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Original Research

rs3918242 variant genotype frequency and increased TIMP-2 and MMP-9 expression are positively correlated with cancer invasion in urinary bladder cancer

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Abstract: To study the role of MMP9 and TIMP2 genotypes and expression in predisposition to bladder cancer and relation with metastasis. 100 urinary bladder cancer patients and 100 healthy controls were included in the study. rs3918242 and rs8179090 genotypes were determined with PCR-RFLP. Quantitative real-time polymerase chain reaction was employed to assess the MMP-9 and TIMP-2 expression in tumors and adjacent healthy tissues. Variant genotype (TT) for rs3918242 polymorphism and rs8179090 variant genotype are not associated with bladder cancer risk. rs3918242 genotype was significantly associated with tumor invasion. In contrast with this, rs8179090 genotype has not shown a significant association with tumor invasion. Both SNPs did not show a significant association with metastatic status. MMP-9 was upregulated in tumors in comparison to cancer free tissues. Significant increase in the expression of MMP-9 was also observed in invasive tumors. TIMP-2 expression was significantly increased in tumors in comparison to cancer free tissues and in metastatic tumors in comparison to non-metastatic tumors. Tissues with rs3918242 variant genotype have shown increased MMP-9 expression. rs3918242 promoter polymorphism of MMP-9 is significantly associated with tumor invasion, however; there is no positive correlation between TIMP-2 rs8179090 promoter polymorphism variant frequency and invasion. MMP-9 and TIMP-2 genes are upregulated in cancerous tissues when compared to normal bladder tissues.

Key words: Bladder cancer; Polymorphism; MMP-9; TIMP-2; rs3918242; rs8179090.

Introduction

Urinary bladder cancer (UBC) is the second most common cancer of all genitourinary cancers after prostate cancer in the United States (1). Phenotypically bladder carcinomas are divided into two categories as non-muscle invasive and muscle invasive carcinomas. Most bladder carcinomas, approximately 75%, are muscle non-muscle invasive (NMSBC–pTa, pTis or PT1) while one third is invasive (T2-4) at the time of diagnosis. Primary treatment of non-muscle invasive bladder cancers is transurethral resection of tumor and intravesical instillation of certain chemotherapeutics and immunotherapeutic agents. In invasive cases more aggressive surgical treatment, radical cystectomy, is required (2).

There is a copious amount of studies focusing on the molecular mechanism of bladder cancer invasion and migration in literature. MMPs and TIMPs are among the most studied molecules. In this study, we aimed to study the role of rs3918242 and rs8179090 variants and MMP9 and TIMP2 expression levels in susceptibility to UBC and their association with UBC metastasis.

The most distinctive features of malign neoplasms are their ability to invade neighbouring tissues and metastasize to other regions of the body. Since invasion and metastasis are the main underlying reasons of cancer related deaths, molecular mechanisms underlying these events should be understood in order to develop new therapeutic approaches. Invasion is a polyphasic process/multistep event which requires the penetration of extracellular matrix and basal membrane by the malignant cells detached from the tumor. During this process, various extracellular matrix (ECM) members including collagens, laminines and fibronectins are degraded/cleaved by the proteases. Therefore matrix metalloproteinases (MMPs) and their inhibitors; tissue inhibitors of metalloproteinases (TIMPs), play crucial roles during invasion and metastasis. The proteins encoded by these genes regulate the connective tissue homeostasis and the balance between the counteracting functions of these genes may indicate to the tumor progression.

MMPs belong to a peptidase family with the ability to cleave almost all basal membrane and extracellular matrix members. So far 19 different MMPs, grouped into 4 different types as collagenases, stromelisins, gelatinases and as membrane type MMPs have been identified. The genomic region encoding the MMP family members is amplified in various cancers (3). The collagenases (MMP-1, MMP-8,MMP-13) comprise of the some of the most studied metalloproteinases. They cleave fibril collagens and the cleavage products are de-

natured to gelatines which are cleaved further by gelatinases (MMP-2, MMP-9) (4).

