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Original Article

SPP1A and *VEGFC* splice isoforms as predictive diagnostic biomarkers for high and low-grade bladder cancer

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Abstract: Objectives: This study aimed to investigate the expression of *Vascular endothelial growth factor (VEGF)* and *Secreted phosphoprotein-1 (SPP1)* isoforms in bladder cancer tissue and their correlation with tumor grade and muscle invasion. Methods: In a prospective study, we examined 40 patients who had been diagnosed with bladder cancer. The diagnosis was confirmed through cystoscopy, biopsy, and histopathology. We used the RT-PCR method to measure the levels of human *SPP1* and *VEGF* splice isoforms. Statistical analysis of the average of three replicates was performed using SPSS for Windows version 23.0. Results: The study revealed a significant correlation between patients' histological grades and muscle invasiveness. Additionally, the investigation of 5 isoforms in patients showed that *SPP1A*, *SPP1B*, and *VEGFA* isoforms were significantly associated with tumor grade. However, the *SPP1C* and *VEGFC* isoforms showed no significant association with tumor grade. A new index was calculated based on the logistic regression model (mean_*SPP1A* - (mean_*VEGFC* * 0.7)), and the cut-off and the Area Under the ROC curve (AUC) were 23.1 and 0.951, respectively. Conclusions: The relationship between the grade of bladder cancer and the two isoforms of SPP1A and VEGFC shows that these indicators could help pathologists differentiate between high and low-grade bladder cancer and potentially be used as predictive markers.

Keywords: *VEGF*, *SPP1*, bladder cancer, tumor grade, invasiveness

Introduction

Bladder cancer is the 10th most common cancer worldwide, with over 573,000 new cases reported in 2020. If not diagnosed and treated promptly, it can lead to a high mortality rate [1, 2]. Bladder cancer is classified into non-muscle invasive bladder cancer (NMIBC: Tis, Ta, and T1) and muscle-invasive (MIBC: T2, T3, T4). This classification based on whether the tumor infiltration extends to the wall of the muscular bladder [3].

On the other hand, bladder cancer has become a prevalent form of cancer among elderly individuals globally. Therefore, it is crucial to identify and develop new markers for effective treatment and management of bladder cancer. As of now, there are no clinical factors that can accurately predict the recurrence or progression of tumors. Despite radical cystectomy for invasive bladder cancer, up to 50% of patients progress to metastases resulting in a low 5-year survival rate [4-6].

Current methodologies for forecasting recurrence and progression predominantly in NMIBC rely on scoring systems that incorporate clinical and histopathological indicators. This approach often overlooks additional biomarkers that may enhance the precision of personalized risk evaluations. The assessment of patient outcomes in NMIBC, especially concerning recurrence, has primarily centered on various biomarkers, including radiomics, histopathology, clinical data, and genomic information. Conversely, pathologists encounter challenges in differentiating "gray zone" tumors from both high-grade

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and low-grade bladder cancer. This has led to ongoing research into alternative markers that could be practical predictive tools [7].

Based on various studies conducted on bladder cancer tissue, blood, and plasma and using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the integrated Differentially Expressed Genes (DEGs), the Vascular Endothelial Growth Factor (*VEGF*) gene has been selected as one of the most important genes. *VEGF* upregulation in bladder cancer associated with poor prognosis, shorter survival, and a higher likelihood of progression [8, 9].

Also, Osteopontin, Secreted phosphoprotein-1 (*OPN*, *SPP1*) is a crucial gene in cancer progression, promoting angiogenesis through activation of the *Brk/NF-κB/ATF-4* signaling pathway [10-12]. Furthermore, *SPP1* is important in mediating the tumorigenic process and can cause *VEGF* accumulation through autocrine and paracrine mechanisms. Subsequently, *VEGF* promotes tumor formation and development and tumor angiogenesis [13-16].

SPP1 has been identified as a factor that enhances the survival of cancer cells. Notably, *SPP1* exhibits differential expression during the initial phases of bladder cancer progression and correlates with a poor prognosis. The interactions between *SPP1* and various proteins or genes, as well as its relationship with immune cell infiltration, may significantly contribute to the disease's pathogenesis, suggesting that *SPP1* could be a promising target for therapeutic intervention in bladder cancer [12, 17].

Researchers have identified the urinary biomarker *VEGF* as a significant differentiator between patients with non-muscle invasive bladder cancer (NMIBC) and healthy individuals. This biomarker achieved an area under the curve (AUC) of 0.968, with a 95% confidence interval ranging from 0.942 to 0.992. Additionally, the biomarker demonstrated a sensitivity of 90% and a specificity of 97%. Additionally, other studies have indicated a correlation between high levels of *VEGF* and both the recurrence of bladder cancer and the grade of primary tumors. Consequently, it has been proposed that urinary *VEGF* could enhance the precision of clinical risk assessments, such as the European Organisation for Research and Treatment of Cancer (EORTC) bladder cancer risk scale [18]. Research has demonstrated that the levels of *VEGF* and *SPP1* are significantly elevated in metastatic tumors compared to non-metastatic tumors in urothelial carcinoma (UCC) of the urinary bladder [19, 20].

SPP1 and *VEGF* are significant prognostic indicators in bladder cancer, highlighting their potential downstream targets. Furthermore, the mRNA levels of *VEGF-A* have been identified as a promising prognostic marker for the progression of bladder cancer, along with the varying expression levels of its splice variants [21]. Additionally, *SPP1* exhibits differential expression during the early stages of bladder cancer development. The interplay between *SPP1*, particularly its C isoform, and various proteins or genes, as well as immune cell infiltration, may play a crucial role in the disease's pathogenesis. This suggests that *SPP1* could represent a viable therapeutic target for bladder cancer [17].

