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Expression of Metallothionein in Oral Cancer

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1. Introduction

Metallothioneins (MTs) are ubiquitous proteins expressed in almost all organisms. MTs were isolated in 1957 for the first time from renal cortex as proteins responsible for binding cadmium (Margoshes & Valee, 1957, Coyle et al., 2002, Vasák et al., 2005). MTs are highly evolutionary conserved between species. Nevertheless, the role of these proteins has not been fully clarified yet and continues to generate interest among researchers. At present, several studies on MTs are focused on, their significance in the process of carcinogenesis, their potential prognostic value, and their involvement in resistance to cytostatic drugs.

1.1 MT structure and synthesis

MTs are low molecular weight of 6 –7 kDa proteins. The molecules of MT demonstrate a highly conserved amino acid sequence: the protein isolated from various organs of various animal species differ only insignificantly (Vasák et al., 2000). The molecule is formed by a single polypeptide chain of 61 to 68 amino acids, depending on the type. About 30% of MTs comprise of cystein residues while aromatic amino acids and histidine are absent. The cystein residues occur in typical tandem sequences of cys-aa1-cys, cys-aa1-aa2-cys, cys-cys, where aa1 and aa2 denotes amino acid other than cystein. The number and distribution of the sequences determine the tertiary structure, stability, and capability for binding metallic ions. The high number of cystein residues is a source of high content of thiol groups (-SH) through which metal ions are bound (Coyle et al., 2002). Their ability to bind heavy metals, such as zinc, copper, mercury, lead, nickel, iron and cadmium has been demonstrated in many studies. One MT molecule may bind up to seven ions of bivalent, and up to twelve ions of univalent metals (Palmiter et al., 1998). In the structure of MT two globular domains can be distinguished, α and β (Zangger et al., 2002). The C-terminal domain α comprises amino acids 31– 68 and binds four Cd ions while the N-terminal domain β, comprises amino acids 1 to 30 and captures three metal ions, including two Zn ions and one Cd ion. Within the MT molecule, the most pronounced antigenicity is shown by regions of the domain β (Dziegiel, 2004). MTs represent a non-uniform group of proteins. Analysis of their structure and function allowed distinction into four principal isoforms: MT-1, MT-2, MT-3 and MT-4 (Mididoddi et al., 1996). Isoforms MT-1 and MT-2 are well recognized and characterized because they are expressed almost in all tissues of the organism. The highest concentrations of these proteins were demonstrated in the kidney, liver, pancreas and intestine (Davis et al., 2000; Coyle et al., 2002). Expression of MT-3 and MT-4 isoforms is tissue specific and are present in the body in much lower amounts. MT-3 can be noted mainly in cerebral neurons of central nervous system (Hidalgo et al., 2001). The

expression of MT-4 is restricted to stratified squamous epithelium of the skin and upper part of the alimentary tract (Quaife et al., 1994). MT are encoded by 17 genes, including 13 genes that code for MT-1, two for MT-2, and individual genes coding for MT-3 and MT-4. There are at least 10 MT genes which encode functional proteins: MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, MT-1X, MT-2A, MT-3 and MT-4 (Palmiter et al., 1992; Quaife et al., 1994). In human, genes encoding for MTs are located on chromosome 16, within the 16q13 region (Mididoddi et al., 1996). MTs are intracellular proteins and their presence was demonstrated in the cytoplasm and in the cell nucleus (Bay et al., 2001). Their synthesis can be induced by several substances, such as heavy metals, hormones, cytokines, growth factors, organic compounds and free radicals (Samson et al., 1998; Haq et al., 2003; Ghoshal et al., 2001). The principal physiological factors, which induce MT synthesis are zinc ions (Zn^{2+}) which are bound by the transcription factor, MTF-1 (*metal response element-binding transcription factor*). The MTF-1 is a protein with domains of zinc finger structure, responsible for interaction with DNA. The MTF-1 binds to the MRE (*metal response element*) sequence within the MT gene promoter. Binding of MTF-1 to MRE initiates the process of MT gene transcription (Langmade et al., 2000; Otsuka et al., 2000; Saydam et al., 2002). The remaining metals (e.g. Pb^{2+}, Ni^{2+}, Fe^{2+}, Cd^{2+}, Bi^{2+}) initiate transcription of MT genes also with mediation of MRE but they are not bound by the MTF-1 transcription factor. They manifest a higher affinity to MT and they replace zinc ions from MT molecules. Released zinc ions are subsequently bound by MTF-1 (Mursta et al., 1999; Koizumi et al., 1999; Lichtlen et al., 2001). Similarly, oxygen free radicals could also replace zinc ions from MT molecule and in this way stimulate MT synthesis. Oxidation of MT by hydrogen peroxide (H_2O_2) leads to release of zinc ions (Andrews, 2000; Nguyen et al., 2003). MT synthesis could also be induced by other factors, such as glucocorticoids, which through glucocorticoid receptors (GR) bind to specific regulatory sequences, GRE (*glucocorticoid response element*) in the promoter region of MT genes (Davis et al., 2000; Hernández et al., 2000).

