Expression of Metallothionein and p53 Antigens are Correlated in Oral Squamous Cell Carcinoma

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Abstract. *Background: Metallothionein and p53 proteins have been associated with tumoral evolution and resistance against therapy. Experimentally, the former may modulate the activity of tumor suppressor protein through zinc exchange. However, there is no information on the relationship of these proteins in oral cancer. Patients and Methods: Immunohistochemical detection of metallothionein and p53 antigens was performed in 100 oral squamous cell carcinomas. Results were compared to evaluate possible relationships between them and the disease-specific survival. Results: Mean cellular indexes of positivity were 66.5% and 52.5% for metallothionein and p53, respectively, and a positive correlation was found between them. Frequent nuclear metallothionein immunolocalization was associated to increased p53 expression. Concomitant overexpression of both antigens predicted shorter survival for patients with advanced disease. Conclusion: These results corroborate the speculated association between metallothionein and p53 and suggest that simultaneous assessment of these proteins may be useful to evaluate aggressiveness of oral cancer.*

Oral cancer figures among the prevalent malignant diseases in countries such as United States, India, Brazil and France (1). Advanced lesions are common but not easily surgically resectable resulting in frequent use of radio and chemotherapy (2). However, tumoral resistance makes such therapy ineffective in many cases. Thus, the study of

molecular markers for patient selection to these therapeutic regimens and prognostication becomes crucial (3).

Metallothionein (MT) is a non-enzymatic protein overexpressed in many cancers, such as in oral squamous cell carcinoma (OSCC), resulting in worse prognosis to the patient (4-6). The cause of this effect is obscure. MT can bind to and neutralize noxious agents (*e.g.* free-radicals generated by radiotherapy; cisplatin), impairing its capacity to kill neoplastic cells (7, 8). Furthermore, MT is mainly associated with zinc, an element with independent anti-oxidant properties (4, 9). MT notably scavenges zinc atoms from other proteins either when the metal is scarce or in oxidant environment (10, 11). Hypothetically, harmful effects on cell metabolism would follow, since an over-activity of MT could impair the normal function of essential apoenzymes (12, 13). In addition, nuclear retention of MT is energy-dependant and likely related to zinc interchange with nuclear proteins (9).

The p53 protein is one of the most important markers for normal cellular growth and proliferation and also mediates many effects of non-surgical cancer therapy. Its inactivation has been linked to tumoral resistance against radiation and chemotherapy, as well as to poorer prognosis to the patients (14-16). Mutations regarding the *TP53* gene have been reported in almost 50% of head and neck squamous cell carcinomas, but many lesions appear in the absence of such phenomenon (17, 18). Alterations in other constituents of the p53 signaling pathway (*e.g.* p16, p21 and mdm2) helps to explain these cases, but other molecular actors may also be enrolled (14). In this way, normal p53 acts as a transcription factor that binds to DNA through well-studied zinc-finger domains. Serial point mutations in *TP53* exemplified the essentiality of zinc for the normal function of the protein (19). In the same way, removal of the zinc atom from p53 tetramers abolishes functional activity (20-22). Moreover, almost all frequent *TP53* mutations relate to an interaction with zinc (19, 23).

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Metal-free MT can disrupt DNA binding activity from p53 through zinc chelation, thus leading to its non-mutational inactivation (10, 12, 20). Control of p53 activity by MT has been reported, as well as a role for p53 in MT gene activation (24, 25). Finally, some authors have proposed that overexpressed MT could significantly impair p53 function, thus facilitating carcinogenesis and tumoral progression (13, 26). Although MT and p53 expression have been studied in OSCC, as well as their isolated effects on patient prognosis (6, 27) it has not been possible to find any previous work on the relationship of these two proteins in this malignant disease.

In the present paper, a significant positive correlation between indexes of MT and p53 expression on OSCC is described. Furthermore, nuclear MT expression was associated to increased indexes of p53 positivity. Finally, there was significant deterioration in survival of patients presenting tumors with high expression of both proteins, corroborating the importance of their interaction on the behavior of the disease.

Patients and Methods

This study was submitted to and approved by Institutional Reviewer Boards.