MMP-9, encoded by a 8800nt long gene with 13 exons located on 20q11.2-q13 (5, 6). It has two transcript variants, one encoding a 72 kDa protein and the other encoding a 92kDa protein. The genomic variants and differential expression of this gene have been reported to be associated with various pathological conditions. So far genomic variations in MMP-9 gene were reported to alter the susceptibility to coronary artery disease (7), pulmonary emphysema (8), pediatric asthma (9) and head and neck cancers (10). In addition to genomic variations, altered MMP-9 expression levels have also been associated with macroprolactinomas (11), endometriosis (12), myocardial infarction (13) and neuroendocrine tumors (14).

TIMP-2 on the other hand is a MMP inhibitor, encoded by a 83 kb long gene located on 17q25 with 5 exons (15). Encoded protein inhibits specific MMPS by binding to them (16). The genomic variants and altered expression levels of this gene have so far been associated with angiogenesis (17), diabetic retinopathy (18), odontogenictumors (19), prostate cancer (20), colorectal cancers (21) and breast cancers (22).

In the present study, we aimed to investigate the relation of rs3918242 and rs8179090 promoter polymorphisms in MMP-9 and TIMP-2 genes with gene expression levels, urinary bladder cancer susceptibility and progression.

Materials and Methods

Study group and nucleic acid isolation

The patient group included 100 patients (89 male, 11 female) with a median age of 61 years, while the control group included 100 healthy subjects (80 male, 20 female) with a median age of 59. Clinical and demographic data was obtained from medical history records.

Bladder tumors were staged according to the 2011 tumor-node-metastasis (TNM) classification (23) and graded according to the World Health Organization (WHO) system (24). All the patients with TCC were newly diagnosed, previously untreated (with chemotherapy or radiotherapy or intravesical instillation therapies) and their diagnoses were histologically confirmed. Control group included the healthy control subjects, who had no previous diagnosis of any cancer type or any other chronic disease.

Genomic DNA was isolated from peripheral blood samples using QIAamp DNA Blood Mini Kit (Qiagen) for genotyping with PCR-RFLP. The suitability of DNA samples for PCR amplification was confirmed by measuring the quantity and purity with the NanoDrop 2000c (Thermo Fisher Scientific). For the determination of mRNA expression levels, total RNA was isolated from 100 surgically extracted tumors and paired cancer free tissues from the patient group. The extracted tissue samples were immediately frozen and stored until RNA isolation. Paired cancer free tissues were extracted from more than 1 cm distant neighboring tissue. All tissue samples were histologically confirmed. RNeasy Mini Kit (Qiagen) was used for total RNA isolation. İsolated RNA samples were stored at − 80 °C after RNA quantity and purity was determined by absorbance measurement with NanoDrop 2000c (Thermo Fisher Scientific). RNA integrity was checked by running on 1.5% agarose gel. All patients and healthy subjects provided an informed consent and the study protocol was approved by local ethics committee (Istanbul Faculty of Medicine Ethics Committee).

Genotyping

rs3918242 and rs8179090 genotypes were determined with PCR-RFLP assays which were designed on Biology-Workbench (ref). 100ng of genomic DNA was used to amplify target regions with specific primers. PCR products were run on 2% agarose gel to confirm the success and specifity of the amplification. Following amplification, PCR products were incubated with polymorphism specific restriction enzymes and restriction products were separated on 3% agarose gels for genotyping. 10 random samples were sequenced to confirm the RFLP results. The genotypes determined by sequencing were the same as the genotypes determined using RFLP for all of the randomly chosen samples. The primer sequences and restriction enzymes used for genotyping each polymorphism are presented in Table 1.

