Nuclear metallothionein in oral squamous cell carcinoma: clinicopathological parameters and patient survival

Abstract: The aim of this study was to identify the immunoexpression of metallothionein in oral squamous cell carcinoma as well as to address the correlation with clinical features, histological grade and patient survival. Samples were collected from 93 patients with tongue squamous cell carcinoma who presented for follow-up. Immunohistochemical expression of metallothionein in all groups was performed. The scoring system has previously been published by Tsurutani in 2005, which is based on intensity and distribution of staining. We used Kappa index to evaluate the degree of observers’ agreement under metallothionein immunostaining and histological grade. Associations between metallothionein expression and clinical parameters (age, gender, smoking, tumor size, lymph node metastasis and disease stage) were examined for statistical significance using the chi-squared test. The overall survival rates were estimated by the Kaplan-Meier method and the relationship between protein expression and survival was compared using the log-rank test (p < 0.05). Our results showed no statistically significant association between metallothionein immunostaining and the selected clinicopathological variables. Immunohistochemistry results showed positive nuclear immunostaining for metallothionein in 62.37% (58/93) and negative for metallothionein 37.63% (35/93). The degree of examiners agreement by Kappa varied from substantial to perfect and both metallothionein immunostaining and histological grade were explored. The present study suggests that positive metallothionein expression found in tongue squamous cell carcinoma may not help to predict survival in the analyzed samples, as well as no relation between the protein and histological grade and clinical features was observed. In conclusion, the present study suggests that metallothionein is not associated with tongue squamous cell carcinoma clinicopathological characteristics and aggressiveness.

Keywords: Mouth Neoplasms; Survival; Immunohistochemistry.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common form of oral cancer, representing more than 90% of all malignant neoplasms in this location.1 Despite the recent advances in surgery and improvements in radio and chemotherapy, OSCC continues to pose a significant therapeutic problem.2,3,4
Metallothioneins (MT) comprise a group of low molecular weight (6–7 kDa), cysteine-rich, intracellular proteins with high affinity for heavy metal ions. This protein is known to act in detoxification of heavy metals, homeostasis of essential metals, antioxidation against reactive oxygen species, protect against DNA damage and oxidative stress as well as in cell survival, angiogenesis, apoptosis and increase proliferation. In addition, its overexpression seems to be related to resistance to radiation and chemotherapeutic agents. Thus, MT has been studied as a prognostic factor for different human cancers. We showed recently a significant increase in positive immunohistochemical staining for MT-1/2 proteins when normal oral mucosa was compared to severe dysplasia and to OSCC as well as a positive correlation with severity of dysplasia of oral leukoplakia, with the lowest MT-1/2 expression found in mild dysplastic lesions and the highest in severe dysplasia.

The transcriptional control of MT induction and changes in its nuclear/cytoplasmic localization during cell proliferation and differentiation suggest that altered levels of nuclear MT can be expected in any situation where there is abnormal cell growth, such as cancer. Moreover, cases characterized by a positive nuclear MT-1/2 immunostaining yielded higher p53 expression levels. MT molecules were found to be able to remove zinc ions from p53 protein, what results in its inactivity. Inactivation of p53 protein in neoplastic cells results in their excessive proliferation and inhibition of apoptotic processes.

A better understanding of the cellular molecules involved in the mechanisms of OSCC pathogenesis is important to identify prognostic markers that reflect disease aggressiveness, to provide more effective forms of therapy and may improve patient survival. In this context, the aim of this study was to examine by immunohistochemistry in samples of OSCC the correlation of MT nuclear expression with selected clinical and pathological variables of the disease as well as with the patient survival. As studies about the expression of MT in OSCC confined to nuclear compartment are limited, we conducted the present study to obtain more accurate information about the clinical importance of nuclear MT expression in OSCC.

