Prognostic significance of nuclear and cytoplasmic expression of metallothioneins as related to proliferative activity in squamous cell carcinomas of oral cavity

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Summary. Metallothioneins (MT) are low molecular weight proteins with high metal and cystein contents. This study was designed to test the hypothesis that cytoplasmic and nuclear MT expression are of prognostic importance in patients with squamous cell carcinomas of the oral cavity, treated by surgery with subsequent radiotherapy. The second aim of the study was to test the potential correlation between the nuclear and cytoplasmic MT expressions as compared to expression of proliferation markers and other clinicopathological variables. Material and Methods: The studies were performed on tumor samples from 50 patients with diagnosis of squamous cell carcinoma of the oral cavity floor or of oral part of the tongue. All the patients were subjected to radical surgery, accompanied by removal of lymph nodes and post-operative radiotherapy. Results: No significant correlation could be detected between percentage and intensity of MT expression on one hand and proportions of cells with Mcm-2 (minichromosome maintenance protein 2), Ki-67 expressions, nor the grade of malignancy (G) on the other. A significantly shorter survival was detected among patients with tumors of MT expression rated 9 or 12 according to the Remmele scale and among patients with a high percentage (> 50%) of nuclear MT staining. In mulivariate analyses, only OTT (Overall Treatment Time), lymph node involvement and high expression of Mcm-2 were found to be independent risk factors for decreased patient's survival. Conclusion: This is relevant evidence that MT overexpression could be related to worse prognosis in patients with oral cancer. We have found no relationship between MT expression and proliferative activity.

Key words: Oral cancer, Squamous cell carcinoma, MT, Mcm-2, Ki-67

Introduction

Metallothioneins (MT) are low molecular weight proteins with a high metal and cystein content. MT's bind to cadmium, zinc and copper (Tapiero and Tew, 2003). MT may protect against certain metal toxicity, and may donate zinc/copper to certain metallo-enzymes and transcription factors (Cherian et al., 2003). It may also protect against oxidative stress (Podhorska-Okolow et al., 2006). Although a number of biological functions have been proposed for MT, most of them are related to metal binding properties (Cherian et al., 2003). Since MT is present in most tissues and cell types in small amounts, it is generally considered a "housekeeping" protein. At the cellular level, MT is mainly distributed in the cytoplasm, and to a lesser extent in the nuclei and lysosomes, but it occurs also in blood (Tapiero and Tew, 2003). MT is mainly a cytoplasmic protein in adult tissues, although it is also detected in nuclei of cells in fetal/early neonatal period (Cherian et al., 2003). A transient localization of MT has been reported in the cell nucleus under certain conditions, such as cell proliferation and differentiation (Cherian et al., 2003). The significance of subcellular localization of MT in nucleus/cytoplasm in certain tumors is also not well understood. Some results suggest that MT induction by zinc in prostate cancer cells is associated with resistance to cisplatin and radiotherapy (Smith et al., 2006). Others have demonstrated that nuclear localization of MT is associated with cisplatin resistance in cancer cell lines (Cherian et al., 2003; Surowiak et al., 2005, 2007). The staining pattern for MT in human tumors is not only dependent on tumor growth but may be influenced by

various factors, such as the type of tumor, cellular origin, histological type, morphological heterogeneity, proliferation rate or degree of differentiation and stage of growth. The majority of prognostic studies have observed an inverse correlation between MT expression and patients survival (Ofner et al., 1994; Douglas-Jones et al., 1995; Haerslev et al., 1995; Jasani and Schmid, 1997; Hiura et al., 1998; Hishikawa et al., 1999; Ioachim et al., 1999; Cardoso et al., 2002; Dziegiel, 2004; Dziegiel et al., 2005). However, there is no consensus in this respect (Jasani and Schmid, 1997). In numerous studies on epithelial tumors a positive correlation was detected between MT and Ki-67 antigen expression (Dziegiel et al., 2003; Dziegiel and Zabel, 2006). Another analyzed marker of proliferation, Mcm-2 protein (minichromosome maintenance protein 2), belongs to the family of 6 minichromosome maintenance 2-7 proteins (Mcm 2-7), engaged in recognition and control of DNA replication, and could be useful as a prognostic factor in patients with squamous cell carcinoma of the oral cavity (Szelachowska et al., 2006). In some types of tumors of epithelial origin other reports demonstrated a strict relationship between MT expression and the grade of histological malignancy – G (Dziegiel et al., 2003).

