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Correlation of Metallothionein Expression with Clinical Progression of Cancer in the Oral Cavity

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Abstract. *This study aimed at finding out whether the expression of metallothionein (MT), laminin, Ki-67 antigen and mnichromosome mantenance-2 (Mcm-2) protein changes with growing invasiveness of the tumour. The expression of these markers in primary tumours with no metastases to lymph nodes (PT N–) was compwred with the expression in primary tumours with metastases in draining lymph nodes (PT N+). The difference in marker expression was also evaluated between metastatic lymph nodes (LN+) and the corresponding primary tumours (PT N+). Patients and Methods: The studies were performed on tumour samples from 39 patients with squamous cell carcinoma of the oral cavity floor or of the oral part of the tongue. All the patients had been subjected to radical surgery, accompanied by the removal of lymph nodes. In 20 patients post-operative histopathology disclosed the presence of metastases in the draining lymph nodes (pN+), while in 19 patients the presence of such metastases was excluded (pN0). Results: The PT N+ group was found to contain a significantly higher percentage of cells with cytoplasmic expression of MT, than the PT N– group. In turn, a significant increase in the intensity of reaction of cytoplasmic MT and an increased percentage of cancer cells demonstrating MT expression in the cell nuclei was demonstrated in the LN+ compared to the PT N+ group. The expression of the remaining parameters did not significantly differ between PT N–, PT N+ and LN+. Conclusion: A gradual increase in MT expression (both cytoplasmic and nuclear) takes place with progression of the tumour and the increased nuclear expression of MT in LN+ cells may suggest a role of MT in metastasis development in the studied tumours.*

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Despite the progress in therapy of patients with cancer of the oral cavity, relapse of the disease is frequently observed. The presence of metastases in lymph nodes represents a recognised unfavourable prognostic index and influences therapeutic decisions (1). Better understanding of the cellular and molecular mechanisms in oral cavity carcinoma may result in the development of better, more effective forms of therapy and may improve patient survival. Markers linked to various mechanisms of tumour progression, laminin, metallothionein (MT) and proliferation markers, such as Ki-67 antigen and minichromosome maintenance-2 (Mcm-2) protein were selected for study.

Laminin is a large glycoprotein of the basement membrane, composed of three sub-units: a heavy chain a and two lighter ones, b and g. At present, at least 10 varieties of these chains and 11 laminin isoforms are known. Laminin plays an important role in the adhesion of cells to the basement membrane, in the extracellular matrix and in the ability of tumour cells to metastasize (2-8). Numerous studies of recent years have provided evidence for the role of laminin in the progression of malignant tumours (8-10). Monoclonal antibody 4C7, which has been acknowledged to be specific for the laminin a-5 chain (11), was used in the present study.

MT are low molecular weight proteins with high metal and cysteine content. Although a number of biological functions have been proposed for MT, most of them are related to the metal binding property (12). MT may protect against certain metal toxicities, and may donate zinc/copper to certain metallo-enzymes and transcription factors (13). It may also protect against oxidative stress because of its high cysteine content (14). Since MT is present in most tissues and cell types in small amounts, it is generally considered as

a "housekeeping" protein (13). The majority of prognostic studies have observed an inverse correlation between MT expression and patient survival (15-20). MT seems to be related to neoplastic resistance to oncological treatment (chemo- and radiotherapy) and therefore has been studied as a prognostic factor for a variety of human malignant tumours (13, 21, 22).

Ki-67 antigen represents one of the most frequently evaluated proliferation markers. Nevertheless, its value as a prognostic index has not been fully proven and the results of studies which evaluated links between cell proliferation and the clinical course of the disease have frequently been contradictory (23-28). Despite routine estimation of the antigen, its biological function remains unclear and most probably it represents no key elements in cell proliferation mechanisms (29). Expression of Ki-67 may appear also in situations when DNA synthesis is blocked or cells undergo the apoptotic process (30). Since both the biological function of Ki-67 antigen and its significance as a prognostic factor remain unclear it was decided to include also a novel marker of proliferation, Mcm-2 protein. At present its estimation is restricted to research purposes and it is not used for clinical purposes. Nevertheless, its clinical function has been well documented. Mcm-2 protein belongs to the family of 6 proteins, Mcm-2 to 7, engaged in recognition and control of DNA replication (31). In the early, G1-phase of the cell cycle Mcm proteins participate in the formation of the pre-replication complex (32-33). Mcm engaged in the pre-replication complex exhibit activity of helicase, which unwinds DNA during replication (34). This allows access to appropriate sites in the DNA for replication. In the course of S-phase Mcm proteins irreversibly detach from the chromatin, which ensures that only one replication cycle takes place in the course of a cell cycle (35). In all eukaryotic cells studied until now Mcm proteins play a key role in DNA replication (36). In several studies Mcm expression has been demonstrated in cells during their division cycle and loss of Mcm expression reflected the cell resting stage. Thus, the expression can be taken as a specific marker of proliferating cells (37-40). With increasing frequency Mcm-2 protein is evaluated as a potential prognostic index, among others in squamous cell carcinoma of the oral cavity (41).

