Original article

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EXPRESSION OF EPIGENETIC SILENCER EZH2 IN EARLY INVASIVE PT1 UROTHELIAL BLADDER CANCER

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Urothelial bladder cancer is the most common malignancy of the urinary tract. Early invasive bladder cancer (stage pT1) is a tumor that has invaded subepithelial connective tissue, without spread to bladder muscle, and represents a major challenge for diagnosis and therapy. EZH2 transcriptional repressor has a crucial role in oncogenesis of the bladder. The aim of this research was to investigate the expression profile of EZH2 in pT1 bladder cancer, to analyze the correlation with clinicopathological factors, and to assess the possible prognostic significance of EZH2 expression. In that purpose 279 tumor samples embedded in tissue microarrays were analyzed immunohistochemically to EZH2 expression. High EZH2 nuclear expression was observed in 44.5% of the tumors. High EZH2 expression was strongly associated with high histologic grade ($p<0.001$). In addition, EZH2 significantly correlated to male gender, and the occurrence of carcinoma in situ in the adjacent urothelium ($p=0.019$, and $p=0.026$, respectively), while divergent differentiation and disease recurrence showed no significant association with EZH2 staining. High EZH2 expression strongly correlated with cancer specific death ($p=0.010$). Kaplan-Meier survival analysis demonstrated that high EZH2 expression predicted worse survival of the patients ($p<0.001$). Impact of EZH2 expression to recurrence free survival was not significant. High EZH2 expression in early invasive urothelial bladder cancer indicates aggressive behavior of the tumor and poor prognosis. EZH2 has promising roles in urothelial bladder cancer as a complementary diagnostic tool in selection of the patients that require closer clinical attention, and as a potential target for anticancer therapy.

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Key words: *urothelial bladder cancer, EZH2, immunohistochemistry, prognosis*

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Introduction

Urothelial bladder cancer (UBC) is the most common malignancy of the urinary tract, and its diagnostics and clinical management represents a major burden for healthcare systems (1). Non-muscle invasive carcinomas account for about 75% of bladder cancer. Early invasive UBC, stage pT1, is a tumor that has invaded subepithelial connective tissue (lamina propria), without evidence of the spread to muscle of the bladder wall. The accurate diagnosis of early invasive UBC is challenging, and is associated with numerous impediments including cautery artefacts, superficial, insufficient or poorly oriented transurethral resection specimens, tangential sectioning, obscuring inflammatory infiltrate (2). UBC pT1 cancer is of particular importance since it comprises a heterogeneous group of tumors in term of patients' prognosis and treatment outcome. High grade early invasive UBC is considered an aggressive and potentially lethal disease, and require more intensive clinical follow up and management (3, 4).

Recent research has highlighted the significance of epigenetic alterations in mutational landscape of bladder cancer (5, 6). It has been found that mutations in chromatin regulatory genes are more frequent in UBC than in any other solid cancer. Via their influence on expression of various transcription factors and signaling molecules, the alterations in epigenetic pathways have profound implications to fundamental features of cancer biology, including tumor growth promotion, invasiveness and capacity to metastasize. EZH2,

enhancer of zeste homolog 2, is one of the most important and most investigated molecules involved in epigenetic control (7). EZH2 acts as enzymatic subunit of Polycomb repressive complex, responsible for silencing of genetic transcription through histone methylation. EZH2 has been found to be overexpressed in various cancers, including UBC (8, 9). However, the prognostic significance of altered EZH2 in UBC has not been unequivocally established.

The aim of this research was to investigate the immunohistochemical expression profile of EZH2 in early invasive pT1 urothelial bladder cancer of low and high histologic grade, and to analyze the correlation with clinicopathological factors, as well as to assess the possible significance of EZH2 expression for disease prognosis and patients' survival.

