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Study of Metallothionein Role in Spinocellular Carcinoma Tissues of Head and Neck Tumours using Brdicka Reaction

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Malignant tumours of head and neck localisation represent serious health problem. All over the world, there is about 650 000 of patients who fall ill with head and neck carcinoma per year. Treatment of this malignity is based especially on the well-timed surgery intervention and radiotherapy. Therefore, alternative ways of diagnostics, which would replace commonly used biopsy in combination with computed tomography, are intensely searched. Attention is devoted especially to molecules, whose levels are changed in body due to malignant process and malignant disease progression. The main objective of this study consisted in determination of metallothionein (/MT)/ levels in tumour tissues of patients suffering from head and neck tumours using differential pulse voltammetry Brdicka reaction. Fifty-five samples of tumour tissue (45 % laryngeal, 40 % oropharyngeal, 9 % hypopharyngeal and 6 % oral cavity cancer) were included in this study. After describing of differences in differential pulse voltammograms of individual tumour localisations, content of MT were determined in the above mentioned localisations. The highest MT level was determined in the tissues of oral tumours (170 ± 70 μg/g) followed by hypopharynx (160 ± 70 μg/g) and larynx (160 ± 70 μg/g). The relatively lowest MT level was determined in tumours of oropharynx (130 ± 50 μg/g). MT level related to the size of

primary tumour focus oscillates with the increasing tumour size. Notwithstanding this fact, the increasing tendency with tumour disease progression is observable. Similar tendency was determined for MT level related to the metastatic activity of tumours, where MT level increased with tumour spreading into local lymph nodes. Our results supported findings on the possible connection of MT and tumour progression.

Keywords: electrochemical detection; cancer; voltammetry; catalytic signal; oral cancer; marker; metastases

1. INTRODUCTION

Malignant tumours of head and neck localisation represent serious health problem. All over the world, there is about 650 000 of patients who fall ill with head and neck carcinoma per year. Treatment of this malignity is based especially on the well-timed surgery intervention and radiotherapy. In the case of progression and/or presence of metastases or in inoperable tumours, pharmacological treatment represents the only therapeutic modality. Platinum-based pharmaceuticals (carboplatin, cisplatin) in combination with 5-fluorouracil or eventually radiotherapy proved effective [1]. It seems that combination of curative methods and connection with the individualization of therapy represents the best approach. Pursuit of finding new effective pharmaceuticals based on molecular-biological approach, which would replace highly toxic metal-based compounds including cisplatin, is well evident, but sooner the tumour is detected, the treatment is better.

Therefore, alternative ways of diagnostics, which would replace commonly used biopsy in combination with computed tomography, are intensely searched. Attention is devoted especially to molecules, whose levels are changed in a body due to malignant process and malignant disease progression .However, it seems that there is no universal tumour marker. This fact is closely connected with particularity and uniqueness of individual types of tumour cells, which demonstrate specific gene expression activity. This fact has been confirmed also in the case of head and neck tumours. The most used tumour markers in these localizations are cyclins, such as cyclin D1, whose increased level was detected almost in 50 % of all cases. Cyclin D1 plays an important role in cell cycle regulation, specifically for transition from G1 to S phase [2,3]. In addition, gene for cortactin, monomeric protein responsible for polymerization and rearrangement of actin cytoskeleton, is localised on the same locus as cyclin D1. Its expression is considered as one of the most important factors affecting cell adhesion and migration. Overexpression of these genes is often simultaneous [4]. Epidermal growth factor receptor (EGFR) belongs to the other tumour markers used in head and neck tumour diagnostics. EGFR is a transmembrane receptor for epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) [5]. Finally, mutation of p53 tumour suppressor gene is commonly detected. However, this mutation is frequently detected also in another tumour cell types [3]. E-cadherin can be considered as another potential tumour marker of head and neck carcinoma. This transmembrane protein is responsible for cell adhesion and metastatic activity of tumour [3,6]. Detection of all above-mentioned

markers is predominantly based on immunohistochemical methods or on mass spectrometry. Protein metallothionein (MT) is in the centre of the interest now. Papers demonstrating connections between metallothionein expression and metastatic behaviour of adenoid cystic carcinoma of the salivary glands has been published [7-9].have Investigation of the epigenetic alterations and gene expression of the MT3 gene in oesophageal adenocarcinomas brought the finding that epigenetic silencing of MT3 is very frequent in adenocarcinoma cells.

