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PREDICTIVE VALUE OF CLAUDIN-4 EXPRESSION IN NON-MUSCLE INVASIVE UROTHELIAL BLADDER CANCER

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Claudin-4 is an integral membrane protein of tight junctions, and its expression is frequently altered in epithelial cancers. Non-muscle-invasive urothelial bladder cancer (NMIBC) is a common neoplasm with an unpredictable clinical course that requires more precise stratification and risk assessment. The aim of this study was to investigate the association between Claudin-4 expression and clinicopathologic features of NMIBC, and to assess the predictive impact of Claudin-4 regarding to disease prognosis. The study comprised tumor tissue samples obtained from 441 patients with urothelial bladder cancer who had undergone transurethral resection. Samples were embedded in tissue microarrays and analyzed immunohistochemically for Claudin-4 expression. High expression was found in 41.6% of pTa and 47.8% of pT1 tumors. High Claudin-4 expression significantly correlated to high histologic grade ($p = 0.002$), and hematuria ($p = 0.038$). High Claudin-4 expression was more frequently observed in tumors with divergent differentiation, early invasive cancers associated with carcinoma in situ, and recurrent disease, however, these associations were not statistically significant. Kaplan—Meier survival analysis failed to indicate a significant difference in overall survival between the patients with high and low Claudin-4 expression. Conversely, recurrence-free survival was significantly associated with Claudin-4 expression ($p = 0.023$). In conclusion, overexpression of Claudin-4 is associated with high tumor grade and shorter recurrence-free survival. As an indicator of aggressive tumor behavior, Claudin-4 may serve as a potentially useful and accessible addition to the pathohistological panel for the prediction of clinical behavior of urothelial bladder cancer, as well as a promising therapeutic target.

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Key words: *urothelial bladder cancer, Claudin-4, tumor grade, recurrence, prognosis*

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Introduction

Claudin-4 is an integral membrane protein of tight junctions in epithelial cells, responsible for maintaining cell adhesion and polarity. A decrease of its expression may be associated with the loss of intercellular bonds and thus contribute to the progression of epithelial cancers, especially in the early stages of tumor invasion (1, 2). However, recent studies have indicated that Claudin-4 is not just a mere adhesion molecule that supports mechanical stability and integrity of the cell, but a much more versatile factor with many important roles in intracellular signalling with an impact on epithelial-mesenchymal transition, cell proliferation andlstemness, DNA repair and genomic instability (1, 3, 4).

Claudin-4 expression is frequently altered in epithelial cancers (5–8). Overexpression of Claudin-4 has been reported in many types of cancer, including breast, ovarian, gastric, pancreatic carcinoma, and oral squamous cell carcinoma. In most of them, Claudin-4 has been found to correlate with aggressiveness of the disease and poor prognosis. However, in some types of cancer, its decreased expression favors tumor invasiveness and progression, including mesothelioma, prostate and thyroid carcinoma (9–11).

Urothelial bladder cancer is a very common neoplasm of the genitourinary tract, frequently associated with exposure to environmental carcinogens, and has a strong association with smoking. It is a heterogeneous disease in terms of clinical behavior that reflects various genetic and epigenetic alterations that underlie the pathogenesis of urothelial carcinoma (12–14). The

majority of patients require life-long cystoscopic surveillance due to frequent recurrence of the disease (13).

Non-muscle-invasive urothelial carcinoma (NMIBC) is an early-stage urinary bladder carcinoma without invasion into the muscle layer of the bladder wall (detrusor muscle of the bladder). It comprises the majority of bladder cancer cases at the time of diagnosis, but this group is quite heterogeneous and associated with a notable risk of recurrence and progression (13, 14). NMIBC groups together the different entities: tumors staged as pTa, tumors with papillary architecture involving only urothelium with preserved basal membrane, tumors staged pT1, that have overt infiltration of lamina propria of the bladder mucosa, and carcinoma *in situ* (CIS). Patients with NMIBC tumors require careful estimation of risk progression and may be treated by several clinically diverse management protocols, from immediate chemotherapy instillation and intravesical bacillus Calmette–Guérin (BCG) immunotherapy to radical cystectomy which is considered in very high-risk patients. Therefore, NMIBC requires as precise as possible risk assessment and stratification (14, 15).

