The prognostic value of mmp-9 expression in lung adenocarcinoma

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SUMMARY

Background: *The increased expression of MMPs in tumor cells is considered immmportant for invasive tumor growth and metastatic potential. The aim of the study was to analyze the expression level of matrix metalloproteinase 9 (MMP-9) in tumor cells, estimate the prognostic value of MMP-9 expression, and correlate the MMP-9 expression to histopathological characteristics of the tumor and the disease stage.*

Methods: *In this study, 107 patients with operable lung adenocarcinoma were examined, including the information obtained in a five-year follow-up. The expression of MMP-9 was determined in paraffin-embedded tissue specimens by immunohistochemical analysis.*

Results: *The research was performed in the patients who underwent a surgery for the removal of lung adenocarcinoma. The patients were divided into three groups based on the stage of the disease: stage I - diagnosed 54 (50.46%) stage II - 30 (28.04%) and stage IIIA - 23 (21.50%) patients. In the total sample, MMP-9 positivity was established in 61 (57.01%) patients, while 46 (42.99%) patients were negative or slightly positive. MMP-9 expression was registered in the disease stage I group, established as positive or negative MMP-9 expression in 27 (50.00%) patients, respectively. In stage II disease group, 17 (56.67%) patients had MMP-9 positive tumors and 13 (43.33%) had MMP-9 negative tumors. In 17 (73.91%) patients of the stage IIIA group, MMP-9 positivity was confirmed in the tumor tissue, while 6 (26.09%) had negative or low MMP-9 expression in tumor cells.*

The log rank analysis showed a significant correlation of a poor survival, higher disease stage (p=0.022), and high MMP-9 expression in tumor cells (p=0.002).

Conclusion: *The study showed that the positive expression of MMP-9 was present in a large number of patients with advanced disease stages. The patients with lung adenocarcinoma who expressed higher MMP-9 levels had shorter survival periods compared to the patients with low MMP-9 expression.*

Key words: *Lung Neoplasms; Adenocarcinoma; Matrix Metalloproteinase 9; Prognosis*

INTRODUCTION

Lung cancer is one of the leading causes of death throughout the world. Approximately one million people, 850,000 men and 330,000 women, die of lung cancer per year (1).

Despite some advances in the diagnosis and treatment of lung cancer in the last several decades, the prognosis of lung cancer remains poor. The overall 5-year survival rate of lung cancer is approximately 12.4% of all newly detected cases in the world, and <9% in developing countries (2, 3).

The MMP family comprises 23 human enzymes that traditionally have long been associated with cancer invasion and metastasis because of their ability to degrade the extracellular matrix. However, recent studies have showed that the roles of MMPs in tumour development and metastasis are much more complex than was originally envisioned. *In vitro* and animal studies have demonstrated that MMPs are also the key mediators of growth factor activation, bioavailability and receptor signalling, cell adhesion and motility, apoptosis and survival mechanisms, angiogenesis, and inflammatory responses and immune surveillance (4).

Matrix metalloproteinases (MMP) are the group of enzymes responsible for degradation of certain extracellular matrix proteins such as collagen, proteoglycan, elastin, laminin and fibronectin. Malignant diseases are accompanied with higher expression of matrix metalloproteinases and lower concentrations of the tissue inhibitors of matrix metalloproteinases (TIMP), also resulting in an increased proteolytic activity. The presence of matrix metalloproteinases was discovered on the surface of invasive tumor cells (5). Degradation of the basal membrane and extracellular matrix presents the key step in the process of intravasation and extravasation of tumor cells (6).

MMP-9 belongs to the gelatinases group, synthesized by keratinocytes, monocytes, alveolar macrophages, polymorphonuclear neutrophil granulocytes, and in many cancer cells. Tumor invasion is a multi-phase process in which the cell motility is associated with controlled proteolysis and includes interaction between tumor cells and extracellular matrix. During the invasion process, malignant tumor cells are detached from the primary tumor, migrate through structural barriers such as the basal membrane and surrounding extracellular matrix rich in collagen. Degradation of the stromal extracellular matrix is also considered one of the key steps in the process of tumor angiogenesis (7). It has been proved that the activity of matrix metalloproteinases is necessary for increased motility of epithelial cells, as well as for the growth of metastatic deposits. Research has confirmed that MMP-2 and MMP-9 have an exceptionally significant role in metastasizing, due to their ability to degrade type IV

collagen. MMP has been confirmed to play an essential role in the process of angiogenesis and intravasation of tumor cells. Each level of MMP expression regulation and activation may be considered a potential target of a therapeutic action in the treatment of malignant tumors.