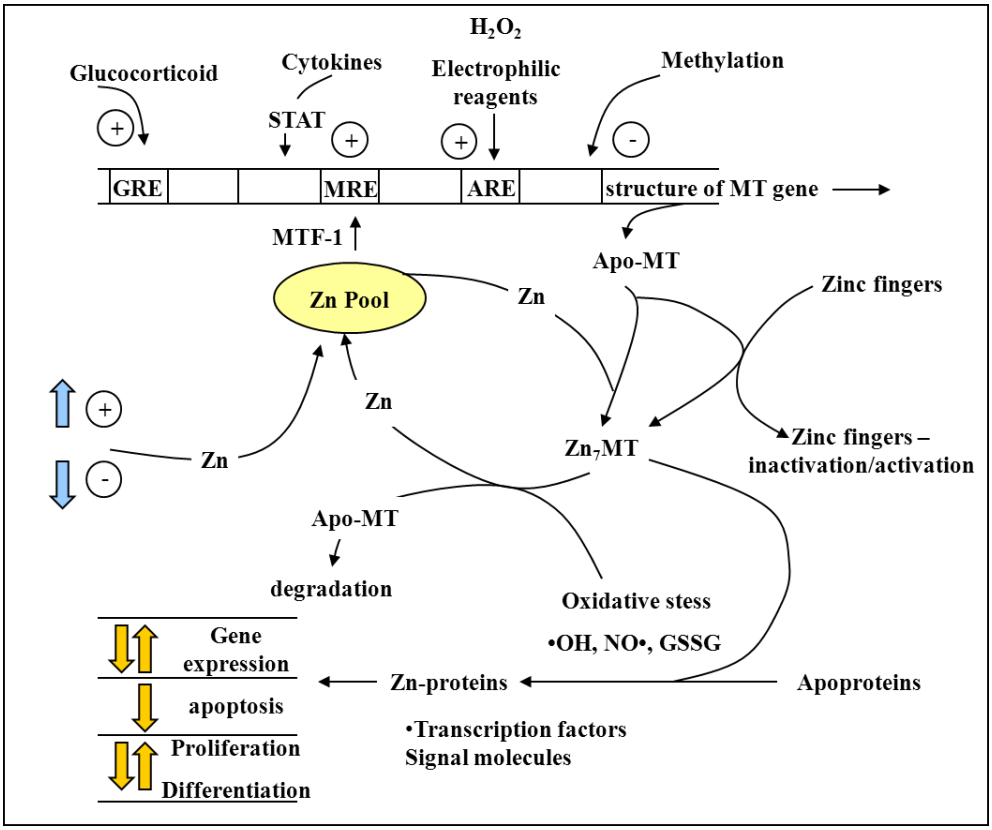

Figure 1. Scheme of MT involvement in cell metabolism with respect of zinc ions distribution. There are some response elements on MT gene promoter: GRE (glucocorticoid response element), STAT elements (signal transducers and activators of transcription), proteins controlled by cytokine signalling, MRE (metal response element), and ARE (antioxidant response element). Zinc(II) ions as an integral part of zinc fingers, small structural motifs, with regulatory function of gene expression can be bound by MT. In addition, they participate in metabolism of reactive oxygen species and regulate processes of apoptosis, which could be other processes to be bound and transported by MT. Modified according to Davis et al. [15].

However, the choice of specific regions in the CpG island is a critical step in determining the functional role and prognostic value of DNA methylation in cancer cells [10]. MTs[10]. were discovered by Margoshes and Valee in 1957 as newly identified proteins isolated from a horse renal cortex tissue [11]. Mammalian MTs are low molecular mass (app. 6 kDa) proteins with unique

abundance of cysteine residues (more than 30 % from all aminoacids). Other interesting structural property is the lack of aromatic amino acids. The main function of MTs in organism is a metal ion transport, maintenance of the oxidative-reducing conditions and regulation of gene expression. MT regulates free radical level directly as a scavenger and also indirectly by binding of metal ions which are potential radical producers, e.g. Cu [12]. As confirmed by several studies [13,14], MT expression in cells is induced also by superoxide and hydroxyl radicals generated by γ-radiation. Beside its scavenger role, MT acts also as a zinc donor for enzymes participating in repairing processes (Fig. 1).

Zinc is together with iron the most abundant metal in human body. In the light of this fact it is not surprising that many proteins are involved in maintenance of zinc homeostasis. Influx/efflux of Zn^{2+} across the cell membranes in mammals is maintained by the family of ZnT1-4 transport proteins [16]. From studies it is evident that ZnT1 and ZnT4 transport zinc ions out of the cell under zinc surplus, whereas ZnT2 and ZnT3 are responsible for deposition of these ions in organelles of the cells of intestine, testes and neurons [17]. Contrariwise, there are many unanswered questions in the processes of intracellular zinc transport. Metallothionein seems to be the crucial protein involved in these processes (Fig. 1). Increased MT level was detected in proliferating cells, which can be closely connected with increased zinc demands [18-20]., Zn^{2+} [15]. Due to its affinity to zinc, MT can regulate activity of zinc fingers. In the case of cadmium-inactivated transcription factors p69 and p88 (repressors of expression of *ftz*, *eve* and *en* genes in the photoreceptor cells of *Drosophila*), MT was able to restore their regulatory function by the exchange of Cd^{2+} for Zn^{2+}. MT serves not only as a zinc reservoir for proteins that need zinc for their correct function (and generally activity), but together with its apo-MT activity also as a regulator of gene expression itself. Its "supply" function has been confirmed by experiments monitoring kinetics of zinc binding into the structures of zinc-dependent proteins. Despite its high affinity to Zn^{2+} ions when compared to other zinc-containing proteins, MT is able to release these ions. MT-Zn bond may be disrupted by the oxidation of sulfhydryl groups, which are crucial for zinc chelation. Molecule of oxidised glutathione (GSSG) has been revealed as one of the agents able to break the MT-Zn bond [21].

