Prognostic Significance of Metallothionein, p53 Protein and Ki-67 Antigen Expression in Laryngeal Cancer

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Abstract. *Background: This study aimed at the evaluation of the expression of metallothionein (MT) in laryngeal carcinoma and its correlation with the expression of Ki-67 antigen and p53 protein and selected clinical and pathological variables in view of their potential prognostic significance. Materials and Methods: Sixty-five laryngeal cancer patients were retrospectively analysed. Expression of MT, Ki-67 and p53 in tumour tissue samples were assessed by immunohistochemistry. Results: In laryngeal cancer a significantly augmented expression of MT, Ki-67 and p53 was noted, as compared to the control group ($p<0.001$) and a significantly increased expression of MT in low malignancy tumours (G1) as compared to the control group ($p<0.001$). The expression of Ki-67 antigen was positively correlated with the expression of p53 protein ($r=0.477$; $p<0.05$) and expression of either marker was positively correlated with malignancy grade ($r=0.47$, $p<0.05$; $r=0.31$, $p<0.05$; for Ki-67 and p53, respectively). Shortened survival was noted in patients with high expression of Ki-67 antigen and p53 protein. Conclusion: The intensity of MT expression was not related to prognosis in laryngeal cancer. Nevertheless, it may provide a significant index indicating the malignant transformation of benign lesions of laryngeal epithelium.*

According to current data of the International Agency for Research on Cancer, in recent years the highest risk of laryngeal cancer incidence in Europe is noted in France, Spain, Italy and Middle-Eastern Europe (the incidence exceeds 15.7/100,000 inhabitants) (1).

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Squamous cell carcinoma represents the most frequent malignant tumour of the larynx. Its development is strongly linked to environmental factors and to life style. The principal risk factors are thought to involve cigarette smoking and alcohol (2-4). Subsequent risk factors include improper diet, exposure to certain gases, dusts, smokes, paints and solvents of potential carcinogenic influence. Reports are also available on the role of oncogenic HPV viruses type 16 and 18 in the promotion of laryngeal cancer (5), as well as of gastroesophageal reflux disease (GERD) (6).

The principal therapeutic techniques in laryngeal cancer include surgery and radiotherapy. Despite the broad range of available diagnostic and therapeutic techniques, results of treatment of the tumour remain unsatisfactory (2). One of the reasons for failure seems to involve inappropriate systems for classifying the advanced disease, which provide grounds for selection of treatment and prognosis. Both TNM classification and evaluation of cancer malignancy grade are burdened by a high dose of subjectivity. Frequently, a definitely distinct course of the disease may be observed in cases at a similar clinical stage and of a similar grade of malignancy. Thus, the need arises to determine new, more objective variables for tumour appraisal, which would provide credible data on prognosis in individual cases, and which would facilitate the selection of the appropriate therapeutic strategy.

Detailed recognition of tumour biology represents a key problem in oncological diagnosis. Modern immunohistochemical techniques allow for identification of several proteins, the expression intensity of which changes in the course of carcinogenesis. Analysis of the expression of the nuclear Ki-67 antigen (a marker of cell proliferative activity) or of the pro-apoptotic p53 protein certainly helps in recognition of proliferation kinetics of tumour cells. Literature on the subject contains reports on prognostic significance of the markers in laryngeal cancers, but the

results are frequently contradictory (7-9).

Metallothioneins (MTs) represent a group of low molecular weight proteins with a high content of cystein in the polypeptide chain (10). Studies on protein structure distinguish of four main types of protein, MT-1, MT-2, MT-3, MT-4, coded by genes localised on chromosome 16 (11). The main function of MT is linked to its effect on cellular homeostasis of Zn and Cu ions and to detoxification from cells of heavy metal ions and free oxygen radicals (12, 10). MTs also participate in proliferation and maturation processes of cells (8). The proteins may promote increased proliferative activity of cells and may augment tumour aggressiveness, unfavourably affecting the course of a neoplastic disease (13). There were numerous publications which discuss the significance of MT expression in various types of human malignant tumours (14-18). The authors demonstrated a positive correlation between the expression of MT on one hand and the expression of the Ki-67 antigen, stage of clinical advancement and grade of malignancy on the other (18, 19).

