Metallothionein immunoexpression in oral leukoplakia

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Abstract

Objectives: to report the immunoexpression of metallothionein in oral leukoplakia and to correlate with histological grade and clinical localization. Leukoplakia is the most common potentially malignant lesion of the oral cavity. As the histological study of oral leukoplakia can not predict precisely the malignant transformation of this lesion, and metallothionein is a protein that has been associated with carcinogenesis, this study could be auxiliar in this histological assessment of this lesion.

Study design: samples of oral leukoplakia (35 cases) and of normal oral mucosa (10 cases) were evaluated. Oral leukoplakia was graded in: hyperkeratosis without dysplasic change (9 cases), mild dysplasia (8 cases), moderated dysplasia (10 cases), and severe dysplasia (8 cases). Immunohistochemistry for the metallothionein was performed and the Mann-Whitney test was used in statistical analysis.

Results: metallothionein was identified in squamous cells of the all samples. The metallothionein stain in all cases exhibit a mosaic pattern and was predominantly in compartments cytoplasmatic and nuclear simultaneously. The total stain was significantly higher in moderate dysplasia when compared with normal oral mucosa, hyperkeratosis, and mild dysplasia.

Conclusion: it was suggested that the metallothionein may be a marker to moderate dysplasia and may play a role in oral carcinogenesis.

Key words: Oral leukoplakia, metallothionein, immunohistochemistry.

Introduction

The oral leukoplakia (OL) is defined by the World Health Organization (WHO) as "a predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion" (1). This disease is widely studied due to the risk of malignant transformation (2-8). The annual transformation rate of OL is proximal to 1% (3).
Clinical and demographic aspects of OL may be correlated with increased risk of malignant change. For example, OL located on the floor of the mouth, tongue, and palate have a higher risk of malignant transformation than OL in other regions, and females with OL have a higher risk of developing oral squamous cell carcinoma (9,10). Histologically, OL may appear as hyperkeratosis, mild dysplasia, moderate dysplasia, and severe dysplasia (11). The ranges of these microscopic features have also been explored as a predictor of malignant transformation (2,8). However, the clinical aspects and histological study of OL can not predict precisely of malignant transformation (12). Studies regarding to markers of potential malignancy, such

as the proliferating cell nuclear antigen (PCNA), Ki-67, argyrophilic nucleolar organizer region (AgNOR) and p53, have been developed as auxiliary in this histological assessment (6,7,10,13-15).

Metallothionein (MT) belongs to group of low molecular weight proteins (6-7 kDa) characterized by high levels of cystein, which are bound to metal ions (16,17). This protein is constitutively found in the cytoplasm of mammalian cells (18). In normal oral mucosa, the MT immunoexpression in squamous epithelium cells was identified within basal and parabasal layers (19).The role of MT in the control of metals homeostasis has stimulated studies which suggest the correlation of MT with protection against oxidative damage caused by free radicals, inhibition of cell apoptosis, and carcinogenesis (18-20).

Up to now, an investigation of MT immunoexpression in OL has not been carried out. The aim of this study was 1) to report the MT immunoexpression in OL, 2) to compare the MT expression among the histological grades, and 3) to correlate the MT expression with clinical localization.

Material and Methods

Institutional ethical board

The protocol of the study was approved by the Committee of Bioethics in Research at Universidade Federal de Minas Gerais (UFMG/COEP - number 207/04).

Specimens and histological evaluation

Samples with diagnosis of the OL (35 cases) and normal oral mucosa (10 cases; Figure 1A) were obtained from the files of the Oral Pathology Service of Universidade Federal de Minas Gerais (UFMG, Belo Horizonte, Brazil) and from the Oral Pathology Service of Pontifícia Universidade Católica of Minas Gerais (PUC-MG, Belo Horizonte, Brazil). The criteria of the WHO (2005) for the histological grading of leukoplakia were used (12,21). The histological degree of epithelial dysplasia was based on the proportion of the height of the epithelial layer that presents the dysplasic changes. Mild dysplasia was characterized by few cytological alterations limited to the lower one-third of epithelium. When the lower two-thirds of the epithelium showed cytological alterations the lesion was classified as moderate dysplasia. When de entire height of the epithelium presented dysplasic changes OL was graduated in severe dysplasia. The architectural changes observed in the classification were: loss of polarity of cells, increase of cellular density, dyskeratosis, basal cell hyperplasia, disordered maturation from basal to squamous cells, bulbous drop-shaped rete pegs, and secondary extensions on rete tips. The cellular changes considered were increase of nucleus-cytoplasm ratio, cellular pleomorphism, cellular and nuclear enlargement, nuclear hyperchromatism and pleomorphism, enlarged and numerous nucleoli, increased mitotic figures, atypical mitotic figures and increased number and size of nucleoli. OL was submitted to histological evaluation on slides stained by haematoxilin-eosin (HE). The 35 cases of OL were graded in: hyperkeratosis without dysplasic change (9 cases), mild dysplasia (8 cases), moderate dysplasia (10 cases; Figure 1B), and severe dysplasia (8 cases).