### Methodology

#### Specimens and inclusion criteria
A total of 93 paraffin-embedded biopsy specimens of tongue squamous cell carcinoma from 67 (72.5%) males and 26 (27.5%) females with a mean age of 60 years (range, 27–85) were selected in the period between January 1999 and December 2014 from the Service of Oral Pathology of the João de Barros Barreto University Hospital (Pará, Brazil) to evaluate the immunohistochemical expression of the metallothionein protein and to correlate with survival. The patients were monitored for up to 62 months after diagnosis. Samples were selected from patients (with a diagnosis confirmed by histopathology) who had primary tumours of the oral cavity with surgery as the only treatment modality. The smokers consumed at least 10 cigarettes per day. The mean follow-up of the patients was 28.02 months (range, 0–62). A total of 15 patients with a change in staging in the period between diagnosis and surgery were excluded. In our study, there were no cases with distant metastasis. The required data were obtained from patient records, summarised on standardized forms and stored in a database. A total of 93 paraffin-embedded biopsy specimens of OSCC were subjected to evaluation of histological grade and clinicopathological features. The primary tumor was clinically staged according to the TNM classification defined by the 2009 International Union Against Cancer (UICC) and the 2010 American Joint Committee on Cancer (AJCC). The histological grade assessment followed the parameters of the World Health Organization and was carried out by two pathologists without prior knowledge of the clinical data of the patients. The relationships between metallothionein protein and clinicopathological features, size of tumour (T), infiltration of metastatic lymph nodes (N), stage, smoking and the histological grade, were assessed. The ethical committee of the João de Barros Barreto University Hospital approved this work under approval number 51641/12.

#### Immunohistochemistry
Sections were dewaxed with xylene and hydrated in an ethanol series. For antigen retrieval, sections that received antimetallothionein antibody were immersed
in 10 mM monohydrated citrate buffer solution (pH 6.0) and heated in a microwave oven at 95°C for 15 min. Peroxidase activity was blocked with 6% hydrogen peroxide and methanol solution in two baths for 15 min each at room temperature. After washing with Tris buffer (pH 7.4), the slides were incubated with the primary antibody anti–metallothionein isoforms 1 and 2 (DAKO Corporation, Glostrup, Denmark) E9 clone, dilution 1:800, incubated for 60 min. The sections were subsequently exposed to avidin–biotin complex (LSAB-Kit + HRP; DakoCytomation, Carpinteria, USA) and to 3, 30-diaminobenzidine chromogen (DAB+; DakoCytomation). The sections were counterstained with Meyer’s haematoxylin, dehydrated in ethanol, cleared in xylene and mounted. Slices of breast adenocarcinoma were used as the positive control, and the negative control was obtained by omitting the primary specific antibody during the reaction. Immunostained sections were examined by light microscopy at 409 magnification, and digital images were captured using an electron microscope model Eclipse Nikon Ci-POL (Nikon Metrology Europe NV, Leuven, Belgium). All sections were evaluated without knowledge of clinical status by two pathologists, under a fixed focus. The staining was considered positive when cells with brown staining were observed in the nucleus compartment, indicating the presence of DAB in the immunohistochemistry reaction.

The scoring system has previously been published in the literature. The analysis was based on intensity and distribution of staining. The distribution of stained cells was analyzed as follows: 0 (0%), 1 (1–50%) and 2 (51–100%). The intensity of staining was rated as follows: 0 (no staining), 1 (mild staining), 2 (moderate staining) and 3 (strong staining). The staining pattern of the specimens was defined by the sum of the values found in the distribution of data for the intensity of immunostaining, thus obtaining the final record (FR) as follows: FR0, FR2, FR3, FR4 and FR5. Using this method, FR0 and FR2 were considered negative staining, while FR3, FR4 and FR5 were considered positive staining. Two independent pathologists blinded to the experimental groups evaluated the immunostained sections. In the event of a disagreement, the two pathologists conferred to achieve a consensus. A record card was used to register the inter-observer agreement among the variables through the Kappa test statistics to compare the agreement among individual pathologists around metallothionein immunostaining and histological grade.

### Statistical analysis

The data was analysed using the Statistical Package for Social Sciences software for Windows, version 18.0 (SPSS Inc, Chicago, IL, USA). Associations between metallothionein expression and clinicopathological parameters were examined for statistical significance using a chi-square test. Overall survival rates were estimated by the Kaplan–Meier method and compared using a log rank test. A p-value of < 0.05 was considered significant.

### Results

**Clinical profiles of patients with OSCC and evaluation of histological grade**

A total of 93 samples were included in the analysis. Patient characteristics are summarised in Table. Moderately differentiated tumours (48,38%), followed by poorly differentiated (35.48%) tumours, were the most represented in our sample. Well-differentiated tumours represented only 15.05%. The chi-square test showed no significant difference for the clinical and histological profiles analyzed.

