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Matrix metalloproteinase 3 and matrix metalloproteinase 9 expression and polymorphisms analysis in bladder cancer

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Background. The matrix metalloproteinases (MMPs) are now being widely studied in a variety of tumour systems to ascertain their role in tumour progression. Ample evidence indicates that MMPs contribute to all the stages of malignant progression in multiple ways, including tumour invasion, blood vessel penetration, metastases and tumour angiogenesis. The aim of the study was to investigate the expression of MMP-3 and MMP-9 in bladder cancer patients' blood and to determine the prevalence of a single nucleotide polymorphism in the promoter of MMP-3 and MMP-9 genes.

Materials and methods. Gene expression of MMP-3 and MMP-9 was assessed by reverse transcription-PCR (RT-PCR) in 44 bladder cancer patients' blood samples. The MMP-3 (–1171 5A/6A) and MMP-9 (–1562 C/T) polymorphisms were determined by the polymerase chain reaction-based restriction fragment length polymorphism (PCR–RFLP).

Results. In 41 cases out of 44, the expression of MMP-9 was found in bladder cancer patients' blood, while the expression of MMP-3 was not detected in patients' blood. All the analysed MMP-3 and MMP-9 gene promoter polymorphism variations were identified.

Conclusions. Significant association was found between the analyzed MMP-3 and MMP-9 polymorphism variations in bladder cancer patients' blood. But no statistical correlations were found among the degree of tumour differentiation and the polymorphisms of gene promoters of MMP-3 and MMP-9.

Key words: matrix metalloproteinase, polymorphism, bladder cancer

INTRODUCTION

The capacity to degrade and break down the basement membrane and extracellular matrix (ECM) is essential for primary tumour cells to invade locally and spread to distant sites. The matrix metalloproteinases (MMPs) comprise a family of 24 zinc dependent endopeptidases with a broad spectrum of enzymatic activity against all the components of the ECM (1). The MMPs are now being widely studied in a variety of tumour systems, including the urinary bladder, to ascertain their role in tumour progression and as potential therapeutic targets (2).

Functional polymorphisms affect the regulation of gene expression and so can contribute to individual differences in susceptibility to and severity of a disease. The effect may be seen by a polymorphism alone, or in combination with other polymorphisms. Polymorphisms of the MMP genes may promote cancer development or progression via alteration of MMP protein expression, resulting in creation and maintenance of the microenvironment for tumour cell proliferation, migration and invasion (3).

MMP-3 is an important MMP interacting with tumour microenvironment and is essentially expressed by fibroblasts and macrophages. MMP-3 is expressed at very low levels in normal adult tissues, and peritumoral expression could be because the host tissue reacted as though the tumour was a wound. This extratumoral MMP expression could influence tumour growth and behaviour toward cytotoxic stress (4).

A common variant in the promoter region of the human stromelysin gene with 1 allele having a run of 5 adenosines (5A) and another one having 6 adenosines (6A) has been recently reported. It has been demonstrated by *in vitro* experiments, that the 6A allele has a lower promoter activity than the 5A allele. Therefore, subjects carrying the 6A allele accumulate extracellular matrix because of the decreased degradation (5).

MMP-9 has been reported to be expressed at a higher level in invasive bladder tumours than in superficial ones. MMP-9 polymorphism influences the binding of a transcription repressor protein; the loss of this DNA-protein interaction is associated with the higher MMP-9 promoter activity (2).

The aim of this study was to identify MMP-3 and MMP-9 genes expression and genetic variations in MMP-3 (–1171 5A/6A) and MMP-9 (–1562 C/T) genes promoters.

MATERIALS AND METHODS

Patients

A total of 44 patients with bladder cancer tumours (30 Ta, 9 T1, 5 T2; 12 G1, 18 G2, 14 G3) were studied.

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RNA extraction and RT-PCR

Total RNA was extracted from the blood samples using EXTRAzol RNA extraction kit (Amplimedical spa, Italy) according to the manufacturer's protocol. Additionally, the final RNA preparations were treated with Rnase-free Dnase (Fermentas, Lithuania) to avoid the contamination with fragments of degraded genomic DNA.

Complementary DNA (cDNA) was synthesized from the total RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Lithuania). To control the quality of isolated total RNA and synthesized cDNA synthesis, the cDNA of the housekeeping gene β-actin was amplified. The primer sequences used for MMP-3, MMP-9 and β-actin detection are summarized in Table 1.