Several studies investigated the expression of Claudin-4 in urothelial bladder neoplasms (16–21). Immunohistochemical studies of Claudin-4 included a limited number of cases (under 100 tumors) and investigated and compared tumors of different pathologic stages. Heterogeneous results have been reported on the correlation between Claudin-4 expression and clinicopathologic features of urothelial cancer, while the prognostic significance varies between different types of urothelial lesions.

The aim of this study was to investigate the association between Claudin-4 expression and clinicopathologic features of NMIBC, and to assess the predictive impact of Claudin-4 in regard to disease prognosis.

Material and methods

Patients and histopathological analysis

This study comprised tumor tissue samples obtained from 441 patients with urothelial bladder cancer who had undergone transurethral resection during a 6-year period in the Clinic of Urology, University Clinical Center Niš, Serbia. All cases were diagnosed at the Center for Pathology, University Clinical Center Niš, according to the WHO classification (WHO, 2022, 5th edition) and staged according to the TNM pathological staging system (TNM classification 2016, 8th edition).

Average patients' age was 65.3 ± 9.6 years. Male patients comprised the majority of the study group, only 25% of the patients were women. Hematuria was the most common clinical symptom precluding the diagnosis, and it was detected in 86.6% of the patients. For every patient included in the study, detailed clinical data were obtained, including recurrence-free survival, as well as overall survival of the patients, and, if a patient died during the 5-year follow-up period, the cause of death was noted.

The study was approved by the Ethics Committee of the Faculty of Medicine, University of Niš, Serbia (Decision No 12-1250/8).

Immunohistochemical analysis

Following the diagnosis of NMIBC, including 173 cases of non-invasive papillary urothelial carcinoma (stage pTa), and 268 cases of superficially invasive urothelial carcinoma (stage pT1), the selected, representative areas of the tumor were incorporated in tissue microarrays, constructed using the manual tissue arrayer (Arraymold Paraffin Tissue Microarrayer, Arraymold, Utah, USA). Two core samples with a diameter of 2mm were selected from each case. Tissue microarray composite paraffin blocks were then cut in 3-micrometer thick sections and immunostained. Immunohistochemical analysis was performed using the primary antibody to Claudin-4, Rabbit polyclonal Anti-Claudin 4 antibody (ab15104, Abcam, Cambridge, UK). The slides were reviewed by two independent pathologists and staining intensity and distribution were assessed. Claudin-4 displayed a membranous staining pattern in urothelial cells, and, rarely, cytoplasmic immunoactivity. Moderate or strong immunostaining intensity in ≥50% of tumor cells was considered a high expression, according to the previously described methodology (18).

Statistical analysis

Analyses were performed using the statistical software for data processing SPSS version 20.0. The frequencies of categorical variables were tested by using the $\chi 2$ test with Yates's correction and Fisher's exact test. Overall survival and recurrence-free survival analysis about Claudin-4 expression were presented with Kaplan–Meier curves. $P \leq 0.05$ values were considered statistically significant.

Results

Immunohistohemical staining to Claudin-4 was found in the majority of investigated tumors, 80.9% of pTa and 87.3% of pT1 tumors (Figure 1). Only 33 tumors staged pTa, and 34 staged pT1 were negative, while 174 NMIBC displayed diffuse membranous staining of low intensity (faint yellowish precipitate), or focal staining in less than 50% of tumor cells with intermediate to strong intensity. These tumors were designated as low expressors. High expression was found in 41.6% of pTa and 47.8% of pT1 tumors, without statistically significant difference between the stages. Strong Claudin-4 expression significantly

correlated to high histologic grade (p = 0.002). Namely, 52.6% of high-grade tumors showed high Claudin-4 expression compared to 41.6% of low-grade tumors. High Claudin-4 expression was more frequently observed in tumors with divergent differentiation, early invasive cancers associated with carcinoma *in situ* in the immediate surroundings, and recurrent disease, however these associations were not statistically significant. High Claudin-4 was associated with hematuria (p = 0.038) (Table 1).

The median follow-up in the study group was 60 months. During that period, 41.3% of the patients had tumor recurrence, most of them had only one (105, 57.7%), while the rest had two or more recurrent tumors. Seventeen percent of the patients succumbed to the disease. The patients with cancer-specific mortality developed aggressive disease with locally advanced growth and metastatic spread. Kaplan–Meier survival analysis failed to indicate significant difference in overall survival between the patients with high and low Claudin-4 expression (Figure 2). Conversely, recurrence-free survival was significantly associated with Claudin-4 expression (p = 0.023). The analysis showed that patients with high Claudin-4 expression had shorter disease-free time and earlier occurrence of novel intravesical tumor growth.

