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AN IMMUNOHISTOCHEMICAL ANALYSIS OF VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR 1 IN HIGH GRADE T1 BLADDER CANCER WITH CONCOMITANT CARCINOMA IN SITU

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Vascular endothelial growth factor receptor 1 (VEGFR1) reduces the angiogenic activity of vascular endothelial growth factor (VEGF), acting like decoy receptor for VEGF and limiting its availability for genuine angiogenic receptors. The purpose of this study was to establish the significance of VEGFR1 expression in high grade T1 (HGT1) bladder cancer with concomitant carcinoma in situ (CIS) and to determine possible immunohistochemical marker helpful in the follow-up of "unpredictable" HGT1 bladder cancer patients. The analysis included 137 HGT1 bladder cancer samples. Concomitant CIS was diagnosed in 21 (15.33%) of these patients. Sections of 137 formalin-fixed, paraffin-embedded materials were incorporated in tissue microarrays and then stained with a rabbit monoclonal antibody against VEGFR1 (N-term: Y103/-Epitomics, diluted 1:250). Immunohistochemical reaction was scored as following: negative if ≤ 10% of cells were stained and positive if > 10% were stained. We considered both membranous and cytoplasmic expression and staining intensity was scored using a scale of 0 to 3 (0, no staining; 1, weak; 2, moderate; and 3, intense). After a mean follow-up of 50 months, in 137 patients diagnosed with HGT1 urothelial bladder cancer, we found that patients who had concomitant CIS had worse overall survival ($p < 0.05$), furthermore, those tumour samples had weakly expressed VEGFR1 ($p < 0.05$). Patients with positive VEGFR1 had longer disease-free ($p < 0.01$) and overall survival ($p < 0.01$). Present investigation has revealed that the estimation of VEGFR1 expression could be diagnostic supplement, selecting the HGT1 bladder cancer patients that would require more intensive follow-up, especially if accompanied with CIS.

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Key words: *angiogenesis, VEGFR1, bladder cancer, carcinoma in situ*

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Introduction

Bladder cancer is the ninth most common cancer worldwide, with an estimated 430 000 new cases per year (1). Approximately 75% of bladder cancers are non–muscle invasive (NMIBC), and of these, roughly 20% to 25% invade the lamina propria (T1). Fifty percent of NMIBC patients who are treated with transurethral resection (TUR) have a recurrence of the disease and 5 % to 25% of these patients will progress to muscle-invasive disease after repeated recurrences (2). The most difficult NMIBC category for treatment planning is high-grade T1 (HGT1) bladder cancer. Treatment with bacillus Calmette-Guerin (BCG) risks recurrence, progression and metastases, however, may preserve the bladder. Cystectomy may offer the best opportunity for cure, but is associated with morbidity and a risk of mortality, and considering the heterogeneous nature of HGT1 it may constitute unnecessary over-treatment. The dilemma facing the urologist is how to treat these tumours the best in a timely manner, so that the chances of bladder preservation and cancer control are maximised, while the risks of over-treatment with radical intervention are minimised (3).

Another obstacle in perceiving properly HGT1 patients is the possible presence of carcinoma in situ (CIS) in surrounding mucosa (urothelium). It is often not possible to distinguish whether the tumour is recurrent due to aggressive tumour biology and implantation of floating cancer cells or due to evolution of non diagnosed in situ lesion. Carcinoma in situ (CIS) of the bladder is a small, flat, high grade,

confined to the mucosa, lesion and it can be easily overlooked in the primary procedure (4). The diagnosis of CIS cannot be made with imaging methods. Cytology is useful, particularly as an adjunct to cystoscopy, if HG/CIS malignancy is present. Positive voided urinary cytology can indicate a urothelial tumour anywhere in the urinary tract; negative cytology, however, does not exclude the presence of a tumour (5). CIS is often multifocal and can occur in the bladder but also in the upper urinary tract, prostatic ducts, and prostatic urethra (6). CIS can present as an area indistinguishable from inflammation, or it may not be visible at all (7). For this reason, the strategy of taking biopsies from abnormal urothelium and random/mapping biopsies from normal-looking mucosa (trigone, bladder dome, and right, left, and anterior and posterior bladder wall) is recommended (8). Some studies have reported worse prognosis in concurrent CIS and T1 tumours compared with primary CIS, in extended CIS (9), and in CIS in the prostatic urethra (10). Relative indications for cystectomy include multifocal disease, associated CIS, T1 disease on repeat resection, deep T1 disease abutting the muscle and poor patient compliance. The only precystectomy prognostic predictor of recurrence is the presence of concomitant CIS (11, 12).