Since *SPP1* increases *VEGF* secretion, it creates a synergistic effect. However, no studies have investigated the relationship and expression of different isoforms of these two genes on the grade and stage of bladder tumors in a patient's tissue. Therefore, this study aimed to investigate the expression of *VEGF* and *SPP1* isoforms in bladder cancer tissue and their correlation with tumor grade and muscle invasion.

Material and methods

This study was approved by the Tehran University of Medical Sciences ethics committee. The inclusion criteria in the study were as follows: patients had to provide informed consent for the use of their medical information, undergo radical cystectomy (RC), and receive a pathological diagnosis of muscle-invasive urothelial carcinoma of the bladder. Preoperative imaging tests must have confirmed the absence of distant metastasis. Furthermore, patients included in the study must be between 18 and 80 years old and should have complete medical records. Exclusion criteria have been established to maintain the integrity of the study. Patients who did not consent to participate were excluded, as were those who had other concurrent malignant tumors. Furthermore, individuals exhibiting acute or chronic inflammatory conditions or diseases affecting the

blood or immune system were disqualified before the preoperative blood draw. Patients who had received neoadjuvant or adjuvant chemotherapy, as well as those with a follow-up period of less than four months, were also excluded from the study. The study was conducted between January 2021 and April 2023. Forty bladder carcinoma tissues were prepared for Real-Time PCR examination.

Ethical approval

The patients provided written informed consent and the study adhered to the principles of the Helsinki Declaration. The Ethics Committee at Tehran University of Medical Sciences approved the study protocol (IR.TUMS.SINAHOSPITAL.REC.1399.081).

Genes selection

Bladder cancer is associated with the *VEGF* gene, according to many research, www.disgenet.org, Human Disease Blood Atlas - SPP1, VEGF - The Human Protein Atlas (HPA) database (https://www.proteinatlas.org/), Gene MANIA report, Application version: 3.6.0 (http://genemania.org), GEPIA database, (http://gepia2.cancer-pku.cn/#analysis) and the KEGG pathway enrichment analysis of the integrated DEGs (https://www.genome.jp/kegg-bin/show_pathway?hsa05219) and (https://www.genome.jp/pathway/hsa04370 +7422).

All patients who were referred to the hospital for examination of their bladder disease and whose pathology report had identified TCC bladder cancer with different grades were included in the study. After obtaining consent from the patients, a part of the tissue sent for pathology was used for examination in this project. In total, 40 patients participated in the study, consisting of 33 men and 7 women aged between 37 and 98.

Based on the formula for determining the sample size n = (16 * s^2)/d^2, and using the estimate of fold change = 2 and the estimate of standard deviation s = ⟦log⟧_2^5 according to the article Rudland, Philip S., et al. "Prognostic significance of the metastasis-associated protein osteopontin in human breast cancer". Cancer research 62.12 (2002): 3427-3417, the sample size that needs to be obtained was estimated to be 37, which, taking into account a 5% attrition and 90% power, the final sample size was considered equal to 40.

Our investigation of bladder cancer patients' samples focused on the isoforms of *SPP1 and VEGF* since these two genes are known to play essential roles in cancer progression and angiogenesis (**Figure 1**). Spearman test analyzed the correlation coefficient of *SPP1* and *VEGF* genes in bladder cancer in the *GEPIA* (Gene Expression Profiling Interactive Analysis) database, and a significant relationship between these two genes was seen in bladder cancer (*p*-value = 0.018). Despite finding a connection between these two genes and bladder cancer, no data was obtained about the isoforms of these genes. Therefore, we investigated the role of both genes in bladder cancer.

Total RNA extraction and cDNA synthesis

We extracted total RNA from bladder cancer tissues using the High Pure RNA Isolation Kit (Cat. No. 11 828 665 001). For cDNA synthesis, we used the Easy cDNA Synthesis Kit according to the manufacturer's protocol. The RNA concentration of each sample was measured using Nanodrop. We calculated the relative expression of genes using the $2^{-\Delta\Delta CT}$ method. The primers used for the Rotor-Gene Q are listed in **Table 1**.

Statistical analysis

SPP1A, SPP1B, SPP1C, VEGFA, and VEGFC expression levels were determined using the cycle threshold and Real-time PCR efficiency in bladder cancer tissues. The *GAPDH* gene was used as the housekeeping gene for data normalization. The Mann-Whitney and Kruskal-Wallis nonparametric tests were used to determine the significance of *SPP1* and *VEGF* isoforms across different cancer grades and stages. Logistic regression was then used to adjust for sex and smoking to identify the most effective model for distinguishing between high-grade and low-grade cancer. In the final step of the analysis, we used a two-graph receiver operator characteristic curve (or two-graph ROC curve) to determine the best cut off indicators, along with their associated sensitivity, specificity, positive predictive value, and negative predictive value. A *P*-value of less than 0.05 was considered significant in all statistical analyses. The statistical analyses were carried