This study aimed at finding out if the expression of these markers changes with the growing invasiveness of the tumour *i.e.* with its increasing tendency to metastasize. Their expression in primary tumours with no metastases to lymph nodes (PT N–) was compared to expression in primary tumours with metastases in the draining lymph nodes (PT N+) and subsequently, expression differences between metastatic lymph nodes (LN+) and the respective primary foci (PT N+), were also examined.

Patients and Methods

Patients. Tumour samples from 39 patients with a diagnosis of squamous cell carcinoma of the oral cavity floor or of the oral part of the tongue were included. All the patients had been subjected to radical surgery, accompanied by the removal of lymph nodes conducted in Wroclaw Medical University (WMU) and in the Lower Silesian Centre of Oncology (LSCO) in 1996-2002. The pathological degree of tumour advancement was established in all the patients in line with the TNM system (42). The grade (G) of histological malignancy was estimated in line with the WHO classification of 1997 (43). The mean age of the examined patients was 56 years, and the group included 7 women and 32 men. In 23 of the patients the primary neoplastic lesion was located in the floor of the oral cavity and in 9 patients in the mobile part of the tongue, while in the remaining patients diffuse infiltration of both structures was observed. In 20 of the patients post-operative histopathology disclosed the presence of metastases in the draining lymph nodes (pN+), while in 19 patients the presence of such metastases was excluded (pN0).

Immunohistochemistry. The tumour samples were fixed in 10% buffered formalin, dehydrated and embedded in paraffin blocks. All the immunohistochemical reactions were conducted on paraffin sections. For the estimation of MT, laminin, Mcm-2 and Ki-67 antigen expressions, mouse monoclonal antibodies were used (clone E9, 1:100, Dako, Denmark A/S, produktionsvej 42, DK-2600 Glostrup, Denmark; monoclonal mouse anti human laminin clone 4C7 isotype IgG2a, kappa, 1:50, Dako; clone CRCT2.1, 1:50, Novocastra Laboratories Ltd, Balliol Bussiness Park West, Benton Lane, Newcastle upon Tyne, NE12 8EW, UK and clone MIB-1, 1:50, Dako, respectively). All the reactions were accompanied by negative controls, in which specific antibodies were substituted by the Universal Negative Control for N-series Mouse Primary Antibodies Kit, N1698 (Dako). The paraffin sections were boiled in Target Retrieval Solution (S 1699, Dako) in a microwave oven for 10 minutes to unblock the antigenic determinants for Ki-67 and Mcm-2. The antigens were visualized using biotinylated antibodies, streptavidin-biotinylated peroxidase complex (Universal LSAB2 Kit, K0675 HRP Rabbit/Mouse, Dako) and diaminobenzidine (Liquid DAB+, K3468, Dako). The intensity of the immunohistochemical reactions was independently evaluated in coded preparations by two pathologists. Intensity of laminin expression in the extracellular matrix was estimated on a four-stage semi-quantitative scale, ranging from 0 to 3. Cytoplasmic MT expression was evaluated using a semi-quantitative scale, taking into account the intensity of the colour reaction (four-grade scale 0 to 3) and the percentage of cells with cytoplasmic MT expression (five-grade scale of 0% as 0; 1-10% as 1; 11-50% as 2; 50-80% as 3; >80% as 4) (44). The nuclear MT expression was evaluated using a semi-quantitative five–tiered grading system, which took into account the percentage of cells manifesting nuclear reactions (0 when no cells were positive, 1 when 1-10% , 2 when 11-50% , 3 when 50-80% and 4 when >80% cells were positive). 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 , 1-10% - 1, 11-25% - 2, 26-50% - 3 and over 50% - 4 (45).