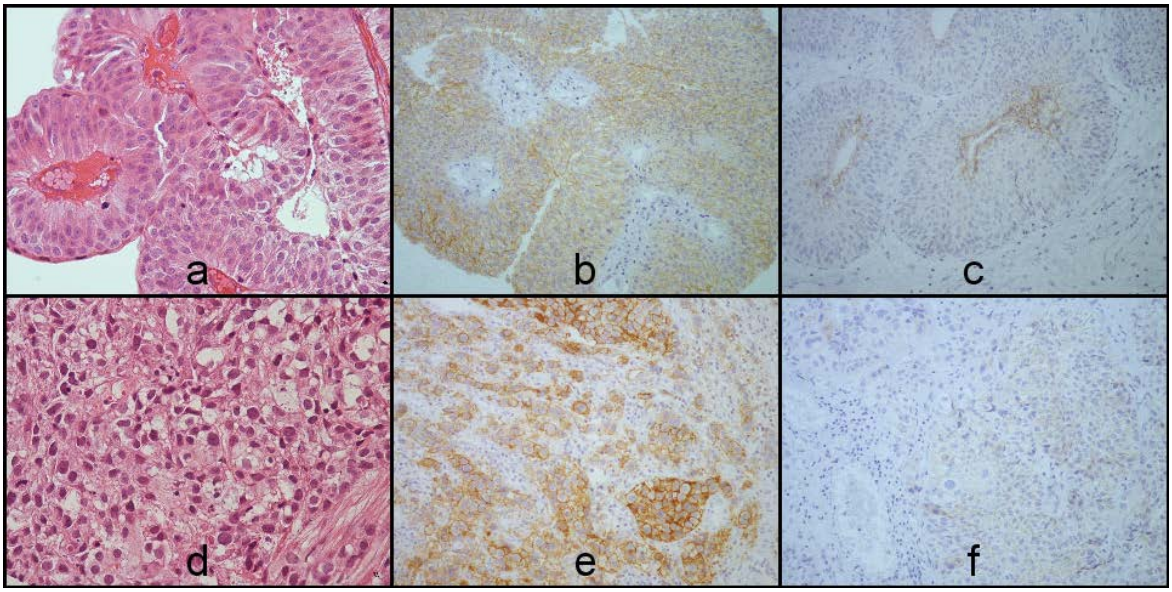

Figure 1. Representative immunohistochemical staining of Claudin-4 in non-muscle-invasive bladder cancer. The upper row shows pTa urothelial carcinoma on H&E stain (a), high Claudin-4 (b) and low Claudin-4 expression (c). The lower row displays superficially invasive pT1 bladder cancer with the infiltration of lamina propria on H&E stain (d), with high Claudin-4 (e) and low Claudin-4 immunohistochemical expression (f).

Table 1. Association of Claudin-4 expression with clinicopathologic features of non-muscle invasive bladder cancer (NMIBC)

Claudin-4 in NMIBC								
Claudin-4				**Low**		**High**		
	Total N (%)	441	(100)	241	(54.6)	200	(66.5)	**P *value***
Histologic grade								
	Low	228	(51.7)	140	(61.4)	88	(38.6)	**0.002**
	High	213	(48.3)	101	(47.4)	112	(68.1)	
Pathological stage								
	pTa	173	(39.2)	101	(58.4)	72	(41.6)	0.121
	pT1	268	(60.8)	140	(52.2)	128	(47.8)	
Carcinoma *in situ*								

	Yes	21	(4.8)	10	(4.1)	11	(5.5)	0.329
	No	420	(95.2)	206	(95.9)	394	(94.5)	
Divergent differentiation								
	Absent	400	(90.7)	222	(92.1)	178	(89.0)	0.169
	Present	41	(9.3)	19	(7.9)	22	(11.0)	
Hematuria								
	Yes	382	(86.6)	202	(52.9)	180	(47.1)	**0.038**
	No	59	(13.4)	39	(66.1)	20	(33.9)	
Recurrence								
	Yes	182	(41.3)	94	(51.6)	88	(48.4)	0.168
	No	259	(58.7)	147	(56.8)	112	(43.2)	
Cancer specific mortality								
	Yes	75	(17.0)	35	(46.7)	40	(53.3)	0.081
	No	366	(83.0)	206	(56.3)	160	(43.7)	

