Original Article

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Vascular Endothelial Growth Factor and Thrombospondin-1 mRNA Expression in Bladder Tumors: Correlation with Histopathology and Prognosis

Bora Özveren MD, Levent Türkeri MD

Acıbadem Mehmet Ali Aydınlar University Faculty of Medicine, Department of Urology, İstanbul, Turkey

Abstract

Objective: The purpose of this study was to determine genetic expression levels of vascular endothelial growth factor (VEGF) and thrombospondin-1 (TSP-1) in fresh bladder tumor specimens and evaluate their relationship with tumor histopathological features and their prognostic value in recurrence and progression in patients with bladder cancer.

Materials and Methods: Patients who were treated for urothelial cancer of the bladder and followed for at least 6 months were included in this retrospective study. Following RNA isolation from fresh tumor tissue samples recovered from transurethral resection or radical cystectomy specimens, VEGF and TSP-1 mRNA expression was analysed by reverse transcription polymerase chain reaction (RT-PCR). The findings were examined in relation to the histopathological parameters and recurrence and progression rates of the respective tumors.

Results: Sixty-eight patients were included in the study. Mean follow-up time was 22.6 months. In patients with non-muscle-invasive urothelial bladder cancer (NMIBC), rates of recurrence and progression were 64% and 35%, respectively. RT-PCR analyses revealed VEGF mRNA expression in 29 patients (43%) and TSP-1 mRNA expression in 22 patients (32%). Recurrence and progression were observed during follow-up in 64% and 24% of the 25 NIMBC patients with positive VEGF expression, while these rates were 63% and 30% among the 30 NIMBC patients with no VEGF expression, respectively. Rates of recurrence and progression during follow-up were 70% and 30% among NIMBC patients with positive TSP-1 expression and 60% and 26% among patients with no TSP-1 mRNA expression, respectively.

Conclusion: In this study, VEGF and TSP-1 mRNA expression was not associated with histological grade or stage of bladder cancer. There was no difference in VEGF expression in tumor tissues from NIMBC patients with or without disease recurrence. Though lacking statistical significance, a positive correlation between TSP-1 expression and tumor recurrence and progression was seen among the NIMBC patients in our study. Although stimulatory and inhibitory factors are known to regulate angiogenesis, no definitive conclusions have been reached regarding their mechanism of action or the prognostic significance of their up-or down-regulation.

Keywords: Bladder, bladder neoplasms, angiogenesis, tumor markers, molecular markers, prognosis

Introduction

Like other solid cancers, bladder cancer depends on angiogenesis for progressive growth and metastasis (1). Tumors need this neovascularization feature in order to weaken the extracellular matrix and meet their migration and nutrition needs. There are many angiogenesis stimulating and suppressing factors in tumor cells and their microenvironment. During tumorigenesis, the angiogenic phenotype emerges as a result of increased expression of angiogenesis stimulating factors, reduced expression of angiogenesis suppressing factors, or a combination/interaction of both these mechanisms. Folkman (2) described this as the "angiogenic switch" (3). This transformation can cause changes in neoplastic cells ranging from accelerated growth to drug resistance, and even invasive and metastatic capabilities. Thus, exploring the complex process of angiogenesis has potential therapeutic benefits in terms of predicting the biological behavior of tumors and revealing ways to prevent angiogenesis. With the recent development of new molecular techniques, there has been an increase in the number of detailed studies on human cells and tissues at the DNA, RNA, and protein level. Beyond the general histologic structure and DNA content of tumors, advances in molecular biology, immunology, and cytogenetics have enabled us to describe tumors' distinguishing features and biological behavior. The most commonly used method for investigating the role of various tumor markers in the diagnosis

Address for Correspondence: Bora Özveren MD, Acıbadem Mehmet Ali Aydınlar University Faculty of Medicine, Department of Urology,ul, Turkey
E-mail: bora.ozveren@acibadem.edu.tr **ORCID ID:** orcid.org/0000-0001-8902-7530
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and prognosis of bladder cancer is immunohistochemistry. Although there are still standardization issues with this method that have not been overcome, it has clinical applications. Other methods such as single-strand conformation polymorphism (SSCP), DNA sequencing, or polymerase chain reaction (PCR)-based investigations are used in experimental research.