The purpose of this study was to establish the significance of vascular endothelial growth factor receptor 1 (VEGFR1) expression in HGT1 bladder cancer with concomitant carcinoma in situ and to determine possible immunohistochemical marker helpful in the follow-up of "unpredictable" HGT1 bladder cancer patients.

Materials and methods

We studied 137 patients with stage T1 urothelial bladder cancer who had undergone transurethral resection (TUR). All cases were diagnosed at the Institute of Pathology, Faculty of Medicine, Niš. The analysis included 137 HGT1 bladder cancer samples. Concomitant CIS was diagnosed in 21 (15.33%) of these patients. The mean patients' age was 68.31 ± 8.92. There were 116 male (84.7%) and 21 female patients (15.3%).

The histological sections were processed from tissue fixed in 10% formalin by standard techniques, and stained with haematoxylin and eosin (H&E). H&E-stained slides were used to assess histological grade (low and high grade), pathologic stage (pT), growth of tumour (papillary/solid), and the presence of CIS, cystitis and squamous differentiation within the tumour, according to the WHO criteria (13). Sections from 137 formalin-fixed, paraffin-embedded materials were incorporated in tissue microarrays and then stained with a rabbit monoclonal antibody against VEGFR1 (N-term: Y103/Epitomics, diluted 1:250). Formalin-fixed, paraffin-embedded tissue samples were deparaffinized in xylene for 10 min, followed by washing in decreasing concentrations of ethanol (95%, 75%, 50%), each for 2 min. After deparaffinization, antigen retrieval was performed by boiling the slides in 0.01 M citrate buffer, pH 6.0, in a microwave for 10 min. The slides were then applied on a semi-automatic IHC diagnostic system (Ventana Inc.) and IHC staining was performed using antigen-specific antibody, as indicated above. Immunohistochemical reaction was scored as follows: negative if ≤ 10% of cells were stained and positive if > 10% were stained. We considered both membranous and cytoplasmic expression and staining intensity was scored using a scale of 0 to 3 (0, no staining; 1, weak; 2, moderate; and 3, intense) according to our previous investigation (Figure 1) (14).

All statistical analyses of the obtained data were performed using Statistical Package for Social Sciences (SPSS version 20.0, Chicago, IL, USA).

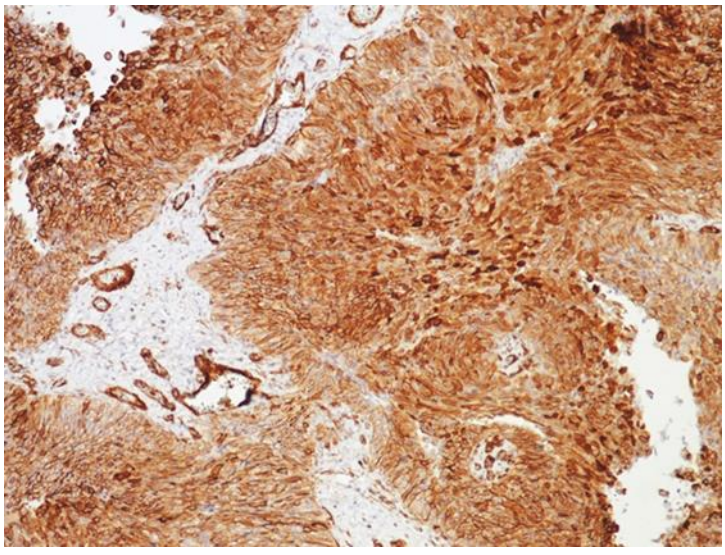


Figure 1. Representative microphotograph of strong, diffuse cytoplasmic and membranous VEGFR1 expression in HGT1 tumour cells and endothelial cells