Ki-67 is a nuclear non-histone protein, the expression of which can be detected in actively proliferating normal and neoplastic cells. The protein is used for the estimation of the so-called growth fraction of the tumour (9). The prognostic significance of Ki-67 antigen has been stressed in various types of malignant tumours (9, 19). Also in cancers of the head and neck, expression of Ki-67 was noted to correlate with clinical and pathological variables of the disease (20).

The p53 protein is a nuclear phosphoprotein responsible for maintenance of genome integrity. Mutation of the *p53* gene represents one of the most frequently encountered genetic abnormalities in human malignant tumours (21). The p53 protein from the mutated gene remains inactive, which significantly disrupts the mechanism of repair of genetic defects and the chance for apoptotic elimination of cells with damaged DNA (22, 23). Due to its long half-life, the inactive p53 protein may be detected using immunohistochemical techniques. Its high expression in tumours of the head and neck has been frequently linked with a higher aggressiveness of the tumours and less favourable prognosis (21). In several publications, attempts have been made to define the prognostic value of p53 protein expression in cancers of the head and neck, but the results have been frequently proven equivocal (21, 24, 25).

Our study aimed at the evaluation of MT expression in laryngeal cancers and at correlating its intensity with selected clinical and pathological variables of the disease, as well as with expression of the Ki-67 antigen and p53 protein in order to define their prognostic significance. Correlation of expression of these two markers with the expression of MT in laryngeal cancers may provide new data which would facilitate prognosis of the course of the disease in individual cases of the tumour.

Table I. *Clinical and pathological characteristics of studied patients.*

Clinical/pathological parameter	N (%)
Mean age in years (range)	60.45 (44-77)
Gender	
Men	56 (86.1%)
Women	9 (13.9%)
Tumour size	
T1	0
T2	7 (10.8%)
T3	32 (49.2%)
T4	26 (40%)
Lymph nodes	
N0	39 (60%)
N1	9 (13.8%)
N2	15 (23.1%)
N3	2 (3.1%)
Clinical advancement	
I	0
II	4 (6.1%)
III	30 (46.2%)
IV	31 (47.7%)
Histological differentiation (Grade)	
G1	15 (23.1%)
G2	33 (50.8%)
G3	17 (26.1%)

Materials and Methods

Patients. Material for the studies originated from 51 patients subjected to surgery due to laryngeal cancer in the Ward of Laryngology, Voivodship Hospital in Wroclaw from 1997 to 2003, and from 14 patients treated for the same reason in the Department of Laryngology, University School of Medicine in Wroclaw from 1996 to 2000. The group studied included 56 men (86.1%) and 9 women (13.9%). The average age was 60.4 years. Before diagnosis and implementation of treatment, none of the patients have been subjected to radio- or chemotherapy. In all cases surgery was performed with the removal of the cervical lymph nodes according to indications. The surgery was supplemented by subsequent radiotherapy according to a conventional irradiation scheme, in line with the binding principles. Clinical and pathological characteristics of the group are presented in Table I. The control group included 26 cases of benign lesions of the laryngeal epithelium. Total survival time was scored in months, elapsing from the time of the diagnosis to the last day of observation or death of the patient.

Tissue analysis. Sixty five biopsies of laryngeal tumours and twenty six of benign lesions were fixed in 10% buffered formalin, dehydrated and embedded in paraffin blocks. The immunohistochemical reactions were performed on paraffin sections, using antibodies and reagents originated from Dako