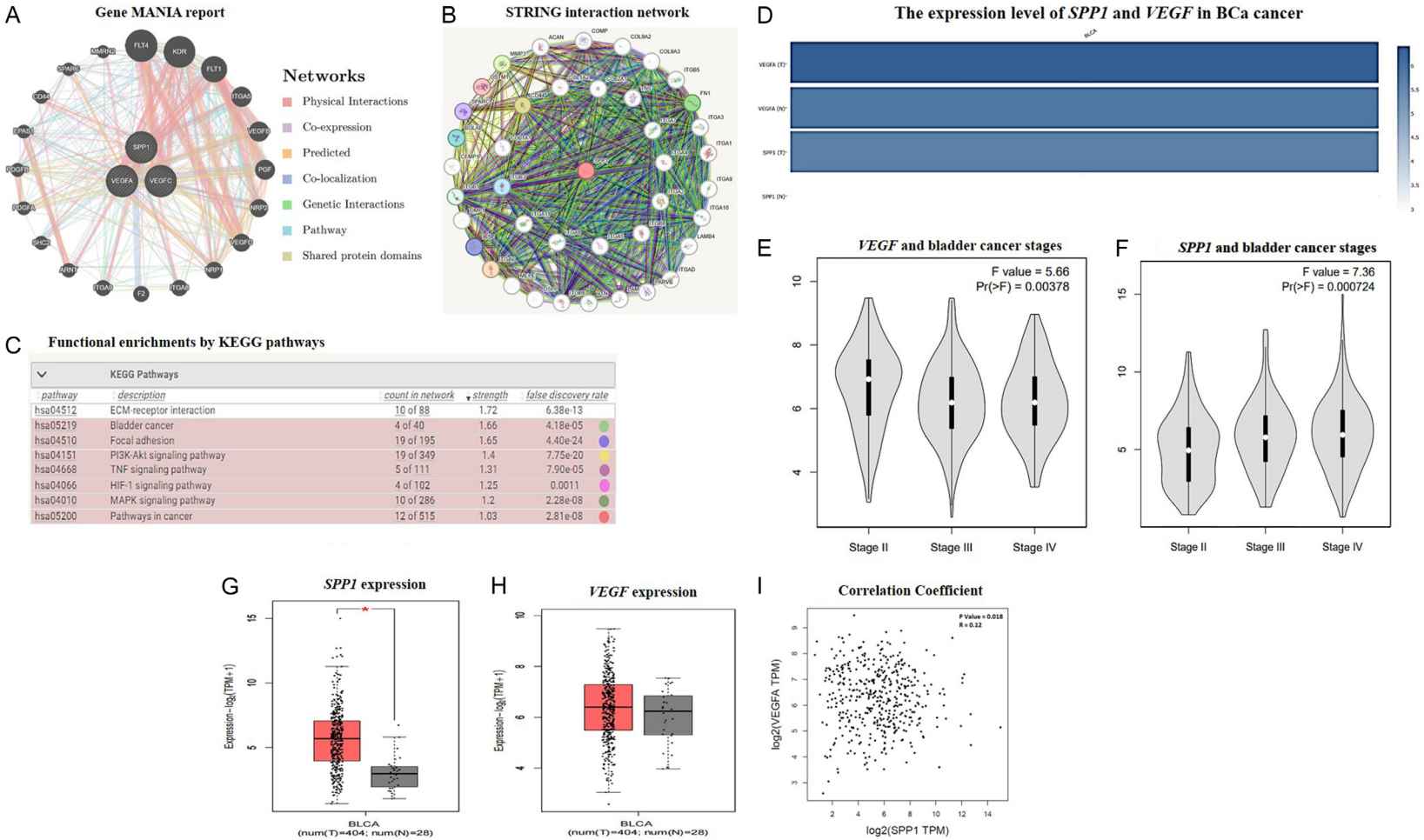

Figure 1. Relationship between *SPP1* and *VEGF* genes. (A) Gene MANIA report, Application version: 3.6.0 (http://genemania.org/search/homo-sapiens/SPP1/VEGFa/vegfc//); (B) STRING interaction network (string-db.org); (C) Functional enrichments by KEGG pathways in B network (https://string-db.org/cgi/network?taskId=bj3pIIPbVnmL&sessionId=bITXqMODcONK); (D) The expression level of *SPP1* and *VEGF* in BCa cancer in GEPIA database (http://gepia2.cancer-pku.cn/#analysis). Pathological Stage Plot: (E) (*VEGF* and Bladder cancer); (F) (*SPP1* and Bladder cancer); (http://gepia2.cancer-pku.cn/#analysis). Box plot-log2 (TPM + 1) for log-scale: (G and H) expression *SPP1* and *VEGF* in tumor and normal bladder tissue(http://gepia2.cancer-pku.cn/#analysis). (I) Correlation Coefficient for *SPP1* and VEGF in Bladder cancer analyzed GEPIA database (http://gepia.cancer-pku.cn/detail.php?gene=SPP1).

Table 1. Primer sequences of targets and normalizer genes

Gene	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')
SPP1A	NM_001040058.1	ATCTCCTAGCCCACAGCAAT	CATCAGACTGGTGAGATCATC
SPP1B	NM_000582.2	ATCTCCTAGCCCCAGACGAC	AAATCAGTGACCAGTTCATCAG
SPP1C	NM_001040060.1	TGAGGAAAAGCAGAATGCCTG	GTCAATGGAGTCCTGGCTGT
VEGFA	NM_001316955.1	CTCACCAAGGCCAGCACATCAG	ATCTGGTTCCGAAAACCCTGG
VEGFC	NM_005429.4	GTCTGTGTCCAGTGTAGCATG	AGGTAGCTCGTGCTGGTGTT
GAPDH	NM-001289746.1	GTGAACCATGAGAAGTATGACCAC	CATGAGTCCTTCCACGATACC

Table 2. Clinical, and histological characteristics and distribution of the patients with bladder carcinoma according to smoking habits

	Low Grade 17(42.5%)	High Grade 23 (57.5%)	Total N (%)
Age	66.85 (59-79)	67.84 (37-98)	-
Sex (Men)	14 (82.4%)	19 (82.6%)	33 (82.5%)
smoking habit (Yes)	5 (29.4%)	18 (78.3%)	22 (55.0%)

