cell carcinoma
Thioredoxin
Metallothionein

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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http://dx.doi.org/10.1016/j.archoralbio.2017.01.020
0003-9969/

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

3. Intensity of nuclear staining (0: no staining, 1: weak, 2: moderate, 3: strong);
4. Extension of cytoplasmic staining (0: no staining, 1: 1%-25% of cells stained, 2: 26%-75%, 3: >75%);
5. Intensity of cytoplasmic staining (0: no staining, 1: weak, 2: moderate, 3: strong).

For each parameter evaluated, the mode of the scores was registered and used for a qualitative evaluation of the results. Additionally, the average scores of the 10 fields was obtained and used for a semi-quantitative analysis. Finally, a *quickscore* was obtained by multiplying the values of extension and intensity of nuclear and cytoplasmic staining, respectively, giving a maximum value of 9.

2.6. Statistical analysis

SPSS software for Windows version 19.0 was used to perform statistics. To compare the means of the immunoexpression indexes between normal lip mucosa, AC, and SCC, ANOVA test was applied, followed by Tukey *post hoc* test. The immunoexpression of Trx and MT (predominant subcellular localization and *quickscore* indexes) in AC was analyzed according to sex, age, and dysplasia with *t* test. The correlation between Trx and MT expression (predominant subcellular localization and *quickscores*) was assessed by Pearson coefficient. "p" values under 0.05 were considered significant.

3. Results

The study comprised 32 samples of AC, 20 of lip SCC, and 5 of normal lip vermillion mucosa. Due to insufficient amount of material, MT analyses were performed in 30 samples of AC and 19 of lip SCC.

Clinical and histological findings are summarized in Table 1. The AC group comprised 25 men (78.1%) and 7 women (21.9%), with mean age of 51.3 years. Most AC lesions presented dysplasia (71.9%). SCC patients were mainly males (68.4%), with mean age of 65.6 years. Most tumors were classified as "T1" (57.9%) and all were histologically well differentiated.

Concerning Trx staining (Table 2, Fig. 1), normal lip mucosa and AC most often presented simultaneous nuclear and cytoplasmic labeling, while in SCC an exclusive cytoplasmic staining was the major pattern ($p < 0.05$). The nuclear staining was widespread and with a moderate to strong intensity in normal lip mucosa and AC, while in SCC only few cells were positive, with a weak intensity ($p < 0.05$). On the other hand, the cytoplasmic labeling was diffuse and weak in all lesions ($p > 0.05$).Overall we can observe that normal lip mucosa and AC show similar scores of Trx staining, especially for the predominant subcellular localization and the pattern of nuclear staining ($p > 0.05$), and these indexes differ from SCC ($p < 0.05$). Accordingly, the *quickscore* indexes reflect this observation ($p < 0.05$ for nuclear staining).

For MT immunexpression (Table 3, Fig. 2), the predominant subcellular localization was nucleus and cytoplasm concomitantly

Table 2
Scores of Thioredoxin staining in normal lip mucosa, actinic cheilitis, and lip squamous cell carcinoma.

		Normal lip mucosa (n = 5)	Actinic Cheilitis (n = 32)	Lip Squamous cell Carcinoma (n = 20)
Predominant subcellular localization	No staining	–	–	–
	Cytoplasm	–	2 (6%)	15 (75%)
	Nucleus	–	–	–
	Cytoplasm and nucleus	5 (100%)	30 (94%)	5 (25%)
	Mean (SD*)	3.0**	2.8 (0.4)	1.5 (0.8)
Proportion of cells with nuclear staining	No staining	–	1 (3%)	7 (35%)
	<25%	–	2 (6%)	9 (45%)
	25–75%	–	5 (16%)	2 (10%)
	>75%	5 (100)	24 (75%)	2 (10%)
	Mean (SD*)	2.7 (0.8)	2.5 (0.7)	1.1 (0.9)
Intensity of nuclear staining	No staining	–	1 (3%)	6 (30%)
	Weak	1 (20%)	11 (34%)	13 (65%)
	Moderate	2 (40%)	7 (22%)	1 (5%)
	Strong	2 (40%)	13 (41%)	–
	Mean (SD*)	2.1 (0.9)	1.9 (0.9)	0.7 (0.5)
Proportion of cells with cytoplasmic staining	No staining	–	–	–
	<25%	–	–	–
	25–75%	–	–	–
	>75%	5 (100%)	32 (100%)	20 (100%)
	Mean (SD*)	3.0 (0.1)	2.9 (0.2)	3.0 (0.2)
Intensity of cytoplasmic staining	No staining	–	–	–
	Weak	2 (40%)	17 (53%)	14 (70%)
	Moderate	3 (60%)	14 (44%)	6 (30%)
	Strong	–	1 (3%)	–
	Mean (SD*)	1.6 (0.5)	1.4 (0.5)	1.3 (0.5)
Quickscore nuclear staining	>=5	3 (60%)	14 (44%)	0
	Mean (SD*)	5.78 (3.3)	5.14 (2.81)	1.16 (1.20)
Quickscore cytoplasmic staining	>=5	2 (40%)	8 (25%)	6 (30%)
	Mean (SD*)	4.56 (1.5)	4.18 (1.47)	3.9 (1.44)