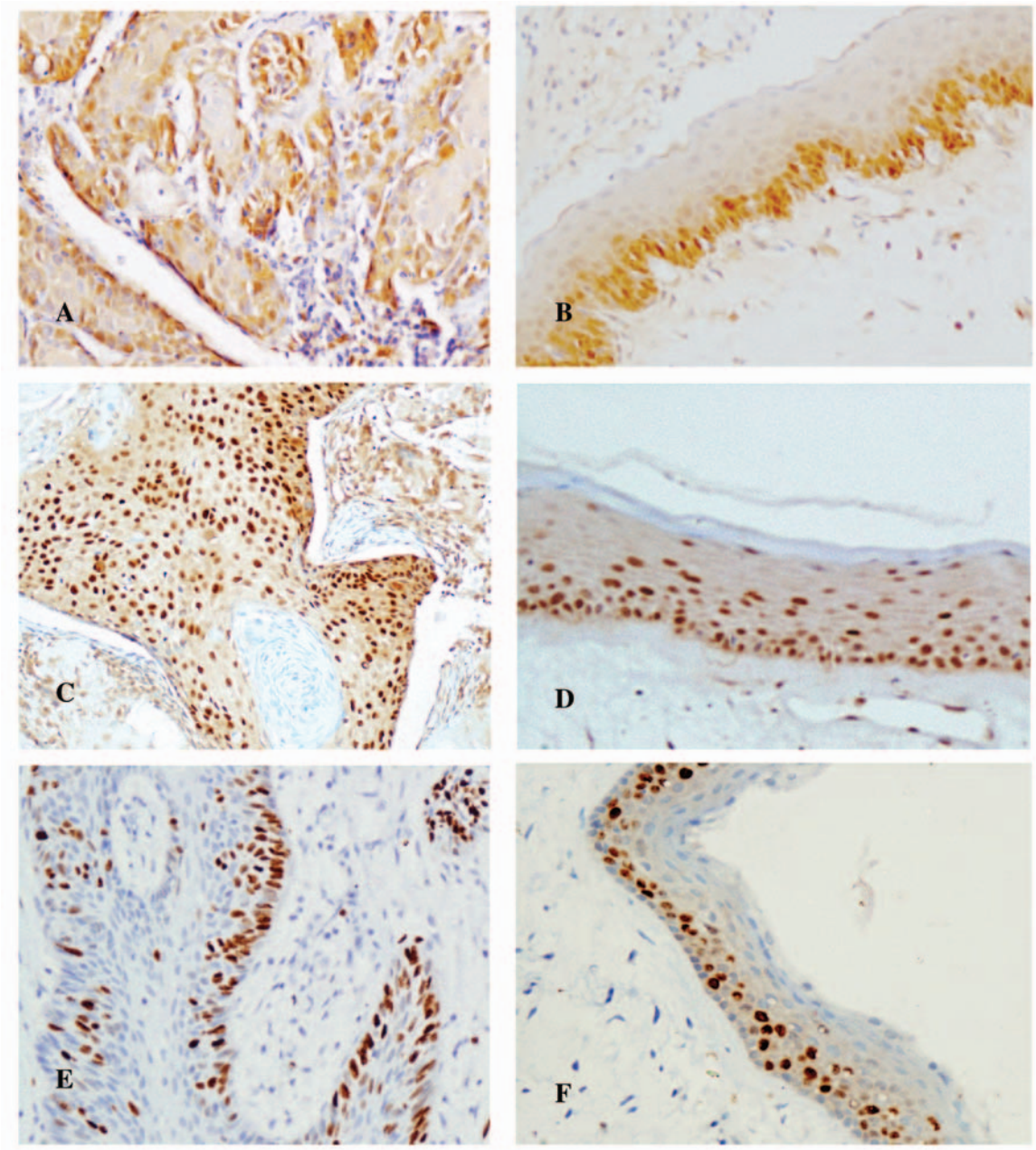

Figure 1. *Immunohistochemical localization of the expression of MT, p53 and Ki-67 in laryngeal cancer and benign lesions of laryngeal epithelium. A) Scattered nuclear - cytoplasmic expression of MT in cells of laryngeal cancer; magnification x200. B) Nuclear - cytoplasmic expression of MT in cells of a benign lesion in laryngeal epithelium. Cells with positive staining are arranged to the basal layer of epithelium; magnification x200. C) Strong expression of p53 protein in cell nuclei of a laryngeal cancer; magnification x200. D) p53 protein expression in cell nuclei of a benign lesion in laryngeal epithelium; magnification x200. E) Nuclear expression of Ki-67 antigen in cells of a laryngeal cancer; magnification x200. F) Nuclear expression of Ki-67 antigen in cells of a benign lesion in laryngeal epithelium; magnification x200.*

(Copenhagen, Denmark). Seven sections of 4 μm thickness of each sample were cut. Three sections were stained for detection of MT, Ki-67 and p53 expression, one with haematoxylin and eosin for traditional light microscopy and the other three were used as a negative control. Immunohistochemical satining was performed using mouse monoclonal antibodies (clone E9 diluted 1:100) specific to MT I and II isoforms (Dako). For detection of expression of the Ki-67 antigen and p53 protein mouse monoclonal antibodies were used (clone MIB-1 and clone DO-7; respectively, diluted 1:100) (Dako). All the reactions were accompanied by the respective control reactions in which specific antibodies were substituted by the Primary Negative Control (mouse