We have been focusing on MT electrochemical detection for several years [22-50]. Besides electrochemistry, other detection methods and procedures based on immune principles or modern separation techniques can be also used [33,34,42,51]. The main objective of this study is[33,34,42,51]. The main objective of this study consisted in determination of MT levels in tumour tissues of patients suffering from head and neck tumours using differential pulse voltammetry Brdicka reaction.

2. EXPERIMENTAL PART

2.1 Tumour tissues

Samples were obtained from Department of Otorhinolaryngology and Head and Neck Surgery, St. Anne´s University Hospital, between the years 2006-2009. All samples originated from patients

suffering from malignant tumour disease in head and neck localization – oropharynx, oral cavity, hypopharynx and larynx. There were no differences in technique of sample storage and preparation. Sample taking and subsequent processing was approved by Ethic Committee of Masaryk University, Brno, Czech Republic.

2.2 Chemicals and material

Rabbit liver MT (MW 7143), containing 5.9 % Cd and 0.5 % Zn^{2+} and PSA, were purchased from Sigma Aldrich (St. Louis, USA). Tris(2-carboxyethyl)phosphine (TCEP) was prepared by Molecular Probes (Eugene, Oregon, USA). MT and PSA stock standard solutions were prepared with ACS grade water (Sigma-Aldrich, USA) and stored in the dark at –20 °C. Working standard solutions were prepared daily by dilution of the stock solutions. To pipette volumes down to micro and nanolitres, pipettes used were purchased from Eppendorf Research (Eppendorf, Germany) with the highest certified deviation (± 12 %). The deionised water was prepared using reverse osmosis equipment Aqual 25 (Czech Republic). The deionised water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

2.3 Differential pulse voltammetry - Brdicka reaction for metallothionein determination

Differential pulse voltammetric measurements were performed with 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder (4 °C). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 and/or 0.25 mm^2 was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and glassy carbon electrode was auxiliary. For data processing GPES 4.9 supplied by EcoChemie was employed. Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer (NH_3(aq) + NH_4Cl, pH = 9.6) was used. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as follows: initial potential of –0.7 V, end potential of –1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude −250 mV, E_{ads} = 0 V, time of accumulation 240 s, volume of injected sample: 5 µl (100 × diluted sample with 0.1 M phosphate buffer pH 7.0). All experiments were carried out at temperature 4 °C employing thermostat Julabo F25 (Labortechnik GmbH, Germany) [25].

2.4 Preparation of tissues samples

Primarily, samples were homogenized according to Fig. 2. The samples were kept at 99 °C in a thermomixer (Eppendorf 5430, Germany) for 15 min with shaking in order to remove ballast proteins

and peptides, which could influence the electrochemical response. The denatured homogenates were centrifuged at 4 °C, 15 000 × g for 30 min. (Eppendorf 5402, Germany).

2.5 Mathematical treatment of data and estimation of detection limits

Mathematical analysis of the data and their graphical interpretation was realized by software Matlab (version 7.11.). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®).

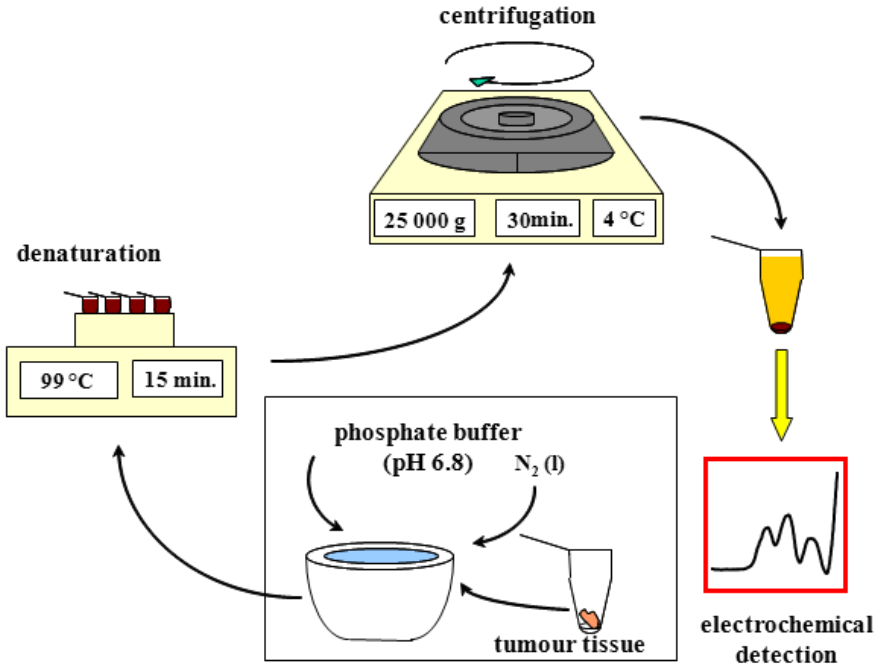

Figure 2. Scheme of the processing of tumour tissues. Firstly, tumour tissue was cut into small pieces and deep frozen by liquid nitrogen. Subsequently, 2 mL of phosphate buffer (pH = 6.8) was added. The homogenized sample was further denatured at 99 °C for 15 min and centrifuged for 30 min under 25 000 g and 4 °C.