Clinical data were obtained from biopsy records. OLs located on the floor of the mouth, tongue, and palate were considered of high-risk region of malignant transformation. Other sites were classified as low-risk region. The sample was composed of 26 male and 9 female; 26 cases were located in the low-risk region and 9 cases in the high-risk region (9). Immunohistochemical evaluation was performed in all 45 cases.

Immunohistochemistry

Immunohistochemical reaction was performed using streptavidin-biotin standard protocol. Sections of 4μm from routinely processed paraffin embedded blocks were deparaffinized and dehydrated. Specimens were immersed in a 1 mM ethylenediamine tetraacetic acid (EDTA, pH= 8.0) buffer (Vetec Química Fina, Rio de Janeiro, 114) and submitted for 30 minutes at 98ºC. The avidin/biotin was blocked in accordance with Miller et al. (22). Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. Sections were incubated with primary antibody for MT at a 1:100 dilution (Dako, Carpinteria, CA, M0639) for 18 hours at 4ºC. A bound primary antibody was detected using LSAB®+system, HRP Peroxidase Kit (Dako Corporation, Carpinteria, CA, K0690) and 3.3'-diaminobenzidine tetrahydrochloride chromogen (DAB, Sigma Chemical, St. Louis, USA, D5637). Positive controls were also used.

Immunohistochemical assessment

The immunohistochemical stain was analyzed by a blind and calibrated examiner (ACBRJ). The number of positive cells was counted in all cases. Indexes for MT expression were constructed based on the percentage of labeled cells among four to six counter fields for each slide. The analysis was carried out taking in count the cell compartment (cytoplasmatic and nuclear, cytoplasmatic only, or nuclear only) and the cellular layer involved (basal and suprabasal).

Statistic analysis

Statistic analysis was performed with a non-parametric method, Mann-Whitney test. BioEstat® software was used and $p < 0.05$ was considered the limit for statistical significance (23).

Results

MT immunoexpression was presented in squamous cells of the all samples. The MT stain in all cases appeared as a mosaic pattern and predominantly in compartments cytoplasmatic and nuclear simultaneously. Immunostain exclusively restrict to the nucleus was not observed.