1.2 Effect of MT on cell proliferation and differentiation processes

MTs are thought to be engaged in the control of cell proliferation and differentiation (Schmidt et al., 1999). The metal binding properties of MT, including binding of zinc ions, allow MT to act as a zinc donor. The zinc-dependent enzymes play a crucial role in DNA replication, transcription and protein biosynthesis. Presumably, in this way MT could modulate the functional activity of many factors controlling the cell cycle (Ostrakhovitch et al. 2007). In the course of cell cycle the distribution of MT in the cell is changed from the cytoplasm to the nucleus, what may indicate the protein's involvement in DNA synthesis. In many studies an increased expression of MT both in the cell nucleus and in the cytoplasm was demonstrated in hepatocytes during liver regeneration, in kidneys undergoing compensatory growth following nephrectomy, and in rapidly growing parabasal cells of stratified squamous epithelium (Zalups et al., 1995; Ioachim et al., 1999; Cherian et al., 2006). The increased expression of MT, correlated to the augmented cell proliferation, was also observed in cells of various human tumours (Theocharis et al., 2004). In mitotically inactive cells (G0 phase) expression of MT can be detected in the cytoplasm while in dividing cells its activity becomes shifted to the nucleus. The high cytoplasmic expression of MT is observed at the end of G1 phase and at the G1/S threshold while the peak accumulation of MT in cell nucleus can be detected in phases S and G2 (Cherian et al., 2000; Levadoux-Martin et al., 2001). The translocation of MT into the nucleus during G1/S phase in tumour cells suggests

that MT facilitates cell proliferation by donating zinc ions to various transcription factors. The stabilization and binding of transcription factors to DNA depend entirely on zinc binding (Ostrakhovitch et al. 2007). In this way MT may control activity of various genes, including the p53 tumour suppressor protein in cells. In *in vitro* studies the transfer of zinc ions from MT to transcription factors was demonstrated (Langmade et al., 2000). The factors involve mainly protein factors with zinc fingers domains in their structures, such as estrogen receptors and MTF-1. Binding of zinc ions by MT was found to be a reversible process: in certain conditions MT may remove zinc from other protein molecules, in this way modulating their biological activity (Davis et al., 2000; Ogra et al., 2001).

1.3 Role of MT in a neoplastic process

Recent investigations confirmed that increased synthesis of MT occurs in neoplastic cells of various origin (Dziegiel, 2004). Investigators have focused on the significance of MT in the process of carcinogenesis and tumour progression but also on the involvement of the proteins in development of tumour chemoresistance as well as the possible role of MT expression as a prognostic and predictive factor. Results of several investigations suggest that the role of MT expression in tumour cells may be linked to the processes of proliferation and apoptosis (Jayasurya et al., 2000; Bay et al., 2001; Shimoda et al., 2003). MT functions as a donor of zinc ions for transcription factors and enzymes involved in the processes of DNA and protein synthesis. This is confirmed not only by elevated MT levels in hyperplasia but also by MT translocation from cytoplasm to cell nucleus during DNA synthesis (S phase) (Woo et al., 1996; Jasani & Schmid, 1997). In neoplastic tissues, the level of MT was shown to be proportionally elevated with the concentration of zinc ions (Jayasurya et al., 2000; Florianczyk et al., 2006). Similar results have been shown in *in vitro* investigations, showing that interaction between MT and p53 protein seems to be highly significant for tumour development, due to regulation of zinc ion homeostasis by MT (Meplan et al., 2000; Ostrakhovitch et al., 2006). p53 protein represents a transcription factor with a DNA-binding domain, stabilized by zinc ions. In normal conditions it inhibits proliferation of cells with a damaged DNA and directs cells toward apoptosis. MT molecules were found to be able to remove zinc ions from p53 protein, what results in its inactivity (Rainwater et al., 1995; Meplan et al., 2000). Inactivation of p53 protein in neoplastic cells results in their excessive proliferation and in inhibition of apoptotic processes. It is suggested that an increased synthesis of MT in tumour cells promotes interaction of MT with p53 protein, resulting in an uncontrolled proliferation (Fan et al., 2002; Ostrakhovitch et al., 2007). The results were confirmed by positive correlation between expression of MT and proliferation antigens, Ki-67 and PCNA. Such a relationship was demonstrated in several tumours, such as breast cancer, cancers of ovary, kidney, and also in tumours of lungs and upper respiratory pathways (Jayasurya et al., 2000; Jin et al., 2001; Hengstler et al., 2001; Harpole et al., 2001; Mitropoulos et al., 2005). Therefore, expression of MT may be of prognostic significance in certain types of tumours. Several reports investigated the relationship between MT expression and other clinicopathological characteristics of various malignancies. In certain tumours, e.g. in colorectal carcinoma overexpression of MT was found more often in cases of tumours of a higher grade of malignancy (Dziegiel et al., 2003). An elevated level of MT was found to correlate with an abbreviated duration of survival and a shorter disease free survival in some tumour types, what may suggest usefulness of MT as a prognostic factor (Dziegiel, 2004). However, some reports did not confirm the prognostic role of MT expression, what does not permit at present to unequivocally specify prognostic value of MT in neoplastic diseases. MT was also shown to be involved in the development of