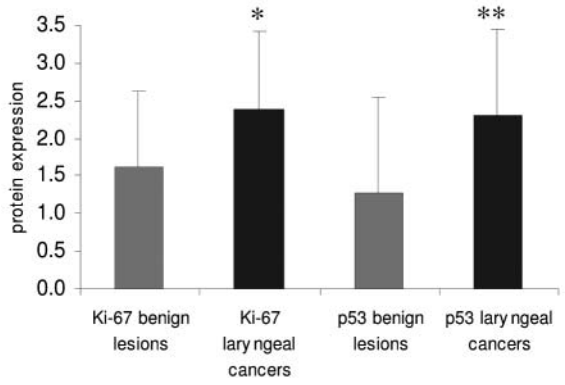

Figure 2. *Intensity of Ki-67 antigen and p53 protein expression in benign lesions and in laryngeal cancers; *benign lesions vs. laryngeal cancers for Ki-67, $p<0.01$; **benign lesions vs. laryngeal cancers for p53, $p<0.001$.*

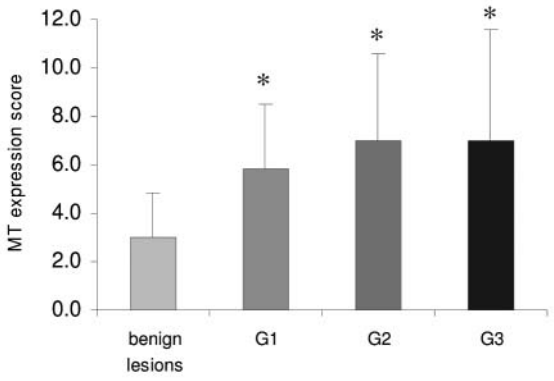

Figure 3. *Intensity of MT expression in benign lesions as related to malignancy grade (G) in laryngeal cancers. *benign lesions vs. G1,G2,G3, $p<0.001$.*

immunoglobulin IgG: Dako). In cases of reactions to detect Ki-67 antigen and p53 protein, the paraffin sections were boiled in the Antigen Retrieval Solution (Dako) in a microwave oven for 20 min to re-establish tissue antigenicity. Visualization of the investigated antigens was conducted using EnVision™+ system (Dako) and diaminobenzidine (DAB).

Coded preparations were independently evaluated by two pathologists. For determination of Ki-67 antigen and p53 protein expression a scale was used taking into account the percentage of cells with a nuclear colour reaction. The results were recorded on a five point scale (0 to 4 points) (19).

The intensity of MT expression was evaluated using the semi-quantitative IRS approach, according to Remmele and Stegner (26), taking into account the percentage of positively stained cells (in the range of 0 to 4 points) and the intensity of the colour reaction (in the range of 0 to 3 points). The results presented were given as the product of points given for individual traits and result in total values ranging from 0 to 12 points.

Statistical analysis. Statistical analysis was carried out using Statistica PL 5.1 software (StatSoft Cracow, Poland) with the following tests: Mann-Whitney, F Cox and Pearson's correlation. Survival curves were calculated using the Kaplan-Meier method. For all statistical tests a *p*-value of <0.05 was considered significant.

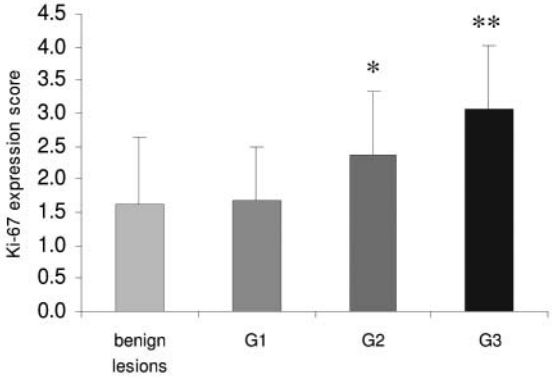

Figure 4. *Intensity of Ki-67 antigen in benign lesions and in laryngeal cancers, as related to malignancy grade (G). *G1 vs. G2, $p<0.05$; **G2 vs. G3, $p<0.05$.*

Results

MT expression both, nuclear and cytoplasmatic, was noted in all 65 cases of laryngeal carcinoma and in 26 cases of the control group (Figure 1 A, B). In the control group, the colour reaction was mainly restricted to the basal layers of the stratified flat epithelium, while in laryngeal cancers positive cells were scattered over the entire section of the tumour, intermingled with cells manifesting no such expression.