This study was designed to test the hypothesis that cytoplasmic and nuclear MT expression are of prognostic importance in patients with squamous cell carcinoma of the oral cavity, treated surgically with subsequent radiotherapy. The second aim of the study was to test a potential correlation between the nuclear and cytoplasmic MT expression, as compared to markers of proliferation and other clinicopathological variables.

Materials and methods

Patients

The studies were performed on tumor samples from 50 patients with diagnosis of squamous cell carcinoma of the oral cavity floor or of the oral part of the tongue. All the patients were subjected to radical surgery, accompanied by removal of lymph nodes and post-operative radiotherapy conducted in Wroclaw Medical University (WMU) and in Lower Silesian Centre of Oncology (LSCO) between 1996-2002. The pathological degree of tumor advancement was established in all patients using the TNM system (Sobin and Wittekind, 1997). On the other hand, the degree of histological malignancy (G) was evaluated in the three-grade scale, in line with the classification of the World Health Organization published in 1997 (Pindborg et al., 1997). The mean age of the examined patients was 56 years, and the group included 9 women and 41 men. In 31 patients the primary neoplastic lesion was located in the floor of the oral cavity and in 11 patients in the mobile part of the tongue, while in the remaining patients diffuse infiltration of both structures was observed. All the patients were postoperatively irradiated.

Fractionation of radiotherapy was 2 Gy per day, with five fractions per week, the total dose was 50-66 Gy (medium dose 58.7Gy). The Overall Treatment Time (OTT, time from operation to the end of radiotherapy) was 71 to 212 days (mean: 121 days). The survival of the patients, (SFLR – survival free of locoregional relapse, DFS – disease-free survival, OS - overall survival and DSS – disease-specific survival) was calculated on the basis of follow-up, performed in both LSCO and WMU and using the data of Lower Silesian Cancer Register (Wroclaw, Poland). The follow-up was terminated in patients living for over 5 years with no traits of progression of disease.

Immunohistochemistry

The tumor samples were fixed in 10% buffered formalin, dehydrated and embedded in paraffin blocks. All immunohistochemical reactions were conducted in paraffin sections. For the estimation of MT, Mcm-2 and Ki-67 antigen expressions, mouse monoclonal antibodies were used (clone E9; 1:100; DAKO, Denmark; clone CRCT2.1; 1:50; Novocastra Laboratories, UK and clone MIB-1; 1:50; DAKO, Denmark, respectively). All the reactions were accompanied by negative controls, in which specific antibodies were substituted by the Primary Negative Control (Dako, Denmark). The studied paraffin sections were boiled in the Antigen Retrieval Solution in a microwave oven to unblock antigenic determinants for Ki-67 and Mcm-2. The investigated antigens were visualized using biotinylated antibodies, streptavidin-biotinylated peroxidase complex (LSAB2 kit, Dako Denmark) and diaminobenzidine (DAB, Dako Denmark). The intensity of immunohistochemical reactions was independently evaluated in coded preparations by 2 pathologists. Cytoplasmic MT expression was evaluated using the semi-quantitative IRS scale according to Remmele, taking into account the intensity of the color reaction and the percentage of cells with cytoplasmic MT expression (percentage in a five-grade scale of 0% scored as 0; 1-10% scored as 1 point; 11-50% scored as 2 points; 50-80% scored as 3 points; >80% scored as 4 points, and intensity of staining in a four grade scale of 0 to 3 points) (Remmele and Stegner, 1987). The final score represented a product of scores representing the two variables (IRS = a x b) and ranged from 0 to 12 points. We assumed that the cytoplasmic MT expression was high when it was scored as ≥ 9 points. The nuclear MT expression was evaluated using a semi-quantitative five–tiered grading system, which took into account percentage of cells manifesting nuclear reactions (0 points when no cells were positive, 1 point when 1-10% , 2 points when 11-50%, 3 points when 50-80%, and 4 points when >80% cells were positive). We assessed nuclear expression of MT as high when it was scored as ≥ 3 points. The evaluation of Ki-67 antigen and Mcm-2 protein expressions was conducted using a scale which took into account the percentage of cells manifesting nuclear reactions: no reaction – 0 points

(pts), 1-10% - 1 point, 11-25% - 2 pts, 26-50% - 3 pts and over 50% - 4 pts (Dziegiel and Zabel, 2006).