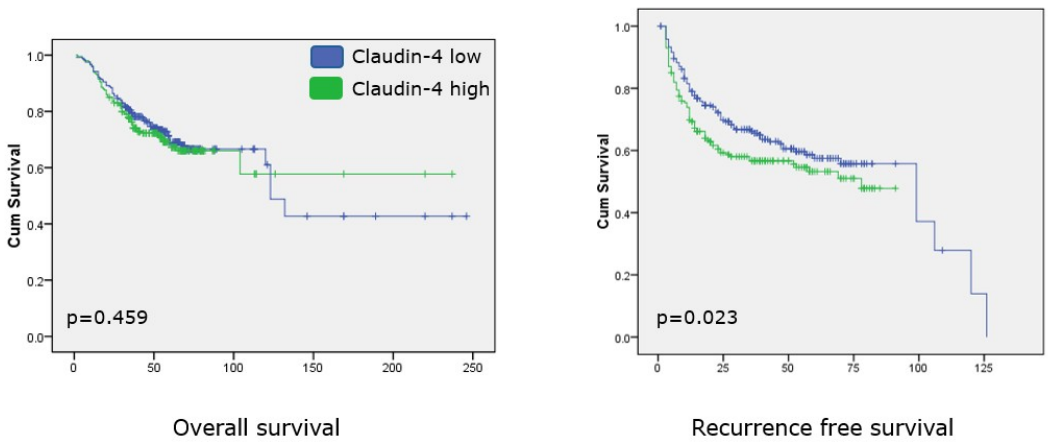

Figure 2. Kaplan–Meier survival curves showing overall survival and recurrence-free survival (the *x* axis represents the time in months) of 441 patients with non-muscle-invasive urothelial bladder cancer with low and high Claudin-4 expression.

Discussion

About 75% of urothelial bladder cancer is diagnosed at the stage of early cancer, NMIBC. The unpredictive nature of NMIBC emphasizes the need for more precise stratification of the disease in order to enhance the recognition of the patients who require more precise follow-up, cystoscopic surveillance or even early aggressive management. The 5-year recurrence rate of 31–78% for NIMBC and 10-20% progression to muscle invasive disease warrants close and expensive clinical monitoring (13–15).

The role of tight junction molecules has been recognized in the carcinogenesis of various epithelial neoplasms (3–8, 22). Claudins are major integral transmembrane proteins of tight junctions which expression profile varies between different tissues. During carcinogenesis, aberrant expression of certain claudin may contribute to invasiveness or impede tumor progression, in a tissue-specific manner. Claudin-4 is predominantly expressed in the kidneys and urinary tract, including the urothelial lining of the pelvis, ureters and bladder (2). In normal urothelium, Claudin-4 has a membrane expression pattern, with strong staining of the upper layers and progressive decrease towards the basal layers.

Accumulated evidence suggests that Claudin-4 overexpression represents an early event in carcinogenesis in many tumors (23, 24). Increased expression and abnormal distribution of Claudin-4 were found in many precancerous lesions, including premalignant changes in the

genitourinary, gastrointestinal and respiratory tract. In tumor cells, the function of tight junctions in polarity maintenance and pericellular trafficking is disturbed. Claudin-4 overexpression in those conditions may contribute to the enforcement of barrier subordinated to retain the tumor microenvironment (1, 3). In addition, Claudin-4 upregulates VEGF and Interleukin-8, thus promoting tumor angiogenesis (25). Recently, the role of Claudin-4 in the suppression of apoptosis and cell survival has been recognized (26, 27).

To our knowledge, this study is the largest investigation of immunohistochemical expression of Claudin-4 in urothelial carcinoma of the urinary bladder. This research comprised 173 pTa and 268 pT1 urothelial carcinomas, while previously published studies analyzed significantly smaller study samples. One of the pivotal studies that investigated Claudin-4 in various low-grade urothelial neoplasms, among other members of the claudin family, indicated that high claudin-4 expression in case of low-grade papillary urothelial cancer is associated with shorter recurrence-free survival (18). This is in accordance with our findings of statistically significant association of high Claudin-4 and recurrence-free survival, but not with the overall survival of the patients as well. Moreover, our results demonstrated a strong correlation between high Claudin-4 expression and high histologic grade of the tumors, indicating markedly worse clinical outcome in high expressor tumors.