Table 1. **Characteristics of RT-PCR reaction**

Primers	Expected RNA size (bp)
MMP-3	
5′-GAACAATGGACAAAGGATACAACA-3′ (forward)	729
5′-TTCTTCAAAAACAGCATCAATCTT-3′ (reverse)	
MMP-9	
5′-ACCGCTATGGTTACACTCGG-3′ (forward)	584
5′-GCAGGCAGAGTAGGAGCG-3′ (reverse)	
β-actin	
5′-GTGGGGCGCCCCAGGGACCA-3′ (forward)	540
5′-CTCCTTAATGTCACGCACGATTTC-3′ (reverse)	

The PCR reaction for MMP-3 starts with 3 min incubation at 95 °C followed by 35 cycles of 50 s at 94 °C, 50 s at 58 °C and 1 min 30 s at 72 °C. The PCR reaction for MMP-9 and β-actin starts with 3 min incubation at 95 °C followed by 35 (β-actin) or 45 cycles (MMP-9) of 50 s at 94 °C, 50 s at 62 °C and 1 min at 72 °C. Final RT-PCR products were electrophoresed onto 1% agarose gel, stained with ethidium bromide and visualized by UV light (Fig. 1).

DNA extraction

Genomic DNA was isolated by modification of the method described by Sambrook J. et al. (6). Blood samples were suspended in 1 ml of erythrocyte lysis solution (320 mM saccharose, 5 mM Mg_2Cl, 1% Triton X-100 and 10 mM Tris-HCl (pH 7.5) and centrifuged at 15,000 × *g* for 15–20 min 400 µl of nucleic lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, (pH 7.5) containing proteinase K (0.1 mg/ml) (Fermentas, Lithuania) and 2% sodium dodecyl sulfate was added to the pellets, the solution was mixed and incubated at +37 °C overnight. After digestion the DNA was extracted with the same volume of phenol, phenol-chloroform and chloroform. The DNA recovered by centrifuging the samples at 15,000 × *g* for 10 min. The concentration and the purity of DNA were determined spectrophotometrically by readings of A_{260} and A_{280}. The extracted DNA was stored at –20 °C.