resistance of cancer cells to cytostatic agents. Chemotherapy was found to induce synthesis of MT while an elevated level of the proteins decreased therapeutic efficacy of certain oncostatic proteins (Cherian et al., 2003). In several investigations it was demonstrated using experimental animals with engrafted tumour cells treated with drugs commonly used in anti-neoplastic therapy, including cisplatin, bleomycin, cyclophosphamide, that synthesis of MT in neoplastic cells increased markedly. In parallel, other drugs, such as mitomycin C, 5-fluorouracil did not alter MT expression level. Animals with engrafted tumour cells with zinc-induced MT, were shown to have significantly higher rate of chemoresitance of tumour cells to cisplatin as compared to the untreated cells. The experiments showed that MT may be responsible for both primary and acquired chemoresistance of neoplastic cells (Florianczyk, 1999; Chun et al., 2004). Mechanism of the cytoprotective MT activity during chemotherapy seems to be related to an anti-oxidative properties of MT. Some anti-neoplastic drugs act by inducing oxidative stress in tumour cells (including anthracyclines, e.g.. doxorubicin, daunorubicin), what results in their damage. MT protect cells from oxidative damage, decreasing therefore the therapeutic efficacy of cytostatic drugs (Cherian et al., 2003). In turn, affinity of MT to metal ions provides grounds for inactivation of alkylating drugs containing heavy metals (e.g. cisplatin, carboplatin). Due to MT direct interaction with the chemotherapeutic agents or their metabolites, MT may protect neoplastic cells from the cytotoxic effects (Shimoda et al., 2003; Theocharis et al., 2004). This hypothesis is confirmed by studies conducted on human malignancies (e.g. ovarian cancer, testicular cancer, colorectal cancer, breast cancer and squamocellular oesophageal cancer), showed that insensitivity of the tumours to chemotherapy was related to their MT over-expression (Yamamoto et al., 1999; Vazquez-Ramirez et al., 2000; Dziegiel et al., 2003; Surowiak et al., 2005; Surowiak et al., 2007). In view of the above, some authors include MT, in parallel to multi-drug resistance proteins (*MDR)*, to significant factors responsible for the lack of therapeutic efficacy of some cytostatic agents (Theocharis et al., 2004). Similar observations were shown regarding resistance of tumour cells to radiotherapy, which is known to generate high amounts of free radicals in tumour cells. MT, playing the function of an intracellular antioxidant inactivate reactive oxygen species, therefore protecting the tumour cells from radiotherapy induced damage, resulting in treatment failure (Cai et al., 1999; Theocharis et al., 2004). Although the role of MT in the process of carcinogenesis, proliferation and resistance of tumour cells to chemo- and radiotherapy still requires further research, MT may be considered as an additional prognostic and predictive marker in some tumour types.

2. Metallothionein in oral cancer

As overexpression of MT-1 and MT-2 was found in many malignant tumours, the studies conducted on cancers of the oral cavity concerning MT expression focused mainly on the expression of these two isoforms in oral squamous cell carcinoma (OSCC), tonque squamous cell carcinoma and tumours of the salivary glands.

2.1 Metallothionein expression in normal and dysplastic oral mucosa and oral squamous cell carcinoma

OSCC is the most frequently diagnosed oral cancer accounting for more than 95% of malignancies originating from the oral cavity with almost 25 000 new cases diagnosed annually in the US (Siegel et al., 2011). Oral leukoplakia (OL) is a premalignant and potentially malignanant lesion of the oral mucosa and proceeds OSCC in some cases (Hunt et al. 2011). On

the basis of the amount of dysplastic cells and the thickness of dysplastic epithelium, this lesion is graded as mild, moderate or severe (Barnes et al., 2005). Until now, in tissues of the oral mucosa and its lesions, only isoforms of the MT-1 and MT-2 family were investigated concerning their expression. In normal oral mucosa MT-1/2 expression is restricted only to basal and parabasal cells with a mosaic cytoplasmic-nuclear expression pattern, whereas in dysplastic lesions additional foci in the spinous layer were noted (Sundelin et al., 1997; Johann et al., 2008; Pontes et al., 2009). MT-1/2 expression intensity was positively correlated 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 (Pontes et al., 2009). Likewise in normal and dysplastic mucosa, cancer cells of OSCC exhibited an cytoplasmic-nuclear pattern of MT-1/2 expression (Szelachowska et al., 2008; Pontes et al., 2009). In OSCC, cancer cells expressing MT-1/2 were found at the centre and the periphery of tumour islands, but interestingly in cases when keratin pearls were present, MT-1/2 expression was restricted to basal and parabasal cells (Pontes et al., 2009, Szelachowska et al, 2009).