Numerous studies have showed that overexpression of the tissue inhibitors of matrix metalloproteinases (TIMP) in pericellular areas results in a decreased metastatic potential of certain tumors (8, 9). Currently, the ongoing clinical studies are testing several synthetic inhibitors of matrix metalloproteinases (hydroxamate and marimastat), by which the extensive degradation of the extracellular matrix might be avoided and the tumor growth and invasion inhibited, as well as the metastatic potential. Blockade of the membrane receptors, regulation of the activity of protein kinases involved in the signal transduction process, and interaction of different transcription and translation factors are potential targets for the development of new therapies of malignant tumors (10, 11). Peroxisome proliferator-activated receptor γ (PPARγ), belonging to the peroxisome proliferator-activated receptors (PPARs) family, is considered to be essential for modulating multiple physical and pathological processes, including lipid and glucose metabolism, inflammation and fibrosis. Additionally, many studies have confirmed that PPARγ plays a critical role in tumor proliferation and differentiation, apoptosis, invasion, angiogenesis and metastasis. Specifically, PPARγ activation was effective in arresting the proliferation of dedifferentiated tumor cells. Matrix metalloprotease-9 (MMP-9) is essential for the cancer cell metastasis and migration, which are the major characteristics of malignant tumors and the most important death-causing reasons. Numerous studies have uncovered the role of PPARγ as a central player in the regulation of MMP-9 expression. Telmisartan, a member of angiotensin II type 1 receptor blockers (ARBs), is usually used for the treatment of cardiovascular diseases, including hypertension and coronary artery disease (CAD). Recently, several studies have indicated that telmisartan and irbesartan have PPARγ–activating properties and they have been considered to be the selective PPARγ modulators. Telmisartan reduces expression of MMP-9 and inhibits the cell proliferation in human non-small-cell lung cancer A549 cells (the human cancer cell line A549 obtained from the American Type Culture Collection, ATCC, Manassas, VA, USA). Thus, inhibition of MMP-9 expression could be a useful therapeutic modality to decrease the growth and invasive properties of tumor cells (12).

The aim of the investigation was to analyze the level of matrix metalloproteinase 9 expression in tumor cells, its correlation to pathohistological characteristics of the tumor and the stage of the disease, and the prognostic value of matrix metalloproteinase 9 expression in the tumor tissue in relation to the clinical disease (occurrence of metastases and death).

PATIENTS AND METHODS

Patients

The study included 107 patients who underwent a surgery for the removal of lung adenocarcinoma. The patients' age was in the range from 38 to 70 years. The gender-related age differences of rhe patients with lung adenocarcinoma were not statistically significant (T- test: p= 0.161).

The examined patients' classification based on the sex, lymph node metastases and lung adenocarcinoma stage in the moment of the surgery is shown in Table 1.

Table 1. Characteristics of lung adenocarcinoma

	number	%
gender		
Females	77	72
Males	30	28
M:F ratio 2,56:1 p= 0,034		
age (yrs) mean (SD) 55,53 (SD ± 7.81)		
metastases		
N0	60	56,07
N+	47	43,93
Lung adenocarcinoma stage		
I	54	50,46
II	30	28,04
IIIA	23	21,50

Post-surgical staging of the disease was performed in all operated patients, selecting for the study the patients with stages I, II or IIIA (Figure 1) determined according to the TNM classification published by World Health Organization (13).

Figure 1. Lung adenocarcinoma stages in the examined population at the moment of surgery

The study inclusion criteria were a radical surgery and availability of the data on the clinical disease obtained in the five-year postsurgical follow-up for all the patients. The groups were mutually compared by the staged and clinical disease, as well as by MMP- 9 expression.