Studies on tumor angiogenesis are expected to lead to crucial advances in our ability to distinguish bladder cancer patients at risk of progression and metastasis and identify novel treatment approaches. It is known that tumors that are initially in the same histopathological category can exhibit substantial differences in terms of recurrence and progression over the course of follow-up. Pioneering studies by Chodak and Summerhayes (4) which demonstrated that pieces of bladder tumor can stimulate angiogenesis and that angiogenic factors are present at high levels in the urine of patients with bladder tumors led to further studies on the role of angiogenesis in bladder cancer (5).

In the present study, we investigated gene expression levels of vascular endothelial growth factor (VEGF), one of the main angiogenesis stimulating factors, and thrombospondin-1 (TSP-1), which is an angiogenesis suppressing factor, in tumor tissues obtained from patients with bladder cancer. These genotypic characteristics were compared with classical histopathological parameters used in bladder cancer in order to evaluate the potential role of angiogenic and anti-angiogenic factors in determining recurrence and progression in urothelial bladder cancer.

Materials and Methods

Patients who were treated for bladder cancer and followed for at least 6 months were included in this retrospective study. Archived patient information, pathology reports, and tumor follow-up records were analyzed in terms of patient age, sex, initial tumor grade, initial tumor stage, tumor recurrence, progression of tumor grade and stage, recurrent tumor grade, recurrent tumor stage, and follow-up time (months).

Changes in tumor grade and stage were evaluated using cystoscopy, transurethral biopsy/resection, computed tomography, magnetic resonance imaging, and chest X-ray. Tumor staging was done according to the TNM classification system defined by the Union for International Cancer Control and histological grade between I and III was determined according to the Mostofi system (6). Tumor tissue samples were obtained from transurethral resection or radical cystectomy specimens of patients operated between 1998-2000. Fresh tumor tissues were sent under sterile conditions in dry tissue containers to the Marmara University School of Medicine Department of Urology, Ergun Özalp Research Laboratory and RNA isolation was performed as soon as possible using Trizol®. After isolating RNA from the bladder tissue samples, reverse transcriptase polymerase chain reaction (RT-PCR) was performed using specially synthesized oligonucleotide primers (Table 1). The presence of VEGF and TSP-1 expression in the samples was investigated using RT-PCR analysis. To demonstrate the efficacy of the PCR technique and to prevent false negatives, positive controls were included in each PCR cycle (Access RT-PCR System, Promega, MI, USA). The presence of separate DNA bands for amplification products expected from PCR in agarose gel electrophoresis was regarded as a positive result. The sizes of visualized bands were estimated using a DNA molecular-weight marker (100-bp DNA ladder, Promega, MI, USA) loaded in the last well of the electrophoresis gel. Seeing no bands was considered a negative result. Because the patients had previously consented to the use of their medical data in scientific research provided that their identities were not disclosed, ethics committee approval was obtained as a retrospective study.

Statistical Analysis

Fisher's exact test was used to determine whether there were any non-random associations between the various histopathological features of tumors with and without VEGF and TSP-1 mRNA expression, and chi-square test was used to compare VEGF and TSP-1 expression levels in the NMIBC patient group ("comparison of proportions"). A p value <0.05 was considered statistically significant.

Results

Clinical and Histopathological Evaluation

There were 68 patients in the study group (47 men, 21 women). The mean age of the patients was 63 (41-80) years. Mean follow-up time was 22.6 (8-48) months.