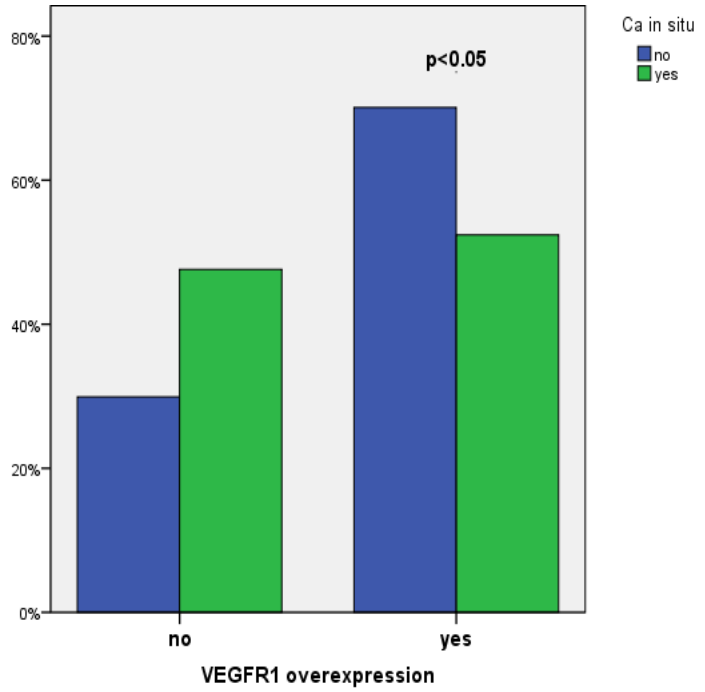
For group comparisons, parametric Student's t-test was performed. The methods of Cox-regression and Kaplan-Meier curves were used to determine survival predictors. The results were considered statistically significant if $p < 0.05$.

Results

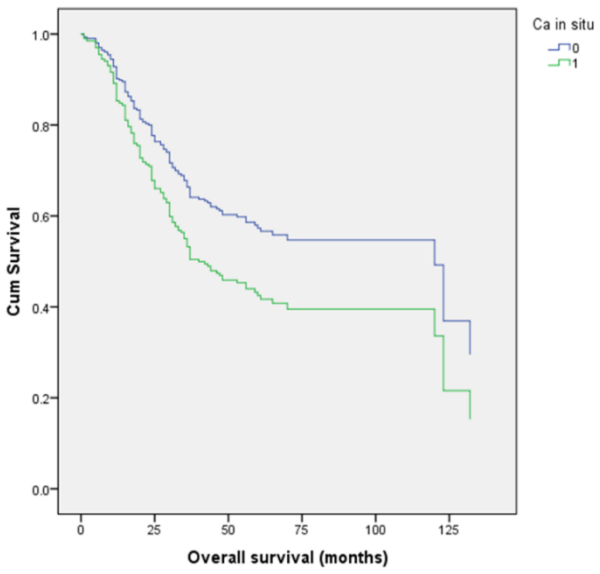
Positive VEGFR1 staining was observed in 11 (52.4%) CIS samples and in 86 (74.1%) samples without CIS (Graph 1), showing that CIS presence in HGT1 tumours was associated with low expression of VEGFR1 ($\chi^2 = 4.072$, $p < 0.05$).

After a mean follow-up of 50 months, mortality was 58.4% (80 patients), and the incidence of relapse was 39.4% (54 patients).

Tumour samples associated with 50 month survival expressed reduced VEGFR1 ($\chi^2 = 4.365$, $p < 0.05$), but no statistically significant association was found between CIS presence and recurrence during the follow-up period ($\chi^2 = 0.000$, $p < 1.000$).