Statistical analysis was performed using software STATISTICA, version 6 (Krakow, Poland). Kaplan-Meier curves were calculated for each variable. The log-rank test and the Cox proportional hazards model were used to examine the relationship between survival and various potential prognostic factors. This analysis was performed on the data pertaining to the cytoplasmatic MT expression, proportion of cells manifesting nuclear MT expression, proportion of cells manifesting Mcm-2 and Ki-67 expression, degree of histological malignancy (G), gender, age, pathological stage of cancer (pTNM), presence of metastases in lymph nodes (N+), overall treatment time (OTT), duration of radiotherapy, interval between surgery and radiotherapy and size of total dose of radiotherapy. The association of all markers with clinical and pathological parameters was evaluated using the Chi-Square Test and Spearman correlation. The level of significance was set at $p<0.05$.

Results

In the entire studied group, the 5-year cumulative SFLR was 72%, DFS was 62%, the DSS was 62%, and the OS was 52%. The 5-year SFLR, DFS, DSS and OS in patient's subgroups are provided in Table 1.

Immunoreactivity for MT was observed in most of the cases. In a single patient only we could not detect MT presence in tumor cells. Frequently, in the same patient, cancer foci with high and other ones with low MT expression were observed. The staining results for MT marker are summed up in Table 2 and Table 3. Cytoplasmic expression of MT was evaluated according to the Remmele scale: in 1 patient the tumor manifested 0 points, in 7 patients it showed 1 pt, in 10 patients 2 pts, in 12 patients 4 pts, in 9 patients it had 6 pts, in 9 patients 9 points and in 2 patients it manifested 12 points. We assumed cytoplasmic MT level as high if the score was 9 or 12 (in 11 cases).The percentage of the cells with nuclear MT staining was appraised at 0 points

Table 1. Cumulative 5-year survival (Survival Free of Locoregional Relapse, Disease Free Survival, Disease Specific Survival, Overall Survival) as related to selected clinicopathological factors.

Parameter		No. of patients	SFLR	DFS	DSS	OS
Gender	female	9	78%	78%	78%	78%
	male	41	70%	59%	58%	46%
p value			NS	NS	NS	NS
Grading	G1	9	75%	63%	63%	56%
	G2	37	70%	61%	60%	49%
	G3	4	75%	75%	75%	75%
p value			NS	NS	NS	NS
Lymph node involvement	N+	31	64%	53%	53%	48%
	N-	19	83%	77%	76%	58%
p value			NS	0.057	0.06	NS
Age	>50	37	66%	57%	56%	43%
	<50	13	85%	77%	77%	77%
p value			NS	NS	NS	<0.05
TNM stage	I	2		unassessed		
	II	6	67%	67%	67%	50%
	III	15	79%	67%	67%	60%
	IV	27	71%	60%	59%	48%
p value			NS	NS	NS	NS
OTT	> 110 days	28	55%	49%	48%	42%
	<110 days	22	94%	80%	80%	63%
p value			<0.05	<0.05	<0.05	NS
Mcm-2	<10%	22	81%	76%	76%	68%
	>10%	28	63%	52%	50%	39%
p value			NS	NS	0.055	<0.05
Cytoplasmic MT expression	high	11	45%	36%	36%	36%
	low	39	78%	70%	70%	56%
p value			NS	<0.05	<0.05	NS
Nuclear MT expression	high	9	39%	33%	33%	33%
	low	41	79%	69.1%	68.7%	56%
p value			<0.05	<0.05	0.07	NS
Ki-67	<10%	18	59%	59%	59%	55%
	>10%	32	79%	64%	64%	50%
p value			NS	NS	NS	NS

in 5 cases, 1 pt in 19 cases, at 2 in 17 cases, at 3 in 9 cases and at 4 in none of the cases. Most of the tumors manifested both the cytoplasmic and the nuclear expression of MT (Table 3). The higher intensity of the cytoplasmic reaction was accompanied by the higher expression of MT in the cell nuclei. A significant correlation (R=0.8; p<0.05) between the cytoplasmic and nuclear expression of MT was detected.

