Expression of MMP-7, MMP-9, TIMP-1 and TIMP-2 mRNA in Lung Tissue of Patients with Non-small Cell Lung Cancer (NSCLC) and Benign Pulmonary Disease

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Abstract. *The expression of matrix metallo-proteinases (MMP-7 and MMP-9) and tissue inhibitors of metallo-proteinases (TIMP-1 and TIMP-2), which are involved in the degradation of the extracellular matrix (ECM) and tumor growth, was investigated in normal lung tissue, tissue of benign pulmonary diseases and non-small cell lung cancer (NSCLC) tissue. Patients and Methods: Tumor tissue and surrounding carcinoma-free lung tissue samples were obtained from 91 patients with NSCLC who had undergone surgery in the years 2005-2007 as well as lung tissue from 12 patients operated on for 'benign' bullous emphysema or interstitial lung disease. The mRNA was isolated from the tissues and the expression of mRNA was assessed using a real-time RT PCR method. Results: Significantly higher expression of MMP-7, MMP-9 and TIMP-1 mRNA was demonstrated in the NSCLC tissue in comparison with the normal lung tissue from the same patients (p=0.0003, p<0.0001 and p=0.0018, respectively). Similar results for MMP-7, MMP-9 and TIMP-1 were found in the histological subgroups: squamous cell lung cancer vs. normal tissue (p=0.0198, p=0.0015 and p=0.0366, respectively), and adenocarcinoma vs. normal tissue (p=0.0045, p<0.0001 and p=0.0140, respectively). The expression of MMP-7 was found to be significantly higher in tumor tissue vs. lung tissue of the benign diseases (p=0.0086) and similar results were also recorded in the histological subgroups: squamous cell lung cancer vs. benign tissue (p=0.0171) and adenocarcinoma vs. benign tissue (p=0.0135). The expression of MMP-9 was*

significantly higher only in the adenocarcinoma subgroup vs. the benign tissue (p=0.0412). No differences in the expression of mRNA between stage IA and stages IB-IIIB of NSCLC were recorded. Conclusion: Significantly higher expression of MMP-7 and MMP-9 in tumor tissue than in the surrounding tissue or in benign lung disease tissue supports the notion of an important role of these metalloproteinases in the growth of lung carcinoma. TIMP-1 expression is increased only in carcinoma, but not in benign lung disease.

Lung cancer is the most commonly diagnosed cancer in the world. In the Czech Republic, lung carcinoma was diagnosed in 91.4 men and 26.8 women per 100,000 people and the mortality rate was 86.0 men and 24.3 women per 100,000 people in 2002 (1). In contrast to the high social importance of non-small cell lung carcinoma (NSCLC), the current possibilities for monitoring this disease with tumor markers seem to be insufficient.

Matrix metalloproteinases (MMPs) are endopeptidases which generally play an important role in the process of extracellular matrix (ECM) and basal membrane degradation in relation to tumor invasiveness (2-6). At present, the most widely known are the roles of MMPs in colorectal carcinogenesis. The studies on metalloproteinases and their inhibitors in NSCLCs are still limited and the results are also heterogeneous (7-10). Well-characterized MMPs in colorectal carcinogenesis are MMP-7 and MMP-9 (11). MMP-7 (matrilysin) cleaves collagen IV, elastin, intactin, fibronectin, gelatin, laminin and tenascin. Some reports have indicated that regulation of the transcription of the MMP-7 gene is controlled by genes which are important in the early stages of carcinogenesis in colorectal cancer. MMP-7 is a target gene transcriptionally activated by beta-catenin-tcf-4 complex, which co-operates with the PEA3 sub-family of the family of ets transcription factors to promote MMP-7 transcription (11). MMP-7 is expressed predominantly by tumor cells in various carcinomas (12-14). MMP-9

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Table I. *The distribution according to histological carcinoma type.*

Histology	No. of patients (%)
Squamous cell carcinoma	49 (54%)
Adenocarcinoma	36 (39%)
Others	6 (7%)

Table II. *MMP-7, MMP-9, TIMP-1 and TIMP-2 mRNA in NSCLC vs. normal lung tissue (n=91).*

mRNA (absolute values)	Normal lung tissue (median, copies)	NSCLC tissue (median, copies)	p-Value
MMP-7	4465.00	15543.00	0.0003
MMP-9	20.50	15365.00	<0.0001
TIMP-1	201229.00	817982.00	0.0018
TIMP-2	180.50	135.50	0.8660

mRNA (normalized values)	Normal lung tissue (median)	NSCLC tissue (median)	p-Value
MMP-7	0.1178	0.6787	0.0002
MMP-9	0.0003	0.5139	<0.0001
TIMP-1	4.7346	15.5793	0.0099
TIMP-2	0.0038	0.0021	0.3211