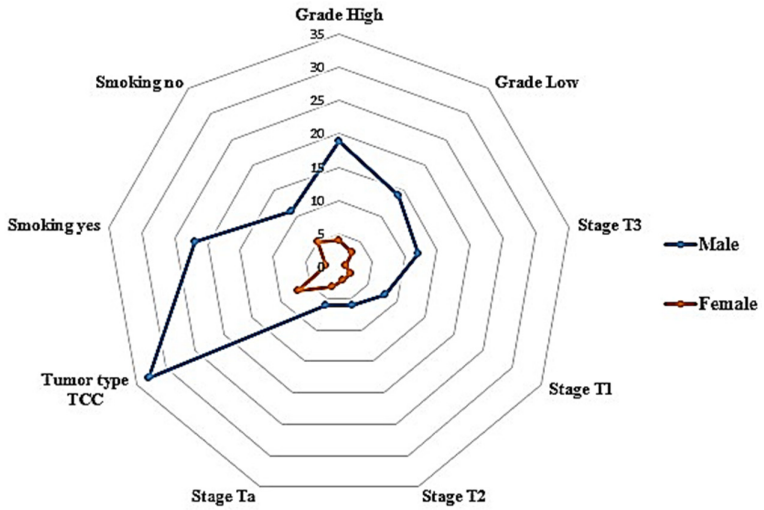

Figure 2. Clinical and histological characteristics and distribution of the patients.

out using SPSS 23.0 software. All tests were repeated three times, and the mean of the data was used in the analysis.

Results

Complete clinical and histological data were available for 40 patients (33 men (82.5%) and 7 (17.5%) women, with a median age 66 years [range: 37-98]). Pathological staging revealed NMIBC in 17 (7 pTa, 10 pT1) and MIBC in 23 patients (9 pT2, 14 pT3). The patients' clinical and histological characteristics and distribution according to smoking habits are presented in **Table 2** and **Figure 2**.

The relationship between the expression of SPP1 and VEGF isoforms with the grade and muscle invasion of bladder carcinoma

Tables 3 and **4** show the distribution of 5 indicators in all samples and separately by grade and stage. As can be seen in **Tables 3** and **4**, it is evident that when analyzing the expression of *SPP1* and *VEGF* isoforms in relation to bladder carcinoma grades and stages, *SPP1A* (p-value < 0.001) and *VEGFA* (p-value = 0.019) were significantly associated with tumor stages, In contrast, *SPP1B* (p-value = 0.080), *SPP1C* (p-value = 0.491), and *VEGFC* (p-value = 0.133) showed no significant association (**Table 3**). In contrast, two of the three isoforms of the *SPP1* gene, *SPP1A* (p-value < 0.000) and *SPP1B* (p-value = 0.012), were significantly associated with tumor grade; however, *SPP1C* (p-value 0.199) was non-significant. In comparison, only *VEGFA* (p-value = 0.003) was significantly associated with tumor grade, whereas *VEGFC* (p-value = 0.432) was not significant (**Table 4**). This study found a statistically significant relationship (p-value < 0.05) between various histological grades, muscle invasiveness, mean_*SPP1A*, and mean_*VEGFA* in all patients (**Table 3**).

Table 5 displays the area under the curve (AUC) for five different indicators. Three of these indi-

Table 3. Distribution of 5 indicators in all samples by stages of bladder carcinoma (n = 40)

		N	Mean	Std. Deviation	PV
Mean_SPP1A	Ta	7	26.6829	2.99132	0.000*
	T1	10	25.3220	2.94865	
	T2	9	21.8244	3.90606	
	T3	14	19.6579	3.24335	
	Total	40	22.7908	4.25674	
Mean_SPP1B	Ta	7	26.9321	2.94676	0.080
	T1	10	27.1445	2.90681	
	T2	9	24.8089	3.98502	
	T3	14	23.5293	4.20085	
	Total	40	25.3165	3.86744	
Mean_SPP1C	Ta	7	25.3536	4.73088	0.491
	T1	10	25.3085	3.64817	
	T2	9	24.6383	3.10856	
	T3	14	23.1464	4.03505	
	Total	40	24.4089	3.85694	
Mean_VEGFA	Ta	7	21.6893	3.64584	0.019*
	T1	10	22.0265	3.42016	
	T2	9	19.4411	3.26820	
	T3	14	17.9014	3.12990	
	Total	40	19.9420	3.66273	
Mean_VEGFC	Ta	7	28.4993	3.72931	0.133
	T1	10	27.1765	2.61656	
	T2	9	28.8183	2.72893	
	T3	14	25.4325	4.57827	
	Total	40	27.1670	3.77649	

*Level of significance.

Table 4. Distribution of 5 indicators in all samples by grades of bladder carcinoma (n = 40)

	Grade	N	Mean	Std. Deviation	PV*
Mean_*SPP1A*	Low	17	25.8824	2.95344	0.000
	High	23	20.5057	3.59624	
Mean_*SPP1B*	Low	17	27.0571	2.83209	0.012
	High	23	24.0300	4.07557	
Mean_*SPP1C*	Low	17	25.3271	3.98496	0.199
	High	23	23.7302	3.69988	
Mean_*VEGFA*	Low	17	21.8876	3.40495	0.003
	High	23	18.5039	3.20360	
Mean_*VEGFC*	Low	17	27.7212	3.08493	0.432
	High	23	26.7574	4.23656	

*Fisher exact test was done to measure the level of significance.

cators showed significant results: 1. mean_*SPP1A* with an AUC of 0.895 (95% CI: 0.788 to 1.00, *p*-value < 0.001); 2. mean_*SPP1B* with an AUC of 0.721 (95% CI: 0.561 to 0.882, *p*-value = 0.018); 3. mean_*VEGFA* with an AUC of 0.777 (95% CI: 0.628 to 0.927, *p*-value = 0.003).

These results indicate a significant level of performance for these indices.

Table 6 presents the results of the logistic regression analysis, both without and with adjustments for sex and smoking. When no adjustments were made, the *SPP1A* (*p*-value = 0.001), *SPP1B* (*p*-value = 0.020), and *VEGFA* (*p*-value = 0.007) index have a significant correlation with the grade.

However, after adjusting for sex and smoking, only the mean_*SPP1A* index showed a significant correlation with the grade. In Model 1, when we adjusted for age and sex, only the mean_*SPP1A* (*p*-value = 0.033) index significantly correlated with the grade.