No significant correlation could be detected between cytoplasmic or nuclear MT expression and proportions of cells with Mcm-2, Ki-67 expressions (Fig. 1) nor the grade of malignancy (G) (Table 1). Among the remaining clinicopathological factors, only lymph nodes involvement (N+) significantly correlated with the percentage of cells expressing cytoplasmic MT reaction (R= 0.32; p<0.05).

All the evaluated clinicopathological variables were

Table 2. Number of patients with cytoplasmic expression of MT (as related to % cells manifesting the expression and intensity of the reaction). Cytoplasmic MT expression according to Remmele's scale represented a product of scores representing the two variables, a and b (IRS = a x b), and ranged from 0 to 12 points.

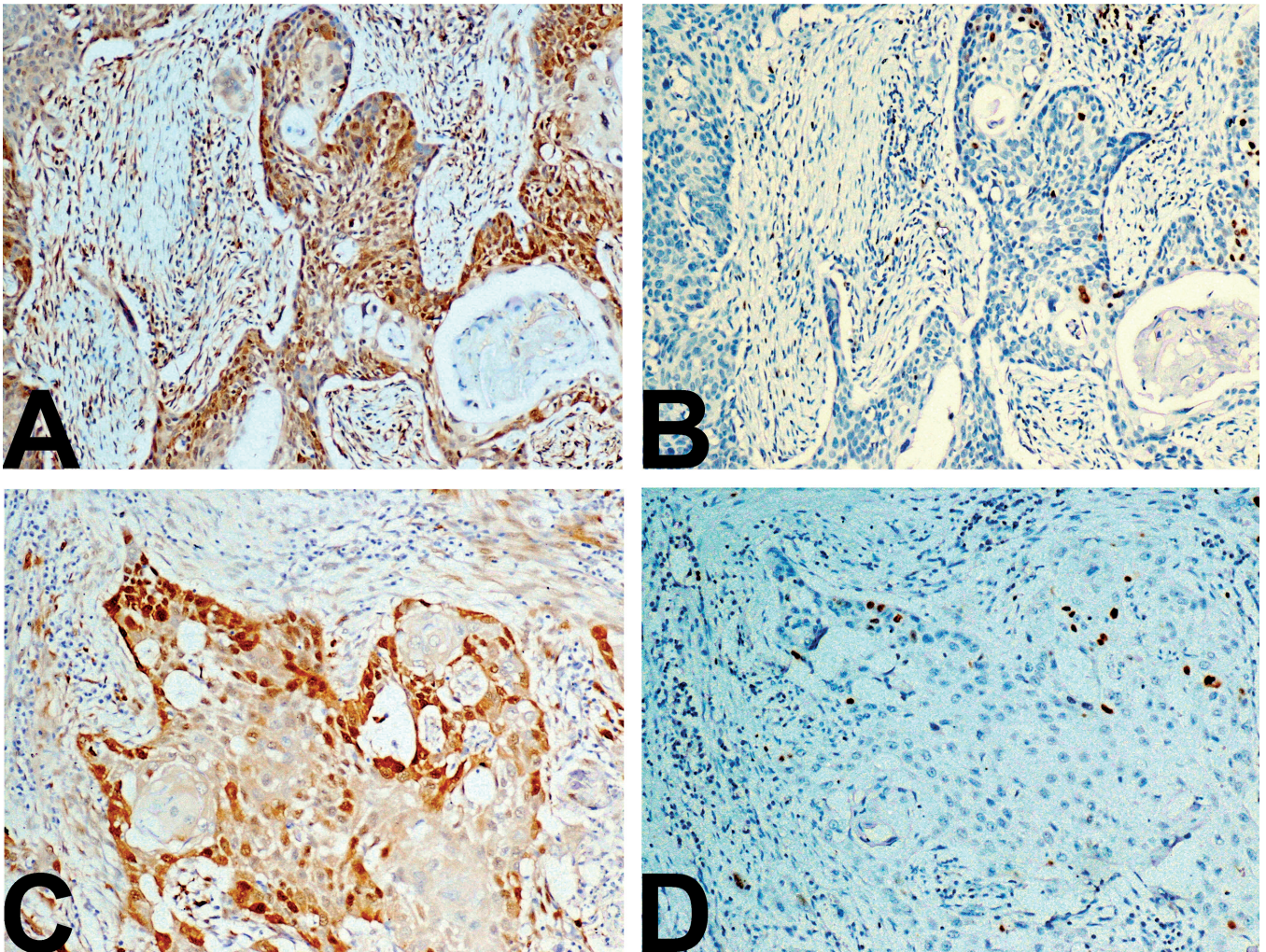| percentage of the cells with cytoplasmic MT reaction (a)	intensity of cytoplasmic MT reaction (b)				number of patients
	0	1	2	3	
0 (0%)	1	0	0	0	1
1 (1-10%)	0	7	10	0	17
2 (11-50%)	0	0	12	1	13
3 (51-80%)	0	0	8	9	17
4 (>80%)	0	0	0	2	2
number of patients	1	7	30	12	50