(gelatinase B, 92 kDa type IV collagenase) can degrade denatured collagen (gelatin) and collagen types IV, V, VII, IX, X, elastin, fibrin, fibrinogen and plasminogen. In addition, MMP-9 is responsible for the processing of cytokines, *e.g.* pro-interleukin-1β and pro-tumor necrosis factor-α into their active forms (15). One study has demonstrated both stromal and cytoplasmic tumor cell MMP-9 expression in NSCLC tissue (16).

MMPs are synthesized as inactive, latent propeptides, which become activated after proteolysis by other proteases. This activation is tightly regulated by many different factors, such as the low weight endogenous tissue inhibitors of metalloproteinases (TIMPs). TIMPs can form complexes with latent or activated enzymes and these bind MMPs in a 1:1 manner to prevent enzymatic activity. The balance of MMPs to TIMPs, therefore, determines the matrix turnover, where either an excess of MMPs or a deficit of TIMPs may result in excess ECM degradation (17, 18). TIMP-1 and -2 are enzymatic inhibitors with broad effects. TIMP-1 has additional biological activities, such as growth stimulation and inhibition of apoptosis, which are independent of any antiproteolytic activity (19-21). The aim of this study was to compare the expression of *MMP-7, MMP-9, TIMP-1* and *TIMP-2* mRNA between tissue samples of NSCLC, surrounding tumor-free lung tissue (normal lung tissue) and benign lung disease tissue. Differences of expression in stage IA *vs*. IB - IIIB stages of NSCLC were evaluated.

Patients and Methods

Patients. A group of 91 patients (median age of 62.4 years, range 43.0-77.8) with NSCLC, stage IA 17 (19%) and IB – IIIB stage 74 (81%) who had undergone lung surgery at the Department of Surgery, University Hospital Pilsen between 2005-2007 were included in the study. The distribution of the histological carcinoma type is shown in Table I. The comparison group consisted of 12 patients (median age of 51.2 years, range 35.7-65.0) operated on for benign disease (bullous emphysema, interstitial lung diseases).

Tissue samples. The lung tissue samples were taken directly from the NSCLC tumor tissue and from adjacent, histologically cancer-free lung tissue (normal lung tissue) in the same patient during surgery and from patients operated on for benign lung diseases. After surgical resection these samples were frozen at –70°C until used. All the samples were histologically verified.

Quantitative estimation of mRNA using real-time RT-PCR. The total RNA was isolated from 100 mg of lung tissue using a Fast RNA Pro Green Kit (Q-BIOgene, Irvine, CA, USA). Three μg of the total RNA were used for reverse transcription (RT) which was performed with Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) and oligo d(T)21 as a primer.

The preparation and design of the primers for *MMP-7, TIMP-2, TIMP-1* and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) real-time PCR has previously been described (22). The sequence of primers used for *MMP-9* was as follows: forward primer, 5'-GCA CGA CGT CTT CCA GTA CC-3'; reverse primer, 5'-CAG GAT GTC ATA GGT CAC GTA GC-3', custom synthesized by GeneriBiotech (Hradec Kralove, Czech Republic). The quantification was performed as *absolute values* (*i.e.* the number of RNA copies per 3 μg of RNA). In all the samples, the expression of *GAPDH* (housekeeping gene) mRNA was also assessed and the results are also presented as *normalized values*, the ratio of the number of copies of the assessed gene *(MMP-9, MMP-7, TIMP-2, TIMP-1)* to GAPDH.

Statistical analysis was performed using SAS 8.02 software (SAS Institute Inc., Cary, NC, USA).

Results

Statistically significantly higher expression of *MMP-7, MMP-9* and *TIMP-1* mRNA was found in the tumor tissue than in the normal tissue, in respect of the absolute values (*p*=0.0003; *p*<0.0001; *p*=0.0018, respectively) and also of the relative values (*p*=0.0002; *p*<0.0001; *p*=0.0099, respectively), see Table II. A statistically significantly higher expression of *MMP-7, MMP-9, TIMP-1* mRNA was also found in the adenocarcinoma than in the normal lung tissue in both the absolute values (*p*=0.0045; *p*<0.0001; *p*=0.0140, respectively) and the relative values (*p*=0.0023; *p*<0.0001; *p*=0.0120, respectively), see Table III. A statistically significantly higher expression of *MMP-7, MMP-9* and *TIMP-1* mRNA was found in the squamous cell carcinoma tissue than in the normal lung tissue in terms of the absolute