Determination of mRNA expression

1 µg of total RNA was used for cDNA synthesis with reverse aid first strand synthesis kit (Fermentas Life Sciences). All amplification reactions were prepared with QuantiTect SYBR Green PCR Kit (Qiagen). Each reaction was performed in triplicates and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the house keeping gene. Obtained Ct values were used to determine the relative expression levels according to Livak's delta delta Ct (ΔΔCt) method. MMP-9 and TIMP-2 expression levels were analyzed using the following primer pairs; 5'-AAGGCCATGCGAACCCCACG-3', 5'-TGGAACCACGACGCCCTTGC-3' for MMP-9 and 5'-GGCAGTGTGTGGGGTCTCGC-3' , 5'-TGGGGCAGCGCGTGATCTTG-3 for TIMP-2.

Statistical analysis

Chi square test was used to test the control samples for Hardy-Weinberg equilibrium (HWE). The association of rs3918242 and rs8179090 genotypes with UBC risk was assessed using logistic regression for the allelic, additive and dominant models. Relative expression levels were evaluated with Student's t test for the comparison between tumors and cancer free tissue. Significant alterations in expression levels in relation to the invasion and metastasis were also assessed with the

Table 1. Primer sequences and restriction enzymes.

SNP	rs3918242	rs8179090
Forward primer	GCCTGGCACATAGTAGGCCC	CGTCTCTTGTTGGCTGGTCA
Reverse primer	CTTCCTAGCCAGCCGGCATC	CCTTCAGCTCGACTCTGGAG
Restriction enzyme	SphI	BsoBI

Table 2. Genotype distributions.

SNP	Genotype	Patients	Controls	Invasive	Non-invasive	Metastatic	Non-Metastatic
	CC (n)	60	56	32	28	27	33
rs3918242	CT (n)	34	40	23	11	15	19
	TT (n)	6	8	5	1	2	4
	GG (n)	65	70	30	35	31	34
rs8179090	CG (n)	31	28	14	17	13	18
	CC (n)	4	2	1	3	2	2

Student's t test. The association of the different genotypes with expression levels were examined using one-way ANOVA. All p-values were two sided and a p-value of 0.05 was considered significant. All analyzes were performed using SPSS v21.

Results

Genotype distributions

HWE held for both SNPs ($p > 0.05$). Variant genotype frequency for rs3918242 was 0.08 and was most similar to Pacific Rim population with a frequency of 0.081 (25). rs8179090 variant genotype had a frequency of 0.02, being most similar to the Korean population (26). Genotype distributions for both SNPs can be seen in Table 2.

Association of rs3918242 and rs8179090 genotypes with bladder cancer susceptibility

Variant genotype (TT) for rs3918242 polymorphism was not associated with bladder cancer susceptibility under any model. rs8179090 variant genotype was also not associated with bladder cancer risk (Table 3).

Association of rs3918242 and rs8179090 genotypes with bladder cancer prognosis

rs3918242 genotype was significantly associated with tumor invasion. This association was most prominent when tumors were grouped according to their allele positivity (dominant model), (OR = 2.667, 95%CI = 1.145-6.210, p = 0.02129). In contrast with this, rs8179090 genotype has not shown a significant association with tumor invasion (Table 4). When the tumors were grouped according to their metastatic status, both SNPs failed to show a significant association (Table 5).

Table 3. Association of rs3918242 and rs8179090 with bladder cancer susceptibility.

rs3918242			
	OR	95%CI	P value
CC		Ref.	
CT	0.793	0.442-1.423	0.43727
TT	0.700	0.229-2.144	0.53074
CC vs (CT+TT)	0.778	0.446-1.356	0.37499
C		Ref.	
T	0.811	0.517-1.271	0.36028
rs8179090			
	OR	95%CI	P value
GG		Ref.	
CG	1.192	0.646-2.200	0.57333
CC	2.154	0.382-12.157	0.37459
GG vs (CG+CC)	1.256	0.694-2.274	0.45034
G		Ref.	
C	1.272	0.760-2.129	0.35966

Table 4. Association of rs3918242 and rs8179090 with bladder tumor invasion.