Material and methods

Patients and tissue samples

Present study comprised tissue samples of 279 patients with urothelial bladder cancer who underwent transurethral resection of bladder tumor in Clinic of urology, Clinical center Niš, Serbia (2007-2012). All cases were diagnosed at the Institute of Pathology, Clinical center Nis, Serbia. The average patients' age was 66.5±9.8 years, with the predominance of male patients compared to women (76% vs. 24%). Patients' clinical history and survival data including survival time, disease-free survival, and recurrence were available for all patients included in the research. The median follow-up was 60 months. Cancer specific death was defined as death caused by bladder cancer, excluding the mortality caused by other neoplasms and non-neoplastic disease (the majority of death during the follow-up period was caused by ischaemic heart disease).

Pathohistologic analysis

The diagnosis of urothelial bladder cancer, pathologic stage and histologic grade were assessed on formalin fixed paraffin embedded tissue samples, processed by standard techniques, and stained with hematoxylin and eosin. For the purposes of immunohistochemical analysis, tissue microarrays were constructed, using manual tissue arrayer. Each tumor was represented in the composite microarray block with two cores of 2mm diameter, carefully selected from the designated area of invasive portion of tumor. The pathohistologic analysis was performed using a light microscope (Olympus BX43, Olympus Corporation, Tokyo, Japan) by two independent pathologists.

Immunohistochemical analysis

Three micrometer thick sections of tissue microarray blocks were first deparaffinized through a series of xylene, and rehydrated in a series of alcohol. Antigen retrieval pretreatment was carried out in a citrate buffer (pH 6), by heating for 20 minutes in a microwave oven at 800W. After thorough washing in phosphate buffered saline (PBS, pH 7.4), endogenous peroxidase activity was quenched by immersion of the slides in 3% hydrogen peroxide solution in methanol for 20 minutes. The slides were incubated with the primary antibody in a water bath at room temperature for 1 hour. The primary antibody used in the study was Rabbit monoclonal antibody to EZH2 (D2C9), Cell Signaling technology, Mariland, United States, at dilution 1:50. The detection of positive immunoreaction was achieved using DAKO EnVision kit (EnVision® + Dual Link System-HRP (DAB +), DakoCytomation), with one hour slide incubation at room temperature. Visualization of the antigen-antibody reaction was performed with chromogene diamino-benzidine-tetrahydrochloride (DAB), which marked the sites of reaction with brown color precipitate. Finally, the sections were rinsed with PBS, counterstained with Mayer's Hematoxylin, dehydrated and mounted. Negative controls were carried out by omitting the primary antibody.

Scoring of immunohistochemical staining. EZH2 nuclear staining was assessed using a semiquntitative combined intensity and percentage/area scoring method, described elsewhere (10). In brief, tumor score values were obtained as sum of products (intensity score x percentage of tumor area) for each tissue microarray spot. Based on the median score value, all tumors were dichotomized in groups with low or high Ezh2 immunohistochemical expression.

Statistical analysis

All data were analyzed using statistical software for data processing SPSS version 20.0. Continuous variables were presented as the mean ± standard deviation. The frequencies of categorical variables were tested by using $\chi 2$ test with Yates's correction. Univariate and multivariate analysis of clinicopathologic variables was performed using a Cox regression analysis. $P \leq 0.05$ values were considered statistically significant.

Results

EZH2 expression in urothelial bladder cancer displayed exclusively nuclear staining pattern (Figure 1). Cancer cell nuclei showed positivity to Ezh2 in a form of golden to dark brown pigmentation. Significant EZH2 stain was not observed in nonneoplastic urothelium of the adjacent normal mucosa, where only small fraction of the predominantly basal nuclei showed EZH2 expression with low to intermediate intensity. High EZH2 immunohistochemical expression was observed in 44.5% of the investigated early invasive UBC, where the majority of tumors with high EZH2 were high grade carcinomas (Graph 1.).