Table III. *MMP-7, MMP-9, TIMP-1 and TIMP-2 mRNA in NSCLC adenocarcinoma vs. normal lung tissue (n=36).*

mRNA (absolute values)	Normal lung tissue (median, copies)	Adenocarcinoma NSCLC (median, copies)	*p*-Value
MMP-7	3501.00	68815.00	0.0045
MMP-9	41.50	63073.00	<0.0001
TIMP-1	209354.00	1482930.00	0.0140
TIMP-2	133.50	131.00	0.6278

mRNA (normalized values)	Normal lung tissue (median)	Adenocarcinoma NSCLC (median)	*p*-Value
MMP-7	0.0535	1.9618	0.0023
MMP-9	0.0007	1.5573	<0.0001
TIMP-1	4.0803	35.9028	0.0120
TIMP-2	0.0024	0.0034	0.5262

Table VI. *MMP-7, MMP-9, TIMP-1 and TIMP-2 mRNA in NSCLC adenocarcinoma (n=36) vs. benign lung disease (n=12).*

mRNA (absolute values)	Benign lung tissue (median, copies)	Adenocarcinoma NSCLC (median, copies)	*p*-Value
MMP-7	2379.50	68814.50	0.0135
MMP-9	574.00	63073.00	0.0412
TIMP-1	276736.75	1482930.25	0.3163
TIMP-2	224.75	131.00	1.0000

mRNA (normalized values)	Benign lung tissue (median)	Adenocarcinoma NSCLC (median)	*p*-Value
MMP-7	0.0510	1.9617	0.0112
MMP-9	0.0188	1.5573	0.0594
TIMP-1	12.4466	35.9028	0.5781
TIMP-2	0.0053	0.0033	0.9904

Table IV. *MMP-7, MMP-9, TIMP-1 and TIMP-2 mRNA in NSCLC squamous cell carcinoma vs. normal lung tissue (n=49).*

mRNA (absolute values)	Normal lung tissue (median, copies)	Squamous NSCLC (median, copies)	*p*-Value
MMP-7	4883.00	15543.00	0.0198
MMP-9	0	12357.00	0.0015
TIMP-1	178449.00	817982.00	0.0366
TIMP-2	246.00	194.50	0.9650

mRNA (normalized values)	Normal lung tissue (median)	Squamous NSCLC (median)	*p*-Value
MMP-7	0.1165	0.5694	0.0182
MMP-9	0	0.4216	0.0010
TIMP-1	7.5468	11.4482	0.2238
TIMP-2	0.0059	0.0022	0.2030

Table VII. *MMP-7, MMP-9, TIMP-1 and TIMP-2 mRNA in NSCLC squamous (n=49) vs. benign lung disease (n=12).*

mRNA (absolute values)	Benign lung tissue (median, copies)	Squamous NSCLC (median, copies)	*p*-Value
MMP-7	2379.50	15543.00	0.0171
MMP-9	574.00	12356.50	0.0893
TIMP-1	276736.75	817981.75	0.3092
TIMP-2	224.75	194.50	0.6706

mRNA (normalized values)	Benign lung tissue (median)	Squamous NSCLC (median)	*p*-Value
MMP-7	0.0510	0.5693	0.0411
MMP-9	0.0188	0.4216	0.1423
TIMP-1	12.4466	11.4482	1.0000
TIMP-2	0.0053	0.0021	0.6706

Table V. *MMP-7, MMP-9, TIMP-1 and TIMP-2 mRNA in NSCLC (n=91) vs. benign lung disease (n=12).*

mRNA (absolute values)	Benign lung tissue (median, copies)	NSCLC (median, copies)	*p*-Value
MMP-7	2379.50	15543.00	0.0086
MMP-9	574.00	15365.50	0.0518
TIMP-1	276736.75	817981.75	0.3440
TIMP-2	224.75	135.50	0.8710

mRNA (normalized values)	Benign lung tissue (median)	NSCLC (median)	*p*-Value
MMP-7	0.0510	0.6787	0.0147
MMP-9	0.0188	0.5139	0.0796
TIMP-1	12.4466	15.5793	0.8818
TIMP-2	0.0053	0.0021	0.7139

values (*p*=0.0198; *p*=0.0015; *p*=0.0366, respectively) and of *MMP-7* and *MMP-9* mRNA in terms of the relative values (*p*=0.0182; *p*=0.0010, respectively) (Table IV).