2.1.1 Role of metallothioneins in the pathogenesis of oral squamous cell carcinoma

Three major risk factors of OSCC development are long term tobacco smoking, alcohol and areca-quid (also known as betel nut) consumption (Shiu et al, 2004). Two components of tobacco: nicotine and 4-(methylnitorosamino)-1-(3-pyridyl)-1-butanone were shown to activate Akt kinase in human airway epithelial cells (West et al., 2003; Shiu et al., 2004; West et al., 2004). Recent studies reported, that MT-1/2 expression correlated positively with phosphorylated Akt (p-Akt) expression and levels of both these proteins were significantly elevated in OSCC in comparison to normal or dysplastic oral mucosa regardless of its severity grade (Sundelin et al., 1997; Johann et al., 2008; Pontes et al., 2009). Akt/PKB (protein kinase B) is a serine/threonine kinase comprised of three isoforms and acts as a downstream target of phosphatidylinositol-3 kinase (PI3K) and is thus involved in many vital cellular pathways and is frequently activated in different human cancers (Carnero 2010; Fayard et al, 2010; Freudlsperger et al., 2011; Grabinski et al., 2011;). Its activation, similarly to MT-1/2 expression, in human cancers was associated with inhibition of apoptosis and promotion of cell proliferation (Diehl et al., 1998; Brunet et al., 1999). Epidemiological studies have also shown, that patients with head and neck cancer and OSCC frequently suffer from zinc deficiency (Doerr et al., 1997; Kleier et al., 1998). These results were also confirmed by a number of *in vivo* studies using an lingual-esophageal carcinogenesis model in mouse and rats (Fong et al., 2005; Fong et al., 2006, Liu et al., 2005). These studies have shown that deficit of zinc in diet induces MT-1 expression, along with other markers related to carcinogenesis e.g. cyclin-B2, carbonic anhydrase II and keratin 14. Moreover, zinc diet restriction potentiated the growth of lingual and esophageal tumours in p53 deficient mice, which were characterized by significantly augmented cell proliferation, keratin 14, COX-2 and MT-1 expression as compared to mice with normal p53 expression level (Liu et al., 2005; Fong et al., 2006). Zinc replenishment in the diet resulted in subsequent reduction of cell proliferation and expression of keratin 14, COX-2 and MT-I (Liu et al., 2005). These experimental results confirm the observations stemming from IHC studies on human specimens, because the carcinogenesis model clearly showed subsequent rise of MT-1 expression correlating with the noted histopathological hyperplasia-dysplasia-carcinoma model (Fong et al., 2006). Nonetheless, little is known about the interaction and possible regulation of MT-1 and MT-2 expression in dysplasia and OSCC cases.