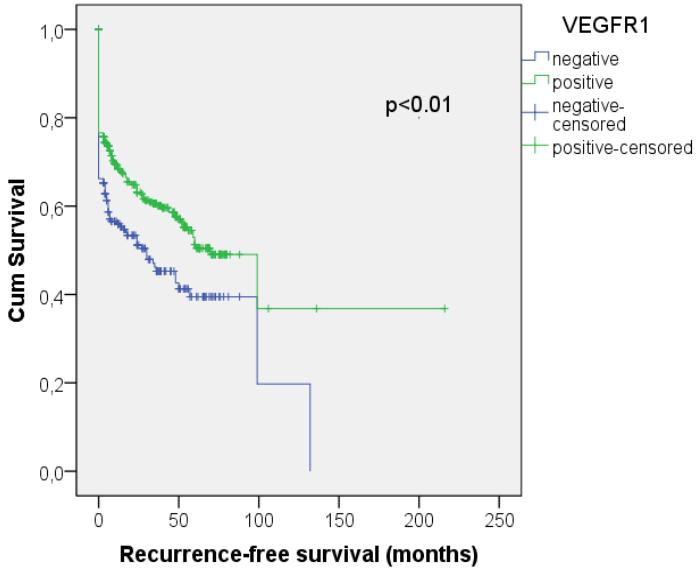


Graph 1. VEGFR1 expression in HGT1 bladder cancer with concomitant CIS

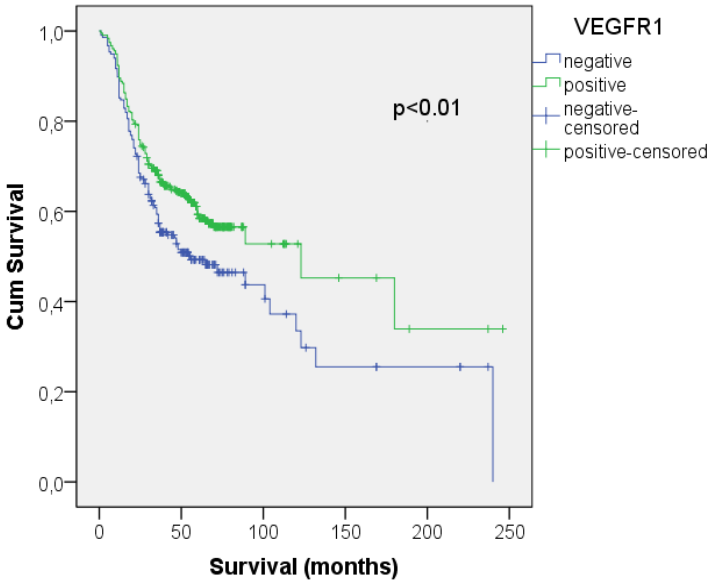


Graph 2. Overall survival in HGT1 bladder cancer associated with CIS

On the contrary, when taking time into the account, we found that patients who had concomitant carcinoma in situ had worse overall survival (HR = 1.537 (1.018-2.320), χ^2 = 4.255, p < 0.05) (Graph 2). Patients with positive VEGFR1 had longer disease-free (HR = 0.663 (0.508- 0.865), χ^2 = 9.305, p < 0.01) (Graph 3) and overall survival (HR = 0.717 (0.565-0.911), χ^2 = 7.258, p < 0.01) (Graph 4).



Graph 3. Recurrence-free survival in VEGFR1 positive HGT1



Graph 4. Overall survival in VEGFR1 positive HGT1

Discussion

Without any treatment, approximately 54% of patients with CIS will progress to muscle-invasive disease (7). The most important prognostic factors for progression are the T category, grade, and the presence of CIS, factors that represent the biological aggressiveness of the disease, and the most reliable

in patients with HGT1 tumours is the presence of concomitant CIS (15). In HGT1 patients without CIS, the probability of progression is 10% in one year and 29% in five years; in HGT1 patients with CIS, the corresponding numbers are 29% and 74%, respectively. Patients with deep lamina propria invasion (T1b/T1c) should be managed more aggressively, especially those with associated CIS (15). For all cases of newly diagnosed HGT1 transitional cell carcinoma (TCC), a secondary TUR 4–6 weeks after the primary TUR is strongly recommended (16). Repeated resection of the previously resected site 4–6 weeks after the initial resection (along with any other cystoscopically suspicious areas) will provide more accurate staging information. This is particularly important because the probability of understaging a HGT1 tumour ranges from 20% to 70%, depending on the presence of muscularis propria in the sample and concomitant CIS obscured by inflammation (17). Molecular markers such as p53, Ki-67, NMP22, and Cox-2 have some promise; however, they have not been sufficiently validated to be used day to day at this time (18). Vascular endothelial growth factor (VEGF), an important protein for triggering and regulating angiogenesis, effects cellular responses by binding to VEGF receptors on the cell surface. Vascular endothelial factor receptor 2 (VEGFR2) mediates most of the known cellular responses to VEGF. VEGFR1 negatively regulates VEGFR2 via high-affinity binding of VEGF, which consequently becomes unavailable for VEGFR2 (19). VEGFR2 expression has been correlated with increasing disease stage and tumour invasion into the muscle, and may be an important determinant for prediction of nodal metastasis in TCC patients (20). Several studies pointed that expression of VEGF and its receptors VEGFR1/VEGFR2 is associated with invasiveness of bladder cancer (21). In this study we found that tumour samples with VEGFR1 overexpression were associated with better recurrence-free and better overall survival, and, explanation lies in decreased angiogenesis. By binding VEGF, soluble VEGFR1 reduces the angiogenic activity of VEGF, acting like decoy receptor for VEGF and limiting its availability for genuine angiogenic receptor VEGFR2. Although tumour cells produce tremendous amounts of VEGF, angiogenic inhibitor VEGFR1 will block angiogenesis disabling the sprout of microvessels and indirectly further nourishment and spreading of tumour cells (14, 22). When observed in tumour associated macrophages, VEGFR1 expression can strongly indicate metastatic potential of the tumour, since VEGFR1 in tumour associated macrophages is required for metastatic tumour outgrowth. This was demonstrated in the breast cancer, but not in TCC (23). However, this new concept of microenvironmental regulation of metastasis through immune cells that express a high level of VEGFR1 points that VEGFR1 is the marker beyond the angiogenic pathway.