In Model 2, we adjusted the indexes only for sex; however, none of the indexes showed a significant correlation with the grade. In contrast, Model 3, which included an adjustment for smoking, revealed significant correlations. Specifically, the following indexes had notable results: mean_*SPP1B* (*p*-value = 0.016, OR = 10.401), mean_*SPP1C* (*p*-value = 0.003, OR = 15.296), mean_*VEGFA* (*p*-value = 0.016, OR = 11.043), and mean_*VEGFC* (*p*-value = 0.002, OR = 16.893). These findings indicate that smoking is a significant factor in angiogenesis, tumor progression, and the grade of bladder cancer.

In **Table 7**, we present the results of logistic regression analysis with the presence of all indicators. The mean_*VEGFC* index was found to be significant, and its direction changed notably (from 0.932 to 2.077). This change underscores the impact of our research. Although mean_*VEGFC* had a confounding effect on mean_*SPP1A* and changed its Odds Ratio from 0.652 to 0.324, the main confounder was mean_*SPP1A*. The interaction effect of these two indicators was investigated, and it was not significant (*p*-value = 0.209). Based on the logistic regression model, the mean_*SPP1A* index - (mean_*VEGFC* * 0.7) was defined, and the area under the curve was calculated as 0.951 (**Table 7**).

Table 5. The area under the curve of the ROC for indicators

Area Under the Curve					
Test Result Variable(s)	Area	Std. Error	Asymptotic Sig.	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
Mean_SPP1A	.895	.054	.000*	.788	1.000
Mean_SPP1B	.721	.082	.018*	.561	.882
Mean_SPP1C	.650	.089	.109	.476	.823
Mean_VEGFA	.777	.076	.003*	.628	.927
Mean_VEGFC	.583	.091	.374	.406	.761
Mean_SPP1A - (mean_VEGFC * 0.7)	.951	.037	.000*	.879	1.000

*Level of significance. The Area Under the ROC curve (AUC) was measured for all indicators and displayed in **Table 5** along with the Asymptotic 95% Confidence Interval. Based on the results obtained in **Table 7**, the formula mean_*SPP1A* - (mean_*VEGFC* * 0.7) was obtained.

Table 6. Mean_*SPP1A* index after adjusting for sex and smoking and a significant relationship with grade

The index entered into the model	Crude		Model 1		Model 2		Model 3	
	Odds Ratio	*P* Value	Odds Ratio	*P* Value	Odds Ratio	*P* Value	Odds Ratio	*P* Value
Mean_SPP1A	0.652	0.001*	0.717	0.033*	1.858	0.653	2.954	0.364
Mean_SPP1B	0.789	0.020*	0.853	0.225	5.198	0.151	10.401	0.016*
Mean_SPP1C	0.894	0.201	0.851	0.243	9.994	0.108	15.296	0.003*
Mean_VEGFA	0.741	0.007*	0.780	0.060	4.898	0.172	11.043	0.011*
Mean_VEGFC	0.932	0.424	0.983	0.866	3.768	0.233	16.893	0.002*

Crude: no adjustment, Model 1: Adjusted model for sex and smoking, Model 2: Adjustment only for sex, Model 3: Adjustment for smoking. *Level of significance.

Table 7. Logistic regression analysis with all indicators

Model number	Variables in the model						
	Odds Ratio (*P* Value) mean_SPP1A	Odds Ratio (*P* Value) mean_SPP1B	Odds Ratio (*P* Value) mean_SPP1C	Odds Ratio (*P* Value) mean_VEGFA	Odds Ratio (*P* Value) mean_VEGFC	Odds Ratio (*P* Value) of smoking	Odds Ratio (*P* Value) Sex
1	0.331 (0.022)*	1.273 (0.489)	0.896 (0.645)	0.667 (0.099)	2.118 (0.020)*	0.347 (0.533)	0.955 (0.983)
2	0.331 (0.022)*	1.274 (0.483)	0.894 (0.602)	0.667 (0.099)	2.114 (0.017)*	0.351 (0.518)	
3	0.384 (0.015)*	1.204 (0.548)	0.915 (0.677)	0.699 (0.116)	2.035 (0.025)*		
4	0.372 (0.012)*	1.136 (0.655)		0.714 (0.124)	2.054 (0.019)*		
5	0.392 (0.013)*			0.746 (0.122)	2.078 (0.021)*		
6	0.324 (0.006)*				2.077 (0.026)*		

*Level of significance. When Logistic regression analysis was done, first all the indicators were checked. Then each indicator that did not have a significant *P* value was removed from the models to check its confounding effect. Finally, we reached two *SPP1A* and *VEGFC* isoforms that were significant in the model. The interaction effect of these two indicators was investigated, and it was not significant

The proposed method is centered around the area under the ROC curve. The optimal cut-point value can be determined by comparing the sensitivity and specificity values to the area under the ROC curve. The chosen value minimizes the absolute difference between the sensitivity and specificity values while remaining closest to the area under the ROC curve. The best cut-off of the two indexes mean_*SPP1A* and mean_*SPP1A* - (mean_*VEGFC* * 0.7) is shown in **Figures 3** and **4**. Based on the cut-off, sensitivity and Specificity can be seen in **Table 8**. Sensitivity and Specificity for mean_*SPP1A* < 23 were 82.6 and 88.2, respectively, while Sensitivity and Specificity for mean_*SPP1A* - (mean_*VEGFC**0.7) < 4.3 were 87.6 and 94.1. We developed a new index based on the logistic regression model, using the fol-