* SD = standard-deviation.
** this index was constant in all samples.

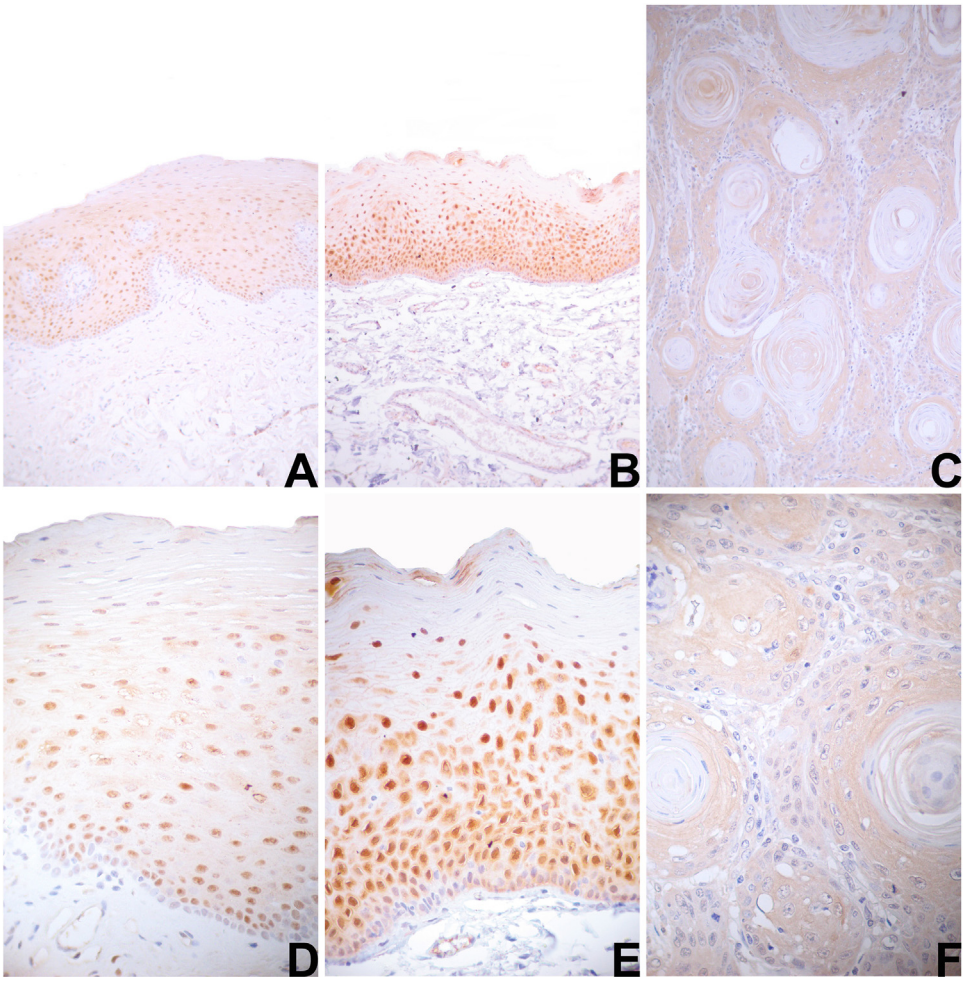

Fig. 1. Thioredoxin immunostaining. A/D- normal lip mucosa and B/E- actinic cheilitis: diffuse positivity for Trx in nuclei and cytoplasm. C/F- lip squamous cell carcinoma: diffuse cytoplasmic positivity with almost negativity in nuclei. Streptoavidin-biotin, original magnification A,B,C: 100×; D,E,F: 400×.