**Metallothionein immunostaining**

Immunohistochemistry results showed positive nuclear immunostaining for metallothionein in 62,37% (58/93) samples and negative immunostaining for metallothionein in 37,63% (35/93) samples. The immunostaining was observed in cells located at the periphery and at the center of the tumours islands; however, wherever keratin pearls were present, MT immunostaining was restricted to the basal and parabasal cells (Figure 1). In order to compare the agreement among individual pathologists the unweighted kappa statistics were calculated which were found to be significant (p < 0.05), evidencing results varying from substantial to perfect when metallothionein immunostaining and histological grade were explored.
Overall survival

Overall survival was defined as the period between the date of diagnosis of the disease until the last follow-up or death (Table). Log rank test showed that there was not a statistically significant association between survival rate and metallothionein immunostaining ($p = 0.0835$) (Figure 2). The Kaplan–Meier curve showed that the probability of survival for a patient after 1 month of monitoring the disease was 96.5% and that the passage of time tended to reduce this probability, as shown in Figure 3. The probability of a patient surviving after 60 months of follow-up was 24.83%.

Discussion

Metallothionein has been implicated in several aspects of cancer pathobiology, such as differentiation, proliferation, apoptosis and invasion. Moreover, the carcinogen from tobacco smoking benzo[a]-pyrene (BaP), a polycyclic aromatic hydrocarbon, also triggers MT-1 overexpression. We showed recently a significant increase in positive immunohistochemical staining for MT-1/2 protein when normal oral mucosa was compared to severe dysplasia and to OSCC as well as a positive correlation with severity of dysplasia of oral leukoplakia, with the lowest MT-1/2 expression found in mild dysplastic lesions and the highest in severe dysplasia, then the present study aimed to assess MT nuclear expression immunohistochemically in oral SCC specimens and to evaluate clinicopathological parameters, including overall survival time.

Table. Association between metallothionein immunostaining and clinicopathological variables.

| Clinicopathological features | Metallothionein nuclear immunostaining |
|-----------------------------|----------------------------------------|
| n = 93                      | Negative | Positive | p-value |
| Age                         |          |          | 0.74    |
| ≤ 40 year                   | 4        | 4        |         |
| > 40 years                  | 31       | 54       |         |
| Gender                      | 1.0      |          |         |
| Female                      | 18       | 29       |         |
| Male                        | 17       | 29       |         |
| Alcohol intake              | 0.61     |          |         |
| No                          | 15       | 28       |         |
| Yes                         | 20       | 30       |         |
| Smoking                     | 0.61     |          |         |
| No                          | 23       | 41       |         |
| Yes                         | 12       | 17       |         |
| Size tumour (T)             | 0.76     |          |         |
| 1 or 2                      | 15       | 23       |         |
| 3 or 4                      | 20       | 35       |         |
| Lymph node metastasis (N)   | 0.74     |          |         |
| 0 or 1                      | 27       | 43       |         |
| 2 or 3                      | 8        | 15       |         |
| Stage                       | 0.94     |          |         |
| I or II                     | 13       | 22       |         |
| III or IV                   | 22       | 36       |         |
| Histological grade          | 0.93     |          |         |
| Well differentiated          | 5        | 10       |         |
| Moderate                    | 18       | 27       |         |
| Poorly differentiated        | 12       | 21       |         |
| Overall survival            | 0.08     |          |         |
| Alive                       | 3        | 40       |         |
| Dead                        | 4        | 46       |         |

Figure 1. Immunoexpression of metallothionein protein in OSCC epithelium, nuclear immunostaining (x400 original magnification) (a). The immunostaining was located at the periphery and at the center of the tumor island (b). The immunostaining around the pearls of keratin (c).
There is no conclusive data on the functional significance of MT distribution in different cellular compartments. In mitotically inactive cells (G0 phase), the expression of MT can be detected in the cytoplasm while in dividing cells its activity becomes shifted to the nucleus. The highest cytoplasmic expression of MT is observed at the end of G1 phase and at the G1/S threshold while the peak of accumulation of MT in the nucleus can be detected in phases S and G2. The translocation of MT into the nucleus during G1/S phase in tumor cells suggests that MT facilitates cell proliferation by donating zinc ions to various transcription factors. In this sense, it is of high importance to distinguish between MT-I+II expression both in subcellular compartments (e.g. cytoplasmic vs. nuclear).