On the contrary, several studies published in bladder cancer patients from the Egyptian population found a correlation between Claudin-4 expression and earlier T stage, and low-tumor grade (19, 20). The discrepancy between the conclusions of these authors and our results may be caused by the differences in the scoring of the immunohistochemical staining. The authors (19, 20) decided to enlist tumors with moderate staining scores in the group of high expressor tumors, while in the present study only the patients that they would designate as strong were considered high Claudin-4 expressors. Moreover, in these studies muscle-invasive cancers were more numerous than NMIBC, which was the subject of the present research. A recently published study of Claudin-4 expression in 50 cases of bladder cancer in the European population (21) indicated significantly higher scores of Claudin-4 immunoexpression in high-grade carcinomas. Moreover, the authors reported that Claudin-4 increases in muscle invasive tumors, suggesting the involvement of Claudin-4 in the progression of bladder cancer.

During the last decade, claudins have become a focus of interest for targeting therapies (1, 3, 25). Claudin-4 is currently being investigated as a possible treatment target, although no clinical trials have started yet. Targeting Claudin-4 can lead to a direct attack of cancer cells with Claudin-4 overexpression, but may also cause the disruption of tight junctions that stabilize and maintain tumor microenvironment, which supports and promotes cancer phenotype. Cancer cells expressing Claudin-4 could serve as a docker molecule for cytotoxic fusion proteins in a targeted therapy approach.

Conclusion

Non-muscle-invasive urothelial bladder cancer is a common neoplasm with an unpredictable clinical course that requires more precise stratification and risk assessment. Overexpression of Claudin-4 is associated with high tumor grade and shorter recurrence-free survival. As an indicator of aggressive tumor behavior, Claudin-4 may serve as a potentially useful and accessible addition to the pathohistological panel for the prediction of clinical behavior of urothelial bladder cancer, as well as a promising therapeutic target.

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PREDIKTIVNI ZNAČAJ EKSPRESIJE PROTEINA KLAUDIN-4 U UROTELNOM KARCINOMU MOKRAĆNE BEŠIKE BEZ ZAHVATANJA MIŠIĆNOG SLOJA

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Klaudin-4 je integralni membranski protein tesnih spojeva epitelih ćelija, čija je ekspresija često izmenjena u karcinomima. Karcinom mokraćne bešike bez zahvatanja mišićnog sloja (engl. *non-muscle-invasive urothelial bladder cancer* – NMIBC) čest je tumor sa nepredvidivim kliničkim tokom koji zahteva precizniju prognostičku stratifikaciju i procenu rizika. Cilj ove studije bio je da se ispita povezanost između ekspresije klaudina-4 i kliničkopatoloških karakteristika NMIBC-a, kao i da se proceni prediktivni značaj klaudina-4 za prognozu bolesti. Studija je obuhvatila uzorke tumorskog tkiva 441 bolesnika sa urotelnim karcinomom bešike, koji su dobijeni transuretalnom resekcijom. Uzorci su inkorporirani u tkivne mikroareje i analizirani imunohistohemijski na ekspresiju klaudina-4. Visoka ekspresija uočena je kod 41,6% pTa i 47,8% pT1 tumora. Visoka ekspresija klaudina-4 značajno korelira sa visokim histološkim gradusom ($p = 0,002$) i pojavom hematurije ($p = 0,038$). Visoka ekspresija klaudina-4 bila je češća kod tumora sa divergentnom diferencijacijom ranih invazivnih karcinoma povezanih sa karcinomom *in situ* i rekurentnom bolešću. Međutim, ove povezanosti nisu bile statistički značajne. Kaplan–Majerova analiza preživljavanja pokazala je da nije bilo značajne razlike u ukupnom preživljavanju između bolesnika sa visokom ekspresijom klaudina-4 i bolesnika sa niskom ekspresijom klaudina-4. Nasuprot tome, preživljavanje bez recidiva bolesti značajno je povezano sa ekspresijom klaudina-4 ($p = 0,023$). Može se zaključiti da je prekomerna ekspresija klaudina-4 povezana sa visokim tumorskim gradusom i kraćim preživljavanjem bez recidiva. Kao indikator agresivnog ponašanja tumora, klaudin-4 može poslužiti kao potencijalno koristan i pristupačan dodatak patohistološkom panelu za predviđanje kliničkog ponašanja karcinoma mokraćne bešike, a i kao i potencijalna meta ciljane terapije.

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***Ključne reči**: urotelni karcinom mokraćne bešike, klaudin-4, gradus tumora, recidiv, prognoza*