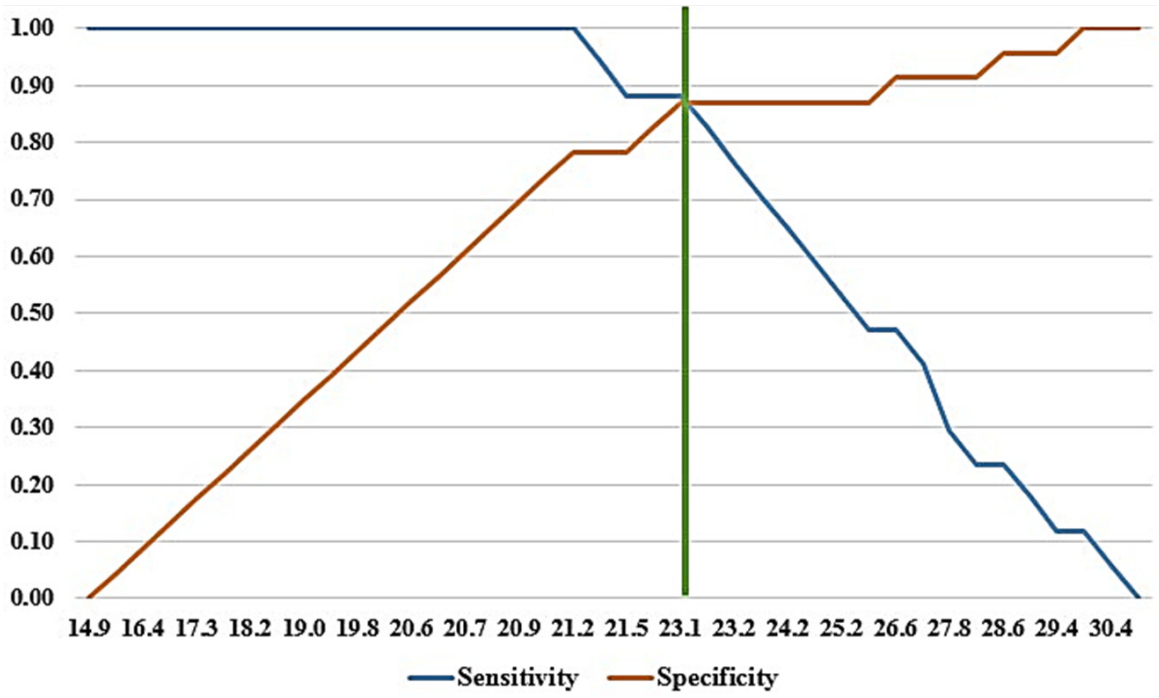

Figure 3. Sensitivity and specificity curve and the best cut-off point according to the mean_*SPP1A* index in High Grade diagnosis. The optimal cut-off for balancing sensitivity and specificity is the point on the curve nearest to (0,1) and the calculated cut-off was 23.1.

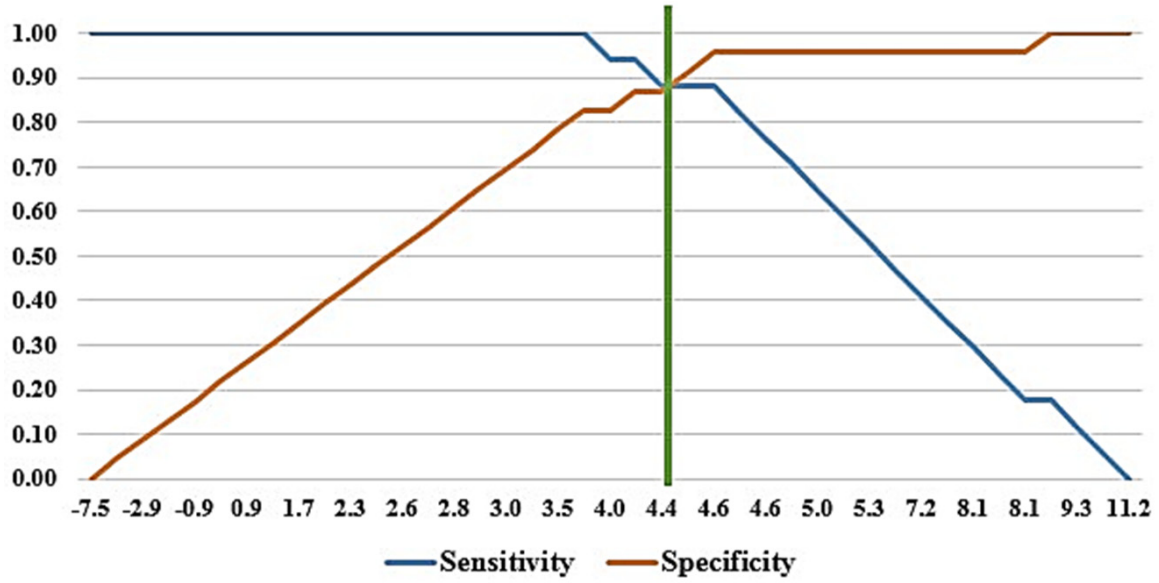

Figure 4. Sensitivity and specificity curve and the best cut-off point according to the mean_*SPP1A* index - (mean_VEGFC * 0.7) in high-grade diagnosis and the calculated cut-off was 4.4.

lowing formula: mean_*SPP1A* - (mean_*VEGFC* * 0.7) The model achieved an AUC of .951 (95% CI .879 to 1.000) and (*p*-value < .001) (**Table 5**). Based on this obtained index, the calculated cut-off was 23.1, and expression values less than the cut-off in patient samples were associated with high-grade samples. Expression values more than those were associated with low-grade samples (**Figures 3** and **4**).