Tumor recurrence was detected during follow-up in 57% (39/68) of all cases. The tumor recurrence rate among patients with non-muscle-invasive urothelial bladder cancer (NMIBC) was 64% (35/55). Of these patients, recurrence occurred in 57% (8/14) of those with initial tumor stage of Ta and 66% (27/41) of those with initial tumor stage of T1. Within the T1 subgroup, recurrence rate was 44% for low-grade tumors and 80% for high-grade tumors. Tumor progression was observed during follow-up in 35% (19/55) of patients with NMIBC. In terms of stage and grade, there were no statistically significant correlation between histopathological characteristics of the bladder tumors and VEGF and TSP-1 mRNA expression (Table 2).

Investigation of VEGF and TSP-1 mRNA Expression Using RT-PCR

VEGF mRNA expression was detected in 29 patients (43%) by RT-PCR (Figure 1). Of these patients, 25 had NMIBC and 4 had

Table 1. Base sequences of oligonucleotide primers synthesized for RT-PCR analysis of genes used in this study (OMIM Genome Database)
VEGF (RT-PCR)
sense primer: 5′ – CGA AGT GGT GAA GTT CAT GGA TG – 3′
antisense primer: 5′ – CCG GAA TTC ACA TTT GTT GTG CTG T – 3′
Thrombospondin (RT-PCR)
sense primer: 5′- CGG GCC GCC GCG CTC CGG TAC ACA C - 3′
antisense primer: 5′- GAG GTC CAG GGT GCC GCC TTG CCA -3′
OMIM: Online Mendelian Inheritance in Man database

Table 2. Distribution of tumors according to histopathological diagnosis in the 68 patients included in the study			
Non-muscle-invasive bladder cancer	**n**	**Muscle-invasive bladder cancer**	**n**
Ta grade 1	9	T2 grade 2	2
Ta grade 2	5	T3 grade 3	9
T1 grade 1	7	T4 grade 3	2
T1 grade 2	20	-	-
T1 grade 3	14	-	-
Low-grade	16	**Low-grade**	_
High-grade	39	**High-grade**	13

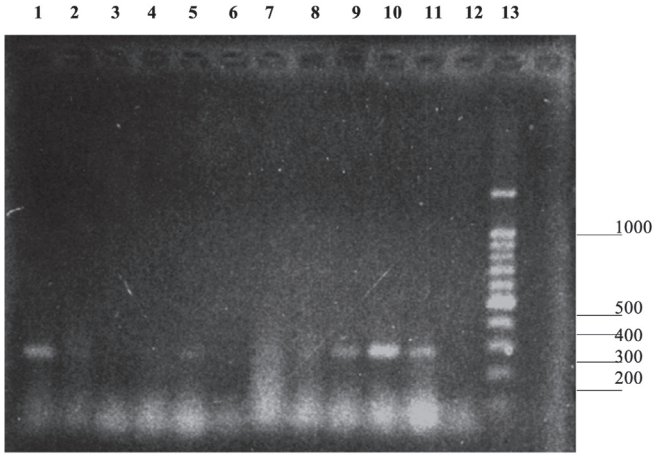

Figure 1. Image of agarose gel electrophoresis of VEGF mRNA RT-PCR products in bladder tumor samples of patient 10. Wells 1, 5, 9-11: patients with positive VEGF mRNA expression; Wells 6 and 12: negative controls; Well 13: 100-bp DNA ladder

VEGF: Vascular endothelial growth factor, RT-PCR: Reverse transcription polymerase chain reaction

MIBC (Table 3). Of the 25 NMIBC patients who were positive for VEGF, tumor recurrence was detected during follow-up in 16 (64%) while progression was detected in only 6 patients (24%). Among the 30 patients with NMIBC who did not have VEGF expression, recurrence rate was 63% (19/30) and progression rate was 30% (9/30) (Table 4). RT-PCR analysis of the tumor samples revealed TSP-1 expression in 22 patients (32%) (Figure 2). Of these patients, 20 had NMIBC and 2 had MIBC (Table 3). Among the NMIBC patients who were positive for TSP-1 expression, the tumor recurrence rate was 70% and tumor progression rate was 30% during follow-up (Table 4). Of the 35 NMIBC patients who tested negative for TSP-1 expression, the tumor recurrence rate was 60% and progression was observed in 26% of the patients.