The MT total labeling was significantly higher in moderate dysplasia (66.7%, Figure 1C) when compared with normal oral mucosa (55.8%; $p < 0.05$; Figure 1D), hyperkeratosis
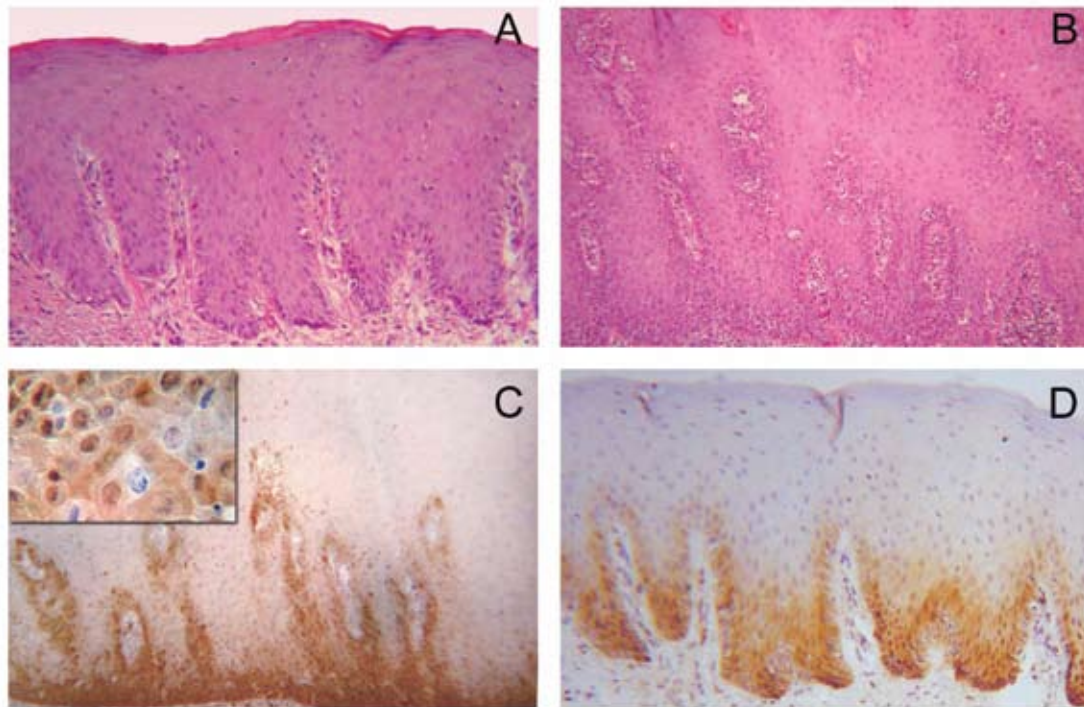
Fig. 1. (A) Normal oral mucosa without dysplasic changes (Haematoxilin-eosin- HE, X200 original magnification). (B) Moderate dysplasia showed alteration in the lower two-thirds of the epithelium layer with loss of polarity, disordered maturation from basal to squamous cells, increased cellular density, and basal cell hyperplasia (HE, X200 original magnification). (C) In moderate dysplasia an increased cytoplasmatic and nuclear stain in the basal layer was observed. In detail, cytoplasmatic and nuclear stains, cytoplasmatic stains only, or the absence of stains may be observed. Also, the mosaic pattern may also be visualized (Streptavidine-biotine, X200 original magnification). (D) In normal oral mucosa, the metallothionein immunoexpression was predominant in the basal layer (Streptavidine-biotine, X200 original magnification).

(56.7%; $p< 0.05$), and mild dysplasia (53.8%; $p< 0.05$). This MT overexpression was due to the high cytoplasmatic and nuclear stain in the basal layer. No significant difference in total MT expression was observed between moderate dysplasia (66.7%) and severe dysplasia (59.7%; $p> 0.05$). Other comparisons among the groups and between layers were performed, but there were not significant differences. No significant difference in MT total labeling was identified between regions of high (61.8%) and low risk (58.0%; $p> 0.05$).

Discussion

This study shows the immunoexpression of MT in OL. Although MT has been associated with carcinogenesis (20,24), there are no registers of this expression in potentially malignant oral lesions. MT is mainly a cytoplasmatic protein found in mammalian cells (18). It was observed that the expression of MT was not restricted to the cytoplasm yet was observed in both cytoplasmatic and nuclear compartment of squamous cells in the basal and parabasal layers (19). Ioachim et al. (25), in a study of the benign, premalignant, and malignant epithelium of the larynx, also observed this double profile of the immunostain.

However, immunolocalization restricted to the nucleus was described in oral squamous cell carcinoma (20). Although MT has been characterized as a cytoplasmatic protein, it may cross the nuclear membrane by passive diffusion. The MT dislocation to nuclei was identified in human malignant lesions, such as lung carcinoma (26), and oral squamous cell carcinoma (26).The significance of nuclei localization is a more effective biological protection against oxidative stress and genomic damage. In addition to its interference in genomic regulation and other proteins linked to DNA (27,28).

The overexpression of MT in moderate dysplasia may mean that the altered cells are more protected with more chances of survival, but it can also indicate alterations in genomic regulation and in other proteins linked to DNA, thus presenting its possible role in carcinogenesis. Furthermore, this overexpression may be correlated with initiation or promotion of carcinogenesis. Ioachim et al. (25) evaluated the expression of MT in potentially malignant lesions of the larynx, but, in contrast with our results, did not find significant differences among the differing grades of dysplasia. The absence of difference in the MT total labeling between moderate dysplasia and severe dysplasia

observed in present study might indicate that the moderate dysplasia is the hallmark point in the process of carcinogenesis. The major requirement for MT function might occur in this phase and do not suffer other alterations with the worsening of the histological features.