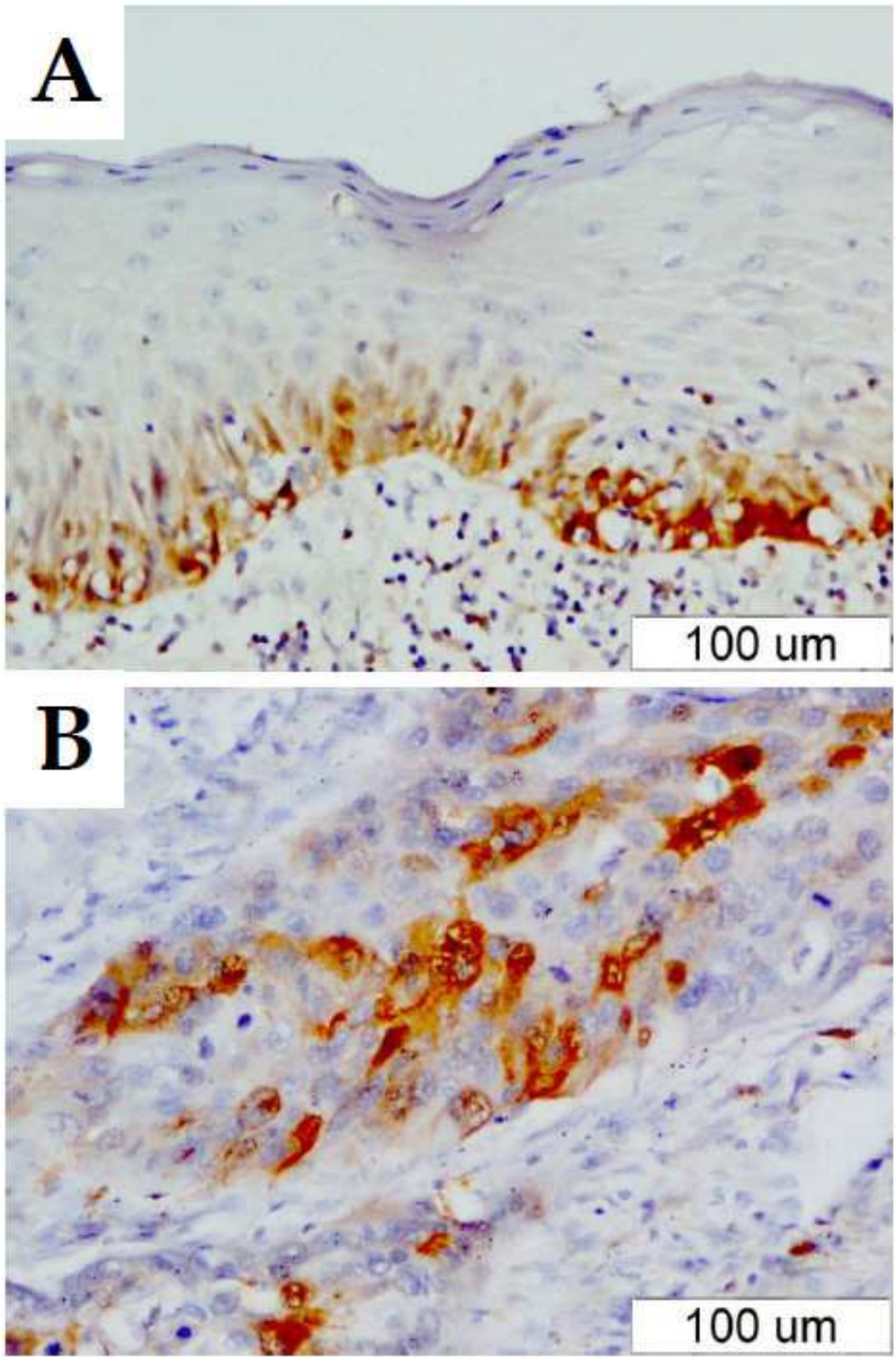

Fig. 1. MT-1/2 expression in basal layer of squamous stratified epithelium with additional foci in the upper parts of oral mucosa (A). Nuclear-cytoplasmic MT-1/2 expression in advanced OSCC (B).

2.1.1.1 Areca nuts, copper ions and reactive oxygen species (ROS)

Areca nuts chewing was shown to be associated with increased risk of OSCC development and progression (Shiu et al., 2004; Merchant et al., 2000). It is estimated that around 200-400 million people, regardless of gender consume areca nuts (Gupta and Warnakulasuriya, 2002). Moreover, almost 80% of OSCC related deaths in Taiwan are associated with areca quid consumption habit (Kwan, 1976; Lee et al., 2008). It was shown, that patients with simultaneous smoking and areca nut chewing habits are characterized be even greater risk of OSCC development, than patients consuming either of these products separately (Ko et al., 1995). The study of Lee et al. showed an upregulated expression of MT-1 in OSCC originating from patients with long-term areca nut consumption (Lee et al., 2008). Areca nuts contain high levels of copper, a metal which is bound by metallotthioneins, and is the source of ROS during chewing (Trivedy et al., 1997; Nair et al., 1992; Chen et al., 2002). This compounds were shown to be responsible for generation of OSCC in areca quid consumers (Warnakulasuriya et al., 2002). The *in vitro* experiments conducted on an oral epithelial cell line GNM originating from a patient with T2N2aM0 gingival carcinoma demonstrated an increase in MT-1 mRNA expression upon arecoline treatment in a dose dependent manner (Lee et al., 2008). Treatment of this carcinoma cell line with benzo[a]pyren (BaP) alone or in combination with arecoline resulted in an increased expression of MT-1 mRNA in GNM cells. The highest MT-1 mRNA expression was seen in the cells treated with both compounds simultaneously. Addition of a glutathione precursor, N-acetyl-L-cyteine, to the cells treated with arecoline reduced the levels of arecoline induced MT-1 mRNA levels. Nonetheless, the results of the *in vitro* experiments are in accordance with the results of the epidemiological studies conducted on Taiwan population, showing an enhanced incidence of OSCC development in patients with simultaneous areca quid and cigarette consumption (Ko et al., 1995, Lin et al., 2011). Moreover, this results also underlie the results of studies showing upregulated expression in response to reactive oxygen species (Iqbal et al., 2003; Reinecke et al., 2006). As areca nuts contain also high amounts of copper ions, high MT-1/2 expression in OSCC originating from areca nut chewers may be partially explained by its interaction with this metal ion. To note, studies have shown, that MT-1/2 expression levels is related to zinc and copper ion content in tumour cells, what points to another mechanism of MT-1/2 upregulated expression in OSCC cells originating from areca-quid consumers (Jayasurya et al., 2000; Florianczyk et al., 2006).