Discussion

The generation of new blood vessels in tumors is dependent on the equilibrium between angiogenic and anti-angiogenic factors in the environment. As in many solid tumors, the development of angiogenesis is also an important step in the pathogenesis of bladder cancer (7). A strong correlation has been detected between tumoral microvessel density and progressive pathological findings and poor prognosis in patients with bladder cancer (8). Among the various mechanisms that can affect the angiogenic switch in bladder tumors, the most

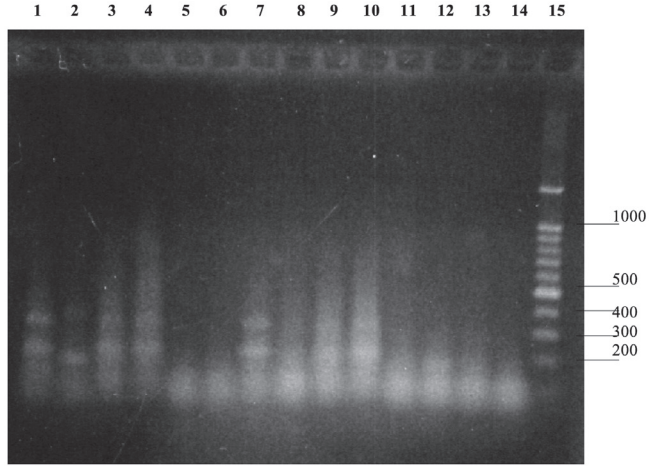

Figure 2. Image of agarose gel electrophoresis of TSP-1 mRNA RT-PCR products in bladder tumor samples of patient 13. Wells 2-4, 7, 10: patients with TSP-1 mRNA expression; Well 12: negative control; Well 15: 100-bp DNA ladder

TSP: Thrombospondin, RT-PCR: Reverse transcription polymerase chain reaction,

Table 3. Histopathologic distribution of tumors with and without VEGF and TSP-1 mRNA expression							
		VEGF (+)	**VEGF (-)**	**p value**	**TSP-1 (+)**	**TSP-1 (-)**	**p value**
Stage	Ta	5	9	-	6	8	-
	T1	20	21	-	14	27	-
	>T2	4	9	-	2	11	-
	NMIBC	25	30	0.37	20	35	0.20
	MIBC	4	9		2	11	
Degree	G1	6	10	-	6	10	-
	G2	15	12	-	11	16	-
	G3	8	17	-	5	20	-
	LG	6	10	0.78	6	10	0.76
	HG	23	29		16	36	

VEGF: Vascular endothelial growth factor, NMIBC: Non-muscle-invasive urothelial bladder cancer, MIBC: Muscle-invasive urothelial bladder cancer, TSP: Thrombospondin, LG: Low grade, HG: High grade

Table 4. VEGF and TSP-1 expression rates in the NMIBC patient group						
	VEGF (+)	**VEGF (-)**	**p value**	**TSP-1 (+)**	**TSP-1 (-)**	**p value**
NMIBC	25	30	-	20	35	-
Recurrence	64% (16/25)	63% (19/30)	0.96	70% (14/20)	60% (21/35)	0.46
Progression	40% (10/25)	30% (9/30)	0.44	30% (6/20)	26% (9/35)	0.73

VEGF: Vascular endothelial growth factor, NMIBC: Non-muscle-invasive urothelial bladder cancer, TSP: Thrombospondin

prominent are overexpression of stimulating factors and/or loss of endogenous suppressive factor production (9). These factors can be produced by tumor cells, secreted from the surrounding extracellular matrix and tumor-related stromal cells, or may be products of inflammatory cells infiltrating the tumor. The most frequently studied stimulants of angiogenesis in bladder cancer are VEGF, thymidine phosphorylase, matrix metalloproteinases (basic fibroblast growth factor b-FGF), carbonic anhydrase 9, and cyclo-oxygenase 2, while the most studied suppressors include angiostatin, endostatin, p53 and thrombospondin-1 (11).