Table 8. Sensitivity, specificity, and confidence limits of 95% for the diagnosis of the High-Grade group

Variable	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
Mean_*SPP1A* < 23	82.6 (66.8-98.4)	88.2 (72.6-100.0)	90.5 (77.7-100.0)	78.9 (60.2-97.7)
Mean_*SPP1A* - (mean_*VEGFC**0.7) < 4.3	87.0 (72.9-100.0)	94.1 (82.7–100.0)	95.2 (85.9-100.0)	84.2 (67.5-100.0)
Mean_ *SPP1A* - (mean_*VEGFC* * 0.7) < 4.0	82.6 (66.8-98.4)	94.1 (82.7-100.0)	95.0 (85.3-100.0)	80.0 (62.1-97.9)
Mean_ *SPP1A* - (mean_*VEGFC** 0.7) < 4.5	91.3 (79.6-100.0)	88.2 (72.6-100.0)	91.3 (79.6-100.0)	88.2 (72.6-100.0)

For the two obtained variables (mean_*SPP1A* < 23 and mean_*SPP1A* - (mean_*VEGFC**0.7) < 4.3), sensitivity and specificity were calculated and as can be seen, variable mean_*SPP1A* - (mean_*VEGFC**0.7) < 4.3 has better sensitivity and specificity than variable mean_*SPP1A* < 23.

Discussion

Bladder cancer is a common urinary tract cancer that can be difficult to treat. Due to its high recurrence rate, patients often require regular surveillance involving biopsies and cystoscopies, which can be financially, mentally, and physically challenging. For this reason, many tests have been developed that can detect bladder cancer at an earlier stage, which helps reduce the need for unnecessary invasive procedures. Tumor grading is a critical factor in determining the prognosis and guiding the management of patients with non-muscle invasive bladder cancer.

In this study, we investigated the correlation between the expression of two main gene isoforms and tumor grade and muscle invasiveness. During our investigation of tissue samples from bladder cancer patients, we found out that univariate analysis revealed a significant association between tumor grade and *SPP1A* and *VEGFA* isoforms. Logistic regression analysis showed a significant association between high-grade tumors and *SPP1A* and *VEGFC* isoforms.

Several studies have investigated the correlation between different gene mutations and bladder cancer grade. A notable study conducted by João Vinagre et al. in 2013. They found that the *TERT* gene mutation is associated with both low-grade tumors (21 out of 14 cases, 67%) and high-grade tumors (61 out of 34 cases, 56%) [22]. On the other hand, Françoise Descotes and colleagues (2017) predicted bladder cancer recurrence non-invasively by identifying *TERT* somatic promoter mutations in urine. They found out that the frequency of *TERT* mutations was higher among high-grade tumors than low-grade tumors [23]. In 2022, Hayashi and his team conducted research to investigate the genomic evolution of cancer in urinary tumor samples from patients with low-grade and high-grade NMIBC. The study identified several somatic mutations in the promoter of *TERT, FGFR3*, and *CDKN2A* genes, specifically in the urothelium of patients with NMIBC. These mutations were found to be associated with malignancy [24].

Since *SPP1* and *VEGF* are among the most important genes involved in cancer recurrence and progression, studies on the expression of these two genes and bladder cancer grading, and subsequently, the importance of examining their isoforms, have been discussed.

In a study by Michail Sarafidis et al. (2022), multiple microarray datasets were collected. The study involved comparing urine and blood-based gene expression data and examining the survival of BCa patients with different stages of MIBC patients receiving neo-adjuvant chemotherapy through bioinformatics analysis. Ultimately, the study identified the expression of nine genes (ANXA5, CDT1, COL3A1, *SPP1*, *VEGFA*, CDCA8, HJURP, TOP2A, and COL6A1) in the urine or blood plasma of patients, which were introduced as diagnostic markers for BCa [25]. As a result, the study confirmed the selection of genes and their isoforms for examination in the biopsy samples of patients with bladder cancer for our research.

According to our findings in this study and the association of VEGF-C levels with disease progression and high grade of bladder cancer, Vlachtsis et al. (2002) demonstrated an association between the main *VEGF* isoforms and the growth characteristics of human head and neck squamous cell carcinoma in nude mice. The researchers concluded that tumors with high proliferation exhibited an expression level of this gene approximately 10 times higher

than tumors with low proliferation [26]. Monica Sankhwar et al. (2015) conducted a study to examine the link between *VEGF* and bladder tumors and their stages. They screened 122 high-risk bladder cancer patients, utilized an immunoassay method to measure *VEGF* levels, and concluded that there exists a direct correlation between *VEGF* levels and tumor stage [27].

According to the results of this research, logistic regression analysis showed that VEGFC was associated with high grade. In a study by Zhuo Li et al. (2011), samples from 93 patients with invasive bladder cancer were collected and the expression of *VEGF-C* protein was examined using immunohistochemistry after surgery. The study concluded that patients with T2 stage, low *VEGF-C* levels, and absence of bladder had high overall and disease-specific survival rates. Additionally, the level of *VEGF-C* can be used to assess disease progression and help determine the appropriate treatment [28].

Together with the results obtained in our research, in 2019, Cui Yu et al. demonstrated that the PVT1 oncogene plays a crucial role in the tumorigenesis of bladder cancer by upregulating the expression of *VEGF-C* through miR-128. This finding suggests that PVT1 could serve as a new biomarker for the diagnosis and treatment of bladder cancer [29]. In another study, Jui-Chieh Chen et al. (2013) stated that high levels of *VEGF-C* expression and *VEGF-C/VEGFR* signaling were significantly associated with poorer prognosis in various malignancies [30].

In a study conducted by Anika Sadaf et al. (2021), it was found that bladder tumors with positive *VEGF* expression are more likely to occur in patients with high-grade and muscle-invasive bladder cancer. The authors suggest that this finding could help identify patients who would benefit from targeted anti-*VEGF* therapy [31].