Fig. 1. Expressions of MT, Mcm-2 protein and Ki-67 in cells of oral cavity squamous cell carcinoma. A, B and C, D are serial sections. **A.** High nuclear and cytoplasmic expression of MT in cancer cells. **B.** Low nuclear expression of Ki-67 antigen in cancer cells. **C.** High nuclear and cytoplasmic expression of MT in cancer cells. **D.** Low nuclear expression of Mcm-2 protein in cancer cells. Counterstained with hematoxylin. x 100.

Table 3. Number of patients demonstrating in parallel variable intensity of nuclear and cytoplasmic expression of MT. Nuclear expression is expressed by the percentage of cell nuclei manifesting MT presence. Cytoplasmic expression of MT is expressed in the scale of Remmele.

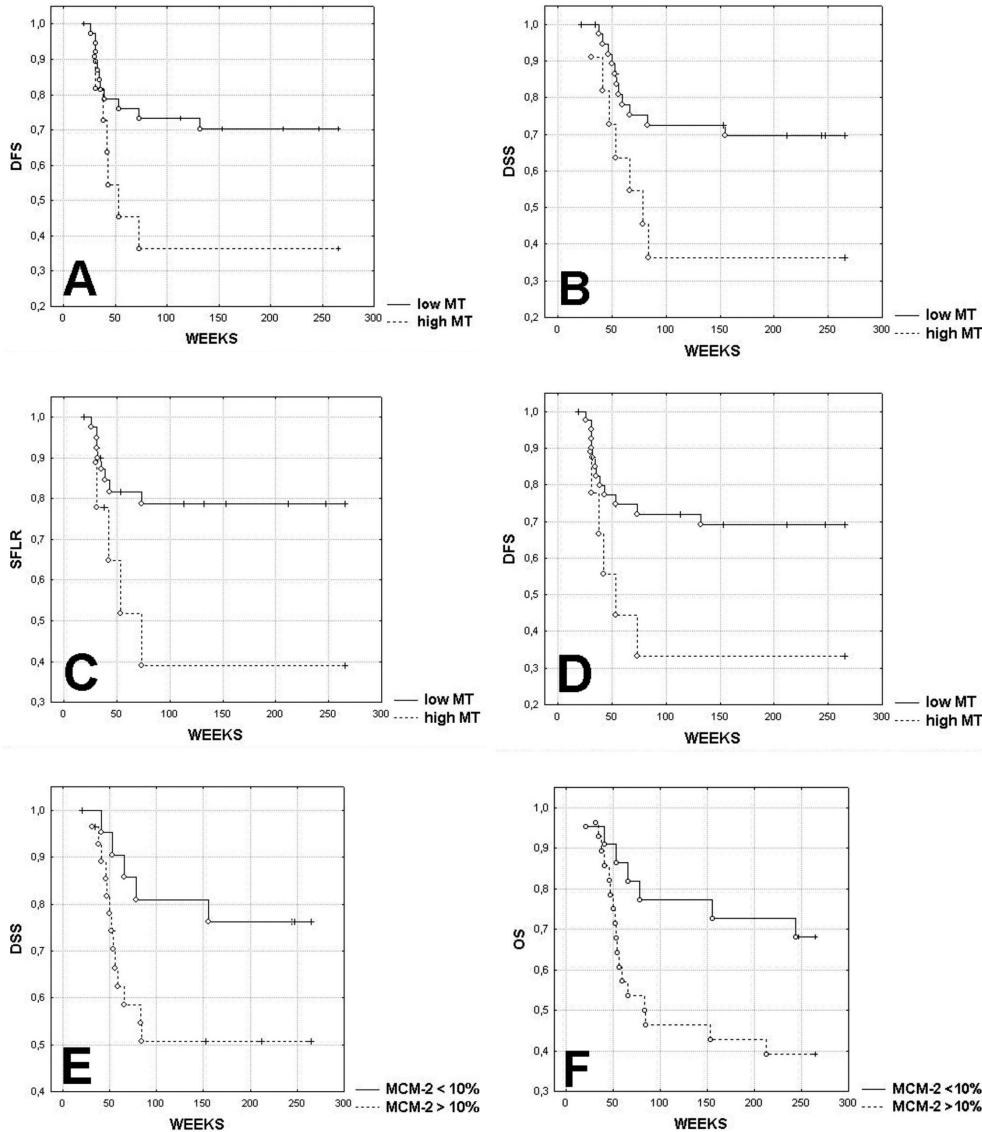| percentage of the cells with nuclear MT expression	cytoplasmic MT expression according to Remmele's scale							number of patients
	0	1	2	4	6	9	12	
0%	1	4	0	0	0	0	0	5
1-10%	0	3	8	7	1	0	0	19
11-50%	0	0	2	4	8	3	0	17
51-80%	0	0	0	1	0	6	2	9
>80%	0	0	0	0	0	0	0	0
number of patients	1	7	10	12	9	9	2	50

Fig. 2. A. Disease free Survival as related to cytoplasmic MT expression. **B.** Disease specific survival as related to cytoplasmic MT expression. **C.** Survival free of locoregional relapse as related to nuclear MT expression. **D.** Disease Free Survival as related to nuclear MT expression. E. Disease Specific Survival as related to Mcm-2 expression. F. Overall Survival as related to Mcm-2 expression.