Studies have explored markers as auxiliary tool in the histological assessment of OL. A gradual increase in PCNA and Ki-67 expression has been positively associated with the grade of dysplasia in OL (7,13,15). In contrast, the relationship between p53 overexpression and OL has been controversial (10). Santos-García et al. (4) observed a p53 overexpression with the advance of the grade of severity histologic of OL. However, Warnakulasuriya (6) claims that p53, when used as a single marker, is an inappropriate immunohistochemical marker for the prediction of tumor development in high-risk patients. Chattopadhyay et al. (14) observed that the mean AgNOR count increase, in accordance with the dysplasia grade, may indeed be an additional tool in distinguishing between OL with dysplasia and without dysplasia.

Ioachim et al. (25) identified a positive correlation between MT and PCNA expression in potentially malignant lesions of the larynx. However, no association was observed between MT and p53. Ostrakhovitch et al. (29) demonstrated, "in vitro" and "in vivo", a possible association between MT and p53 in breast cancer epithelial cells and suggested that the co-expression of these proteins may be related to the control of apoptosis. The p53 binds to DNA impede transcription through a zinc-dependent process. Metal-chelanting agents, such as MT, can remove zinc, therefore inducing a reversible conformation change in wild type p53, blocking its action (30). Future evaluations are necessary to clarify the association of these markers with MT in OL.

The mosaic stain pattern, or the heterogeneity for MT labeling intensity observed, was also verified in oral squamous cell carcinoma (20), and renal cell carcinoma (17). Cardoso et al. (20) suggested that this pattern may be due to the phenotypic variations among neoplasic cells. This application to dysplastic cells might be worthy of a more meticulous investigation.

Although the clinical data of OL has been correlated with increased risk of malignant change, in this study, no significant difference was observed in MT total indexes between regions of high and low risk. This study only detected a tendency of MT overexpression in regions of high risk.

In conclusion, this study suggests that the MT may be a marker to moderate dysplasia and may play a role in oral carcinogenesis.