3. RESULTS AND DISCUSSION

Experimental results of metallothionein level in blood of patients suffering from head and neck tumours have been summarized in our previous work. Significant increase of MT levels in patients with tumours in all localizations compared to control group have been demonstrated [52]. Some patients underwent surgery intervention with the aim to reduce tumour mass (cytoreduction). Unfortunately, it was not possible to take a sample for metallothionein analysis from all tumours included into the previous study. Fifty-five samples of tumour tissue (45 % laryngeal, 40 % oropharyngeal, 9 % hypopharyngeal and 6 % oral cavity cancer) were obtained for analysis (Fig. 3).

These samples were processed for electrochemical analysis in accordance with the procedure described in the Experimental section.

3.1 Electroanalytical detection of MT by Brdicka reaction

Electrochemical determination of metallothionein using Brdicka reaction was described and discussed in our previously published works, which are summarized in the following papers [35,36,41,53]. Current accepted nature of the electrochemical signals and description of the electrode reactions were published by Dr. Biserka Raspor and others [54-57] . Briefly, MT gives very well separated and distinguishable signals: RS2Co at the potential of −1.1 V, which represents current response of MT complex with components of Brdicka's supporting electrolyte, Cat1 signal (potential about −1.25 V) and Cat2 signal (potential about −1.45 V) are catalytic signals of hydrogen that are generated from the supporting electrolyte by the presence of MT. In addition to these well distinguishable signals, there can be occasionally found another one, called "R", at potential app. −1 V, which nature is unclear. Due to sample processing, we were able to separate thermostabile part of tissues (metallothionein) from high-molecular mass thermolabile proteins. Sample (5 μl) was injected directly into the electrochemical cell containing supporting electrolyte.

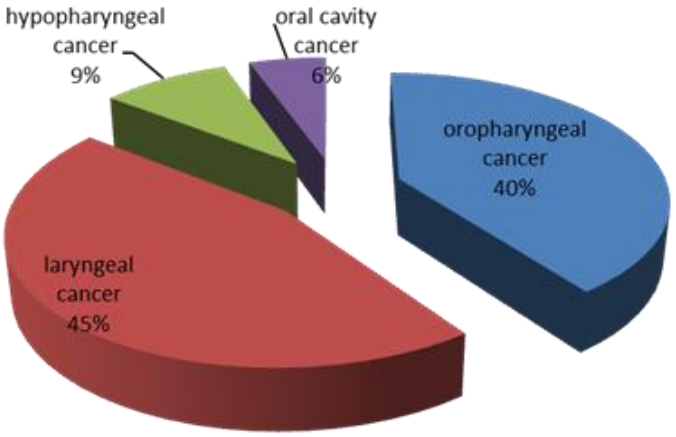

Figure 3. Distribution diagram of tumour tissue samples of newly diagnosed patients suffering from head and neck tumours obtained from 2006 to 2009 in South Moravian region. The patients are divided according to their diagnoses.

3.2 Differential pulse voltammograms

Typical voltammograms obtained by the analysis of tumour tissue samples prepared according to procedure mentioned in the Experimental section are shown in Fig. 4. Fig. 4A demonstrates

voltammogram of oropharyngeal tumour tissue with significantly deformed RS2Co signal, very well developed Cat1 signal, well-identifiable R signal and typical Cat2 signal.