Table 4. The results of multivariate Cox analyses for 5-year Survival Free of Locoregional Relapse, Disease-Free Survival, Disease-Specific Survival and Overall Survival.

Survival	HR	p value
Survival Free of Locoregional Relapse		
OTT > 110 days	13.7	0.01
Disease Free Survival		
OTT > 110 days	3.7	0.02
Lymph node involvement	3.2	0.05
Disease Specific Survival		
OTT > 110 days	3.9	0.01
Lymph node involvement	3.1	0.04
Mcm-2 >10%	3.0	0.04
Overall Survival		
Mcm-2 >10%	2.7	0.04
age > 50 years	3.8	0.04

examined in respect to their relationship to patient survival. A significantly shorter DFS and DSS duration was detected among patients with tumors where MT showed expression of 9 or 12, according to the Remmele scale (Fig. 2A,B). A significantly decreased SFLR and DFS survival was shown among patients with a high percentage (>50%) of nuclear MT staining (Fig.2C,D). Despite marked differences in the shape of survival curves, the differences in DSS were not significant. A significantly shorter OS duration was detected among the patients older than 50 years, and among patients with tumors in which over 10% cells manifested expression of the Mcm-2 protein (Fig. 2E,F). In the groups of patients whose OTT was longer than 15 weeks (more than 110 days) we observed significantly shorter DSS, DFS and SFLR survival. In addition, we found a close to significant decrease in DFS and DSS among the patients with the lymph node involvement (N+) (Table 1). None of the remaining studied parameters could be shown to exert significant effects on SFLR, DFS, DSS or OS.

