

## Original Article

# Immunohistochemical expression of matrix metalloproteinase-9 and inhibitor of matrix metalloproteinase-1 in prostate adenocarcinoma

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**Abstract:** An immunohistochemical study on the cells proliferative activity by Ki-67 protein and localization of the matrix metalloproteinase-9 and the inhibitor of matrix metalloproteinase-1 was carried out at the benign prostatic hyperplasia (BPH) and the adenocarcinoma (AC) of different Gleason's grades. A significant decrease of the MMP-9 and TIMP-1 level in the AC of different gradations was observed. A moderate positive correlation between the Gleason score and cell proliferation Ki-67 index ( $r_s = 0.674$ ) and a moderate negative correlation with the level of such a score and expression of MMP-9 ( $r_s = -0.660$ ) was detected. A weak negative correlation exists also between the level of proliferative activity of secretory cells and the expression of MMP-9 by tumor cells ( $r_s = -0.369$ ). The invasive properties of AC cells that promote a degradation of the basal membrane and connective tissue in prostate may be explained by the imbalance between the MMP-9 and TIMP-1, which expression is significantly reduced in AC, in comparison with BPH.

**Keywords:** Adenocarcinoma of the prostate, Ki-67, collagen IV, MMP-9, TIMP-1

## Introduction

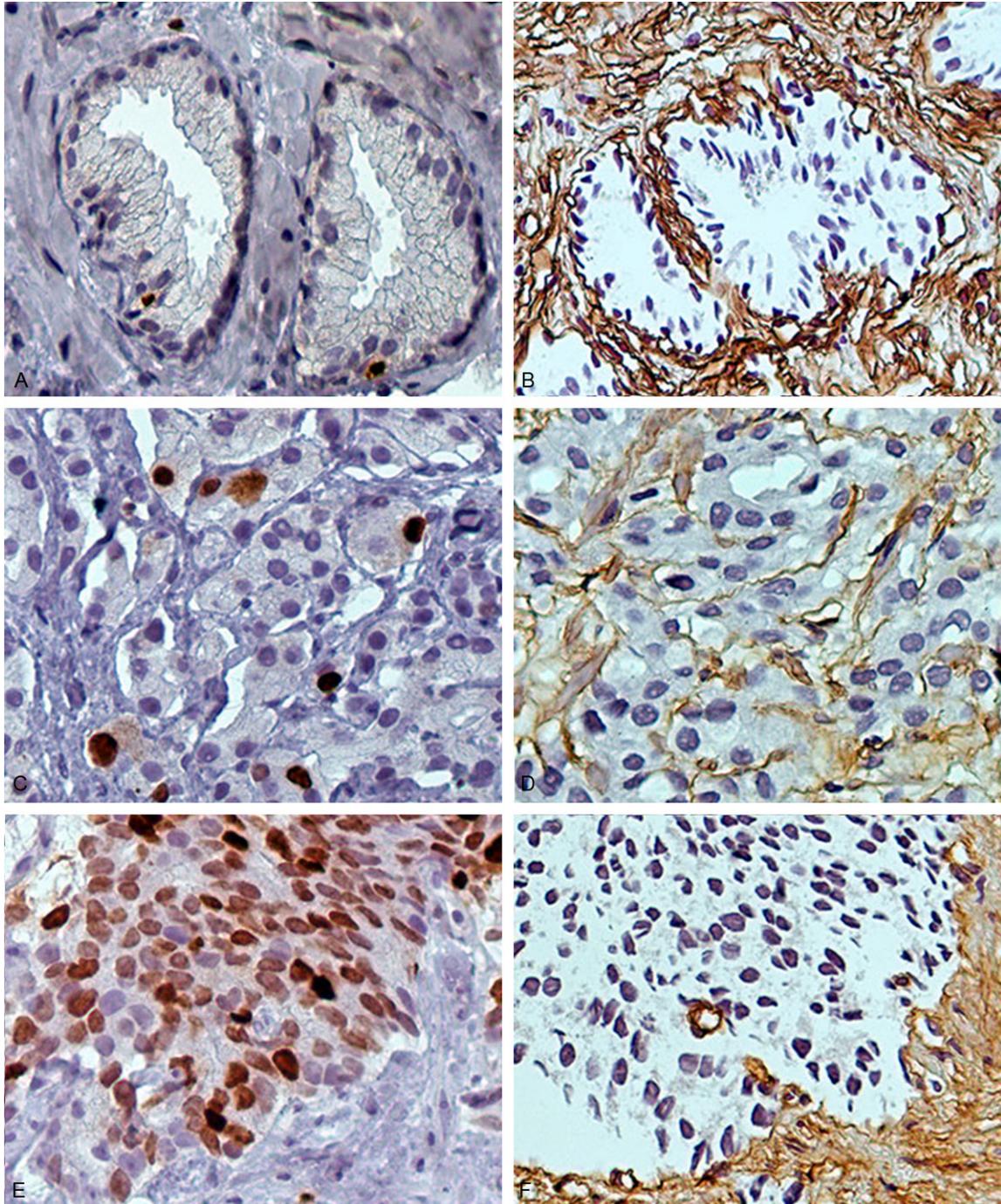
Prostate cancer is the fifth most common cancer in the world and is the second incidence in men. Infiltrating growth of glandular structures in stroma, the absence of basal cell layer and atypia of cell nuclei are the main criteria that refer to the prostate adenocarcinoma [1].