2.1.1.2 Cancer cell proliferation

Few studies compared the proliferative activity of OSCC tumour cells with expression levels of MT-1/2 (Cardoso et al., 2002; Szelachowska et al., 2008; Szelachowska et al., 2009). None of the studies performed on OSCC cases succeeded in noting a positive correlation between MT-1/2 expression and expression of proliferation markers. The study of Cardoso et al. found no correlation between the expression of MT-1/2 and Ki-67 antigen (Cardoso et al., 2002). Similar results were obtained in both studies performed by Szelachowska et al. on a subset of patients with OSCC (Szelachowska et al. 2008; Szelachowska et al., 2009). In the first study no correlation with Ki-67 and MCM-2 protein expression was found, when the MT-1/2 nuclear and cytoplasmic expression was analyzed separately (Szelachowska et al. 2008). Similarly, no correlations were found in the second study performed on 39 patients with OSCC between MT-1/2 expression intensity and cancer cell proliferation measured by the Ki-67 and MCM-2 expression levels (Szelachowska et al., 2009). MT-1/2 expression

seems not to exert pro-mitotic effects in OSCC, which was observed in other tumour types, including the squamous cell carcinoma of the head and neck (Jayasurya et al., 2000; Dziegiel et al., 2005; Szajereka et al., 2008).

2.1.1.3 p53

Recent study performed on 100 cases of OSCC disclosed a positive correlation between the expression of MT-1/2 and p53 protein. Moreover, cases characterized by a positive nuclear MT-1/2 immunostaining yielded higher p53 expression levels (Cardoso et al., 2009). This underlies the earlier observed role of MT-1/2 in regulation of p53 expression by influencing zinc ion cell homeostasis (Rainwater et al., 1995; Meplan et al., 2000; Ostrakhovitch et al., 2006; Pastuszewski et al., 2007).

2.1.1.4 Invasiveness

Numerous studies showed the involvement of laminin in progression of different malignancies, including these of the oral cavity (Ono et al., 1999; Patarroyo et al., 2002; Stolzfus et al., 2004; Lyons and Jones, 2007). Until now, only one study analyzed the impact of MT-1/2 expression levels on the expression of invasiveness related markers in OSCC. In this immunohistochemical study a positive correlation was disclosed between the expression of MT-1/2 and laminin-5 (Szelachowska et al., 2009). Lamin in normal conditions is expressed in the epithelial basement membrane and its expression increases with severity of the dysplasia and may regulate cell motility (Kainulainen et al., 1997; Décline & Roussel, 2001; Décline et al., 2003, Lyons and Jones, 2007). This might explain the higher expression of MT-1/2 in tumours with the presence of lymph node metastases in comparison to tumours without lymph node involvement (Szelachowska et al., 2009).

2.1.1.5 Chemoresistance

As metallothionein isoforms were suggested to play a role in cancer cells chemoresistance, Nakano et al. studied the impact of cisplatin treatment of human tongue squamous cell carcinomas on MT expression (Nakano et al., 2003). These cells expressed the MT-1, MT-2 and MT-4 isoforms, whereas no expression of MT-3 was noted. The subsequent treatment of the cells with cisplatin resulted in a significant increase of expression of only the MT-1 and MT-2 isoforms in cisplatin-resistant cells (Nakano et al., 2003). Role of MT-1/2 in OSCC chemoresistance was also issued in the work of Muramutsu et al., but no difference in MT-1/2 expression levels between the treated and untreated patients was noted (Muramutsu et al., 2000).

2.1.2 Clinical implications of MT expression in OSCC

Until now six studies (Table 1) investigated the impact of MT expression regarding patients clinicopathological characteristics (Muramatsu et al., 2000; Cardoso et al., 2002; Lee et al., 2008; Szelachowska et al., 2008; Cardoso et al., 2009; Szelachowska et al., 2009). All those studies were performed on archival paraffin blocks from patients whose cancers originated mainly from oral mucosa, but in some cases specimens from cancers of the mobile part of the tongue were included (Szelachowska et al., 2008; Szelachowska et al., 2009). In case of only one study three additional tumours from the maxilla region were investigated (Muramutsu et al., 2000). Except one study of Lee et al., a primary antibody directed against MT-1/2 isoforms was used. None of the mentioned studies showed correlations with

primary tumour size, grade of tumour differentiation and proliferation markers (Ki-67 and MCM-2). In three studies, a positive correlation was noted between lymph node involvement and the intensity of MT expression (Lee et al., 2008; Szelachowska et al., 2008; Szelachowska et al., 2009). Studies which analyzed the impact of metallothionein expression on patients outcome, showed that elevated MT-1/2 expression in patients with OSCC was generally an unfavourable prognostic marker (Cardoso et al., 2002; Szelachowska et al., 2008; Cardoso et al., 2009). In the study of Cardoso et. al., MT-1/2 cell positivity (cytoplasmic-nuclear staining) was counted and enrolled in the survival analysis. Univariate analysis, as well as multivariate analysis showed that MT-1/2 overexpression was an unfavorable prognostic factor in the studied patient cohort (Cardoso et al., 2002).