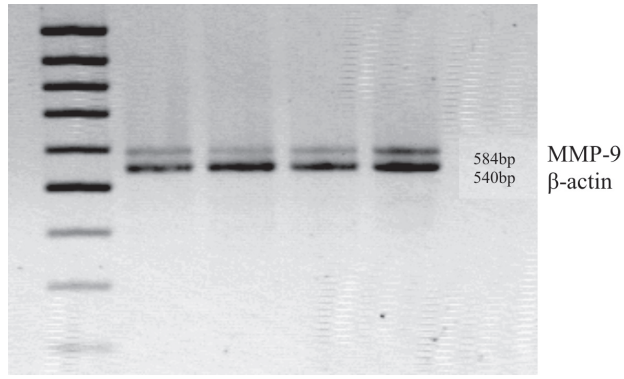

Fig. 1. RT-PCR analysis of β-actin and MMP-9

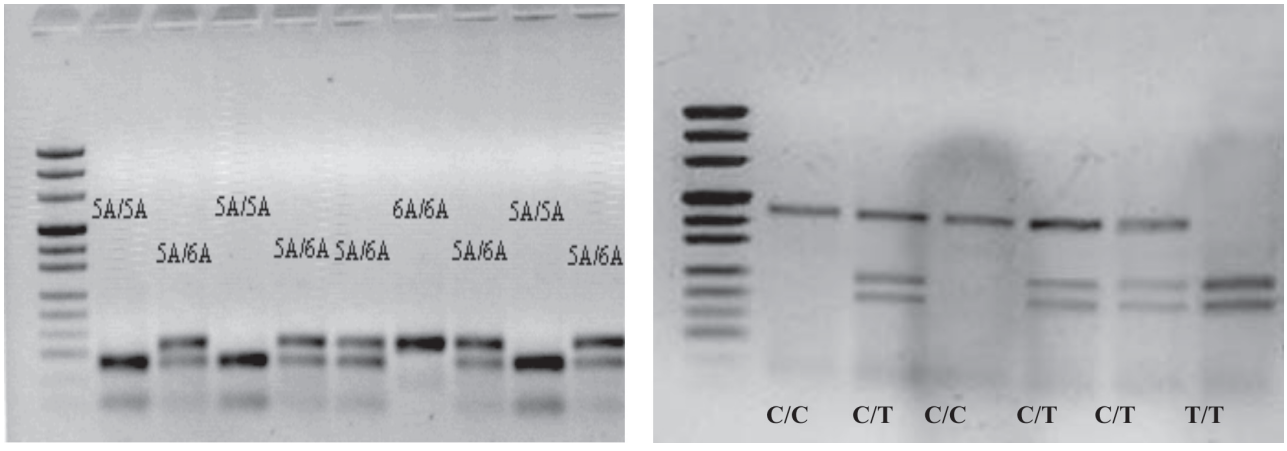

Fig. 2. PCR-RFLP analysis of the MMP-3 promoter –1171 5A/6A polymorphism and MMP-9 promoter –1562 C/T polymorphism

Table 2. **PCR primers for MMP-3 and MMP-9 PCR-RFLP assays**

Polymorphism	Primers	Restriction enzyme	Fragment length
MMP-3	5′-GGTTCTCCATTCCTTTGATGGGGGGAAAGA-3′ (for)	Psy I	129bp (6A)
(–1171 5A/6A)	5′-CTTCCTGGAATTCACATCACTGCCACCACT-3′ (rev)		97 + 32bp (5A)
MMP-9	5′-GCCTGGCACATAGTAGGCCC-3′ (for)	Pae I	436bp I
(–1562 C/T)	5′-CTTCCTAGCCAGCCGGCATC-3′ (rev)		240 + 196bp (T)

Genotype analysis

The genotypes of MMP-3 (–1171 5A/6A) and MMP-9 (–1562 C/T) were determined by the polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP). The PCR cycling conditions were 2 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C, and with a final step at 72 °C for 2 min to allow complete extension of PCR products. The primers and restriction enzymes are summarized in Table 2.

Digested PCR products were loaded on a 2% agarose gel, stained with ethidium bromide and visualized by UV light (Fig. 2).

Statistical analysis

Statistical analysis was performed using computer program SPSS. The proportions of groups were compared by a χ^2 test. Threshold for significance was $P < 0.05$.

RESULTS AND DISCUSSION

MMP-9 gene expression was examined by RT-PCR analysis in bladder cancer patients' blood. Amplification of cDNA fragments of MMP-9 confirmed an induction of the gene expression in blood cells of bladder cancer patients. In our study, in 41 cases out of 44, the expression of MMP-9 was found in bladder cancer patients' blood. The expression of MMP-3 was not detected in total RNA isolated from patients' blood.

According to the data of polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) analysis, all the patients were divided into the groups according to genotypes of the MMP-3 and MMP-9 detected (Table 3).

The MMP-3 and MMP-9 genotype was successfully determined in all of the patients, and significant association was found between polymorphism variations. In literature we do not find any experiments in which this MMP-3 (–1171 5A/6A) polymorphism was subject to research with bladder cancer patients. But there are experiments in which the authors demonstrate that MMP-3 mRNA localization in stromal cells are associated with poor patients' disease-free and overall survival (7).

Approximately 70% of all the newly diagnosed bladder cancer are well- or moderately differentiated superficial papillary tumours, with recurrence taking place after transurethral resection in the majority of cases. Studies demonstrate that MMP-9 degrades extracellular matrix (ECM) and basement membrane (BM) components and helps tumour cells to reach distant sites. In view of this important role of MMP-9, it could be useful to determine MMP-9 (–1562 C/T) polymorphism variation to learn about the expected enzyme expression (higher or lower) sooner during the course of the disease. More importantly, most MMPs are synthesized not only by the genetically altered cancer cells but also by adjacent and intervening stromal cells. There is also growing evidence to support an expanded role of MMPs in creating and maintaining a microenvironment that facilitates the initial stages of tumour development.

Functional polymorphisms affect the regulation of gene expression and so can contribute to individual differences in susceptibility to and severity of a disease. The effect may be seen by a polymorphism alone or in combination with other polymorphisms. Kader AK et al. (9) recently published that the MMP-9 microsatellite > or = 24 CA repeat and MMP-12 –82 G alleles were associated with a higher risk of bladder cancer invasiveness.

In this study no statistical correlations were found among the grading (G) and the polymorphisms of the gene promoters of MMP-3 and MMP-9 (Table 4). Wallard MJ et al (1) showed that MMP-3 was only moderately expressed in normal tissue and tumour tissue, with no relationship with increasing grade.