The research was partly a retrospective and partly a prospective analytical study due to the survival period follow-up.

Immnuhistochemistry analysis

The samples were obtained by a surgical resection and fixated in 10% formalin. They were embedded in paraffin blocks, and semi-serial cuts of 4 μm thickness were made. After deparaffinization, the samples were dyed with the routine hematoxylin-eosin (HE) method, and analyzed in light microscopy for histological type of the lung tumor and presence of metastases in regional lymph nodes.

Based on HE dyed samples, one representative cut was selected for each patient, of the border area between necrotic tumor tissue and lung tissue, and immunohistochemical analysis was performed. The cuts
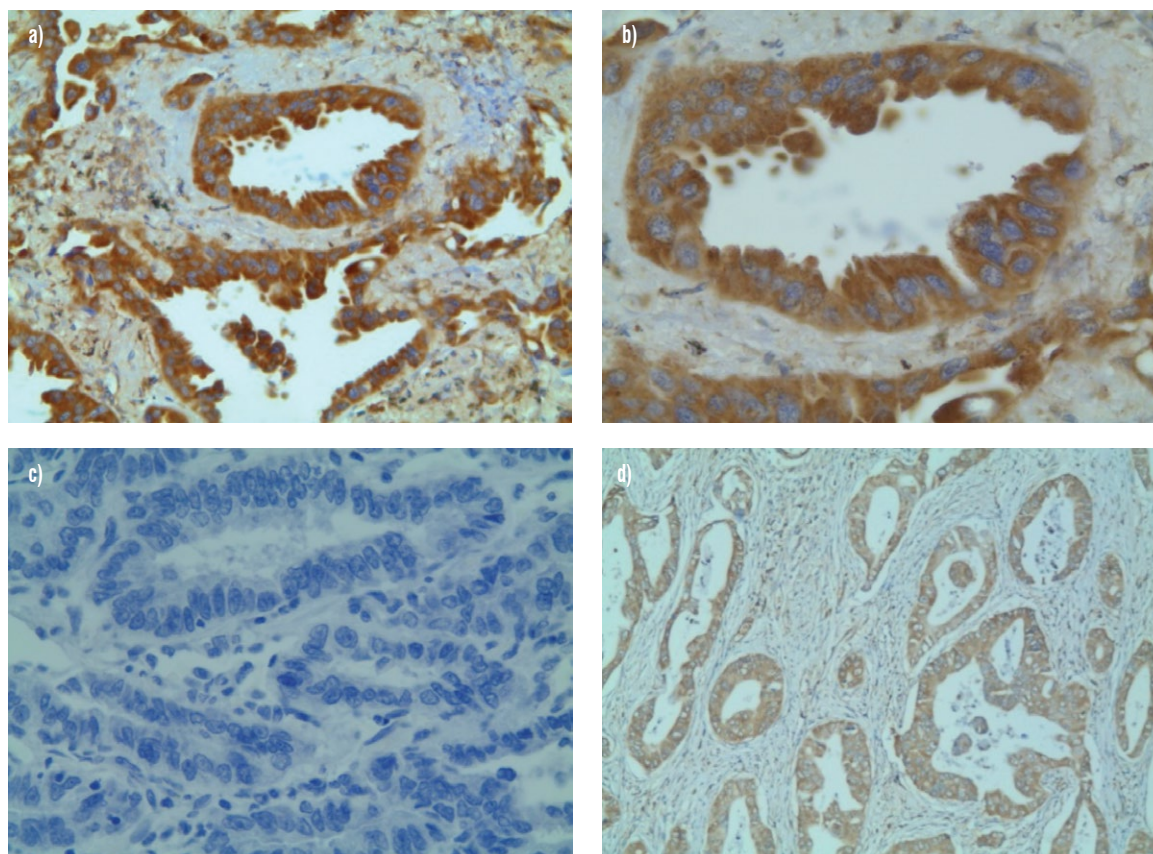
Figure 2. MMP-9 expression in lung adenocarcinoma; a) High MMP-9 expression in lung adenocarcinoma × 20, b) High MMP-9 expression in lung adenocarcinoma × 40,
c) Negative MMP-9 expression in lung adenocarcinoma MMP-9 × 40, d) Low MMP-9 expression in lung adenocarcinoma MMP-9 × 10