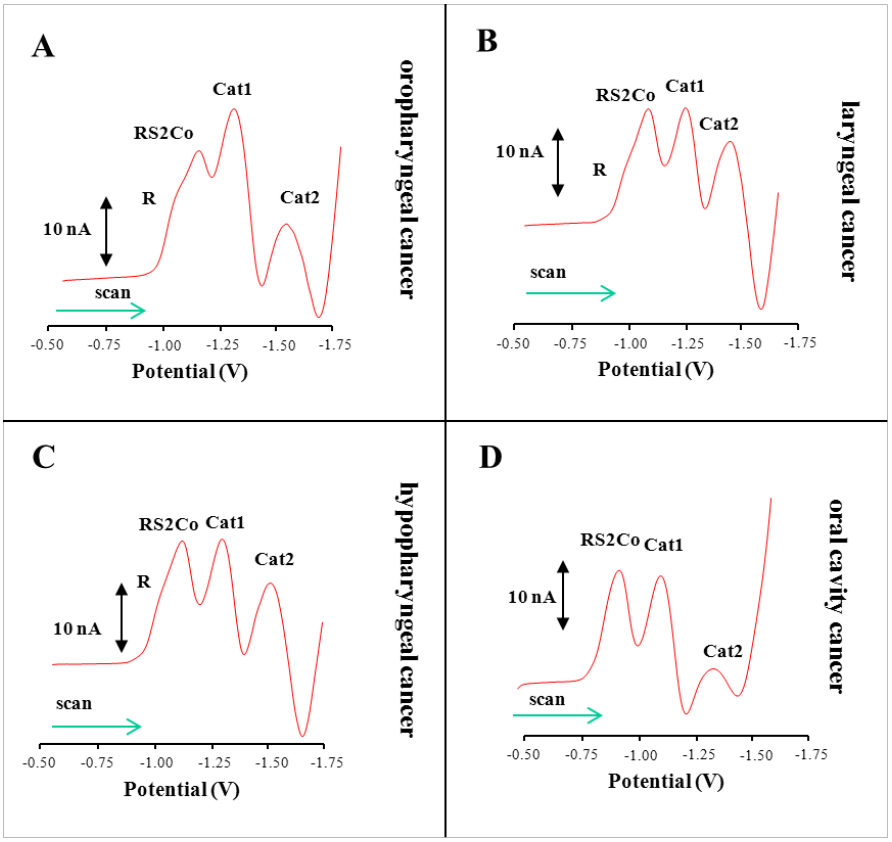

Figure 4. Typical DP voltammogram of tissues homogenate from patient suffering from (**A**) oropharyngeal cancer, (**B**) laryngeal cancer, (**C**) hypopharyngeal cancer, (**D**) oral cavity cancer. Experimental conditions were as follows. Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer ($NH_3(aq)$ + NH_4Cl, pH = 9.6) was used. The supporting electrolyte was changed after each analysis. The parameters of the measurement were as follows: initial potential of –0.7 V, end potential of –1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude -250 mV, E_{ads} = 0 V, time of accumulation 240 s, volume of injected sample: 5 µl.

Samples of laryngeal cancer demonstrated differences in RS2Co, Cat1 and Cat2 signals (Fig. 4B). Hypopharyngeal cancer signals were similar to signals of laryngeal cancer samples; however, heights of Cat1 and RS2Co signals were different compared to laryngeal cancer samples (Fig. 4C). Completely different shapes of voltammetric curves were observed in samples of patients suffering from oral cavity tumours (Fig. 4D). Well-separated signals RS2Co and Cat1 were observed. In addition, significantly changed Cat1/Cat2 rate in comparison with other samples was determinable. Moreover R signal was not detectable (Fig. 4D). Changes in shapes of voltammetric curves records

seem to be specific with respect of tumour localisation, respectively localisation of surgically taken tumour tissues. Samples of tumour tissues were analysed also histochemically. They were confirmed as spinocellular carcinomas. Changes in shapes of voltammetric curves are probably caused by the presence of other low-molecular thermostabile compounds, which modify surface of the measuring electrode and are responsible for changes in recorded currents [23].

3.3 Content of metallothionein in tumour tissues

Knowledge about MT levels in tumour tissue is crucial for understanding of molecularly-biological mechanisms in tumour cells, and also for diagnostic and predictive purposes. Utilization of commonly used analytical techniques as spectrometry, liquid chromatography and electrophoresis in MT detection and quantification suffers from several disadvantages, but electrochemical methods are able to overcome these disadvantages [41,42,53,58]. The reason why[41,42,53,58]. The reason what is metallothionein of such interest for analytical chemist is that MT level significantly depends on the type of cells (original and malignant), stage of tumour disease and next factors, which are presently in the centre of scientists´ interest [36,52,59]. Our aim consisted in determination of MT in tumour tissue of patients suffering from malignant disease in head and neck localization. Foregoing works focused on these questions have demonstrated fact that MT level is significantly increased in both tumour and surrounding tissues (in the case of the presence of metastases into adjacent lymph nodes) in this localization [60]. In addition, the paper aimed at MT expression in malignant tumours of larynx indicated connection between MT expression and tumour disease progression at early stages of disease [61]. In this study, tumours of oropharynx, oral cavity, hypopharynx and larynx were analysed using differential pulse voltammetry Brdicka reaction.

3.3.1 Oropharyngeal cancer

Twenty-two samples were analysed. MT level varied from 30 to 250 μg MT per g of analysed tumour tissue (Fig. 5A). Relative standard deviation of measurement was up to 5 %. The increased MT level was well evident with the increasing size of primary pharyngeal tumour marked as “T” (Fig. 5B). In the same type of tumour with metastases into adjacent regional lymph nodes called “N” increased MT level was also observable, namely in tumour cells, which are probably more invasive (Fig. 5B). Interesting relation was found in patients with nodal metastases, where MT level in primary tumour was significantly lower compared to patients with primary tumour only. The increasing tendency of MT levels is well evident up to the stage IV. However, sub-stages of this stage demonstrate decreasing tendency. In accordance with theory, dependence of MT level in tumour cells on the stage of cell differentiation has been confirmed, i.e. MT levels did increase with the increasing rate of dedifferentiation (Fig. 5).