We investigated the expression levels of SPP1 isoforms in relation to the grades and stages of bladder carcinoma. Our analysis revealed that *SPP1A* had a significant correlation with tumor stages ($P < 0.001$), whereas SPP1B (P 0.080) and SPP1C (P 0.491) did not demonstrate significant associations. Additionally, we identified a statistically significant relationship ($P < 0.05$) between different histological grades, muscle invasiveness, and the mean levels of *SPP1A* across all patients. Furthermore, we reported three indices, specifically mean SPP1A ($P < 0.001$) and mean SPP1B (P 0.018), which showed significant levels under the curve, indicating their potential relevance in the assessment of bladder carcinoma. Furthermore, In 2012, Min-Gu Park et al. introduced plasma *SPP1* levels as a potential predictor of disease stage and recurrence in patients with bladder urothelial carcinoma (UC). Blood tests were conducted on 50 UC patients before and after transurethral resection of bladder tumor (TURBT) to examine the plasma *SPP1* level. The study found that *SPP1* levels were higher in the group of patients who experienced a relapse. There was a significant difference in plasma *SPP1* expression levels between the groups with and without muscle invasion. Additionally, plasma *SPP1* levels decreased after TURBT but increased following radical cystectomy. The researchers suggested that plasma *SPP1* levels could serve as a promising marker for predicting risk and clinical prognosis in patients with bladder urothelial carcinoma [32]. They also demonstrated that plasma levels of SPP1 are linked to disease burden in bladder urothelial carcinoma and muscle invasion. Also, Ke et al. (2011) highlighted that the over-expression of *SPP1* suggests it could serve as a potential biomarker for other types of urothelial carcinomas in the urinary tract, including those affecting the renal pelvis and ureter [13, 33]. Consistent with this research, Wong JPC et al. (2017) exhibited that the proteins *SPP1, MMP9,* and *S100A8* play a crucial role in bladder cancer progression. Studies have shown that higher levels of *SPP1* in bladder cancer specimens are associated with advanced tumor stages and grades. Based on these findings, researchers suggest that *SPP1*, MMP9, and S100A8 could be potential prognostic markers and therapeutic targets for bladder cancer [34].

In our investigation of the expression levels of *VEGF* isoforms related to the grades and stages of bladder carcinoma, we observed a significant association of *VEGFA* (P 0.019) with tumor stages. In contrast, *VEGFC* (P 0.133) showed no significant correlation. Furthermore, only *VEGFA* (P 0.003) exhibited a statistically significant relationship when analyzing tumor grades, while *VEGFC* (P 0.432) remained non-

significant. This research identified a notable statistical correlation ($P < 0.05$) between different histological grades, muscle invasiveness, and mean_*VEGFA* across all patients. Additionally, the mean_*VEGFA* index (P 0.003) showed a significant level under the curve. Notably, without any adjustments, the *VEGFA* index (P 0.007) also revealed a significant correlation with tumor grade.

In a study that focused on two isoforms, *VEGF-A* and *VEGF-B*, researchers investigated the correlation between *VEGF-A* and *VEGF-B* mRNA levels and clinical pathology parameters to evaluate their prognostic value in bladder cancer. They analyzed the total RNA from 37 bladder cancer samples and concluded that *VEGF-A* transcript levels were higher in cancer tissues. Levels in pT2-T4 tumors were significantly higher than in pTa and pT1 urinary tumors, indicating a correlation with the pathological stage. This research confirmed that *VEGF-A* mRNA levels can serve as a potential prognostic indicator of progression in bladder cancer [21].

A study investigated four secreted *VEGF-A* splice forms used RT-PCR to identify four *VEGFA* isoforms related to *VEGF A121, A165, A189,* and *A206* in bladder cancer tissue. The *VEGF A206* and *VEGF A189* levels in pT2 or higher grade tumor samples were significantly lower than pT1 or lower grade tumors [35].

In 2022, Jiang et al. reported that cigarette smoke increases *SPP1* expression via *JAK2/STAT3* signaling and leads to lung cancer metastasis [36]. In a study by Rafał Suwiński et al. (2019) on blood serum proteins, including *SPP1* and *VEGF*, as biomarkers for predicting survival, metastasis rate, and resistance to radiotherapy and radio-chemotherapy in lung cancer, it was indicated that smokers had significantly higher expression levels of these proteins [37]. Our research found that the levels of *SPP1B* increased ten-fold, *SPP1C* fifteenfold, *VEGFA* eleven-fold, and *VEGFC* sixteen-fold, respectively, in smokers with high-grade bladder cancer. We observed a significant relationship between the grade of bladder cancer and the two isoforms of *SPP1A* and *VEGFC*. This suggests that these two indicators can assist pathologists in distinguishing between high-grade and low-grade bladder cancer. In the future, these two isoforms could be used as additional predictive markers.

Further investigation is needed to understand the mechanistic link between the three SPP1 isoforms and the two VEGF isoforms in bladder cancer progression. Future studies that include more patient samples and follow-up and treatment information will lead to better decision-making.

Conclusion

The relationship between bladder cancer grader and the two isoforms, *SPP1A* and *VEGFA*, indicates that these indicators could help pathologists differentiate between high-grade and low-grade bladder cancer and potentially be used as predictive markers. The data obtained in the present study suggest that the index signatures mean_*SPP1A* and mean_*SPP1A*-(mean_*VEGFC**0.7) are prognostic and predictive markers.

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Disclosure of conflict of interest

None.

Abbreviations

AUC, Area Under the ROC curve; BCa, Bladder cancer; EORTC, European Organisation for Research and Treatment of Cancer; GEPIA, Gene Expression Profiling Interactive Analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; MIBC, Muscle-invasive Bladder Cancer; NMIBC, Non-muscle-invasive Bladder Cancer; RNA, Ribonucleic acid; SPP1, Secreted Phosphoprotein 1; TURBT, Trans urethral resection of bladder tumor; UC, Urothelial carcinoma; UCC, Urothelial carcinoma; VEGF, Vascular endothelial growth factor.

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