The intensity of MT expression in laryngeal cancer tissue, according to the IRS scale, amounted on average to 6.69±3.67 (SD), and in the control group to 2.62±1.24 (SD). This difference proved significant ($p<0.001$).

In all the cases of laryngeal cancer and of control individuals manifesting positive immunohistochemical reaction with anti-p53 and anti-Ki-67, nuclear expression of the proteins studied was detected (Figure 1 C-F).

In the control group cases manifested absent or only faint positive reaction to p53 [mean of 1.27±1.28 (SD)]. In 64 cases of laryngeal carcinoma, expression of p53 protein was present [mean of 2.26±1.18 (SD)]; it was absent in only a single case.

Expression of the Ki-67 antigen was detected in most benign lesions in the larynx. In two cases, no positive reaction for Ki-67 was noted [mean: 1.62±1.02 (SD)]. On the other hand, in all 65 cases of laryngeal carcinoma, a positive reaction for the Ki-67 antigen was noted [mean: 2.38±1.04 (SD)].

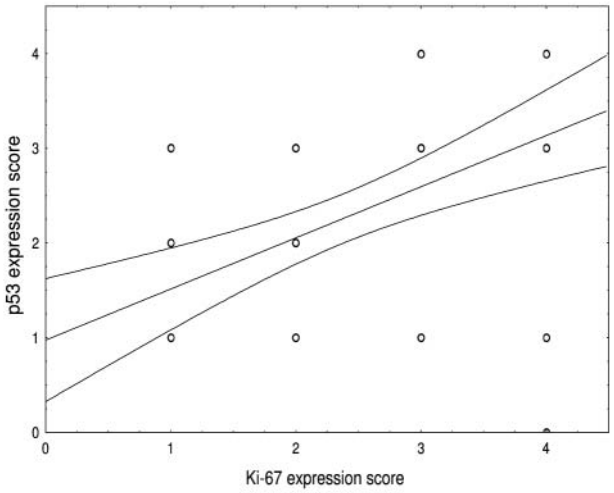

Figure 5. *Correlation between intensity of Ki-67 antigen expression and p53 protein expression in laryngeal cancers. r=0.477, p<0.05.*

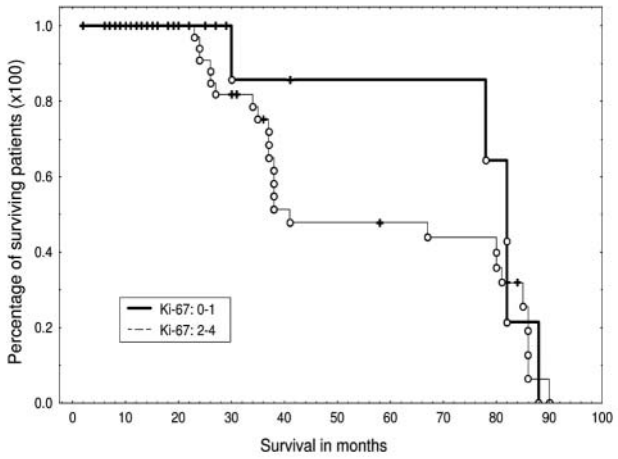

Figure 6. *Survival analysis in patients with low (0-1 pt.) or high (2-4 pt.) expression of Ki-67 antigen in laryngeal cancer; p<0.05.*

Significant differences were observed in the expression of both p53 protein and Ki-67 antigen between the control group and the group of patients with laryngeal carcinoma ($p<0.001$, $p<0.01$, respectively) (Figure 2).

The intensity of MT expression was compared between the different grade (G) groups and the benign lesions. G2 laryngeal cancers demonstrated the highest intensity of MT expression [mean of 7.03±3.59 (SD)]. Differences between cancers of different malignancy grade proved insignificant. However, a significant increase in MT expression was noted in groups G1-G3 as compared to the control group ($p<0.001$) (Figure 3).