Statistically significantly higher expression of *MMP-7* mRNA was observed in the NSCLC tissue than in the benign disease lung tissue in both absolute and relative values (*p*=0.0086; *p*=0.0147, respectively) (Table V). The expression of *MMP-7* and *MMP-9* mRNA was statistically significantly higher in the tissue of the adenocarcinoma subgroup than in the benign disease lung tissue in absolute values (*p*=0.0135; *p*=0.0412), but only for *MMP-7* (*p*=0.0112) for relative values (Table VI). The *MMP-7* mRNA expression was also statistically significantly higher in the squamous cell carcinoma subgroup tissue than in the benign lung tissue (absolute values, *p*=0.0171; relative values, *p*=0.0411) (Table VII).

No statistically significant differences in the expression of *MMP-7, MMP-9, TIMP-1* and *TIMP-2* were found between the benign disease lung tissue and the normal lung tissue (absolute values *p*=0.2164, 0.6223, 0.5722 and 0.9618, respectively and relative values *p*=0.4150, 0.5246, 0.2576 and 0.9202, respectively). The expression of *MMP-7, MMP-9, TIMP-1* and *TIMP-2* mRNA in the stage IA and stage IB-IIIB tumors did not register any statistically significant difference either in the absolute values (*p*=0.4765, 0.9614, 0.6498 and 1.0000, respectively) or in the relative values (*p*=0.6034, 0.9529, 0.9957 and 0.9479, respectively).

Discussion

The current view of the role of MMPs in carcinomas is complex. They are known to have effect on neoangiogenesis of carcinoma, and the origin and growth of metastatic lesions in distant organs. It has been demonstrated that MMPs are produced not only by tumor cells (MMP-7), but even by surrounding, stromal tissue (MMP-9), including fibroblasts and inflammatory cells. The effect of MMPs is not only the proteolytic degradation of the ECM and basal membrane it also includes influence upon changes in the growth, apoptosis, and migration of healthy cells (5, 23). Through remodelling or destruction of the ECM, they even work during processes of tumor cells migration (9, 24, 25). In consequence of the degradation of the ECM by metalloproteinases, healthy cells can also be affected by proliferation, apoptosis, or pathological morphogenesis. MMPs can also change the activity, for example, of growth factors and their receptors (26). Finally, they can even change the gene expressions of other proteins.

In this study using both the normalized and absolute values in the mRNA expression, generally similar results were obtained and, apart from two outcomes (*p*=0.0366 *vs. p*=0.2238, Table IV and *p*=0.0412 *vs. p*=0.0594, Table VI), at the same statistical significance. In our previous study in patients with colorectal cancer where the expression of CEA and also MMPs and TIMPs in liver tissue was assessed, an inconsistency was recorded between normalized and absolute values of expression (27). As with a number of other reports, this demonstrated a changed expression of *GAPDH* in some tumors (28, 29) and indicated a limitation in the use of *GAPDH* as a housekeeping gene.

The present study demonstrated a statistically significantly higher expression of *MMP-7, MMP-9* and *TIMP-1* mRNA in NSCLC tumor tissue and in both the histological subgroups (squamous carcinoma and adenocarcinoma) in comparison with the normal non-tumor lung tissue of the same patients. The expression of *TIMP-1* was enhanced in the lung tumor tissue as shown in some other tumor tissues, for example in colorectal carcinomas and their liver metastases (27). The expression of *TIMP-2* mRNA was not increased in the carcinoma or in the benign disease or the normal lung tissues.

Higher expression of *MMP-7* in NSCLC in general was found and also in both the squamous and adenocarcinoma subgroups in comparison with the tissue of the benign lung diseases. On the other hand, it was only in the adenocarcinoma subgroup that the expression of *MMP-9* was significantly higher than in the benign lung disease tissue. This result may be related to the hypothesis that MMP-7 is produced mainly by carcinoma cells and MMP-9 mainly by stromal cells (30). This result did not completely conform with our previous findings for the expression of MMP-7 and TIMP-1 (31), which may have been related to the size of the group of patients, this study having more than 90 patients.

No statistically significant differences in the expression of *MMP-7, MMP-9, TIMP-1* or *TIMP-2* between the benign disease and normal lung tissue or between stage IA and stages IB-IIIB, NSCLC were recorded.

The significantly higher expression of *MMP-7* and *MMP-9* in carcinoma than in the surrounding tissue or in benign lung disease tissue supports an important role of these metalloproteinases in the processes of growth and progression of NSCLC. *TIMP-1* expression is increased in carcinoma, but not in a lung with benign disease.

Acknowledgements

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