MT could exert its anti-apoptotic effects in oral squamous cell carcinomas by blocking the action of p53 protein. Douglas-Jones et al. suggested that MT could remove Zn of p53 by blocking the activity of inducing programmed cell death. Ostrakhovitch et al. showed, in breast cancer cell culture, the interaction between MT1 and p53, suggesting that co-expression of MT1 and p53 may be involved in the regulation of apoptosis in these cells. In addition, it has been shown that combined Zn and p53 proteins deficiency allows extreme sensitivity to malignant transformation, using 4NQO (4-nitroquinoline 1-oxide) at low concentrations at an experimental model of oral carcinogenesis conducted in mice. Finally, cases characterized by a positive nuclear MT-1/2 immunostaining, yielded higher p53 expression levels. This underlies the earlier observed role of MT-1/2 in regulation of p53 expression by influencing zinc ion cell homeostasis.

Despite the immunostaining observed in this study being sometimes restricted to the nucleus, sometimes restricted to the cytoplasm and sometimes found in both compartments, in view of the above considerations, the present study focused only in specimens with positive nuclear staining or nuclear and cytoplasmic staining simultaneously. To our knowledge, the present study is the only investigation in which the intensity and distribution of MT nuclear expression in OSCC is analyzed together and related with clinicopathological characteristics.

A mosaic staining pattern for the MT protein was observed in the squamous cell carcinomas investigated in our study, with strongly immunostained cells in some areas, while other areas, on the same blade, showed negative results. We believe this mosaic pattern may be the result of cell phenotypic differences acquired during neoplastic progression.

MT immunoreactivity was frequently detected in the peripheral cell layers around keratins pearls, where the tumour cells were less differentiated, whereas the cell layers inside the keratin pearl,
where the tumour cells were more differentiated and keratinized, showed weak or negative MT immunoreactivity (Figure 1C). For Sundelin et al., peripheral staining of MT reproduces the staining pattern found in normal epithelium and can help divert these cells from programmed cell death. The pattern of immunostaining observed in the present study was similar to the one found by Cardoso et al., and Theocharis et al.

The tongue carcinoma has been shown to have high local failure and poor survival rates compared with other anatomical sites in the oral cavity. In this context, the present study focused on anatomical site to assess overall survival time. Analysis of the MT protein expression did not promote statistically significant results when associated with survival of patients with squamous cell carcinoma involved in this study, similar results to those found by Szelachowska et al., where MT nuclear staining was associated with overall survival time. In another study of Cardoso et al., no influence on patients survival was observed, when MT-1/2 expression was analyzed alone. Interestingly, a combined analysis of this protein with p53 expression, showed that high expression of both these markers predicted poor outcome of patients with OSCC.13

It is worth mentioning that the study by Theocharis et al. found a statistically significant relationship when evaluating the intensity of MT staining with overall survival time. Their study evaluated the staining intensity separately of the staining distribution, finding no statistically significant results when the relation of MT immunostaining with the staining distribution were evaluated singly. In our study, we evaluated the intensity and the distribution of staining together. Therefore, there is no methodological parameters for comparison between the two studies.

No significant statistical correlation was observed among the clinicopathological parameters examined and MT immunostaining in malignant cells. Similar results to those found by Theocharis et al. 2011 which separately assessed MT distribution and staining intensity. Some studies in the literature did not show correlations with primary tumour size and grade of tumour differentiation. However studies of Lee et al., Szelachowska et al., and Szelachowska et al. showed a positive correlation between lymph node involvement and the intensity of MT expression. Szelachowska et al. analyzed cytoplasmatic and nuclear MT-1/2 expressions separately.

Zavras et al. demonstrated that certain MT-1 allotypes may be genetic risk factors which increase susceptibility to OSCC and were consistently associated with a higher risk for advanced stage, increased involvement of lymph node and dedifferentiation of the tumor.

The significance of subcellular localization of MT at the nucleus/cytoplasm in OSCC is also not well understood. In our study, we also compared the exclusive cytoplasmic localization of MT with the clinical/histological variables (data not shown), but no statistically significant results of MT expression with any of the investigated characteristics were found, which means that, in our sample, the sublocation of MT was unrelated to the aggressiveness of the OSCC.

The differences between our study and the others which developed associations between histological grade, clinical features and positive prognostic with MT may be related to the number of analyzed samples and the heterogeneity of the samples.

In conclusion, the present study showed that whilst MT is highly expressed in human OSCC, it is not correlated with clinicopathological characteristics. Therefore, this protein does not seem to exert a crucial role in the aggressiveness of this tumor in the cases evaluated. The accumulated knowledge of MT expression in relation to clinicopathological parameters has provided contradictory results, possibly due to the methodology of evaluation; the specificity of OSCC affected sites used in the studies, or because of tumor-specific characteristics. Further studies are necessary to understand the participation of MT in oral carcinogenesis.

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