Patients and tissue specimens. This study was performed on 100 samples of oral squamous cell carcinomas (ICD C01-C06), obtained from the first surgical intervention on 78 men and 22 women without any previous treatment. Mean age of the patients was 58.9 years old (SD±12.4). Primary sites included tongue (36 cases), floor of the mouth (22 cases), gums and hard palate (20 cases), oral mucosa (3 cases), and soft palate, tonsils and oropharynx (19 cases). From 92 lesions with available TNM data, only 23 (25%) presented at initial stages of the disease. Seventy-seven lesions were classified as well-differentiated tumors.

Immunohistochemistry. All specimens were fixed in formalin and embedded in paraffin. Three-micrometer sections were exposed to monoclonal antibodies anti-metallothionein and anti-p53 (clones E9 and DO7, respectively, both from DAKO Corp, Carpinteria, CA, USA), using a streptavidin-biotin-peroxidase immunostaining system (LSAB+, DAKO, USA). Briefly, after deparaffinization and dehydration in a graded ethanol series, sections were treated for 30 minutes with EDTA buffer (10 mM, pH 8.2) in a steamer. Incubation with primary antibodies was then conducted using dilutions of 1:100 for MT and 1:75 for p53, at 4˚C, for 18 and 24 hours, respectively. After amplification, diaminobenzidine was used as chromogen followed by counterstaining with Harris' hematoxilin. Squamous cell carcinoma and breast ductal carcinoma with previously demonstrated immunostaining for both antigens were used as positive controls. Negative control consisted of the omission of the primary antibodies.

Evaluation of the immunohistochemical staining. Immunohisto-chemical staining, independently of intensity, was quantified by two observers by mean indexes of labeled cells among 500 tumoral cells counted for each sample. When required, overexpression was determined by cut-off values of 76% for MT or 10% for p53 (6,

16). Since sub-cellular restriction of MT has been linked to its function, two subgroups were delineated among 50 samples depending on whether in each one of the lesions there were more cells with cytoplasmic restricted localization or larger number of cells with concomitant nuclear and cytoplasmic positivity, followed by statistical comparison between respective indexes of p53 staining of these groups.

Disease specific survival. From hospital files of the original sample, it was possible to consistently retrieve both disease specific survival time (DST, defined as the time interval between the first surgical intervention and cancer-related death or the last follow-up before preparing this manuscript) and TNM (Tumor-Node-Metastasis) clinical staging of 50 patients. Cases staged as TNM I or II (n=10) were excluded from specific survival comparison according to MT and p53 expression. In addition, these patients also presented better DST than those with advanced disease (*p*=0.05, log-rank test). Therefore, 40 patients with advanced disease (TNM III and IV) were included in survival analysis. Follow-up was completed from first surgical intervention to cancer related death in 25 cases (62.5% of patients assessed in this part of the study), using death from other causes and loss of follow-up (15 cases, 37.5%) as censoring variables. DST of patients with complete observation (first surgery to cancer-related death dates) ranged from 1 to 37 months (11.0±3.5 months).

Statistical analyses. Statistical significance was defined at a level of 5%. Spearman test was applied to investigate the possible correlation between the immunohistochemical indexes of staining of MT and p53. Mann-Whitney test was employed to compare mean p53 expression between lesions according to subcellular MT expression. These statistical analyses were performed with the software BioEstat 3.0 (28). Survival was compared by Kaplan-Mayer estimates and log-rank (Cox-Mantel) test for equality of survival curves (software KMSurv, as cited by Campos-Filho and Franco) (29).

Results

Immunoreactivity for MT was observed as brown pigmentation in cytoplasm, or nucleus, or both, while staining of p53 was only nuclear, as shown in Figure 1. Mean indexes of reactivity were 66.5% (±22.4%) for MT and 52.5% (±33.6%) for p53. MT index varied from 1% to 98% (33 cases ≥76%), while p53 localization ranged from zero to 98% (77 cases ≥10%). These indexes were positively correlated to each other (R=0.26, *p*=0.01).