3.3.2 Laryngeal cancer

Twenty-five samples of tumour tissue of this localisation were analysed. MT level varied from 60 to 300 μg MT per g of analysed tumour tissue (Fig. 6A). No dependence between MT level and T classification was observed (Fig. 6B). Decrease in MT levels in II stage is probably associated with statistical data analysis and in lack of samples of this stage compared to other ones. Correlation between MT level and tumour spreading into local lymph nodes showed at the increasing tendency of MT levels with the increasing invasiveness of tumours (Fig. 6B).

The comparison of MT levels in tumour cells and tumour disease stage had almost similar tendency. This fact is probably caused by accentuation of tumour disease classification during evaluation of tumour disease progression. Based on the obtained results it cannot be concluded relation between MT level and higher tumour cell dividing due to the presence of a tumour. However, with the exception of III stage of tumour disease we can assume that the tendency could be moderately decreasing with tumour cell dedifferentiation (Fig. 6).

3.3.3 Hypopharyngeal and oral cavity cancer

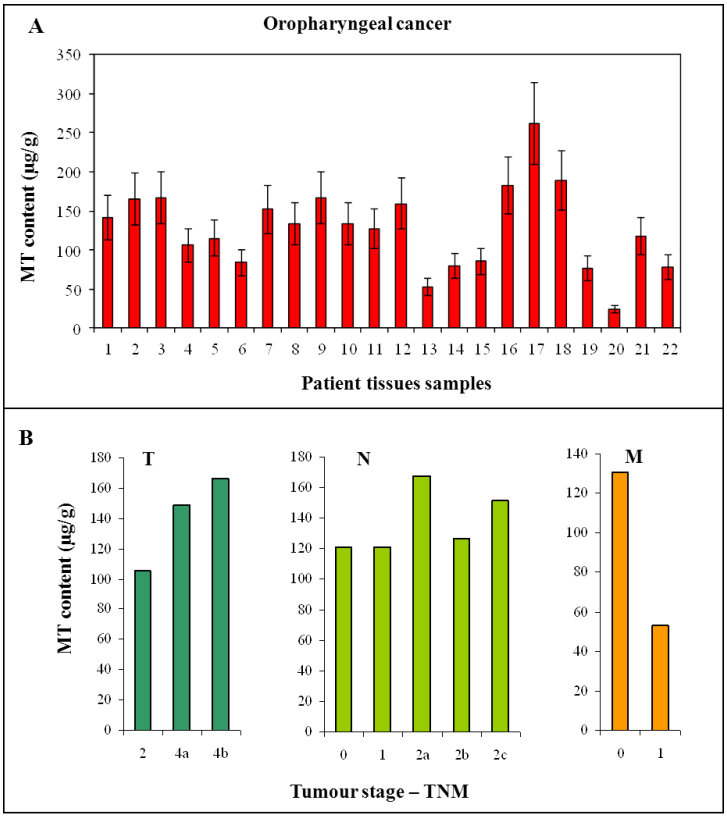

Figure 5. (**A**) MT content determined in tissue samples (n = 22) obtained from the patients suffering from oropharyngeal cancer. (**B**) The average content of MT according to tumour classification as T, N and Stage. For other details see Fig. 4.

Five samples of hypopharyngeal tumour tissue and three samples of oral cavity tumour tissue were analysed (Figs. 7A and 7B, respectively). MT level varied from 100 to 270 μg MT per g of analysed tumour tissue (Fig. 7A). However, detailed evaluation could not be performed due to limited number of analysed samples, but relatively high MT levels are well evident from obtained results, which is promising for further studies (Fig. 7).