for normal lip mucosa, AC, and SCC ($p > 0.05$). The intensity of nuclear staining was moderate for all lesions ($p > 0.05$), nevertheless SCC showed higher proportion of labeled cells than normal lip mucosa and AC ($p < 0.05$). Similarly, the cytoplasmic labeling was weak in three lesions, but SCC showed more proportion of positive cells than normal lip mucosa and AC ($p < 0.05$).Concerning MT staining, we notice that the difference observed between SCC and normal lip mucosa and AC relies mainly in the proportion of cells with nuclear and cytoplasmic staining (higher for SCC), as the intensity of staining is not so different among lesions. This difference is highlighted when evaluating the higher *quickscore* found for SCC in comparison with normal lip mucosa and AC ($p < 0.05$).

Interestingly, for both proteins the semi-quantitative analysis provided a numerical evidence of the results described in the qualitative evaluation, showing a concordance between the analyses.

No difference in the expression of Trx and MT in AC according to age, gender, and dysplasia was observed ($p > 0.05$).

The correlation between Trx and MT indexes of predominant subcellular localization, and nuclear, and cytoplasmic *quickscores* was direct but weak in AC ($r = 0.202, 0.381, 0.301$, respectively) and SCC ($r = 0.168, 0.206, 0.336$). In normal lip mucosa, a direct and moderate/strong correlation was observed for the nuclear ($r = 0.675$) and cytoplasmic ($r = 0.800$) *quickscores*.

4. Discussion

Trx is normally highly expressed in a wide range of non-cancerous cells and tissues, including non-proliferating ones (Arnér & Holmgren, 2006). Accordingly, we found that the lip vermillion epithelium showed a diffuse, moderate to strong positivity for Trx in cytoplasm and nuclei. In addition, the differences of indexes between normal lip mucosa and AC were not statistically different. As the UV light can induce the expression of Trx, this high expression of Trx could be linked to the antioxidant role of this system (Söderberg, Sahaf, & Rosén, 2000).

Trx can prevent cell apoptosis process via ALK1 (Holmgren & Lu, 2010; Lu & Holmgren, 2012), supply electrons for deoxyribonucleotide and DNA synthesis (Arnér & Holmgren, 2006), and modulate transcription factor or protein kinase signaling cascades (Arnér & Holmgren, 2006), which in turn would favor cancer cells (Holmgren & Lu, 2010; Söderberg et al., 2000). Moreover, the Trx system plays a central role in established cancers particularly for distant metastasis and angiogenesis, as it is also involved in

Table 3
Scores of Metallothionein staining in normal lip mucosa, actinic cheilitis, and lip squamous cell carcinoma.

		Normal lip mucosa (n = 5)	Actinic Cheilitis (n = 30)	Lip Squamous cell Carcinoma (n = 19)
Predominant subcellular localization	No staining	–	–	–
	Cytoplasm	–	–	1 (5%)
	Nucleus	1 (20%)	6 (20%)	–
	Cytoplasm and nucleus	4 (80%)	24 (80%)	18 (95%)
	Mean (SD*)	2.8 (0.4)	2.7 (0.5)	2.8 (0.3)
Proportion of cells with nuclear staining	No staining	–	–	–
	<25%	4 (80%)	17 (57%)	1 (5%)
	25–75%	1 (20%)	12 (40%)	2 (11%)
	>75%	–	1 (3%)	16 (84%)
	Mean (SD*)	1.2 (0.4)	1.4 (0.5)	2.5 (0.5)
Intensity of nuclear staining	No staining	–	–	–
	Weak	–	5 (17%)	6 (32%)
	Moderate	3 (60%)	14 (46%)	10 (53%)
	Strong	2 (40%)	11 (37%)	3 (15%)
	Mean (SD*)	2.4 (0.5)	2.0 (0.7)	1.9 (0.7)
Proportion of cells with cytoplasmic staining	No staining	–	6 (20%)	–
	<25%	5 (100%)	12 (40%)	–
	25–75%	–	11 (37%)	–
	>75%	–	1 (3%)	19 (100%)
	Mean (SD*)	1.0**	1.3 (0.7)	2.7 (0.4)
Intensity of cytoplasmic staining	No staining	–	6 (20%)	–
	Weak	4 (80%)	12 (40%)	7 (36%)
	Moderate	1 (20%)	11 (37%)	6 (32%)
	Strong	–	1 (3%)	6 (32%)
	Mean (SD*)	1.3 (0.4)	1.3 (0.7)	1.8 (0.6)
Quickscore nuclear staining	>=5	1 (20%)	4 (13%)	16 (84%)
	Mean (SD*)	2.96 (1.73)	2.96 (1.56)	7.05 (1.81)
Quickscore cytoplasmic staining	>=5	0	0	10 (56%)
	Mean (SD*)	1.30 (0.4)	1.96 (1.38)	5.05 (2.04)

* SD = standard-deviation.
** this index was constant in all samples.

integrins and adhesion molecules pathways (Arnér & Holmgren, 2006).