Multivariate analyses were performed with the Cox proportional hazards regression model. Only the parameters which revealed statistical significance (or were close to such a significance) in the univariate analysis were entered. Among the factors, only OTT was found to be an independent risk factors for SFLR, DFS and DSS, with no influence on OS (Table 4). Lymph node involvement influenced DFS and DSS. High expression of Mcm-2 was revealed to be an independent risk factor for DSS and OS. Also, the older age of patients was an independent risk factor for OS (Table 4).

Discussion

In our studies we have failed to demonstrate any relationship between MT expression, independently evaluated in cytoplasm and in cell nuclei of cancer cells, and the two recognized proliferation markers, Ki-67 and Mcm-2. This might indicate that MT expression in squamous cell carcinoma of oral cavity is not linked to proliferative activity. Most of the data suggesting the linkage between MT activity and proliferation originated from material distinct from cancers of head and neck region (Nagel and Vallee, 1995; Hiura et al., 1998; Hishikawa et al., 1999). Also, the two reports published on nasopharynx cancers, and the other on oral cavity carcinomas have provided conflicting results (Jayasurya et al., 2000; Cardoso et al., 2002). In the first one, concerning nasopharyngeal cancer, a significant positive correlation was observed between Ki-67 and MT expression (Jayasurya et al., 2000). In the second, involving patients with oral cancer, no significant correlation was detected between MT index and other studied variables, nor between MT immunolabeling and the proliferative activity (Ki-67) of the tumors (Cardoso et al., 2002).

Some authors have observed a correlation between the MT levels and grade of differentiation of the tumors (Meskel et al., 1993; Moussa et al., 1997; Dziegiel et al., 2003). Then, we compared MT immunolabeling with the grade of differentiation of the tumors, but no statistically significant correlation has been found in our study.

In our study we have demonstrated that patients overexpressing both cytoplasmic and nuclear MT had significantly shorter survival than those presenting lower levels of MT expression. Our results are in agreement with the results of Cardoso's study (Cardoso et al., 2002). However, it remains unclear what the mechanisms are by which a higher content of MT in cancer cells results in an abbreviated survival of the patients.

MT seems to be related to neoplastic resistance to oncologic treatment and, therefore, it has been studied as a prognostic factor in a variety of human malignant tumors. Previous studies have demonstrated that overexpression of MT can confer resistance to radiation (Bakka et al., 1981; Cherian et al., 2003) and to antineoplastic drugs such as cisplatin (Satoh et al., 1994; Cherian et al., 2003). Nevertheless, the biological significance of MT remains to be fully recognized.

Relationships between MT on one hand and apoptosis, proliferation and differentiation of cells are still not understood (Cherian et al., 2003). The two basic mechanisms through which cell numbers are controlled in tissues involve apoptosis and proliferation. Neoplastic progression, in turn, is acompanied by a disturbed balance between these processes. Sundelin (Sundelin et al., 1997) observed that within the normal squamous cell epithelium of the oral cavity MT is located mainly in the basal and parabasal layers, characterized by active proliferation and absence of apoptosis. At the same time he has observed no MT in the more differentiated layers (Sundelin et al., 1997). In cases of squamocellular carcinoma of oral cavity, MT was noted mainly in tumor cells at the periphery of the tumor rather than in its central portion, in which apoptosis was more frequent (Sundelin et al., 1997; Muramatsu et al., 2000; Cardoso et al., 2002). On this basis Sundenlin concluded that MT may inhibit apoptosis (Sundelin et al., 1997). Because

MT may donate zinc/copper to certain metallo-enzymes and transcription factors, it can influence the process of proliferation or apoptosis, and modulate the behavior of the cell (Cherian et al., 2003). This might explain the translocation of MT to the cell nucleus in the course of G1-S phase during proliferation or, in cases of cell chemoresistance, following application of cisplatin (Apostolova and Cherian, 2000; Surowiak et al., 2007). Inhibition of apoptosis in cells subjected to radiation therapy results in an increase in proportion of surviving cells and, therefore, deteriorates the results of the treatment. Results of in vitro studies and of studies on animals prove that cells with high MT content are resistant to radiotherapy, and less frequently enter apoptosis than cells which are devoid of MT (Deng et al., 1999; Cai et al., 1999, 2004). Nevertheless, the mechanism in which MT can prevent apoptosis remains unclear.