Statistical analysis. This was performed using software Statistica, version 6 (Krakow, Poland), for the cytoplasmic MT expression, the proportion of cells manifesting nuclear MT expression, the proportion of cells manifesting Mcm-2 and Ki-67 expression, the

Table I. *Comparison of clinical and pathological data in patients with squamous cell carcinoma of the oral cavity with no metastases (PT N–) or with metastases to lymph nodes (PT N+).*

	PT N–	PT N+	*p*-value
Age of patients	40-78 years (mean: 56.6)	39-73 years (mean: 56.2)	NS
Number of patients	19	20	
Gender			
Women	2	5	NS
Men	17	15	
cT			
1	2	2	NS
2	6	6	
3	6	7	
4	5	5	
grading			
G 1	3	3	NS
G 2	15	16	
G 3	1	1	

CT: primary tumour size.

Table II. *Comparison of marker expression in primary tumours with no metastases (PT N–) metastases (PT N+) and in lymph node metastases (LN+).*

Marker expression	PT N– number of patients	PT N+ number of patients	LN+ number of patients	*p*-value
Ki-67				
0%	1	2	1	NS
1-10%	4	5	6	
11-25%	9	9	5	
26-50%	3	3	5	
>50%	2	1	3	
Mcm-2				
0%	1	3	2	NS
1-10%	8	5	4	
11-25%	4	7	6	
26-50%	4	5	5	
>50%	2	0	3	
laminin				
0	9	9		NS
1	3	3		
2	5	3		
3	2	5		

extracellular laminin expression, the degree of histological malignancy (G), gender, age, the pathological stage of the cancer (pTNM) and the presence of metastases in the lymph nodes (N+). The association of all markers with clinical and pathological parameters was evaluated using the Mann–Whitney *U*-test, Wilcoxon test and Spearman correlation test. The level of significance was set at $p<0.05$.

Results

In 6 patients squamous cell carcinoma was G1 in 31 patients it was G2 while in 2 patients it was G3. No significant differences were disclosed between the group of 20 (pN+) patients and the group of 19 (pN0) patients in respect to age, sex, size of the primary tumour (cT) or grade of malignancy (Table I). Therefore, the patient groups were assumed to be comparable in respect to the principal clinico-pathological traits.

A strong correlation was disclosed between the intensity of expression of the proliferation markers, Ki-67 antigen and Mcm-2 protein ($r=0.76$; $p<0.05$) and a moderate correlation between the expression of laminin and the percentage of cancer cells manifesting the presence of MT ($r=0.34$. $p<0.05$). Within the PT N+ group a significantly higher percentage of cells manifested cytoplasmic expression of MT, but the intensity of the reaction showed no differences (Figure 1).

The expression of the remaining markers in the PT N– group showed no significant differences as compared to expression in the PT N+ samples (Table II).

No difference of Ki-67 antigen and Mcm-2 protein expression was noted between the PT N+ and LN+ samples (Table II). On the other hand, a significantly increased intensity of MT cytoplasmic reaction and an increase in the percentage of cancer cells with MT expressed in the cell nuclei were noted in the LN+ samples (Figures 1 and 2).

Discussion

In the present study only MT expression was found to change significantly with the increasing capacity of the squamous cell carcinoma of the oral cavity to develop metastases to the lymph nodes. It was assumed that the studied material represented subsequent stages of tumour progression, beginning at the less aggressive form of PT N– (without metastases), through PT N+ (with metastatic potential) to LN + (metastatic cells in lymph nodes). Neoplastic progression was accompanied by a gradual increase in the percentage of cells manifesting cytoplasmic MT and increased intensity of reaction and more frequent expression of MT in the cell nuclei were noted in the LN+ cells (Figures 1 and 2).

The results of various authors investigating links between MT expression and the presence of metastases to lymph nodes have been equivocal (16, 46-50). Nevertheless, results similar to ours, pointing to a correlation between high expression of MT in the primary tumour and more frequent metastases to lymph nodes were presented in several studies (16, 46-48). Lee *et al*. (46) observed that high expression of MT in cells of squamocellular carcinoma of the oral cavity was accompanied by the development of metastases to