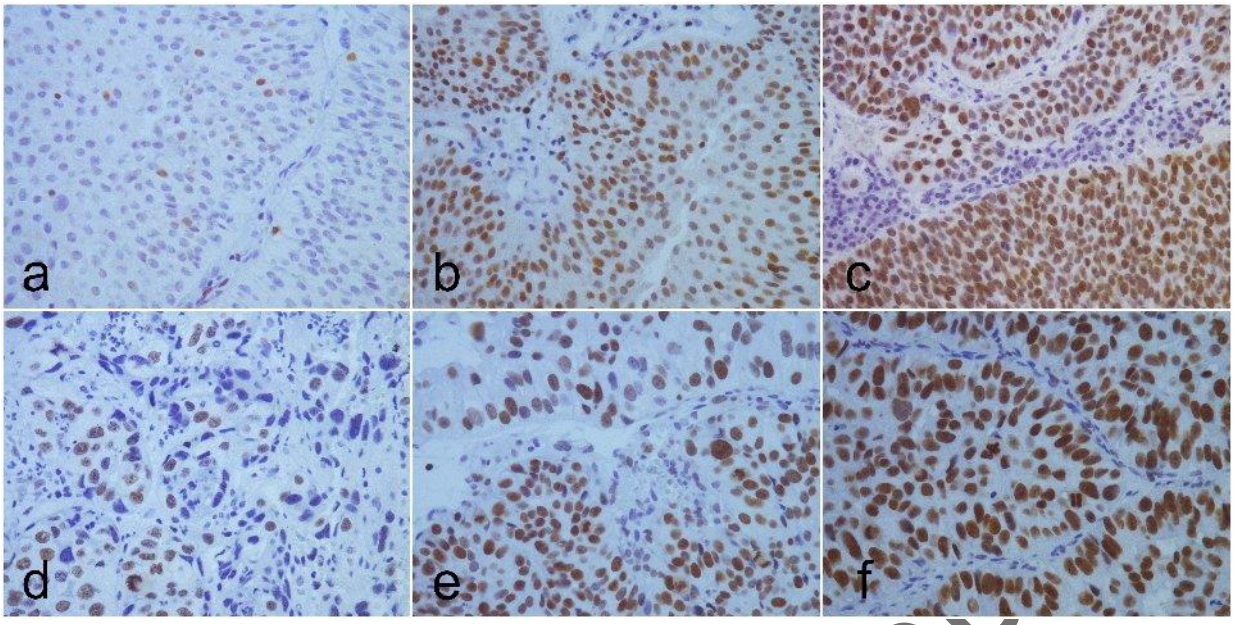
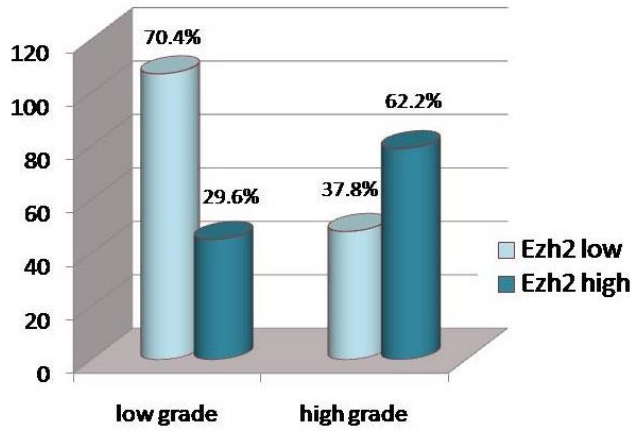


Figure 1. Immunohistochemical expression of EZH2 in low grade (a, b, c), and high grade pT1 urothelial bladder carcinoma (d, e, f);
Low EZH2 expression with scattered brown stained tumor nuclei (a, d);
High EZH2 expression with nuclear staining of intermediate to strong intensity in majority of tumor cells (b, e);
High EZH2 expression with intense diffuse nuclear staining (c, f).
Original magnification x400.



Graph 1. EZH2 immunohistochemical expression in early invasive urothelial bladder cancer of low grade (N=152) and high grade pT1 tumors (N=127).