With reference to sub-cellular localization of MT immunostaining, the group of lesions with predominant cytoplasmic restricted localization (10 cases) was associated to a statistically significant lower mean p53 immunostaining index (15.9%±26.0% *versus* 47.6%±32.0% from the other 40 lesions depicting more frequent concomitant cytoplasmic and nuclear MT immunolocalization, *p*=0.01). Interestingly, only nine out of the 40 (22.5%) cases with more frequent nuclear/cytoplasmic reaction for MT were not negative for p53, compared with seven out of the 10 (70.0%) remaining lesions with cytoplasmic restricted staining (*p*=0.01, χ^2 test).
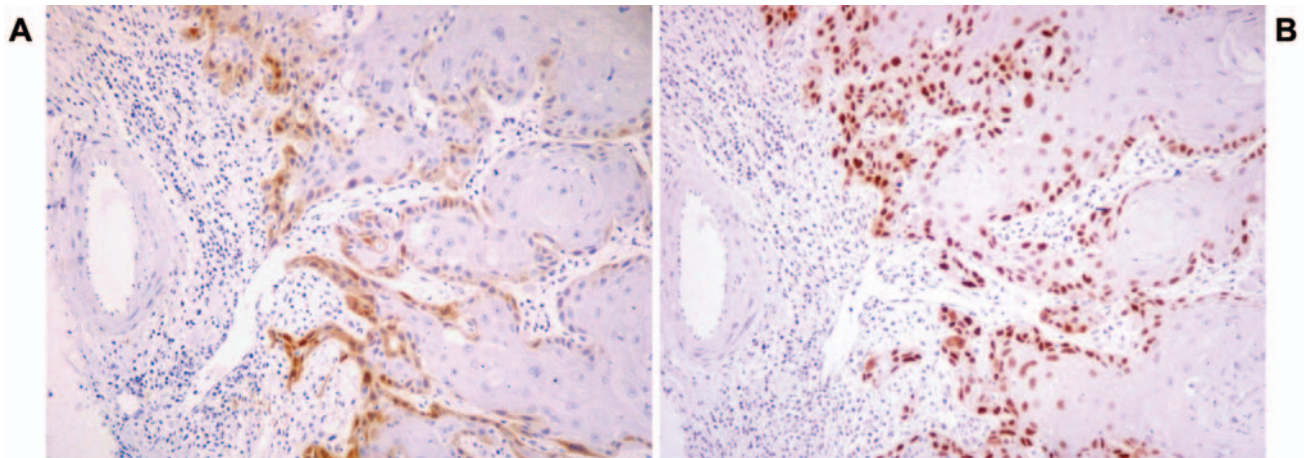
Figure 1. *Immunohistochemical localization of the metallothionein (A) and p53 protein (B) in oral squamous cell carcinoma. Note the heterogeneity in intensity for metallothionein, and distribution of staining for both antigens (streptavidin-biotin-peroxidase, original magnification=500×).*

MT indexes of immunostaining of these groups were not significantly different (61.1% *versus* 57.8%, *p*=0.9).

Cases with concomitant overexpression of MT and p53 were significantly associated to faster deterioration of cumulative survival than the other patients (*p*=0.02), as shown in Figure 2. Isolated effects of MT or p53 expression on patient prognosis were only near to statistical significance (*p*=0.06 or 0.14, respectively).

Discussion

The presented data indicate that immunohistochemical assessment of MT and p53 expression in OSCC supports the proposed interaction between these proteins (13, 26) and suggests an interesting pathway for oral cancer development. In addition, there are no previously reported studies examining the concomitant effect of MT and p53 on the survival of patients with OSCC and these results suggest that this interaction may result in significant prognostic deterioration for those patients with clinically advanced lesions which present an increased expression of these two antigens.

These results are similar to previous reports on the cytological pattern of expression of MT and p53, as well as indexes of staining (6, 27). A significant correlation was observed between the labeling indexes in the studied cases, indicating that some direct or indirect relationship may exist between these two proteins. Positive correlation was also previously found between MT and p53 immunostaining in endometrial and small cell lung carcinomas (30, 31). Inverse correlation was found in colitis-associated colorectal carcinoma (32). Other studies did not find any significant correlation in breast and larynx (33-35). The use of semi-quantification instead of objective investigation through
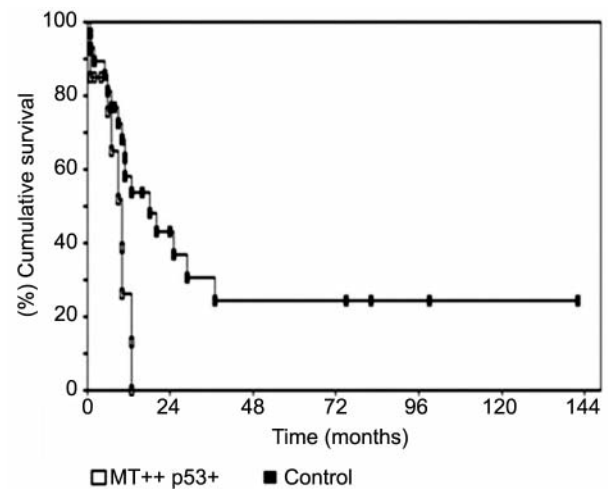