rs3918242			
	OR	95%CI	P value
CC		Ref.	
CT	2.390	0.992-5.758	0.04978
TT	5.714	0.629-51.888	0.08677
CC vs (CT+TT)	2.667	1.145-6.210	0.02129
C		Ref.	
T	2.410	1.178-4.928	0.01426
rs8179090			
	OR	95%CI	P value
GG		Ref.	
CG	0.961	0.407-2.269	0.92728
CC	0.389	0.038-3.938	0.40908
GG vs (CG+CC)	0.875	0.382-2.003	0.75195
G		Ref.	
C	0.818	0.402-1.662	0.57818

MMP-9 expression

MMP-9 was upregulated in tumors in comparison to cancer free tissues with a fold change of 7.078 (95%CI: 3.08-11.07, p=0.0264). Significant increase in the expression of MMP-9 was also observed in invasive or metastatic tumors in comparison to non-invasive or non-metastatic tumors, in association with worsening prognosis (Table 6). Finally the control samples were

Table 5. Association of rs3918242 and rs8179090 with metastatic status.

rs3918242			
	OR	95%CI	P value
CC		Ref.	
CT	0.965	0.414-2.250	0.93411
TT	0.611	0.104-3.595	0.58301
CC vs (CT+TT)	0.903	0.403-2.025	0.80512
C		Ref.	
T	0.867	0.445-1.690	0.67468
rs8179090			
	OR	95%CI	P value
GG		Ref.	
CG	0.792	0.334-1.879	0.59658
CC	1.097	0.146-8.264	0.92854
GG vs (CG+CC)	0.823	0.360-1.882	0.64356
G		Ref.	
C	0.886	0.438-1.793	0.73641

Table 6. Fold changes in MMP-9 and TIMP-2 genes.

	MMP-9			TIMP-2		
Comparison	**FC**	**95%CI**	**P**	**FC**	**95%CI**	**P**
Polymorphic vs Nonpolymorphic	1.7859	1.63-1.95	0.00015	0.9482	0.83-1.07	0.470
Tumorvs Cancer Free	7.078	3.08-11.07	0.0264	2.434	1.21-3.66	0.0375
Invasive vs Non-Invasive	3.0951	1.50-4.69	0.0374	2.3729	1.10-3.64	0.071
Metastatic vs Non- Metastatic	4.0935	3.51-4.68	0.00027	1.9453	1.79-2.11	0.00083

categorized in two groups according to the existence of variant allele and samples with homozygote wild-type genotype were compared to samples with homozygote variant or heterozygote genotype (CC vs (CT+TT)). This comparison indicated a significant upregulation of MMP-9 in samples with a variant allele (Figure 1).

TIMP-2 expression

TIMP-2 expression was significantly increased in tumors in comparison to cancer free tissue and in metastatic tumors in comparison to non-metastatic tumors. No significant deregulation was observed for the comparison of polymorphic / non-polymorphic samples and invasive/ non-invasive tumors (Table 6).

Discussion

Differential diagnosis between muscle non-muscle invasive and invasive bladder carcinoma of urinary bladder is very important in terms of deciding the appropriate treatment, because the first one requires relatively minimally invasive intervention, while the second one necessitates a more aggressive surgical approach. In this study we aimed to study the role of MMP9 and TIMP2 promoter polymorphism and mRNA expression in bladder cancer tissues. Invasion is an important step that occurs in all solid cancers against which the extracellular matrix (ECM) provides an important barrier. A family of extracellular matrix degrading enzymes, namely the matrix metalloproteinases (MMPs), are able to degrade the components of the ECM and basement membranes (collagen, elastin, and gelatin) and facilitates tumor cell dissemination. The MMP family includes at least 28 members, representing a large class of multi-domain, zinc-dependent endopeptidases. All MMPs share homologous amino acid (AA) sequences, with conserved specific domain structures, related to their substrate specificity (27). MMP 2 and 9 (gelatinase) degrade type IV collage, which is the major component of the basement membrane. These MMPs are frequently associated with the malignant pheno¬type, and expression of these has been found to be elevated in several human malignancies and associated with aggressive behaviour and low overall survival (28, 29).