References

1. Axéll T, Pindborg JJ, Smith CJ, Van der Waal I. Oral white lesions with special reference to precancerous and tobacco- related lesions: conclusions of an international symposium held in Uppsala, Sweden, May 18-21 1994. International Collaborative Group on Oral White Lesions. J Oral Pathol Med. 1996 Feb;25(2):49-54.
2. Fischer DJ, Epstein JB, Morton TH, Schwartz SM. Interobserver reliability in the histopathologic diagnosis of oral pre-malignant and malignant lesions. J Oral Pathol Med. 2004 Feb;33(2):65-70.
3. Scheifele C, Reichart PA. Is there a natural limit of the transformation rate of oral leukoplakia. Oral Oncol. 2003 Jul;39(5):470-5.
4. Santos-García A, Abad-Hernández MM, Fonseca-Sánchez E, Cruz-Hernández JJ, Bullón-Sopelana A. Proteic expression of p53 and cellular proliferation in oral leukoplakias. Med Oral Patol Oral Cir Bucal. 2005 Jan-Feb;10(1):5-8; 1-5.
5. Chimenos-Küstner E, Font-Costa I, López-López J. Oral cancer risk and molecular markers. Med Oral Patol Oral Cir Bucal. 2004 Nov-Dec;9(5):381-4; 377-80.
6. Warnakulasuriya S. Lack of molecular markers to predict malignant potential of oral precancer. J Pathol. 2000 Mar;190(4):407-9.
7. Takeda T, Sugihara K, Hirayama Y, Hirano M, Tanuma JI, Semba I. Immunohistological evaluation of Ki-67, p63, CK19 and p53 expression in oral epithelial dysplasias. J Oral Pathol Med. 2006 Jul;35(6):369-75.
8. Lee JJ, Hong WK, Hittelman WN, Mao L, Lotan R, Shin DM, et al. Predicting cancer development in oral leukoplakia: ten years of translational research. Clin Cancer Res. 2000 May;6(5):1702-10.
9. Waldron CA, Shafer WG. Leukoplakia revisited. A clinicopathologic study 3256 oral leukoplakias. Cancer. 1975 Oct;36(4):1386-92.
10. Reibel J. Prognosis of oral pre-malignant lesions: significance of clinical, histopathological, and molecular biological characteristics. Crit Rev Oral Biol Med. 2003;14(1):47-62.
11. Lippman SM, Hong WK. Molecular markers of the risk of oral cancer. N Engl J Med. 2001 Apr 26;344(17):1323-6.
12. Bouquot JE, Speight PM, Farthing PM. Epithelial dysplasia of the oral mucosa: Diagnostic problems and prognostic features. Curr Diag Pathol 2006;12:11-21.
13. Liu SC, Sauter ER, Clapper ML, Feldman RS, Levin L, Chen SY, et al. Markers of cell proliferation in normal epithelia and dysplastic leukoplakias of the oral cavity. Cancer Epidemiol Biomarkers Prev. 1998 Jul;7(7):597-603.
14. Chattopadhyay A, Ray JG, Caplan DJ. Agnor count as objective marker for dysplastic features in oral leukoplakia. J Oral Pathol Med. 2002 Oct;31(9):512-7.
15. Fan GK, Chen J, Ping F, Geng Y. Immunohistochemical analysis of P57(kip2), p53 and hsp60 expressions in premalignant and malignant oral tissues. Oral Oncol. 2006 Feb;42(2):147-53.
16. Takeda A, Hisada H, Okada S, Mata JE, Ebadi M, Iversen PL. Tumor cell growth is inhibited by suppressing metallothionein-I synthesis. Cancer Lett. 1997 Jun 24;116(2):145-9.
17. Mitropoulos D, Kyroudi-Voulgari A, Theocharis S, Serafetinides E, Moraitis E, Zervas A, et al. Prognostic significance of metallothionein expression in renal cell carcinoma. World J Surg Oncol. 2005 Jan 17;3(1):5.
18. Kägi JH, Schäffer A. Biochemistry of metallothionein. Biochemistry. 1988 Nov 15;27(23):8509-15.
19. Sundelin K, Jadner M, Norberg-Spaak L, Davidsson A, Hellquist HB. Metallothionein and Fas (CD95) are expressed in squamous cell carcinoma of the tongue. Eur J Cancer. 1997 Oct;33(11):1860-4.
20. Cardoso SV, Barbosa HM, Candellori IM, Loyola AM, Aguiar MC. Prognostic impact of metallothionein on oral squamous cell carcinoma. Virchows Arch. 2002 Aug;441(2):174-8.
21. Gale N, Pilch BZ, Sidransky D, Westra WH, Califano J. Epithelial precursor lesions. In: Barnes L, Eveson JW, Reichart PA, Sidransky D, eds, World Health Organization Classification of tumors. Pathology and Genetics of Head and neck tumours. Lyon: IARC Press; 2005. p. 140-3.
22. Miller RT, Kubier PHT, Reynolds BHT, Henry TB, Turnbow HB. Blocking of Endogenous Avidin-Binding Activity in Immunohistochemistry: The Use of Skim Milk as an Economical and Effective Substitute for Commercial Biotin Solutions. Appl Immunohistochem Mol Morphol 1999;7:63-5.
23. Ayres M, Ayres JR, Ayres DM, Santos AS. Bioestat 3.0: aplicações estatísticas nas áreas das ciências biológicas e médicas. Lithera Maciel, Belém (2003).
24. Cherian MG, Jayasurya A, Bay BH. Metallothioneins in human tumors and potential roles in carcinogenesis. Mutat Res. 2003 Dec 10;533(1-2):201-9.

25. Ioachim E, Assimakopoulos D, Peschos D, Zissi A, Skevas A, Agnantis NJ. Immunohistochemical expression of metallothionein in benign premalignant and malignant epithelium of the larynx: correlation with p53 and proliferative cell nuclear antigen. Pathol Res Pract. 1999;195(12):809-14.

26. Theocharis S, Karkantaris C, Philipides T, Agapitos E, Gika A, Margeli A, et al. Expression of metallothionein in lung carcinoma: correlation with histological type and grade. Histopathology. 2002 Feb;40(2):143-51.

27. Zeng J, Vallee BL, Kägi JH. Zinc transfer from transcription factor IIIA fingers to thionein clusters. Proc Natl Acad Sci U S A. 1991 Nov 15;88(22):9984-8.

28. Chubatsu LS, Meneghini R. Metallothionein protects DNA from oxidative damage. Biochem J. 1993 Apr 1;291 (Pt 1):193-8.

29. Ostrakhovitch EA, Olsson PE, Jiang S, Cherian MG. Interaction of metallothionein with tumor suppressor p53 protein. FEBS Lett. 2006 Feb 20;580(5):1235-8.

30. Hainaut P, Milner J. A structural role for metal ions in the "wild-type" conformation of the tumor suppressor protein p53. Cancer Res. 1993 Apr 15;53(8):1739-42.