of 4 μm thickness were deparaffinized by the routine method, treated with citrate buffer and heated in a microwave oven for 20 minutes, in order to unmask the antigen. After the blocking of endogen peroxidase with hydrogen peroxide (H_2O_2) in methanol, the cuts were rinsed in Tris buffered saline (TBS) solution pH 7.6, and the antibody MMP-9 was applied (Lyophilised monoclonal NCL-MMP9, Clone 2C3, produced by Novocastra Laboratories Ltd.) in 1:50 dilution for 30 minutes. Immunohistochemical identification of the proteins was performed by EnVision technique. 3,3´-Diaminobenzidine (DAB) was used as a chromogenic substrate. The contrasting was performed by hematoxylin.

The level of MMP-9 expression of was determined based on the clear cytoplasmic brown staining by a chromogenic substrate. A semiquantitative analysis of MMP-9 expression was performed according to the percentage of the positive tumor cells and the intensity of cytoplasmic staining. MMP-9 expression was shown through the following score that represents the sum of: the percentage of positive cells (score 0 = 0% positive cells; score 1 = 1- 20% positive cells; score 2 = 21- 50% positive cells; score 3 = > 50% positive cells) and the intensity of staining (0 = no staining; 1 = slightly positive; 2 = moderately positive; 3 = very positive).

The samples with a moderate and high expression in more than 20% of the cells were grouped as MMP-9 positive tumors (Figure 2a and Figure 2b), and the patients with a negative (Figure 2c), slight (Figure 2d), moderate or high staining in less than 20% of the cells were grouped as MMP- 9 negative cases.

The semiquantitative determination of MMP-9 expression is in accordance with the recommendations given by Cox G, et al. and it was applied on NSCLC (non- small-cell lung carcinomas) (14).

Statistical analysis

All the data were described with the appropriate methods of descriptive statistics. Quantitative characteristics were described with the arithmetic mean, median and standard deviation, and qualitative ones with frequencies, and percentages. The analysis of differences was based on relevant parametric and nonparametric statistical tests, and univariate and multivariate statistical methods. The survival analysis was used (Kaplan-Meier method of survival assessment, log rank test for determining the significance of the difference of survival depending on the selected prognostic or risk factor).

The statistically significant difference was defined at the level of 0.05, and the difference of a high statistical significance at the level of 0.01.

The statistical analysis of the data was performed by the software for Windows XP on PC Statistica 11.0 (StatSoft Inc USA).

RESULTS

In the total sample, MMP-9 positivity was found in 61 (57.01%) patients, while the negative MMP- 9 expression was established in 46 (42.99%) patients (Figure 3).
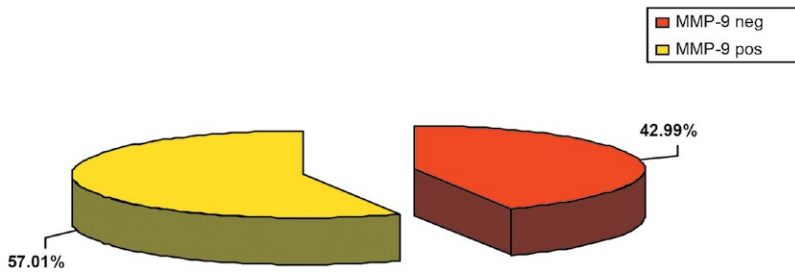
Figure 3. MMP-9 expression in lung adenocarcinoma: an overview

Figure 3

Almost the same percentage of tumors exhibited a high MMP-9 expression in male and female patients. A high MMP- 9 expression was found in 17 (56.67%) female patients and 44 (57.14%) male patients, while a low expression was confirmed in 13 (43.33%) female and 33 (57.14%) male patients. The analysis of MMP-9 expression levels and gender distribution did not reveal any statistically significant difference. The average age of the patients with a positive MMP-9 expression was 54.82 (SD± 8.14) years, while the average age of the patients with a negative MMP-9 expression was 56.48 (SD± 7.35) years. The difference obtained by the analysis of MMP-9 categories in relation to ages was not statistically significant (T- test: p= 0.279).