Matrix metalloproteinases are enzymes that degrade the extracellular matrix proteins, they play a leading role in the embryogenesis, in the tissue repair after injury, during inflammation and angiogenesis [2]. At the oncopathology, matrix metalloproteinases provide cancer cell invasion and metastatic processes [3]. Metalloproteinase-9 (MMP-9) lyses main components of the extracellular matrix: collagen type IV, fibronectin and laminin, and it is commonly used as a tumor marker of malignant phenotype [4, 5]. MMP expression is regulated at the level of gene transcription, inhibition of the activation of proteolytic enzymes, and tissue inhibitors of metalloproteinase activity-TIMP [6, 7]. TIMP binds MMP and inhibits their activity by

forming a complex with pro-MMP-9 that blocks activation of the latter by stromelysins [8].

TIMPs, which have been described as potential MMP inhibitors, currently are regarded as proteins with pro-oncogenic and pro-metastatic activity, as well as factors of tumors growth [8]. High expression of TIMP-1 found in many tumors, including cancers of the breast, colon, stomach, lung, and lymphomas [9-12]. However, the role of TIMP-1 in the prostate cancer remains controversial [13]. Several investigators have shown that the expression of TIMP-1 is reduced in the tumor tissue as compared to unchanged one [14, 15], accordingly the TIMP-1 blocking effect on tumor vascularisation is also reduced [16]. Another group of scientists observed the increase of the level of TIMP-1 in tumors that testified to a high metastatic potential of anaplastic cells [3].

Therefore, the role of matrix metalloproteinases and inhibitors in molecular mechanisms of prostate adenocarcinoma invasive growth is



**Figure 1.** IHC reaction in benign hyperplasia (A, B) and prostate adenocarcinoma with the Gleason's grade 2 (C, D) and 4 (E, F) with antibodies to Ki-67 (A, C, E) and collagen type IV (B, D, F). Staining: DAB-Mayer hematoxylin (Magnification  $\times 400$ ).

still poorly understood. The purpose of this study was to investigate the expression of the proliferation marker Ki-67, type IV collagen, MMP-9 and TIMP-1 in the unaltered secretory epithelial cells of patients with benign prostatic

hyperplasia, and prostate adenocarcinoma cells of different scores accordingly to Gleason's grades, in order to study the molecular mechanisms of the invasive tumor cell growth in the prostate gland.