Figure 2. *Oral squamous cell carcinoma cumulative survival in 40 patients with advanced disease, as segregated by the concomitant evaluation of metallothionein (MT) and p53 immunostaining indexes. "MT+p53+", cases overexpressing MT and positive for p53 immunostaining (n=13); "control", other patients (n=27). Respective cut-off values=76% and 10% for MT and p53.*

indexes of immunohistochemically stained cells may be partially responsible for these inconsistencies. Tissues with different embryologic origin also differentially express isoforms of MT (5) and this fact may be reflected in its interaction with p53. Also, double-staining techniques have not been used up till now to actually demonstrate co-expression of MT and p53, and results obtained from such investigations would be of value to clarify the interaction between these proteins. In this sense, formation of complexes between MT and p53 has been recently clarified (24). On the other hand, weak correlation found between MT and p53

indexes (R=0.26) indicate that an interaction between these proteins is not the only factor responsible for their expression. This is as expected since it has been widely shown that other proteins and conditions are associated with p53 function in the cell (14, 23). Although the most important source of p53 disruption is mutation in *TP53*, which is found in more than 50% of all OSCC (18), some tumors evolve in absence of such alteration. This fact calls for alternative explanations for oral carcinogenesis. In this sense, further studies should be carried out to specify strength, ways and biological implication of MT and p53 association in neoplastic and normal cells. The status of the *TP53* gene should be studied in lesions presenting a high level of MT and p53 expression, to determine whether the stabilization of the p53 protein is linked to mutation or to an epigenetic phenomenon such as zinc removal by MT.

The biological basis for this hypothesis is founded upon studies that have proposed the chelation, caused by MT, of zinc from other proteins (10, 12). In the p53 context, this situation would impair its tumor suppressing activity, contributing to neoplastic transformation. Indeed, the association between the two proteins has been reported in experimental studies showing that chelation, caused by MT, of zinc from p53 may result in the structural inactivation of the former, abrogating its DNA-binding ability (20). It could also theoretically impair the p53-mediated effects of oncologic treatments over tumoral cells. Moreover, moderate quantities of zinc-depleted p53 may cause a dominant negative phenotype in the cell, since the altered protein is able to aggregate to wild-type protein, thus impairing its DNA-binding activity (21). Breast cancer cells that do not produce p53 express significantly lower levels of MT than wild-type cells (36). In view of these findings, some authors have proposed that MT could in fact regulate p53 stability and DNA-binding activity (13, 20). Another interesting finding of the present work was that the presence of MT in the nucleus was found to be associated with p53 positivity, suggesting that co-localization may be relevant to the interaction between them. Frequent nuclear MT localization was recently associated to worse disease free survival for patients with oral cancer (37).

Besides supporting a biological association, the present study observed that concomitant MT overexpression and p53 positivity led to a poor prognosis of patients with advanced-stage disease. Prospective studies should be carried out to support this result. It was not possible to locate any other study on other cancers regarding this prognostic association. Neoplastic lesions that are usually associated with overexpression of MT (*e.g.* breast cancer) should also be searched for a likely prognostic deterioration in cases also associated with p53 stabilization. Clinically, patients with concomitant expression of MT and p53 should be investigated to determine whether this phenotype is associated to resistance against radiation or chemotherapy. Since there is a hypothetical MT impairment over the wild-type p53 activity,

therapies should be developed that do not cause overexpression of the former (5, 13). This is also highlighted by observation of an anti-apoptotic role for MT, as well as increased proliferative activity in malignancies with high expression levels of this protein (13).

In conclusion, MT and p53 expression seems to be associated in OSCC, as suggested in previous experimental studies. This fact should be further studied to clarify its clinical applications and relevance for carcinogenesis. In addition, this was the first study to indicate a prognostic impact of this interaction, since patients with advanced-stage disease and overexpression of both antigens presented a lower survival rate. Of greater interest would be a larger and prospective study, as well as investigations in a -more anatomically defined subpopulation of patients. Subsequently, therapeutic schedules should be adjusted to do not to cause an increase in the MT levels in neoplastic cells, which has shown to be hazardous to patients with cancer.

Acknowledgements

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