In stage I of the disease, the equal percentage of the patients showed a positive and negative MMP-9 expression, i.e. both groups had 27 (50.00%) patients each. In stage II of the disease, 17 (56.67%) patients had MMP-9 positive tumors and 13 (43.33%) had MMP-9 negative tumors. In 17 (73.91%) patients with stage IIIA adeno lung cancer, MMP-9
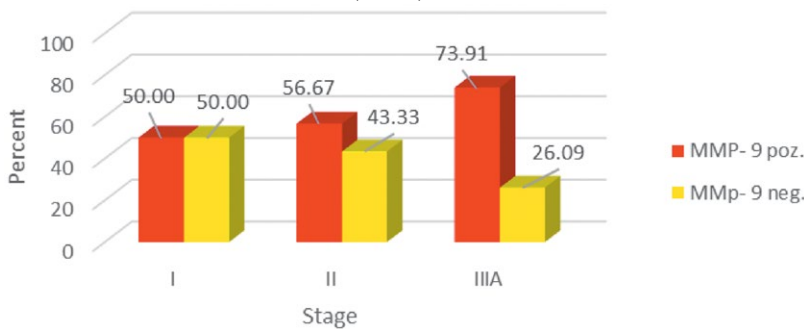

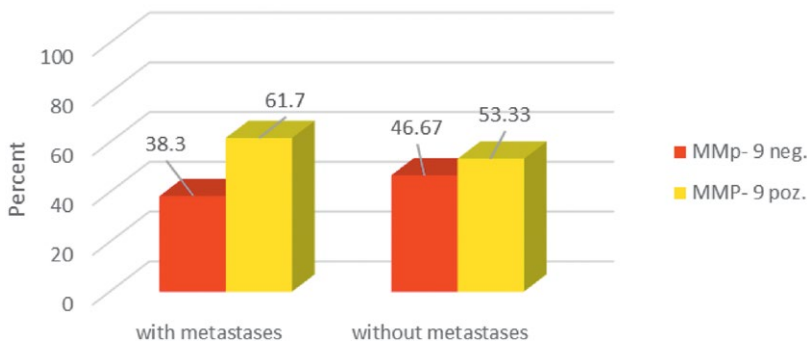Figure 4.The ratio between MMP-9 and disease stage

Figure 5. The ratio between MMP-9 expression and the presence of metastases in regional lymph nodes

positivity was confirmed in the tumor tissue, while 6 (26.09%) patients had negative or low MMP-9 expression levels in tumor cells.

The difference obtained by the analysis of MMP-9 categories in relation to the disease stage was not statistically significant (χ^2 test: p= 0.152; p>0.05) (Figure 4).

The group of MMP-9 positive lung adenocarcinomas without metastases in regional lymph nodes contained 32 (53.33%) patients, while there were 29 (61.70%) patients with metastases in regional lymph nodes. In the analyzed sample, negative and low levels of MMP-9 in tumor cells were confirmed in 28 (46.67%) patients who did not have metastases in regional lymph nodes, while negative MMP-9 expression was found in 18 (38.30%) patients with present metastases in regional lymph nodes. The difference obtained by the analysis of MMP-9 categories in relation to the presence or absence of metastases in regional lymph nodes was not statistically significant (χ^2 test: p= 0.385; p>0.05) (Figure 5).

Based on the Kaplan-Meier curve, it can be seen that 5 (10.87%) MMP-9 negative and 3 (4.92%) MMP-9 positive patients survived 60 months after the surgery regardless the MMP-9 expression level. However, a highly significant difference was registered in the proportion of 5-year survival of lung adenocarcinoma patients depending on the MMP-9 expression level (log rank test; p= 0.0002) (Figure 6).
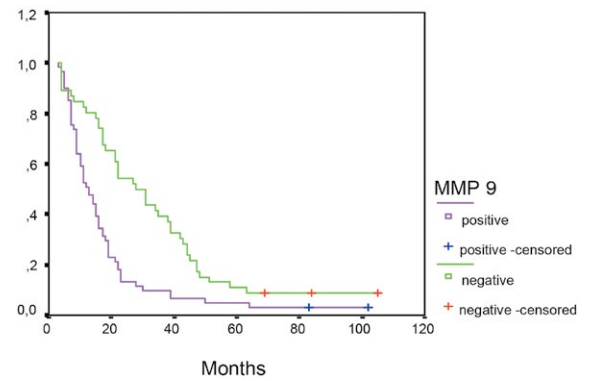