Expression of p53 and Ki-67 in tumour cells increased in line with increasing malignancy grade, reaching peak levels in the least differentiated cancers (G3).

In turn, the most faint colour reaction to either of the proteins was observed in the benign lesions. Significant differences were observed in the expression of Ki-67 antigen between individual grades of cancer malignancy ($p<0.05$ for Ki-67 expression in G1 *vs.* G2; $p<0.05$ for Ki-67 expression in G2 *vs.* G3) (Figure 4). Similar differences were noted in the expression of p53 protein, but in this case the differences did not reach a level of statistical significance. These results were reflected by a significant positive correlation between the expression of Ki-67 and that of p53 ($r=0.477$, $p<0.05$) (Figure 5), as well as by a positive correlation between the expression of the two proteins and malignancy grade ($r=0.47$, $p<0.05$ for Ki-67, and $r=0.31$, $p<0.05$ for p53).

No significant relationships were detected between the intensity of expression of Ki-67, p53 protein and MT on the one hand and consecutive stages of tumour clinical advancement, tumour size (T), or presence of metastases in regional lymph nodes (N) on the other. Also no

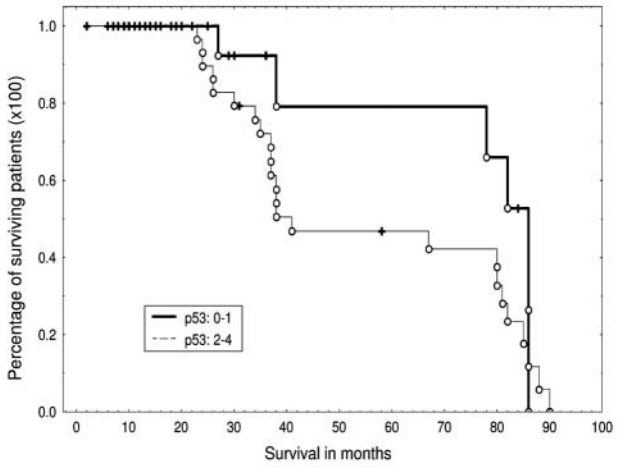

Figure 7. *Analysis of survival of patients with low (0-1 pt.) or high (2-4 pt.) expression of p53 protein in laryngeal cancers; p<0.01.*

relationships were detected between the expression of MT and that of the remaining markers (Ki-67, p53).

Analysis of survival demonstrated a significantly shortened survival in patients with a high expression of Ki-67 as compared to patients with a low expression of Ki-67 ($p<0.05$) (Figure 6). Similarly, patients with a high expression of p53 protein exhibited a significantly shorter survival as compared to patients the tumours of which demonstrated a low level of expression of the protein ($p<0.01$) (Figure 7). Even though expression of MT was not found to significantly affect the survival of patients with laryngeal carcinoma, shortened survival was observed in cases with high MT expression (Figure 8).

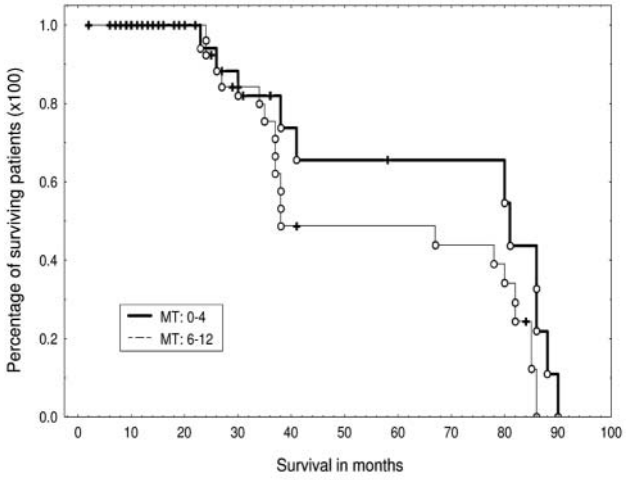

Figure 8. *Analysis of survival of patients with low (0-4 pt.) or high (6-12 pt.) expression of MT in laryngeal cancer.*