In this study, we investigated mRNA expression levels of VEGF and TSP-1, which are known to have important roles in angiogenesis stimulation and suppression mechanisms, in bladder tumor tissues and its relationship with histopathological classification and prognosis of bladder cancer. In our study, RT-PCR analysis of tumor tissues with histologic features of NMIBC and MIBC obtained from a total 68 patients revealed VEGF expression in 43% of the tumors. The proportion of tumors positive for VEGF expression was higher in patients with NMIBC (45% vs 31%) and in higher grade tumors (44% vs 37.5%). When NMIBC patients were evaluated separately, it was found that tumors with and without VEGF expression had tumor recurrence rates of 64% and 63%, and progression was observed in 40% and 30%, respectively. Comparison of these proportions yielded no statistically significant differences. Campbell et al. (12) observed no significant difference in VEGF levels determined using immunostaining in normal urothelium versus NMIBC and invasive bladder cancer tissues. However, Crew et al. (13) detected higher VEGF concentrations in the urine of bladder cancer patients compared to controls and reported that urine VEGF levels identified using ELISA correlated with recurrence rates in patients with Ta and T1 tumors. Another study of 62 patients with long-term follow-up showed that high initial serum VEGF level had predictive value for overall and cancer-related mortality and could identify high-risk patients who would benefit from preventive treatment (14). However, in another study on 185 patients with Ta/T1 tumor, VEGF expression detected using immunohistochemical method was not associated with bladder cancer recurrence risk or survival (15). In a multivariate analysis of 55 patients with NMIBC who were treated with neoadjuvant MVAC chemotherapy and radical cystectomy, Inoue et al. (16) reported that VEGF expression detected using *in situ* hybridization was an independent prognostic factor for disease recurrence. In studies by O'Brien et al. (17,18), it was reported that VEGF expression was correlated with more aggressive phenotype in the non-invasive tumor subgroup, and that high VEGF expression levels increase the probability of recurrence in low-grade T1 tumors. In our study, RT-PCR analysis of fresh tumor tissues from NMIBC patients revealed no difference in VEGF mRNA positivity between tumors with and without recurrence (46% vs 45%). When evaluated in light of data from the literature cited above, these results suggest that the prognostic value of VEGF expression in bladder cancer has not been clarified to date. There may be several reasons for this. Expression of angiogenesis stimulating factors in bladder cancer can be measured both at the transcriptional and protein level. However, when interpreting results obtained *in vitro*, it must be kept in mind that any contributions from other factors that mediate the angiogenic process or the effects of complex stromal-epithelial interactions and enzymes that cause matrix degradation cannot be taken into account. Furthermore, increased expression (up-regulation) of an angiogenic factor alone is not sufficient for a tumor to become angiogenic. Reduced expression (down-regulation) of certain negative regulators or vascular growth suppressing factors is required. The mechanisms involved in changing the balance between angiogenesis stimulating and suppressing regulators have not been clearly established.

TSP-1 is an extracellular matrix glycoprotein known to be a potent inhibitor of angiogenesis. The role of TSP-1 in tumor angiogenesis and its mechanisms of action are both complex and controversial. In our study, TSP-1 mRNA expression was detected in one-third of all patients. Further analysis of patients with NMIBC showed that recurrence and progression rates were 60% and 26% in patients negative for TSP-1 expression, whereas these rates were 70% and 30% among those positive for TSP-1 expression. In the NMIBC group, TSP-1 mRNA expression was detected in 40% of recurrent tumors and in 30% of tumors that did not recur during follow-up. Although the results of our study were not statistically significant, they suggest that TSP-1 expression may be correlated with tumor recurrence and risk of progression.