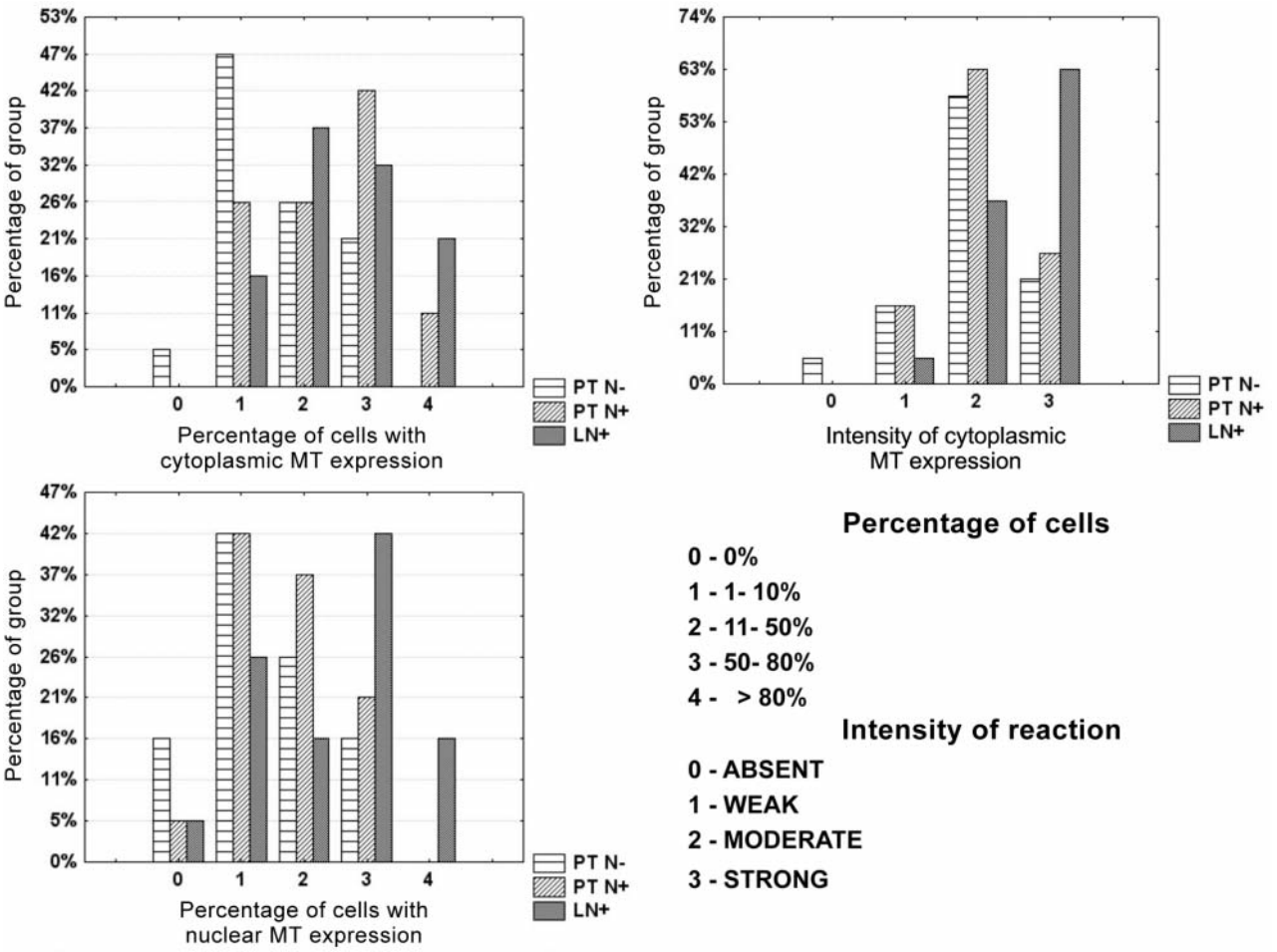

Figure 1. *Comparison of intensity of cytoplasmic MT reaction, percentage of cells with cytoplaqsmatic MT and percentage of MT cell positive nuclei in primary tumours with no metastases (PT N–), primary tumours with metastases (PT N+) and in metastases to the lymph nodes (LN+). Percentage of cytoplasmic MT positive cells was significantly higher in PT N+ vs. PT N– tumours ($p<0.05$). Intensity of cytoplasmic MT reaction and percentage of MT positive cell nuclei were significantly higher in PT N+ vs. LN+ tumours ($p<0.05$).*

lymph nodes and concluded that MT expression may be a marker distinguishing tumours with the potential to form metastases (46). However, the mechanism remains unknown by which high MT levels affect metastatic potential.

In numerous studies a possible relationship between MT and cell proliferation was suggested which could explain the less favourable prognosis in patients with higher MT expression (16, 17, 23, 51). However, in the present study no correlation was disclosed between MT level and the tumour proliferative activity measured by the intensity of Ki-67 antigen or Mcm-2 protein expression. Also no differences in expression of the proliferation markers were detected between the PT N–, PT N+, and LN+ groups. Therefore, it seems improbable that increasingly aggressive carcinomas of the oral cavity, which are accompanied by growing MT expression, reflect proliferation.