As reported before, most cancer cells have a high level of expression of Trx (Cha, Suh, & Kim, 2009; Lincoln, Ali Emadi, Tonissen, & Clarke, 2003; Kim et al., 2003; Lincoln et al., 2010; Lo et al., 2007; Raffel et al., 2003), however some malignant cells have low or undetectable level of Trx and in these cells probably glutaredoxins are involved in DNA synthesis (Holmgren & Lu, 2010). In the present study, we found Trx to be more expressed in AC than SCC of the lip. The difference was mainly related to the nuclear staining, which was present in AC samples but almost absent in SCC. The nuclear localization of Trx could be related to its function other than redox regulation (Zhu et al., 2011), for example as a modulator of transcription factors (Holmgren & Lu, 2010; Yoshioka et al., 2006). Indeed, under oxidative stress conditions, Trx1 is found to be translocated from the cytosol to the nucleus (Lu & Holmgren, 2012). On the other hand, similar proportion of cells with cytoplasmic expression in AC and SCC could indicate that Trx could benefit those malignant-committed AC cells to progress to carcinoma.

It is known that lip SCC has a more favorable course and prognosis than intra oral SCC. Interestingly, Zhu et al. (2011) reported that moderate and high-Trx-expressing tumors indicated poorer survival as compared with low-Trx-expressing cases. Moreover, these authors observed a relation between Trx and tumor differentiation. Surprisingly, normal oral mucosa was negative or with a slight and weak staining within their study, whereas we found strong and diffuse staining. This discrepancy could be due to different antibodies used but also due to different etiopathogenesis of lip and intra oral SCC. Likely, Lincoln et al. (2003) demonstrated that Trx was highly overexpressed in aggressive invasive mammary carcinoma and advanced malignant melanomas, as compared with less aggressive tumors.

Induction of MTs has been observed in the human skin by UVB radiation, indicating their physiological protective role in human skin cells (McGee, Woods, Bennett, & Chung, 2010; Tapiero & Tew, 2003). Accordingly, Zamirska, Matusiak, Dziegiel, Szybejko-Machaj, and Szepietowski (2012) reported higher MT immunoexpression in skin SCC than actinic keratosis. Similarly, in the present study we have demonstrated a higher proportion of cells with MT labeling when comparing AC with lip SCC. This finding was also observed for intra oral leukoplakia compared with intra oral SCC (Pontes et al., 2009), as well as in oral leukoplakia with different degrees of dysplasia (Johann et al., 2008). Interestingly, Szelachowska et al. (2009) found an increased intensity of cytoplasmic MT and the percentage of nuclear MT in oral SCC with lymph node metastasis and Theocharis et al. (2011) and Cardoso et al. (2002) reported that MT immunolabeling was an independent prognostic factor for intraoral SCC.

Although MT is thought to be a cytoplasmic protein, many studies suggest that it can be translocated transiently to the cell nucleus under certain conditions such as cell proliferation and differentiation, being present in the cytoplasm and nucleus of normal and malignant cells (Pontes et al., 2009). Indeed, we found a higher extension of nuclear staining in SCC than in AC.

Herein, a direct strong correlation was found between Trx and MT in normal lip mucosa. This is in consonance with intact and possibly complementary functions of these proteins, protecting cells from damage processes. With the development of AC and SCC, the correlation between Trx and MT was weakened. From the