Different forms of MMPs and their specific inhibitors have been studied in bladder cancers and found positively associated invasive phenotype and poor survival. In most of these studies serum and urine samples have been used (30-37). Ricci et al. evaluated MMP-2, MMP-9, TIMP-1, TIMP-2, NGAL and MMP-9/NGAL complex in urine and sera from patients with bladder cancer and found that urinary TIMP-1 and serum NGAL levels may be useful non-invasive biomarkers for bladder cancer disease management (84), similarly Ramón de Fata et al found a positive correlation between the TIMP-2 and MMP-9 levels in sera of UBC patients and concluded that both serum MMP-9 and TIMP-2 levels would have an utility in the prediction of the development and progression of bladder cancer, and a potential utility as molecular markers of the disease (31). In our previous study we showed that MMP-1 promoter polymorphism might be linked to susceptibility for bladder

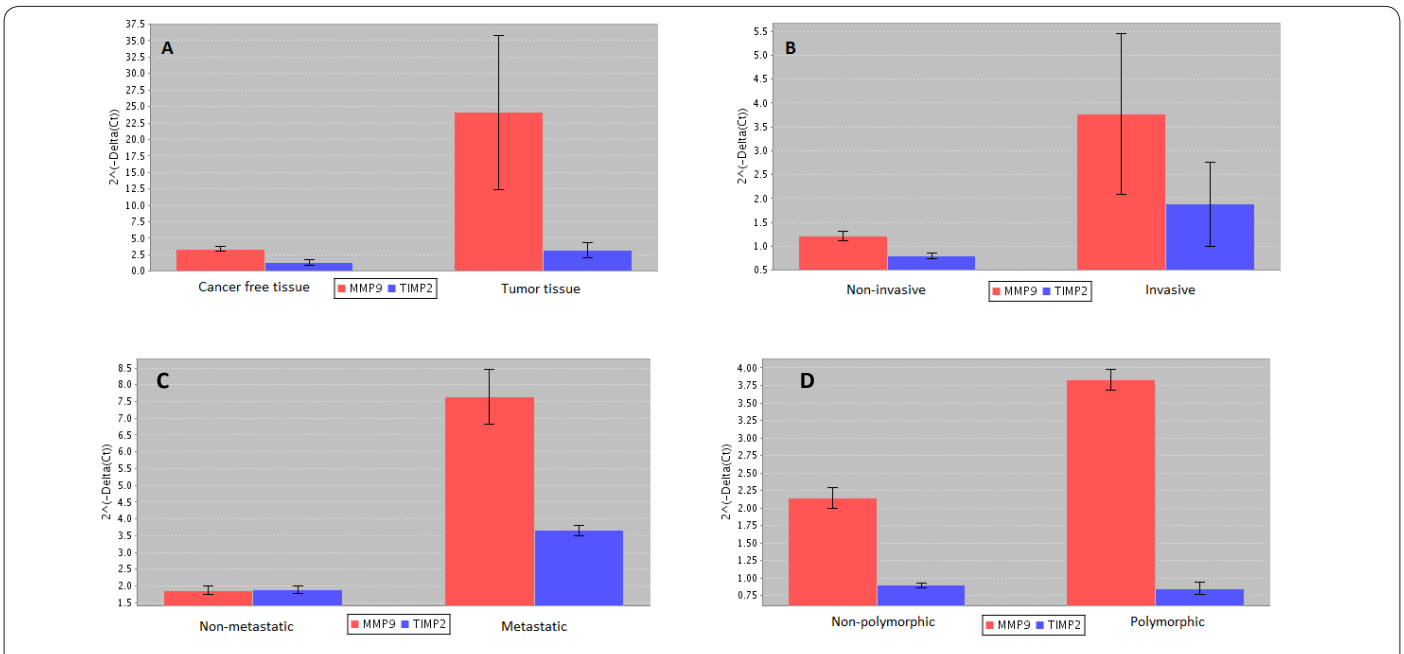

Figure 2. Normalized expression levels of MMP-9 and TIMP-2 in tumors and cancer free tissues (A), in invasive and non-invasive tumors (B), in metastatic and non-metastatic tumors (C) and in polymorphic and non-polymorphic samples (D).