## Matrix metalloproteinase-9 and its inhibitor in prostate adenocarcinoma

**Table 1.** Characteristic of expression of Ki-67 PI, MMP-9 and TIMP-1 in the prostate benign hyperplasia and adenocarcinoma of different Gleason's grades

BPH/adenocarcinoma Gleason grading	No. of patients	IP Ki-67 (M ± SD) %	MMP-9 (M ± SD) points	TIMP-1 (M ± SD) points
BPH (control)	10	0	3.0	3.0
Grade 2	10	5.3±1.1	1.8±0.4	0.7±0.5
Grade 3	18	7.2±1.4*	1.8±0.5	0.9±0.5
Grade 4	20	10.0±3.9*.*.*	0.9±0.4#,#	0.6±0.5
Grade 5	5	17.2±9.4*.*.*	0.8±0.4#,#	0.8±0.4
Total	63			

\*-Significant differences between 2-3, 2-4, 2-5; \*\*-Significant differences between 3-4, 3-5. #-Significant differences between 2-4, 2-5; ##-Significant differences between 3-4, 3-5.

### Materials and methods

The surgical tissue material of 53 patients diagnosed with adenocarcinoma of the prostate, the average age of patients was 67 with an interval of 52 to 83 years, was investigated. Biopsies in 10 patients of the similar age, diagnosed with benign prostatic hyperplasia, were used as control.

Gleason's grades in tumors were histologically evaluated [17] and a correlation with the most pronounced anaplastic changes in the tumor cells biopsy in this patient was observed. Level of total prostate-specific antigen (PSA) in the blood serum in all patients was also determined. Analyses of tissues based Gleason's grades revealed 4 groups of patients: grade 2-10 patients (median PSA 9.2 + 8.4 ng/ml), grade 3-18 patients (PSA 13.3 + 12.0 ng/ml), grade 4-20 patients (PSA 10.7 + 5.3 ng/ml) and grade 5-5 patients (PSA 16.3 + 6.2 ng/ml).