Retrospective analysis of MMP expression in cancer patients indicates that the presence or elevated expression of many MMPs, including MMP-1, –2, –3, –7, –9, –13, –14, in both primary tumours and/or metastases, are positively associated with tumour progression, i.e. poor tumour differentiation, invasive stage of cancer, poor prognosis, metastasis to secondary organs and shorter survival time (8). In bladder cancer most widely documented MMPs are MMP-2 and MMP-9, which have been shown to correlate with increasing tumour grade. Other proteinases, such as MMP-7, MMP-11 and MMP-13 have been detected

Table 3. **Distribution of MMP-3 and MMP-9 genotypes and frequencies in blood samples of patients with bladder cancer**

MMP-3 polymorphism				MMP-9 polymorphism			
Genotype	Detected (frequency)	Not detected	Total	Genotype	Detected (frequency)	Not detected	Total
5A/5A	9 (0.20)	35	44	CC	29 (0.66)	15	44
5A/6A	28 (0.64)	16	44	CT	12 (0.27)	32	44
6A/6A	7 (0.16)	37	44	TT	3 (0.07)	41	44
χ^2		27.48		χ^2		35.66	
p		<0.0001		P		<0.0001	

Table 4. **Distribution of MMP-3 and MMP-9 genotypes in blood samples of patients with bladder cancer by degree of tumour differentiation**

MMP-3 polymorphism				MMP-9 polymorphism			
Degree of tumour different.	5A/5A	5A/6A	6A/6A	Degree of tumour different.	CC	CT	TT
G1	4	7	1	G1	8	3	1
G2	2	13	3	G2	12	6	0
G3	4	7	3	G3	9	3	2
χ^2		3.24		χ^2		2.86	
p		>0.5		p		>0.5	

in increased levels in bladder cancer, but have in general failed to demonstrate statistically significant positive relationships with pathological end points (10).

CONCLUSIONS

All MMP-3 (–1171 5A/6A) polymorphism variations (5A/5A, 5A/6A, 6A/6A) and MMP-9 (–1562 C/T) variations (CC, CT, TT) were detected in bladder cancer patients' blood. Significant association was found between the analyzed MMP-3 and MMP-9 polymorphism variations. No correlation was shown between the different MMP-3 and MMP-9 genotypes and degree of tumour differentiation. In conclusion, our study suggests that MMP-3 and MMP-9 promoter polymorphisms may be a facilitating factor for cancer growth in bladder cancer patients. The reported data need to be validated in larger studies.

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MATRIKSO METALOPROTEINAZĖS-3 IR MATRIKSO METALOPROTEINAZĖS-9 RAIŠKOS IR POLIMORFIZMO TYRIMAI SERGANT ŠLAPIMO PŪSLĖS VĖŽIU

S a n t r a u k a

Įvadas. Šio darbo tikslas buvo nustatyti matrikso metaloproteinazių MMP-3 ir MMP-9 raišką sergančiųjų šlapimo pūslės vėžiu kraujyje ir identifikuoti vieno nukleotido polimorfizmo variantus šių matrikso metaloproteinazių genų promotorinėse dalyse.

Medžiaga ir metodai. Tyrime dalyvavo 44 pacientai, sergantys šlapimo pūslės vėžiu. MMP-3 ir MMP-9 raiška kraujyje nustatyta atvirkštinės transkriptazės polimerazės grandininės reakcijos (AT PGR) metodu. MMP-3 (–1171 5A/6A) ir MMP-9 (–1562 C/T) vieno nukleotido polimorfizmo variantų analizė atlikta restrikcijos fragmentų ilgio polimorfizmo (RFIP) metodu.

Rezultatai. Sergančiųjų šlapimo pūslės vėžiu kraujyje 41 atveju (iš 44 tirtųjų) identifikuota MMP-9, MMP-3 raiška nebuvo nustatyta. Sergančiųjų kraujyje nustatyti visi MMP-3 (–1171 5A/6A) ir MMP-9 (–1562 C/T) geno promotorinėje dalyje galimi polimorfizmo variantai.

Išvados. Įvertinus MMP-3 ir MMP-9 polimorfizmo variantus sergančiųjų šlapimo pūslės vėžiu kraujyje, tarp nustatytųjų gautas statistiškai patikimas skirtumas, tačiau statistiškai patikimo skirtumo negauta įvertinus analizuotų polimorfizmų variantus ir šlapimo pūslės navikų diferenciacijos laipsnį.

Raktažodžiai: matrikso metaloproteinazės, polimorfizmas, šlapimo pūslės vėžys