cancer (38). In some studies mRNAs of certain MMPs and their inhibitors have been evaluated in cancerous tissues and adjacent normal bladder samples. Xu et al studied the level of matrix metalloproteinase-2 (MMP-2) mRNA and the tissue inhibitor of metalloproteinase-2 (TIMP-2) mRNA in transitional cell carcinoma of the bladder and reported a high level of the MMP-2 mRNA and suggested that MMP-2 to TIMP-2 ratio may play an important role in the determination of the aggressiveness and prognosis of bladder cancer (39). The expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases are also related with the recurrence of superficial transitional cell carcinoma of the bladder. Another study investigating the role of MMP-9 and TIMP-2, showed MMP-9 and TIMP-2 upregulation in the tumors of patients with recurrence compared to those without recurrence, indicating that MMP-9 and TIMP-2 expression levels may be used as marker of recurrence in patients with superficial transitional cell carcinoma of the urinary bladder (40). In our present study we have determined the mRNA expression levels of MMP-9 and TIMP-2 in bladder cancer and compared results with normal bladder tissues adjacent to the cancer in muscle-invasive and muscle-noninvasive cases. mRNA expression levels of MMP-9 and TIMP-2 are significantly higher in tumors in comparison to normal tissues. This marked upregulation was also observed in invasive tumors when compared to superficial bladder tumors.

There is a variety of studies showing the relation between the functional polymorphisms of MMPs and their specific inhibitors and solid organ cancers (21, 41-44). Basically, almost all of these studies are focused on cancer invasiveness, recurrence and cancer related survival. During the last decade there were also some clinical studies investigating the polymorphisms in MMPs and TIMPs in association with transitional cell carcinoma of the bladder (37, 45-52). However, to the best of our knowledge there is no previous study investigating the relation between MMP-9 and TIMP-2 promoter polymorphism and UBC. In our previous study we evaluated the association between MMP-1 promoter polymorphisms and invasion and metastasis in UBC (38). In our present work we investigated the rs3918242 and rs8179090 promoter polymorphisms in MMP-9 and TIMP-2 genes as well as mRNA expression levels. Variant genotype (TT) for rs3918242 polymorphism was not associated with bladder cancer susceptibility under any model. Similarly rs8179090 variant genotype was also not associated with bladder cancer risk (Table 1, 2). rs3918242 genotype was significantly associated with tumor invasion. This association was most prominent when tumors were grouped according to their allele positivity. In contrast with this, rs8179090 genotype has not shown a significant association with tumor invasion. When the tumors were grouped according to their metastatic status, both SNPs failed to show a significant association (Table 3, 4). When it comes to mRNA expression, MMP-9 was upregulated in tumors in comparison to cancer free tissues with a fold change of 7.078 (95% CI: 3.08-11.07, p=0.0264). Significant increase in the expression of MMP-9 was also observed in invasive or metastatic tumors in comparison to non-invasive or non-metastatic tumors, in association with worsening prognosis. TIMP-2 expression was significantly increased in tumors in comparison to cancer free tissue and in metastatic tumors in comparison to non-metastatic tumors. (Table5). In addition to that, samples with rs3918242 variant allele have shown 1.8 fold increased expression when compared to samples with homozygous wild type genotype. This finding is in accordance with previous studies, reporting an increased expression associated with rs3918242 MMP-9 promoter polymorphism (7). In contrast to this finding, TIMP-2 expression was not significantly different in samples with rs8179090 variant genotype.

Our findings indicate rs3918242, but not rs8179090 is significantly associated with bladder tumor invasion and increased expression. We believe that this study should be replicated with a larger sample size in addition to in vitro transcription studies to confirm our findings and provide a better understanding of the effects of MMP-9 and TIMP-2 promoter variants.

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Interest conflict
The authors declare no conflict of interest.

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