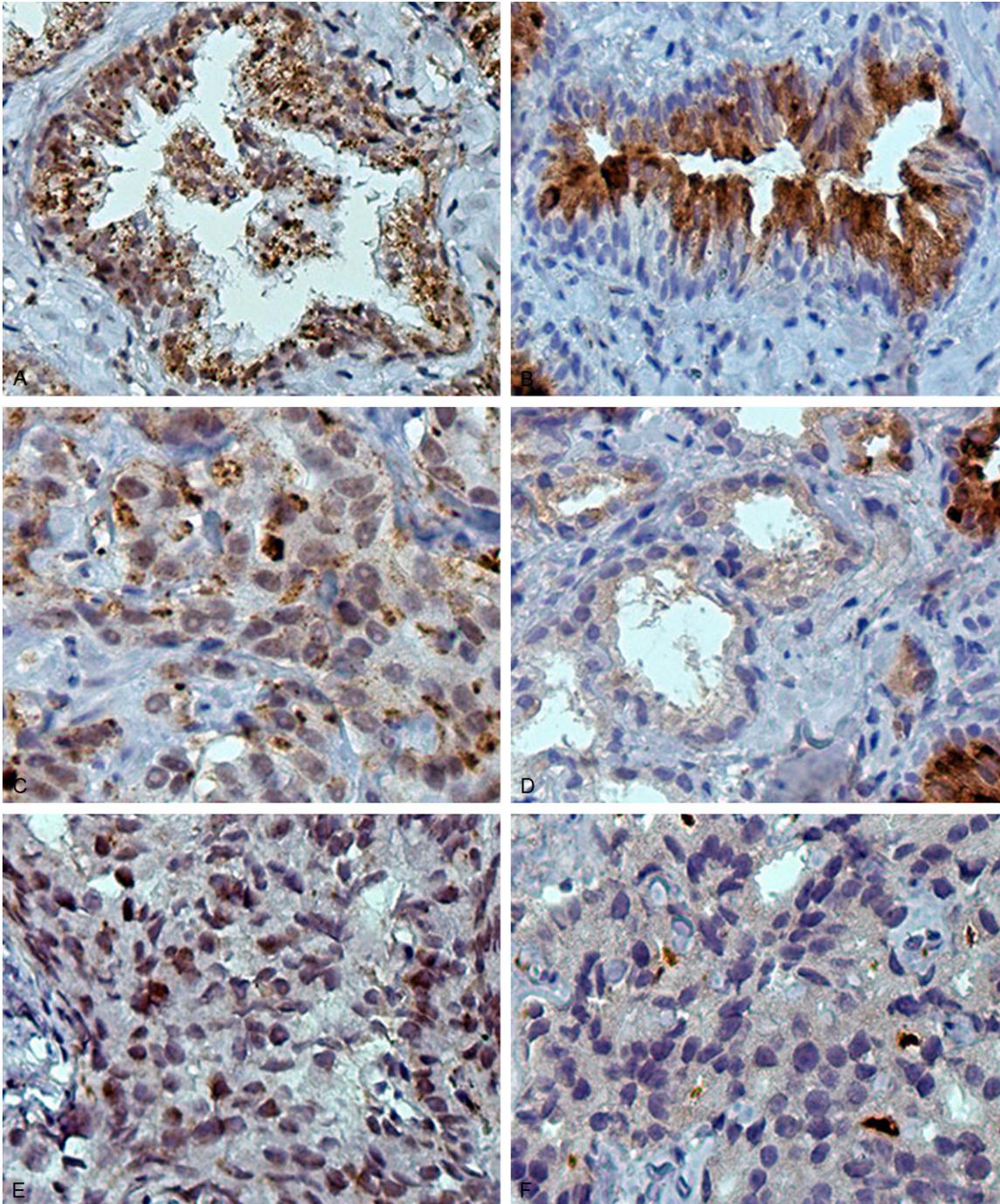
The material was fixed in 10% neutral buffered formalin (pH 7.4) and embedded in the paraffin wax (melting point +54°C) using the histocentre. For histological and immunohistochemical (IHC) studies the 5 µm serial tissue sections were mounted on the slides coated with poly-L-lysine. Identification of tissue antigens was performed using rabbit antibodies against MMP-9 (Thermoscientific) 1:400; rabbit monoclonal antibodies against TIMP-1 (EPR 1550, EPITOMICS) 1:100; murine monoclonal antibodies against collagen type IV (PHM-12, Thermoscientific) 1:100 and Ki-67 (MM1, Diagnostic Biosystems) 1:100. Detection of immune complexes was performed using unbiotinated detection system based on the horseradish peroxidase (N-Histofine, Japan), sections were stained with Mayer's hematoxylin.

Intensity of the immunohistochemical reactions with MMP-9 and TIMP-1 was evaluated according to the following parameters: 0-the absence of brown granules in the cell cytoplasm, 1-presence of small granules in the cytoplasm, 2-distribution of large granules in the single cells, 3-presence of large granules in the cytoplasm of most cells. Distribution of the type IV collagen in the basement membrane of the prostatic glands was evaluated semi-quantitatively in compliance with the following parameters 0-lack of collagen along the basement membrane, 1-the presence of a thin strip of collagen, 2-thick band of collagen along the basement membrane, comparable to the vascular wall in the thickness and staining intensity. Proliferation index Ki-67 (Ki-67 PI) was determined by evaluating the percentage ratio of immunoreactive cell nuclei to the total number of nuclei. Basic statistical analysis (calculation of arithmetic mean [M] and standard deviation [SD]) was carried out using the STATISTICA ver. 10.0 software package. In considering the non-normal distribution of the certain statistical figures, the comparison of two independent groups was performed by using the nonparametric Mann-Whitney U test. The difference between the arithmetic means was considered reliable when the *p*-value, indicating the statistical significance, remained less than 0.05. Relationships between Gleason's grade, index of cell proliferation, immunohistochemical staining intensity of MMP-9 and TIMP-1 was evaluated using Spearman's correlation coefficient (*r*<sub>s</sub>).