Publication	**Muramatsu et al., 2000**	**Cardoso et al., 2002**	**Lee et al., 2008**	**Szelachowska et al., 2008**	**Szelachowska et al., 2009**	**Cardoso et al., 2009**
Patients	28 OSCC and NPC; 3 cancers of the maxilla region	60 OSCC	*34 OSCC	50 OSCC	39 OSCC	100 OSCC
MT isoform	MT-1/2	MT-1/2	MT-1	MT-1/2	MT-1/2	MT-1/2
pT	-	-	-	-	-	NA
pN	-	-	+	+ cytoplasm	+	NA
Grade of malignancy (differentiation)	NA	-	-	-	-	-
Clinical stage	NA	-	-	not analyzed	not analyzed	-
Survival	NA	poor prognosis	NA	*poor prognosis	not analyzed	*poor prognosis
Proliferation	(-) Ki-67	(-) Ki-67	NA	(-) Ki-67, (-) MCM-2	(-) Ki-67, (-) MCM-2	-
Other markers	NA	NA	NA	NA	(+) Laminin-5	(+) p53
Notes			*Areca quid consumers	*shorter DFS and DSS in patients with high MT-1/2 expression; lack of significant impact on SFLR and OS		*combined high expression of MT-1/2 and p53 on OS

Abbreviations: SFLR – survival free of locoregional relapse; DFS – disease free survival; OS – overall survival; DSS – disease specific survival; NA – not analyzed

Table 1. Summary of studies on MT expression with regard to patients clinicpathological characteristics. (-) represents lack of correlation or relationship between the analyzed variables, whereas (+) represents positive correlations.

A more detailed analysis of MT-1/2 expression on patients outcome was performed in the study of Szelachowska et al., where cytoplasmatic and nuclear MT-1/2 expressions were analyzed separately (Szelachowska et al., 2008). For the nuclear evaluation, cells showing positive reaction were counted, whereas, for the evaluation of cytoplasmatic reaction an immunoreactive score (IRS) of Remmele and Stegner based on evaluation of number of positive cells and the intensity of colour reaction took advantage (Remmele & Stegner, 1986). The authors noted a significant correlation between the cytoplasmatic and nuclear MT-1/2 expression, but none of the evaluated types of the reaction correlated with tumour size, grade of malignancy or expression of both proliferation markers (Ki-67 and MCM-2). Interestingly, only in cases with lymph node involvement, a significant increase in MT-1/2 expression was noted when compared to cases without lymph node involvement (Szelachowska et al. 2008). Also differences regarding patients survival differed among the both analyzed expression patterns. The univariate analysis showed that cases characterized by high cytoplasmatic MT-1/2 expression had a significantly shorter disease specific survival (DSS) and disease free survival (DFS), whereas cases with high nuclear MT-1/2 expression had a significantly shorter DFS and tended to have also a shorter DSS, but this trend did not reach statistical significance. No impact of MT-1/2 expression in the cytoplasm or nucleus affected significantly patients overall survival (Szelachowska et al., 2008). 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 (Cardoso et al, 2009). Differences in the studies concerning lymph node involvement and patients survival, may by caused be heterogenous origin of specimens used in the study (Szelachowska et al., 2008; Szelachowska et al., 2009) In summary, the above mentioned studies highlight and underlie the potential role of MT-1/2 expression in the development and tumour progression of OSCC.

2.2 Metallothionein expression in normal salivary gland and its lesions

MT were shown to be expressed in normal salivary glands as well as in benign and malignant tumours mainly in cells resembling the phenotype of myoepithelial cells (Sunardhi-Widyaputra et al., 1995; Gao et al., 1997; Hecht et al., 2002; Ogawa, 2003; Alves et al., 2007). As in the case of OSCC, the majority of studies concerning the role of MT expression in salivary glands were based on immunohistochemistry (Sunardhi-Widyaputra et al., 1995; Gao et al., 1997; Ogawa, 2003; Alves et al., 2007).

2.2.1 Role of MT in salivary gland development

MT seem to be involved in salivary gland development as shown in the studies of Hecht et al. (Hecht et al., 2002). It was found that MT is mainly the only upregulated family of genes, when human salivary gland (HSG) cells, derived from a human submandibular tumour, were cultured on a laminin-1 gel in different conditions. In gel cultures lamin-1 stimulates HSG cells to form acinar-like structures within 24-48 hours, whereas in the absence of laminin-1 in culture conditions results in a monolayer growth type. Also in microgravity culture conditions laminin-1 affects HSG cells growth by facilitating acini formation (Hoffman et al., 1998). It was seen that under laminin-1 culture conditions mRNA expression of three members of the MT family was significantly upregulated: MT-1F, MT-1B and MT-2. Subsequent immunostaining of these cells revealed a higher cytoplasmic

MT-1/2 expression in comparison to HSG cells cultured in the absence of laminin-1. An overexpression of MT-1F in HSG cells did not affect cell proliferation as compared to native and control mock-transfected cells, but MT-overexpressing cells were characterized by an augmented growth after addition of low concentrations of zinc and copper to the medium (Hecht et al., 2002). MT-overexpressing cells grew in aggregates, were larger and had more pronounced adhesive properties than the parental cells. Moreover, MT-overexpressing cells formed acini-like structures larger and faster. Despite the strong impact on cellular differentiation, MT-overexpression did not affect amylase expression or mucin production. Interestingly, when these cells were injected s.c. to nude mice, tumours formed by MT-overexpressing HSG cells were significantly smaller and more differentiated than the tumours formed by the parental cells (Hecht et al., 2002). Although, no differences in cell proliferation were observed *in vitro*, tumours originating from MT-overexpressing cells seemed to have more mitotic cells in the tumour mass (Hecht et al., 2002).