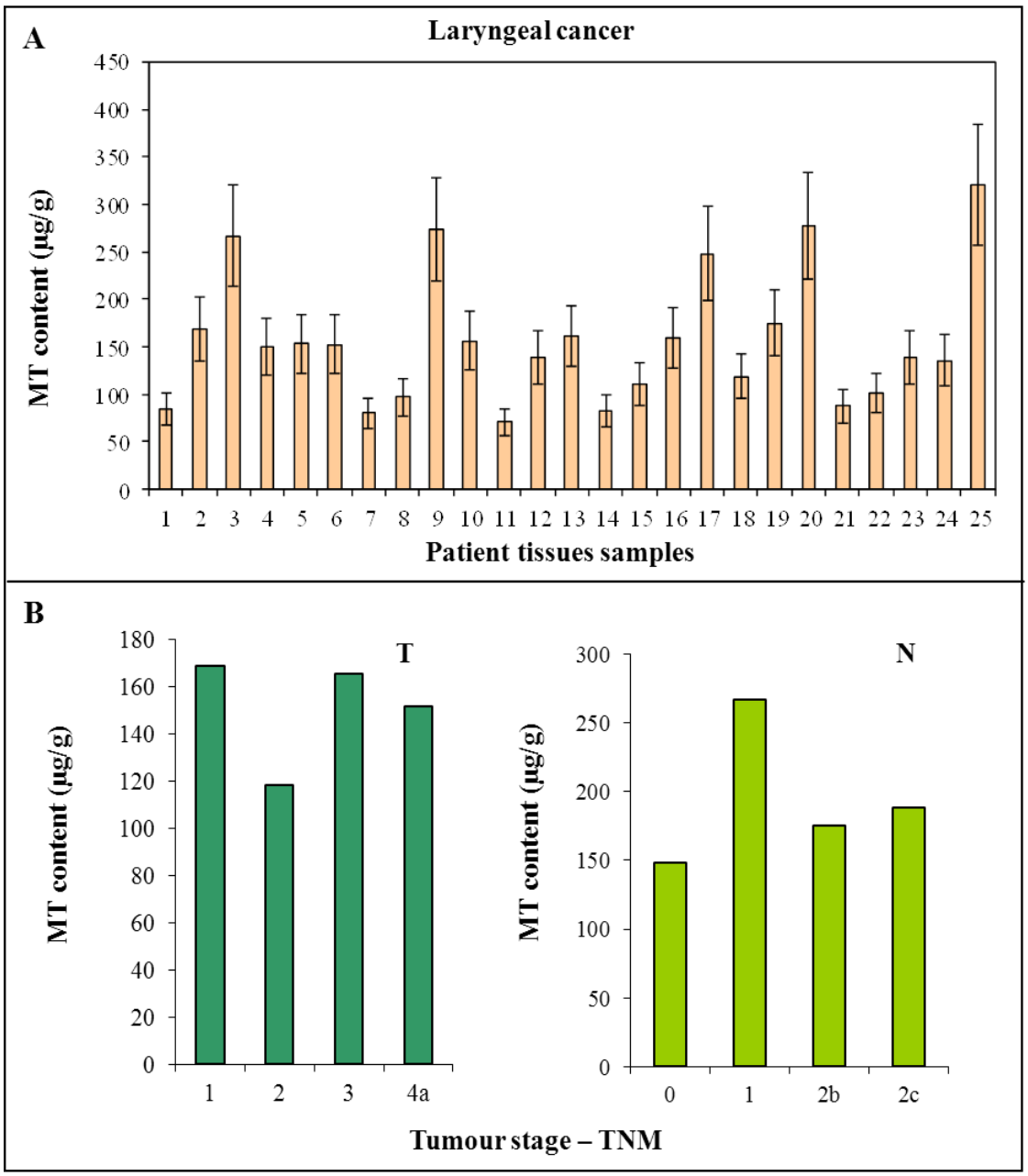

Figure 6. (A) MT content determined in tissue samples (n = 25) obtained from the patients suffering from laryngeal cancer. (B) The average content of MT according to tumour classification as T, N and Stage. For other details see Fig. 4.

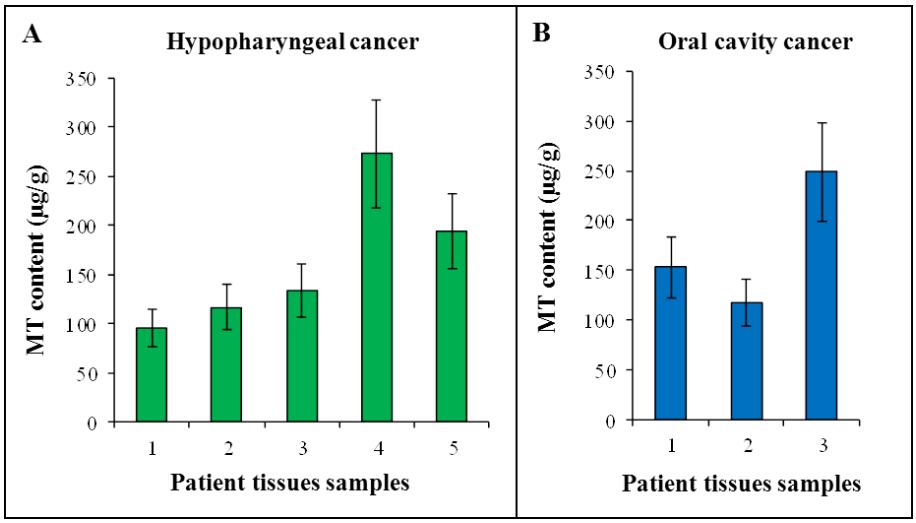

Figure 7. MT content determined in tissue samples obtained from the patients suffering from (**A**) hypopharyngeal (n = 5) and/or (**B**) oral cavity cancer. For other details see Fig. 4.