### Results and discussion

Immunohistochemical (IHC) study of the prostate tissue revealed special aspects of the

## Matrix metalloproteinase-9 and its inhibitor in prostate adenocarcinoma



**Figure 2.** IHC reaction in benign hyperplasia (A, B) and prostate adenocarcinoma with the Gleason's grade 2 (C, D) and 4 (E, F) with antibody to MMP-9 (A, C, E) and TIMP-1 (B, D, F). Staining: DAB-Mayer hematoxylin (Magnification  $\times 400$ ).

expression of proteins that play a considerable role in proliferative processes, in the distribution of either collagen type IV, MMP-9 and its inhibitor, depending on the Gleason's grades.

Examination of the control material-benign prostatic hyperplasia, showed that individual

cells, stained with antibodies against Ki-67, were located only in the basal cell layer, and the proliferative activity of cells in the secretory layer was not observed (**Figure 1A**). Meanwhile, a wide range of the proliferative activity was detected in secretory glandular cells in patients with adenocarcinomas of different Gleason's

## Matrix metalloproteinase-9 and its inhibitor in prostate adenocarcinoma

**Table 2.** An correlation relationship between clinical, morphological and immunohistochemical characteristics of prostatic adenocarcinomas

	Data	Age	PSA	Gleason grade	Ki-67 proliferation index	Expression of MMP-9	Expression of TIMP-1
Age	$r_s p$	-	-	-	-	-	-
PSA	$r_s p$	0.290* (0.034)	-	-	-	-	-
Gleason grade	$r_s p$	0.164 (0.239)	0.276* (0.046)	-	-	-	-
Ki-67 proliferation index	$r_s p$	0.065 (0.642)	0.1313 (0.348)	0.674* (0.0001)	-	-	-
Expression of MMP-9	$r_s p$	-0.009 (0.948)	0.022 (0.875)	-0.660* (0.0001)	-0.369* (0.006)	-	-
Expression of TIMP-1	$r_s p$	0.088 (0.529)	0.012 (0.932)	-0.057 (0.684)	-0.137 (0.328)	0.268 (0.052)	-

\*-Significant indicators.

grades (**Figure 1C, 1D; Table 1**). We observed an increase in the proliferative activity of atypical cells in highly invasive adenocarcinomas (scores 4 and 5 of Gleason grading system). Thus, the proliferative activity of anaplastic adenocarcinoma cells of different Gleason's grades is a sensitive test for the differential diagnosis of these tumors.

Collagen type IV is a major component of the basal membrane of the epithelial cells; it has been also detected in the wall of blood vessels and connective tissue. Invasive properties of tumor cells exhibit dependencies in the destruction of this important component of the connective tissue. In the immunohistochemical study, collagen type IV was detected along the basement membrane of glandular cells in the form of thin brown bands of the different staining intensity. **Figure 1B** shows an immunohistochemical reaction of antibodies against collagen type IV in the stromal tissue of BHP (2 points of intensity), where thick brown bands of collagen type IV are distributed along the basement membrane of glands. In the examination of the distribution of the type IV collagen in sections of adenocarcinoma of the prostate, the intensity of immunohistochemical staining correlated with a score of the Gleason patterns. **Figure 1B** shows the IHC reaction in the stromal tissue of prostatic adenocarcinoma (score 2 of Gleason grading), and staining intensity was less pronounced in comparison with BPH and corresponded to point 1, however some thin collagen fibers embraced individual cell elements. With an increase of Gleason's grades, collagen fibers around tumor cells completely disappeared. **Figure 1E** shows the distribution of the type IV collagen in the stroma of adenocarcinoma 4 th of Gleason grading, and there were no staining of the basement membrane around glandular tumor cells observed, subsequently collagen remained only in the stromal

tissue. Thus, the distribution of the type IV collagen was descriptive of benign and malignant prostate tumors.

Further investigations were focused on mechanisms of the proteolytic activity of prostate adenocarcinoma that characterized an invasive tumor growth and determined their malignancy potential. MMP-9 was detected in the cytoplasm of tumor cells in the form of small or large brown granules with different staining intensity by DAB reaction. **Figure 2A** shows the distribution of MMP-9 in the glandular cells in benign prostatic hyperplasia, an immunohistochemical reaction in secretory cells was prominent as came amid numerous large, medium and small MMP-9 granules (intensity 3 points).