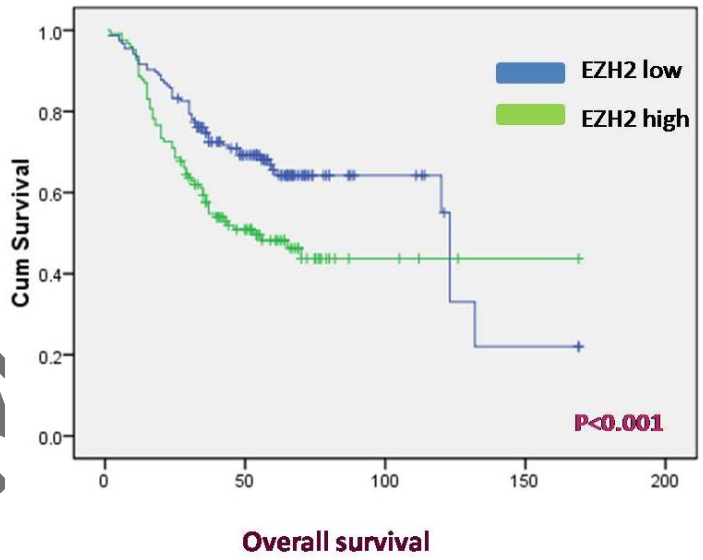
High histologic grade of the tumor correlated with nuclear EZH2 overexpression with high statistical significance ($p<0.001$) (Table 1). High EZH2 significantly associated to male gender, and the occurrence of carcinoma in situ in the urothelium surrounding the superficially invasive tumor ($p=0.019$, and $p=0.026$, respectively). Tumors with divergent differentiation (squamous, glandular, micropapillary, microcystic) demonstrated no significant variability in EZH2 staining.

High EZH2 expression strongly correlated with cancer specific death ($p=0.010$). Almost 60% of the patients who died from direct consequences of disease progression and dissemination showed strong EZH2 positivity. Conversely, EZH2 was not associated with tumor recurrence.

Kaplan-Meier survival analysis showed that high EZH2 expression in early invasive UBC is strongly associated with worse survival of the patients and poor clinical outcome ($p<0.001$) (Graph 2). Impact of EZH2 expression to recurrence free survival was not statistically significant ($p>0.05$).

Table 1. Association of EZH2 expression with clinicopathologic features of early invasive pT1 bladder cancer

Characteristics	Ezh2	Low	High	*p value*
Total (n (%))	279 (100)	155 (55.5)	124 (44.5)	
Gender				
Female	67 (24.0)	45 (67.2)	22 (32.8)	***0.019*** *
Male	212 (76.0)	110 (51.9)	102 (48.1)	
Tumor grade				
Low	152 (54.4)	107 (70.4)	45 (29.6)	***0.000****
High	127 (45.6)	48 (37.8)	79 (62.2)	
Carcinoma in situ				
Yes	19 (6.8)	6 (31.6)	13 (68.4)	***0.026****
No	260 (93.2)	149 (57.3)	111 (42.7)	
Divergent differentiation				
Negative	247 (88.5)	138 (55.9)	109 (44.1)	*0.456*
Positive	32 (11.5)	17 (53.1)	15 (46.9)	
Recurrence				
Yes	114 (40.9)	63 (55.3)	51 (44.7)	*0.516*
No	165 (59.1)	92 (55.8)	73 (44.2)	
Cancer specific death				
Yes	75 (26.9)	31 (41.3)	44 (58.7)	***0.010****
Other	42 (15.1)	23 (54.8)	19 (45.2)	
Live	162 (58.0)	101 (62.3)	61 (37.7)	



Graph 2. Kaplan Meier survival curves showing overall survival in 279 patients with pT1 urothelial bladder cancer with low and high Ezh2 expression.

In multivariate Cox regression analysis that tested the influence of clinicopathologic parameters on overall patients' survival, only histologic grade was statistically significant determinant ($p<0.001$), while the predictive value of EZH2 expression was not established as statistically significant.