Another explanation, as described by Haga *et al*. (52) could involve the activity of MT as an activator of gelatinase A, which belongs to the MMP (matrix metalloproteinases) family of enzymes. Gelatinase A (MMP-2) plays a significant role in the invasion of the tumour sublayer and in the development of tumour metastases, mainly through the degradation of extracellular matrix components, including laminin. As a result, tumours with high MT expression might be accompanied by an increased degradation of extracellular matrix components and facilitated invasion by tumour cells. However, in the present study evaluation of α-5 laminin chain expression disclosed no differences in the extent of extracellular matrix degradation between PT N– and PT N+ tumours. Furthermore, a positive correlation was documented between the percentage of cells with MT

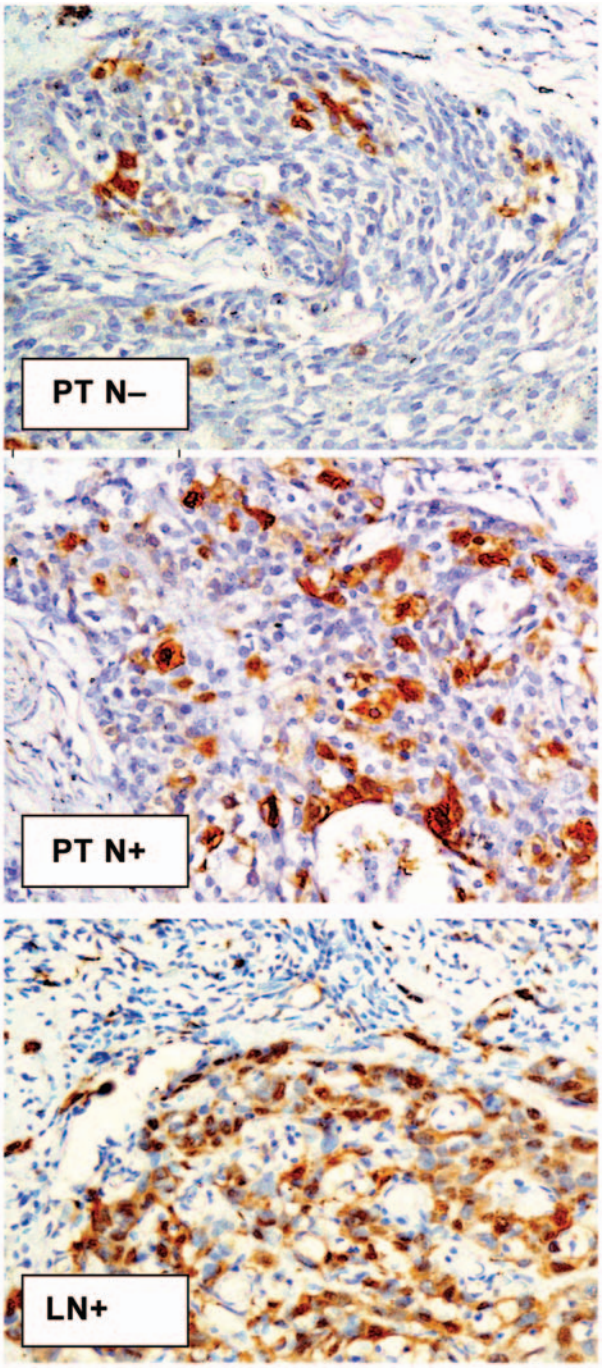

Figure 2. *Expression of MT in cells of oral cavity squamous cell carcinoma (magnification ×100; counterstained with hematoxylin). PT N–: Low nuclear and cytoplasmic MT expression in primary tumour with no metastases. PT N+: Moderate nuclear and cytoplasmic MT expression in primary tumour with metastases. LN+: High nuclear and cytoplasmic MT expression in metastatic lymph nodes.*

expression and the presence of laminin (α-5 chain) in the extracellular matrix and thus, the concept of Haga *et al.* was not confirmed.

Probably the less favourable prognosis in patients with higher expression of MT may be linked to the potential anti-apoptotic activity of MT, due to modulation of p53 protein activity or nuclear factor-kappa B (NF-κB) activity (53, 54). Earlier studies demonstrated that the activation of NF-κB was responsible for the overexpression of pro-metastatic and anti-apoptotic genes in cells of breast cancer (55). Since tumour progresssion was linked to an increased content of MT in the cell nuclei in the present study interactions of MT with NF-κB can not be excluded.

In conclusion a gradual increase in MT expression parallels progression of the tumour. Increase nuclear expression of MT in LN+ cells might suggest a direct effect of MT on transcription factors and thus the modulation of metastatic activity. A relationship between the intensity of MT expression and tumour progression is possible, but the mechanism by which the higher MT expression is translated into increased metastatic activity of the tumour remains unknown and requires further studies.

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