In the study of adenocarcinoma, we showed a reduction in the intensity of immunohistochemical staining of MMP-9. **Figure 2C, 2E** demonstrate immunohistochemical reaction in the prostate adenocarcinoma (scores 2 and 4 of Gleason's grade) with separate small MMP-9 granules in the cytoplasm of secretory cells. It should be noted that the staining intensity of adenocarcinomas of various Gleason's grades was lower than in BHP (**Table 1**).

As it can be seen from the data, the level of expression of MMP-9 in tumor cells decreases with the growth of anaplastic changes in them, herein the level of expression of this enzyme was trustworthy 2-fold higher in adenocarcinomas of Gleason's grade 2 and 3 than in adenocarcinomas of grades 4 and 5. We did not found significant differences in the expression of MMP-9 between adenocarcinomas of scores 2 and 3, as well as between scores 4 and 5 th. These groups of tumors demonstrated similarity in the level of the MMP-9 expression. Similar studies on the expression of TIMP-1, which

## Matrix metalloproteinase-9 and its inhibitor in prostate adenocarcinoma

inhibits activity of MMP-9 in tumor cells, were undertaken for further investigation on mechanisms of the adenocarcinoma invasion. Separate unchanged glandular structures that served as controls for immunohistochemical studies were identified by us in the vast majority of the examined histological material, except the previously described pathological conditions of glandular epithelium.

**Figure 2B** demonstrates the distribution of TIMP-1 in glandular cells in benign prostatic hyperplasia. It shows the pronounced immunohistochemical reaction in the secretory cells of the major glands with a numerous large, medium and small granules of TIMP-1 (intensity-3 points). However, a reduction in the intensity of the immunohistochemical staining for TIMP-1 was observed when studying adenocarcinoma. **Figure 2E, 2F** represent the immunohistochemical reaction with prostate adenocarcinoma of scores 2 and 4 according to Gleason's grades with separate small TIMP-1 granules (1-intensity point) in the unaltered glands, which in this case is a valid control, though the staining and coloration of 3 points was also detected. Results of semi-quantitative analysis of staining of adenocarcinomas of different Gleason's grades are summarized in **Table 1**. As it may be seen from the data, the level of the TIMP-1 expression in adenocarcinomas decreases, thus the level of expression of the enzyme in adenocarcinomas of different Gleason's grades was even more than 3 times less than in cells in BHP or in unchanged glands. We did not discover any significant differences in the TIMP-1 expression in adenocarcinomas with different Gleason patterns since the level of the TIMP-1 expression in these groups was similar.

Thus, current investigation demonstrated that the high expression of MMP-9 in BHP was inhibited by the high content of TIMP-1. A decrease in the MMP-9 expression was observed in adenocarcinomas, though it degrades collagen type IV in the basement membrane of glandular structures due to the lack or weak expression of TIMP 1 that blocks the action of the enzyme on collagen of the connective tissue. A low TIMP-1 expression in adenocarcinomas, could likely explain invasive properties of these tumors.

**Table 2** shows a correlation between clinical and morphological characteristics of prostate

tumors, the cell proliferation and the expression of MMP-9 and TIMP-1. As it can be seen from the presented data, there is a moderate positive correlation between Gleason scores and a cell proliferation index Ki 67 ( $rs = 0.674$ ), and a moderate negative correlation with tumor scores and the level of expression of MMP-9 ( $rs = -0.660$ ). There is a weak negative correlation between the level of the cell proliferative activity and expression of secretory tumor cell MMP-9 ( $rs = -0.369$ ). The lack of significant correlation between an expression of TIMP-1 and other clinical, morphological and immunohistochemical indicators was likely due to the reduced level of the protein in secretory adenocarcinoma cells.

The study of molecular characteristics of the carcinogenesis provides new options for diagnosis and treatment of tumors of prostate glands, reveals the mechanisms of their biological potential and invasive properties. Although the prognostic value of the measures such as Gleason scores and a total PSA level in the blood serum has been recently demonstrated in patients with prostate adenocarcinomas, many authors indicated that the proliferative activity of tumor cells is an important diagnostic indicator.