Discussion

In bladder cancer genomic landscape mutations in chromatin remodeling and histone modifying genes are frequent (5). EZH2, histone methyltransferase subunit of a Polycomb repressor complex 2, is a prominent target of oncogenetic alteration. Increased EZH2 activity leads to epigenetic silencing of numerous genes, including tumor suppressors, thus contributing to neoplastic phenotype (6, 7). E-cadherin gene (CDH1) is one of

the critical EZH2 targets (11). Its downregulation is crucial for epithelial-mesenchymal transition and metastasis. This association of increased EZH2 and inhibition of E-cadherin expression has been confirmed in urothelial bladder cancer (12). EZH2 exerts its function through canonical pathway of H3K27 methylation, however, emerging evidence indicate that EZH2 methylates non-histone substrates, and may also have functions in carcinogenesis that are methylase independent (13).

EZH2 plays a major role in oncogenesis of the bladder, and has been found to be associated with aggressive disease (8, 10, 14). Increased levels of both EZH2 mRNA and protein product were detected in cancerous tissue of transitional cell carcinoma compared to adjacent nonneoplastic bladder mucosa (8). Moreover, this increase was significantly higher in high grade lesions. Similarly, our study showed that in low grade early invasive bladder cancer high EZH2 expression is present in less than one third of the cases, while high grade tumors exhibited EZH2 overexpression in more than 60% of the cases. In addition, prevalent finding of EZH2 expression in high grade lesions was homogeneous and diffuse nuclear staining with strong intensity, while the majority of low grade superficially invasive tumors that were classified as high EZH2 expressors showed heterogeneous stain, with areas of low to intermediate nuclear signal intensity. EZH2 stain was more intense in invasive tumor areas of deepest infiltration to lamina propria, than in superficial, often papillary segments.

The study that analyzed EZH2 in a large cohort of cystectomy specimens indicated that EZH2 expression is more frequent in invasive carcinoma than noninvasive papillary lesions (15). In addition, the authors concluded that high EZH2 expression was most consistent with carcinoma in situ, implying the best diagnostic utility of EZH2 in diagnosis of flat urothelial carcinoma in situ. We found that the majority of analyzed cancers associated with the finding of carcinoma in situ in adjacent mucosa in transurethral resection specimens aligned in the group with high EZH2 expression, and this association was statistically significant. This may support the notion that deregulation of EZH2 expression is an early event in bladder carcinogenesis, thus invasive tumor shares the same alteration of epigenetic factor with in situ precursor lesion.

One of the hallmarks of non muscle invasive UBC is a relatively high recurrence rate, often associated with tumor progression (1, 2). About 40% of the patients included in present study developed one or more recurrent bladder tumor during a follow-up period. However, EZH2 expression was not associated with recurrence of the disease, and provided no significant prognostic information in analysis of recurrence-free survival. Recent study described a model for high grade superficial urothelial carcinoma, where Rb-E2F-Ezh2 axis disruption provided a genetic base for tumor development (9). This study verified significant increase of EZH2 expression in tumor samples showing progression in recurrence. Moreover, increased EZH2 activity that mediates the increased progression risk of non muscle invasive UBC can be precluded by increased PIK3CA expression that acts as functional opponent to EZH2 (16). This implies that high EZH2 overexpression may not indicate relapse of the superficially invasive tumor, but its finding indicates more aggressive phenotype of the recurrent neoplasm.

Among all forms of UBC, early invasive pT1 bladder cancer, especially high grade non-muscle invasive carcinoma, is the most significant problem for clinical treatment. Therefore, numerous approaches to better stratify patients that would benefit from early radical cystectomy have been made (3, 4, 17). The use of immunohistochemical biomarkers is widely available and reliable method for analysis and classification of bladder cancer to prognostic groups. The prognostic significance of EZH2 in non muscle invasive bladder cancer yielded discrepant findings (15, 18). Our results indicate that high EZH2 expression in pT1 UBC is a strong predictor of shorter overall survival and is significantly associated to cancer specific death. Although multivariate analysis of a significant model failed to establish EZH2 expression as the independent predictor of poor patients' outcome, further studies are warranted to clarify the prognostic potential of EZH2 expression in urothelial neoplasms of the bladder.