Discussion

Despite the significant progress in contemporary surgery, radio- and chemotherapy, laryngeal carcinoma continues to pose a significant therapeutic problem. The broad scope of techniques for conservative treatment stimulates patients' hopes for the preservation of the organ and its principal functions. In parallel, this forces doctors to develop a precise system of qualifying the patients for appropriate therapeutic modalities (27). Evaluation of the clinical stage, based on the TNM classification, and evaluation of the tumour malignancy grade, based on the relatively subjective analysis of the level of tumour histological differentiation, (*G*), allows conclusion to be made on tumour aggressiveness and prognosis. Nevertheless, prognoses as to the disease course based on the afore-mentioned parameters frequently fail. Recognition of tumour cell biology may help in solving the problem. To date, several molecular factors have been recognized – linked to proliferative processes, apoptosis or angiogenesis – which carry prognostic significance for various types of malignant tumours (9, 20, 22, 23).

MT is a low molecular weight protein linked to proliferation and differentiation of both healthy and neoplastic cells and, therefore, it may mark cells of a high proliferative potential (10, 11, 14, 18, 28-30). Using an immunohistochemical technique, MT expression was detected in various types of malignant tumours (31-38). In the tumours studied a high expression of MT was found to be linked to intensified cell divisions, malignancy grade and progressive clinical advancement of the disease. The effect of high MT expression was stressed to be indicative of deterioration of prognosis and shortening of survival in patients with tumours (31, 36, 39). In the present study, the expression of MT was significantly stronger in laryngeal cancer as compared to the control group, but MT failed to correlate with the expression of p53 protein, malignancy grade and clinical stage. Based on our results, we could not confirm that high MT expression is associated with features indicative of tumour aggressive behaviour and accelerated progression of the disease. In contrast to former studies (19, 20, 40), we did not find a significant correlation between the expression of MT and Ki-67 antigen which may indicate that MT has no influence on a cell proliferative potential.

Evaluation of MT expression intensity in laryngeal carcinomas has been the subject for few reports (32, 37). Ioachim *et al.* (32) noted a significantly increased intensity of MT and PCNA expression in tumour tissue as compared to benign lesions, as well as an absence of such differences in the case of p53 protein. They also demonstrated positive correlation between expressions of MT and PCNA in laryngeal carcinoma and, similar to our results, the absence of such correlations with p53 protein. Other authors stressed the significance of MT in the process of division and tumour progression (a positive correlation with Ki-67, PCNA, p53), suggesting that it might provide an additional marker of proliferative activity of neoplastic cells (19, 38-40).

To our knowledge, the present study is the only investigation in which the intensity of MT expression in laryngeal cancers is analysed in relation to selected clinical and pathological parameters of neoplastic disease (G and TNM). The results obtained demonstrated no significant relationships between MT expression and the mentioned variables, though in other types of tumours MT expression has been noted to increase in line with the increasing grade of malignancy and with the stage of clinical advancement (34, 35). Nevertheless, we observed unequivocal correlations between the expression of Ki-67 antigen and p53 protein on the one hand and grade of malignancy on the other. The results corroborate the prognostic significance of the markers in laryngeal carcinoma. Both proteins point to a significant proliferative potential of tumour cells and to their increased survival and, consequently, to higher aggressiveness and faster progress of the tumour. The conclusions are in accordance with those presented by other authors who examined a similar group of tumours (23, 41). The prognostic value is reflected in the analysis of survival of patients with a high *vs.* low level of Ki-67 and p53 protein expression. A significantly shortened patient survival was observed when their tumours manifested high intensity of expression of both proteins. However, other results published on the prognostic significance of Ki-67 antigen and p53 protein in various types of malignant tumours are not so unequivocal (19, 25, 42, 43).

Many of the available contributions confirm the prognostic value of MT expression in the neoplastic process. This stems from the well documented correlation between a high expression of the protein in neoplastic cells and a

shortened survival of the patients (19, 44, 45). Our survival analysis for patients with a high *vs.* low MT expression pointed to shortening of total survival time in cases of tumours with a high level of MT expression, but these differences failed to reach a level of statistical significance.

Conclusion

Both Ki-67 and p53 proteins represent significant indices of tumour aggressiveness and form parameters for prognostic evaluation in squamocellular cancers of the larynx. Expression of MT in the tumours does not significantly affect survival of the patients and, thus, carries no prognostic significance. Immunohistochemical evaluation of MT expression in laryngeal epithelial cells could provide an accessory marker in the differentiation of benign and malignant lesions of laryngeal epithelium.

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