In the present study, we evaluated the levels of MMP-9 and TIMP-1 expressions in adenocarcinomas of prostate. Yet a significant number of investigations on the role of MMPs in the invasion and angiogenesis of various cancers has been documented, the role of these enzymes in the progression of prostate cancer remains controversial [13]. Furthermore there are discrepancies in reports in regards to the expression of MMP-9 in the prostate cancer tissue. Protein expression has been reported to be either absent [18] or present [19]; and reports about localization of the MMP-9 expression were also inconsistent. Most authors agree on the opinion, that there is an imbalanced expression of MMPs and TIMPs in the prostate cancer tissue, manifested as a general loss of TIMPs and an up regulation of MMPs. As such, it is generally thought that MMPs are more active in advanced stages of prostate cancer, as indicated by the fact that most MMPs display a higher expression in higher Gleason score tumors [20, 21]. Assaying freshly frozen malignant tissue specimens from patients with clini-

## Matrix metalloproteinase-9 and its inhibitor in prostate adenocarcinoma

cally localized PC by quantitative real-time polymerase chain reaction (qRT-PCR), Reis et al [22] demonstrated that MMP-9 was overexpressed by 9.2 times, and TIMP-1 was underexpressed 0.75 times. In this study, MMP-9 was also overexpressed in benign tissues of patients with prostate cancer. However, a number of researchers using immunohistochemical techniques noted a decrease in the activity of MMP-9 with increasing Gleason score of prostate cancers [23, 24], or the complete absence of such activity [18]. Such differences can be explained by the presence of inflammatory infiltrates, because the expression of the MMP-9 mRNAs was detected only in macrophages in inflammation areas in prostates [25].

Recent findings from in-vitro models and animals studies that proposed the MMP overexpression could promote the tumor progression stimulated the development of MMP inhibitors as a cancer therapy. However, several clinical trials were disappointing since they revealed unwanted side effects, i.e. MMP inhibitors interfered with the normal development processes and compromised the host defense system [26].

TIMPs are secreted by epithelial and stromal cells and the unregulated expression of TIMP has been involved in the tumor invasion and metastasis. Biochemical analysis revealed that a considerable amount of TIMP protein was secreted by normal prostate tissue, but the expression was either significantly reduced or not detected in the conditioned medium from the tumor tissues [27]. Our results are consistent with those of other authors who also showed a decrease in levels of TIMP-1 expression in prostate adenocarcinomas [20, 23]. TIMP-1 is well known for its role as a negative regulator of angiogenesis [24].

Brehmer et al [15] recognized that the imbalance in MMPs and TIMPs was primary caused by a significant loss of TIMP-1 in malignant epithelium and an up regulation of MMPs. Nevertheless, in this study palpable tumors (T2, T3) expressed significantly less MMP-9 than T1c tumors. These data are in agreement with our observations that the tumors with a higher Gleason' score are characterized by a low level of MMP expression.

Taking into account the negative effect of TIMP-1 on angiogenesis [28], the decrease in the

level of TIMP-1 in cancer cells might be considered as a negative feature in the development of prostate cancer, since the reduced expression of TIMP-1 in adenocarcinomas compared to BPH and normal tissue could serve as a basis of neoangiogenesis in tumor tissues and metastases formation.

### Conclusion

Thus, current study demonstrated a decrease production of MMP-9 with increase of the Gleason's grade and progress in the proliferative activity of secretory cells in prostate adenocarcinoma. Furthermore, the invasive properties of tumor cells, which lead to the degradation of the type IV collagen in the basement membrane and stromal tissue of the prostate, could be explained by an dysregulation between the MMP-9 and TIMP-1-a protein TIMP-1 that inhibits the enzyme action of MMP-9, and which expression is significantly reduced in adenocarcinomas of the different Gleason's grade.

### Disclosure of conflict of interest

None.

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## Matrix metalloproteinase-9 and its inhibitor in prostate adenocarcinoma

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