In fact, the definitive role of TSP-1 in tumor angiogenesis and progression is a controversial issue: both stimulating and suppressive effects of TSP-1 have been reported in the literature (19-21). In their study on 163 cystectomy specimens, Grossfeld et al. (22) demonstrated using immunohistochemical methods that TSP-1 expression was an independent marker of disease recurrence and overall survival in patients classified according to bladder tumor stage, lymph node status, and histological grade. In addition, it was shown that TSP-1 expression in invasive bladder cancer patients was negatively correlated with p53 expression and microvessel density, and it was suggested that reduced expression of TSP-1, which is a suppressive factor, increases the generation of new vessels in these patients. In line with these findings, Bochner et al. (23) demonstrated that increasing TSP-1 expression in bladder cancer cell cultures resulted in reduced microvessel density and tumor growth arrest. Mutations in oncogenes and tumor-suppressor genes in tumor cells are usually associated with decreased TSP-1 expression. On the other hand, TSP-1 produced by stromal fibroblasts, endothelial cells and immune cells also inhibits tumor progression (24). However, in studies by Qian and Tuszynski (25) it was reported that high levels of TSP-1 mRNA and protein expression in particular have a stimulating effect on invasive tumor biology. The effect of TSP-1 on angiogenesis depends on TSP-1 level, the presence and level of angiogenic stimulants such as bFGF in the tissues, as well as the location of TSP-1 (26). There are various in vitro and in vivo studies in the literature demonstrating that TSP-1 can be negatively correlated with poor prognosis or has no prognostic value (27-29). Our results, although not significant, suggest that TSP-1 expression in NMIBC can indicate poor prognosis. These contradictory observations may be attributable to the complex structure of the TSP-1 protein and its different behaviors specific to various

cell types. The up-regulation of matrix degradation enzymes and inhibitors by TSP-1 may explain both its stimulatory and inhibitory effects. The interaction of signals reaching different tumor and host cells may result in varying response to TSP-1 (30). Discrepancies in the results of these studies demonstrate that there are not yet enough data to elucidate the mechanisms through which TSP-1 expression affects angiogenesis and the biological behavior of tumors.

Inconsistencies in the results of both the current study and other studies in the literature may be related to the limited numbers of patients with various tumor types or to potential methodological flaws such as patient selection criteria, heterogeneous treatment methods, insufficient follow-up time, selection of antibodies used in endothelial staining, the tumor section examined, the researchers' experience, and statistical methods used.

Study Limitations

Limitations of our study include the short mean follow-up time, small patient population, and its retrospective design. Treatment methods used in our heterogeneous patient group might have affected prognosis. Moreover, characteristics of the tumor specimen (peripheral or central sampling of the mass), specimen preparation, and other technical procedures might have affected the results of RT-PCR analyses.

Conclusion

As with other tumors, angiogenesis is profoundly important for the nutrition, growth, invasion, and progression of tumor cells in bladder cancer. The mechanisms of action of the angiogenesis stimulating and suppressing factors involved in the angiogenetic switch, as well as the prognostic value of their up- or down-regulation, have not been definitively determined. However, future studies conducted in bladder tumor tissues may enable identification of tumors with angiogenic phenotype in bladder cancer. In this way, not only will angiogenic factors gain value as prognostic markers, but the mechanisms involved in this process may become targets for novel therapeutic approaches targeting the prevention of cancer progression and metastasis.

Ethics

Ethics Committee Approval: Retrospective study.

Informed Consent: Retrospective study.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: L.T., B.Ö., Design: L.T., B.Ö., Data Collection or Processing: B.Ö., Analysis or Interpretation: B.Ö., L.T., Literature Search: B.Ö., Writing: B.Ö.

Conflict of Interest: No conflict of interest was declared by the authors.

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