During the recent years the inhibition of EZH2 expression has been recognized as an exciting novel therapeutic approach in cancer treatment (19 – 21). Inhibitors developed against EZH2 achieve their goal through direct or indirect mechanisms. Anticancer therapies targeting EZH2, now well established oncogene in many malignancies, have already imparted promising results in clinical trials in treatment of solid tumors and lymphoma. However, studies of EZH2 inhibitors in bladder cancer treatment are scarce, and, in spite of the profound understanding of EZH2 roles in bladder carcinogenesis, it will require a lot of work and solid evidence of efficacy to fully establish the clinical settings of using these therapeutics in management of urothelial bladder cancer.

Conclusion

Urothelial bladder cancer is one of the most common cancers worldwide and a major healthcare issue due to expensive process of diagnostics and clinical management. Early invasive bladder cancer is challenging to diagnose and very difficult to define in terms of behavior and evolution prediction. High EZH2 expression is found in one third of low grade early invasive UBC, while in high grade pT1 lesions it is twice as often. High EZH2 expression in early invasive urothelial bladder cancer indicates aggressive behavior of the tumor and poor prognosis. EZH2 has promising roles as a complementary diagnostic tool in selection of the patients that require closer clinical attention and second transurethral resection, and as a potential target for anticancer therapy.

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EKSPRESIJA EPIGENETSKOG PRIGUŠIVAČA EZH2 U RANO INVAZIVNOM PT1 UROTELNOM KARCINOMU MOKRAĆNE BEŠIKE

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Urotelni karcinom mokraćne bešike je najčešća maligna neoplazma urinarnog trakta. Rano invazivni karcinom bešike (stadijum pT1) je tumor u kojem je prisutna invazija subepitelnog vezivnog tkiva, bez zahvatanja mišićnog sloja, i predstavlja veliki izazov za dijagnozu i lečenje. EZH2 transkripcioni represor igra ključnu ulogu u onkogenezi mokraćne bešike. Cilj ovog istraživanja bio je da ispita profil ekspresije EZH2 u pT1 karcinomu bešike, analizira korelaciju ekspresije EZH2 sa kliničko-patološkim faktorima i da proceni njen prognostički značaj. U tom cilju je imunohistohemijski analizirano 279 tumora ukalupljenih u tkivne mikroareje na ekspresiju EZH2. Visoka jedarna ekspresija EZH2 zabeležena je u 44,5% tumora. Visoka EZH2 ekspresija bila je udružena sa visokim histološkim gradusom ($p < 0,001$). Pored toga, EZH2 značajno je korelirao sa muškim polom i pojavom carcinoma in situ u okolnom urotelu ($p = 0,019$ i $p = 0,026$), dok divergentna diferencijacija tumora i pojava recidiva bolesti nisu pokazale značajnu udruženost sa ekspresijom EZH2. Visoka EZH2 ekspresija bila je snažno povezana sa specifičnim mortalitetom uzrokovanim karcinomom ($p = 0,010$). Kaplan-Majer analiza preživljavanja pokazala je da visoka ekspresija EZH2 predviđa lošije preživljavanje obolelih ($p < 0,001$). Uticaj EZH2 na vreme bez razvoja relapsa tumora nije se pokazao statistički značajnim. Visoka ekspresija EZH2 u rano invazivnom karcinomu mokraćne bešike ukazuje na agresivno ponašanje tumora i lošiju prognozu. EZH2 ima potencijalno značajne uloge kao komplementarno sredstvo u dijagnostici za selekciju bolesnika koji zahtevaju intenzivniju pažnju kliničara i kao potencijalna meta antikancerske terapije.

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Ključne reči: *urotelni karcinom bešike, EZH2, imunohistohemija, prognoza*