3.4 Average MT levels in tumour cells related to TNM classification

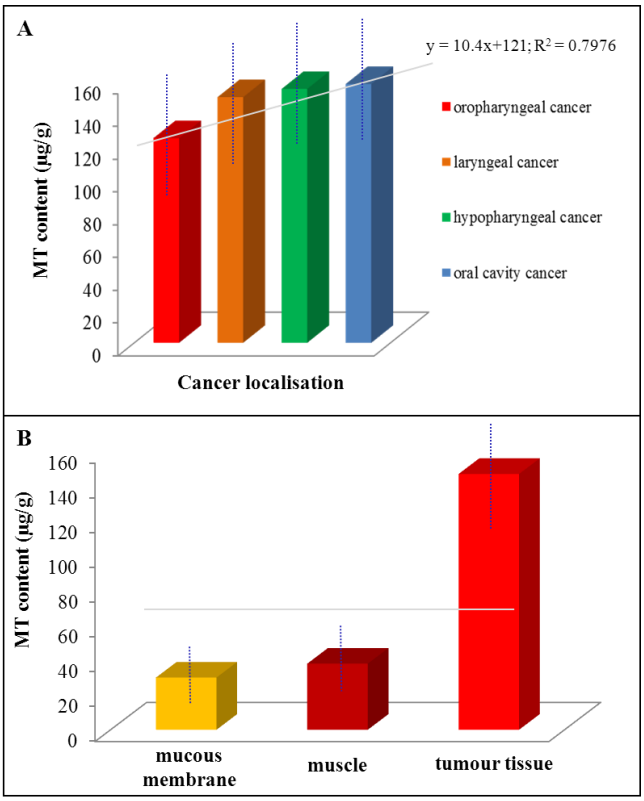

Figure 8. (**A**) Average MT level in tumour tissues of all analysed samples of oropharyngeal, laryngeal, hypopharyngeal and oral cavity cancers (n = 55, mean). (**B**) Average MT level in mucous membrane, muscle and cancer tissues. For other details see Fig. 4.

Content of intracellular MT can inform us about molecular- biological processes proceeding inside a tumour cell. MT level related to the size of primary tumour focus oscillates with the increasing tumour size. Notwithstanding this fact, the increasing tendency with tumour disease progression is observable. Similar tendency was determined for MT level related to the metastatic activity of tumours, where MT level increased with tumour spreading into local lymph nodes. Difference in 2b classification can be caused probably by statistical data processing because of lack of samples in some classes. MT level related to the stage of tumour disease evidenced decreasing tendency except of differences in the first two stages. Content of MT in cells distributed according to tumour differentiation correlates with our expectation, i.e. less differentiated cells produce higher amount of MT. Fig. 8A summarizes average MT levels for individual malignant tumour localizations. All tumour localizations demonstrated increasing trend in MT content, which can be expressed by the following equation $y = 10.4x+121$; $R^2 = 0.7976$. The highest MT level was determined in the tissues of oral tumours (172.8 ± 68.2 μg/g) followed by hypopharynx (162.9 ± 71.8 μg/g) and larynx (156.30 ± 69.6 μg/g). The lowest MT level was determined in tumours of oropharynx (127.2 ± 53.0 μg/g). There is one important question, what can MT bring to tumour cells and how can it make tumour cells more favourable compared to non-tumour cells? Some advantages of the increased MT expression can be defined. Increased apo-MT concentration (apo-MT is metallothionein without naturally contained metal ions) leads to the increased zinc binding from tumour suppressor gene p53 [62]. This effect causes limited zinc availability including limited binding to DNA with subsequent deactivation of apoptic cascade. In addition, MT is important in intracellular metal homeostasis and detoxification of xenobiotics and has significant antioxidant properties. These antioxidant properties are responsible for cell protection, which can lead to the lowering of the effectiveness of antitumour agents, such as metal-based cytostatics including cisplatin or carboplatin [63,64]. However, relation between MT and tumour disease remains unclear and is in the focus of interest of many scientific workplaces. It is evident from obtained results that prognostic significance of MT must be further investigated.

3.5 Analysis of surrounding tissues

Surrounding tissues were analysed in the case of four patients suffering from laryngeal tumour. These tissues included muscle and mucous parts. Compared to surrounding tissues, MT level was significantly higher in tumour tissue compared to the surrounding tissue (Fig. 8B). Different results were found in the case of two patients, where MT level is similar in both tumour and surrounding tissues. This phenomenon can be related to the rate of higher cell differentiation (G2) compared to previous undifferentiated cases with elevated MT levels (G3). These cells are less differentiated, however, conclusions cannot be pronounced due to limited number of samples. In the well-differentiated oral carcinomas MT level was increased in the peripheral tumour cells, i.e. in cells with high mitotic activity. On the other hand, MT level was significantly lower in well-differentiated cells in the central part of tumour. Higher apoptotic activity was also demonstrated in these cells [65]. These

findings support presumption of chelatation properties of MT and apoptosis delay mediated by p53 protein.

4. CONCLUSIONS

The increased expression of MT has been demonstrated in the tumour cells of larynx [66], oral cavity and pharynx [67], oesophagus [68], and nasopharynx [69]. MT immunopositivity was more frequently evidenced in cells of tumours of higher clinical stages. Longer time of survival was recorded in the case of patients with lower MT levels treated with cisplatin. This fact could be used in the future for the prediction of effectiveness of cytostatic treatment [68]. Resistance to radiotherapy has been demonstrated in nasopharyngeal tumour cells with the increased MT expression. This fact may be closely connected with significant antioxidant properties of MT [69]. Increased MT levels independently on tumour aggressiveness were observed in the case of laryngeal cancer [61,66]. Our results supported the above mentioned findings and electrochemistry can be used in this type of studies.

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