Figure 6. Kaplan-Meir curve of the five-year survival of lung adenocarcinoma patients in terms of MMP-9 expression

DISCUSSION

In our study, MMP-9 expression levels were determined based on the clear cytoplasmic brown staining by a homogenous substrate. The semiquantitative analysis of MMP-9 expression was performed according to the percentage of positive tumor cells and the intensity of cytoplasm staining.

A positive MMP-9 expression was confirmed in 57.01% of the patients in our study. The MMP-9 expression level in non-small-cell lung carcinomas ranged between 52% and 95% (15-17).

The reported level of MMP-9 expression in lung cancer cells and its relation to the disease stage and survival of patients varies among authors (18-20).

Hoikkala et all. have found no significant difference in the level of MMP-9 expression in tumor cells of patients who underwent a surgery for non-small-cell lung carcinoma with respect to the stage of the disease. The

study included 59 patients with squamous [30], adenocarcinoma [21] and macrocellular lung carcinoma [8] (20).

The data from the Ylisirnio et al. study of 90 patients who underwent a surgery for non-small-cell lung cancer and were followed up for 12 months showed that the MMP-9 level in the serum was inversely proportional to the survival period (21).

Cox et al. study was performed on 167 samples from patients who underwent a surgery for non-small-cell lung cancer and were followed up for 24 months, concluding that the level of MMP-9 in tumor cells, the density of blood vessels in the tumor, and the stage of the disease presented independent prognostic parameters (18).

The study by Wang et al. included 68 samples of non-small-cell lung carcinomas and 38 samples of the normal lung parenchyma. They found that the level of MMP-9 expression was significantly higher in tumor cells compared to the normal lung parenchyma. They also confirmed that the level of MMP-9 expression was in correlation with the level of tumor differentiation and stage of the disease (22).

Our study found an increase of MMP-9 expression with the disease advancement. The equal number of stage I lung adenocancer patients had a positive or negative MMP-9 expression; of stage II lung adenocancer patients, 56.67% were positive for MMP-9, and high MMP-9 expression levels were confirmed in 73.91% of stage III A patients.

The comparison of the disease-stage-related expression levels showed there was no significant difference in MMP-9 expression levels between patients in disease stages I and II (p= 0.843). Also, no significant difference in the level of MMP-9 expression was found between the operated patients with disease stages II and IIIA (p= 0.461), nor between the patients with disease stages I and IIIA (p= 0.158).

In their study of 152 patients who underwent the surgery for lung adenocarcinoma, Pinto et al. confirmed that MMP- 9 expression in the tumor tissue correlated with the stage of the disease (p= 0.012), and with the size of the primary tumor (p= 0.0076), while the presence of metastases in regional lymph nodes was approaching the statistically significant value (p= 0.06) (23). Cox et al. and Komaki et al. published contradictory data and showed that the level of MMP-9 expression in tumor cells correlated neither with the disease stage, nor with the size of the primary tumor, presence of metastases in regional lymph nodes, histological grade or gender (14, 24).

We did not find a significant difference between the level of MMP-9 expression and the presence of metastases in regional lymph nodes .

Sienel et all. performed a study of 143 patients who underwent a surgery for non-small-cell lung cancer, confirming that the high MMP-9 expression level correlated with a shorter period of the disease relapse (17). On the sample of 119 patients with non-small-cell lung cancer, Shou et all. showed that the patients with high MMP-9 expression had a shorter survival period than the patients with lower expression levels (15). Similar results were obtained in the study by Pinto and Cox (14, 24).

CONCLUSION

Our results showed the positive MMP-9 expression was present in the majority of patients with advanced disease. The patients with a high

MMP-9 expression in lung adenocarcinoma cells had a shorter survival period than the patients with a low MMP-9 expression.

Conflict of interest

We declare no conflicts of interest.

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