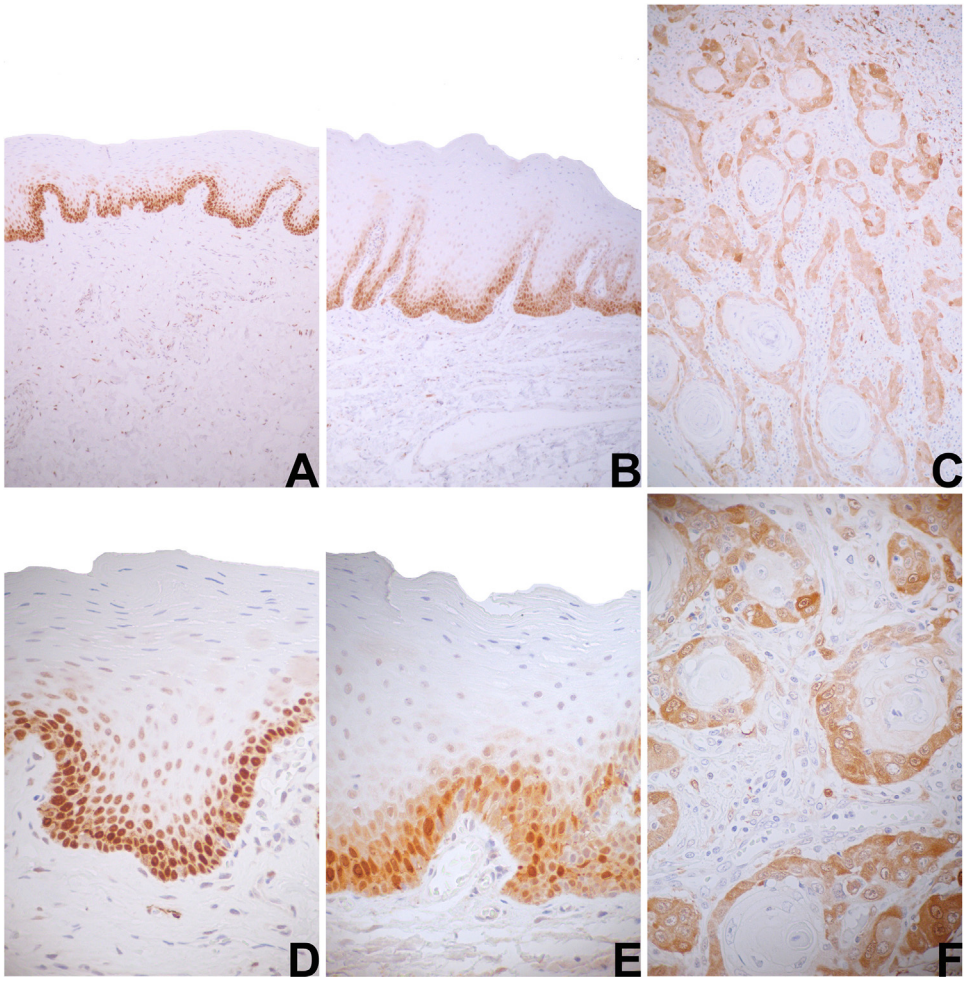

Fig. 2. Metallothionein immunostaining. A/D- normal lip mucosa and B/E- actinic cheilitis: positivity for MT in nuclei and cytoplasm mainly in basal and parabasal layers. C/F- lip squamous cell carcinoma: diffuse cytoplasmic and nuclear positivity. Streptoavidin-biotin, original magnification A,B,C: 100×; D,E,F: 400×.

biological point of view, it may be interpreted that Trx and MT functions are disrupted trough the carcinogenic process, thus losing their correlation. It is known that Trx and MT may interact with p53, a major tumor suppressor protein involved in oral carcinogenesis. Events of apoptotic p53-inducing stimuli seems to require an intact Trx system (Arnér & Holmgren, 2006). The presence of MT in the nucleus was found to be positively associated with p53 positivity, suggesting that co-localization may be relevant to the reported interaction between them (Cardoso et al., 2009). On the other hand, maybe the anti-apoptotic function of Trx would protect cells in pre-cancer stages, while MT could confer cell resistance for cancer cells.

In conclusion, the present study showed a different immunopattern of Trx and MT between normal lip mucosa, AC, and lip SCC. Overall, the results indicate a consistent expression of Trx in normal lip mucosa and AC, with a remarkable nuclear labeling, but not in SCC. Conversely, MT immunodetection seems to be more expressive in SCC than normal lip mucosa and AC. The cellular compartment-based analyses evidenced differences that can be related to the proteins function. Considering the relevant roles of Trx and MT in cellular homeostasis, especially in protection against UV-induced damage, these proteins could play an important role in lip carcinogenesis.

Conflict of interests

None declared.

Acknowledgements

This study was supported by grants of Fundação de Amparo à Pesquisa do Estado de Minas Gerais (PPM-00221-13), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, and Conselho Nacional de Desenvolvimento Científico e Tecnológico (150144/2014-8).

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