Conclusion

Present investigation has revealed that the estimation of VEGFR1 expression could be diagnostic supplement, selecting the HGT1 bladder cancer patients that could require more intensive follow-up, especially if accompanied with CIS. Vascular endothelial growth factor receptor 1 is potentially reliable marker and could be a trustful prognostic predictor of surveillance and recurrence of the disease. Diverse angiogenic pathways occurring in tumours with and without accompanying CIS lead to unequivocally different expression of VEGFR1. Increased VEGFR1 expression was associated with HGT1 tumours without CIS, and longer disease-free and overall survival and vice versa, HGT1 tumours accompanied with CIS were associated with decreased VEGFR1 expression, and worse overall survival in those patients.

Acknowledgment

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The authors declare no conflict of interest.

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IMUNOHISTOHEMIJSKA ANALIZA RECEPTORA 1 ZA VASKULARNI FAKTOR RASTA KOD T1 KARCINOMA MOKRAĆNE BEŠIKE VISOKOG GRADUSA I KONKOMITANTNIM *IN SITU* KARCINOMOM

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Receptor 1 za vaskularni faktor rasta (VEGFR1) smanjuje angiogenetsku aktivnost vaskularnog faktora rasta (VEGF), ponašajući se kao lažni receptor za VEGF i ograničavajući njegovu dostupnost za prave angiogene receptore. Cilj rada je da se ustanovi značaj VEGFR1 ekspresije kod T1 karcinoma mokraćne bešike visokog gradusa (HGT1) sa konkomitantnim *in situ* karcinomom (CIS) i da se odredi imunohistohemijski marker koristan za praćenje "nepredvidivih" HGT1 bolesnika. Analizirano je 137 HGT1 karcinoma mokraćne bešike. Konkomitantni CIS dijagnostikovan je kod 21 bolesnika (15,33%). 137 parafinskih uzoraka uklopljeno je u tkivne mikroareje i obojeno zečjim monoklonalnim antitelom na VEGFR1 (N-term: Y103/Epitomics, razblaženje 1 : 250). Imunohistohemijska reakcija procenjivana je na sledeći način: negativna ekspresija ukoliko je obojeno ≤ 10% ćelija, a pozitivna ukoliko je obojeno > 10% ćelija. Procenjivana je i membranska i citoplazmatska ekspresija i skorirana prema skali od 0 do 3 (0 - nema bojenja; 1 - slabo bojenje; 2 - umereno; i 3 - intenzivno). Nakon praćenja u trajanju od 50 meseci, utvrđeno je da bolesnici sa HGT1 karcinomom mokraćne bešike i konkomitantnim CIS imaju ukupno manju stopu preživljavanja ($p < 0,05$), kao i manju ekspresiju VEGFR1 ($p < 0,05$). Bolesnici sa pozitivnim VEGFR1 imali su duži vremenski period bez recidiva ($p < 0,01$) i duže ukupno preživljavanje ($p < 0,01$). Istraživanje je pokazalo da procena VEGFR1 ekspresije može biti dijagnostička dopuna za odabir bolesnika sa HGT1 urotelnim karcinomom mokraćne bešike, kojima je neophodno intenzivnije praćenje, posebno ukoliko je prisutan i CIS.

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Ključne reči: *angiogeneza, VEGFR1, karcinom mokraćne bešike, carcinoma in situ*