2.2.2 MT expression in normal salivary gland tissues and neoplasias

Normal salivary glands and its tumours exert a broad expression of MT-1/2 in myoepithelial cells (Sunardhi-Widyaputra et al., 1995; Gao et al., 1997; Ogawa, 2003; Alves et al., 2007, Prasad et al., 2008). In the study of Sunardhi-Widyaputra et al., performed on 21 benign and 4 malignant lesions of salivary glands, MT expression was compared with parathyroid hormone-related peptide (PTHrP) expression. In benign changes (pleomorphic adenoma, Warthin's tumour), both these proteins were coexpresed. In myoepthelioma majority of the cells expressed MT, while few have shown PTHrP reactivity. In oncocytoma, peripheries of oncotytic islands showed MT expression, while only few oncotytic PTHrP positive cells were noted. In cases of mucoepidermoid carcinomas MT and PTHrP expression was the most heterogenous. MT positivity was seen in epithelial cells and PTHrP in cyst-like structures and squamous cells (Sunardhi-Widyaputra et al., 1995). Similar results regarding MT expression were noted by Gao et al. in myoepitheliomas and myoepithelial carcinomas (Gao et al., 1997). Although these studies did not quantified MT expression, they clearly showed that MT expression may vary in regard to tumour degree of differentiation with the most heterogenous expression in the most dedifferentieted and immature tumours (Sunardhi-Widyaputra et al., 1995; Gao et al., 1997). The results of these studies are also supported by the findings of Ogawa, which demonstrated MT expression in a subset of myoepithelial cells during salivary gland development (Ogawa, 2003). MT expression in myoepithelial cells of adenoid cystic carcinomas and polymporphous low-grade adenocarcinomas was also studied as a potential marker of differentiation of these two malignancies as they may pose problems in the pathological examination (Prasad et al., 2008). This study showed, that diffuse MT expression in combination with smooth muscle acitin, calponin and smooth muscle myosin heavy chain was strongly predictive for adenoid cystic carcinoma (Prasad et al., 2008). MT expression in adenoid cystic carcinomas varied according to histological subtype (Alves et al., 2008). The most pronounced MT-1/2 staining was seen in solid and cribiform as compared to tubular subtypes of this tumour. This results support the results stemming from studies conducted on other malignanancies, showing that elevated MT-1/2 expression is linked to patients poor clinical outcome (Dziegiel, 2004). High expression of MT-1/2 in solid and low in tubular subtypes, may partially explain their distinct clinical outcome (Nascimento et al., 1986; Perez et al., 2006; Alves et al., 2008).

3. Conclusion

MT expression in OSCC seems to be of importance for the development and progression of these cancer type. Although the results of the clinical studies were not concordant (due to small sample size, heterogenous patient cohort) it is worth to mention that some of them noted a relationship between high MT expression and cancer cell metastasis and associated MT overexpression with poor patients outcome. Despite the few reports concerning MT expression in salivary glands and its lesions, one might consider MT as an interesting point of future research, mainly due to the impact of MT-1F expression on salivary tumour cells differentiation and growth. Nonetheless, further studies are needed to better characterize the role of MT expression on OSCC and tumours of the salivary glands.

4. Acknowledgment

This work was supported by the scientific grant of Wroclaw Medical University, No. ST-442.

5. References

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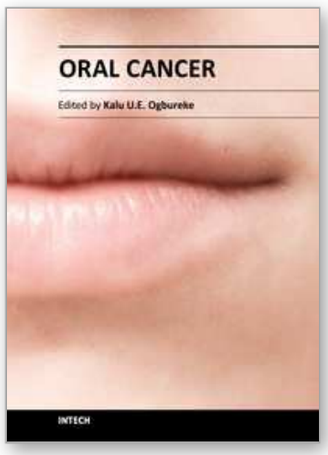

Oral Cancer

Edited by Dr. Kalu U. E. Ogbureke

ISBN 978-953-51-0228-1

Hard cover, 388 pages

Publisher InTech

Published online 14, March, 2012

Published in print edition March, 2012

Oral cancer is a significant public health challenge globally. Although the oral cavity is easily accessible, early diagnosis remains slow compared to the enhanced detection of cancers of the breast, colon, prostate, and melanoma. As a result, the mortality rate from oral cancer for the past four decades has remained high at over 50% in spite of advances in treatment modalities. This contrasts with considerable decrease in mortality rates for cancers of the breast, colon, prostate, and melanoma during the same period. This book attempts to provide a reference-friendly update on the etiologic/risk factors, current clinical diagnostic tools, management philosophies, molecular biomarkers, and progression indicators of oral cancer.

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