Radiotherapy induces ionization of the irradiated environment and formation of the very reactive free radicals. The latter damage anything they encounter, including lipid cell membrane, DNA and proteins of importance for the cell. The probability of cell death increases with the number of lesions, their location and capacity for repair (Steel, 1997). Free radicals may become scavenged by sulfhydryl group-containing compounds. MT protein is rich in cystein (30%), an amino acid containing sulfhydryl (-SH) groups. MT is a very effective scavenger of free radicals (Cai and Cherian, 2003). In cases of a high content of sulfhydryl group-containing compounds (among others, MT) scavenging of free radicals takes place and, as a consequence, the sites of damage in the cell are less numerous and effects of radiotherapy are reduced (Cai and Cherian, 2003). Since cell death develops mainly due to damage to DNA (Warters et al., 1977) the nuclear localization of MT should exert a particularly pronounced radioprotective effect. Most probably, MT prevents against post-irradiation cell death by scavenging free radicals and a reduction of DNA injuries, and by its direct effect on apoptosis. It seems to us that MT protects against ionizing radiation mainly due to its antioxidative properties. It seems generally accepted that induction of MT results in an increased resistance to radiotherapy and chemotherapy (particularly with cisplatin) (Bakka et al., 1982; Cai et al., 1999). Induction of MT has been suggested to represent one of adaptive processes to LDR (Low Dose Rate Radiotherapy) exposure (Cai et al., 1999). Most studies have confirmed an increase in MT expression following chemotherapy and radiotherapy (Bakka et al., 1982; Cai et al., 1999; Smith et al., 2006; Surowiak et al., 2007). On the other hand, Muramatsu has noted no differences in MT level in tumor cells originating from patients with squamous carcinoma of head and neck subjected to neoadjuvant therapy (chemotherapy and/or radiotherapy) and those from patients treated by surgery only. Since both groups manifested a high MT index, the author suggested that perhaps overexpression of MT in tumor cells has no effect on the efficacy of the treatment (Muramatsu et al., 2000). We cannot agree with the author, since in our studies we have noted more frequent relapses of the disease and abbreviated survival of patients with high MT expression, and all our patients have been subjected to postoperative radiotherapy. It is probable that a high MT level in cancer cells has reduced the effects of radiotherapy and has deteriorated results of treatment in our patients.

Moreover, in our study we have found a significant positive correlation between the percentage of cells with cytoplasmic MT expression and the presence of lymph node metastases. But there was no correlation between the other parameters of MT expression (intensity of cytoplasmic reaction, MT expression according to Remmele scale, nuclear expression of MT) and lymph node involvement. In our opinion, this represents an interesting observation and it would be worth while to investigate the cause of the relationship between high content of MT in the primary tumor and presence of metastases in lymph nodes, observed by us. Until now, such a correlation has been noted only in the studies of Lee (Lee et al., 2008).

The presence of lymph node metastases is a very important prognostic factor in head and neck carcinoma (Leemans et al., 1994; Beenken et al., 1999). Among our patients, even if lymph node involvement caused marked alterations in the shape of survival curves, the alterations in DFS and DSS were not significant. But in multivariate analysis, the presence of metastases in lymph nodes represented an independent prognostic factor, decreasing DFS and DSS duration.

OTT longer than 110 days was negatively correlated with patients' survival. Both in univariate and multivariate analysis, OTT longer than 110 days was an independent prognostic factor for SFLR, DFS and DSS, with no affect on OS. This corroborates the results of previous studies (Muriel et al., 2001; Langendijk et al., 2003; Dietl et al., 2005).

Conclusion

In general, the results provided evidence that MT overexpression could be related to worse prognosis in patients with oral cancer. In the patients we could not find any relationship between MT expression and proliferative activity.

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