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ORIGINAL ARTICLE

High Grade T1 Papillary Urothelial Bladder Cancer Shows Prominent Peritumoral Retraction Clefting

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Abstract Differentiation of noninvasive from invasive papillary urothelial carcinoma can be challenging due to inability of proper orientation and thermal damage of transurethrally obtained material. The aim of this study was to analyze the presence and extent of peritumoral retractions in pT1 compared to pTa papillary urothelial carcinoma. Since peritumoral retractions may result from altered expression profiles of extracellular matrix proteins, we additionally analyzed the expression of matrix metalloproteinase 2 (MMP-2) and interleukin 8 (IL-8) in these tumors. The study comprised 50 noninvasive (pTa) and 50 invasive (pT1) cases of transurethrally obtained primary papillary urothelial carcinomas. The invasive nature of nests showing peritumoral retractions was confirmed immunohistochemically using antibody against collagen IV. Staining for MMP-2 and IL-8 was evaluated semiquantitatively using immunohistochemical staining index, calculated by multiplying the percentage of positive cells and staining intensity. Peritumoral retractions were found in 32% of pT1 carcinomas but in none of the pTa carcinomas. All tumors showing peritumoral retraction were high grade tumors. There was no statistically significant correlation between the expression of MMP-2 or IL-8 and the presence of peritumoral retractions or stage of the tumor (pTa vs. pT1). A statistically significant but weak correlation was found between MMP-2 and IL-8 expression (χ2-test, p=0,015). There was no statistically significant correlation between the presence of peritumoral retractions or MMP-2 expression and tumor recurrence and progression. Our study shows that, in doubtful cases, when differentiating between pTa and pT1 stages of papillary urothelial carcinoma, the presence of peritumoral retractions could favor the diagnosis of invasive neoplasm.

Keywords Urinary bladder · Papillary urothelial carcinoma · Peritumoral retraction clefting · Matrix metalloproteinase 2 · Interleukin 8

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Introduction

Owing to the highest lifetime treatment costs per patient of all cancers, urinary bladder cancer makes up a significant financial burden in the health system [1]. It is estimated that around 70% of patients present with a noninvasive or early invasive bladder cancer (stages Ta, Tis, T1) [2]. Beside the grade of disease, the TNM stage has the highest significance in choosing treatment options and estimating biological behaviour [3]. The most prevalent histological type of bladder cancer is that of urothelial origin. One of the major pitfalls in assigning the stage to papillary urothelial carcinoma in transurethrally obtained material is the differentiation between Ta and T1 stages, due to tangentially oriented tumor papillae, thermal damage of the material or strong inflammatory response in the stroma. Therefore, it is helpful to pay attention to additional histologic clues favoring tumor invasiveness, such as a single cell infiltration, finger-like projections of epithelium, paradoxical differentiation, retraction clefting or pseudosarcomatous stroma [4]. Retraction clefts are thought to be caused by loss of basal epithelial cells, lower expression of adhesion molecules and higher expression of proteins involved in extracellular matrix remodeling. Most extensive studies of peritumoral retractions

had been made on breast and prostate cancer, where their occurence has been proved to have both diagnostic and prognostic significance [5–8]. A few studies analyzing peritumoral retractions in urinary bladder cancer aimed at differentiating it from lymphovascular invasion or micropapillary variant of urothelial bladder cancer, both using immunohistochemistry [9–11].

The extracellular matrix (ECM) undergoes constant remodeling which is tightly regulated in order to preserve tissue homeostasis [12]. Although in normal conditions the ECM is able to localize the progression of tumor, it can also create susceptible microenvironment for tumor growth. Collagen IV is one the main components of basal membranes and the antibodies aimed against it are often used to confirm the invasiveness of the tumor. Matrix metalloproteinases (MMPs) play an important role in ECM remodeling and are thought to promote tumor growth, invasiveness and metastatic potential by participating in all steps of carcinogenesis – loss of tumor cohesiveness, basal membrane degradation, cell migration and blood vessel penetration [13]. Some MMPs are specific for certain tissues. A high expression of MMP-2, MTI-MMP and MMP-28 was found in urothelial carcinoma of urinary bladder [14]. Interleukin 8 (IL-8) is a proinflammatory cytokine which can improve tumor growth and survival through autocrine signaling, and induce tumoral blood vessel formation [15, 16]. Recently, IL-8 has emerged as a potential urinary biomarker for detection of both primary and recurrent bladder cancer [17, 18].

In our study we analyzed whether peritumoral retractions occur more frequently and are more pronounced in pT1 than pTa tumors. We also analyzed whether pT1 tumors have higher expression of MMP-2 and IL-8 than pTa tumors, particularly those with peritumoral retractions.

Patients and Methods

Patient's Selection and Clinical Data

The study was made on archival material taken from files of the Department of Pathology, Clinical Hospital Centre Sestre milosrdnice, Zagreb, comprising 100 consecutive cases of transurethrally obtained samples of primary papillary urothelial carcinoma, 50 stage pTa and 50 stage pT1. Micropapillary variant of urothelial carcinoma and papillary urothelial neoplasm of low malignant potential (PUN-LMP) were not included in the study. For all samples, tumor grade was assessed according to 2004 WHO classification of tumors. In the meanwhile, the 4th edition of the WHO classification was published (2016), but no significant changes were introduced regarding the grading of papillary urothelial carcinomas of urinary bladder [2, 19]. The follow-up data was available for overall 44 patients who developed recurrent disease (20 stage pTa, 24 stage pT1). The progression of disease was found in 22 patients. Table 1 shows the clinicopathological characteristics of analyzed cases.

Histopathology

Hematoxylin and eosin stained tissue sections from tumors were available for review in all cases. The slides were scanned for presence of peritumoral retractions under low magnification (× 40) and the extent of retractions was further analyzed under medium (× 100) and high (× 400) magnification of light microscope. As peritumoral retractions we considered empty spaces that partially or completely encircled tumor nests and separated them from the surrounding stroma. Artefactual retractions resulting from thermal damage of material, as well as areas of lymphovascular invasion, were not considered to be peritumoral retractions. According to extent of retractions, three groups were formed, as we previously described [5]: 1 – no peritumoral retractions; 2 – peritumoral retractions in less than 50% of tumor nest circumference; 3 – peritumoral retractions in 50% or more of tumor nest circumference.

Immunohistochemical staining was performed using standard procedures on a DAKO TechMate Horizon automated immunostainer (DAKO, Copenhagen, Denmark). The pretreatment of sections was performed using Dako PT link (deparaffinization, rehydration and epitope retrieval). After blocking the endogenous peroxidase activity by 5 min incubation with 3% hydrogen peroxide, the sections were incubated at room temperature with primary monoclonal mouse antibody against collagen IV (code M078501, clone CIV 22;

Table 1 Clinicopathologic characteristics of analyzed cases of papillary urothelial carcinoma of urinary bladder

	Noninvasive carcinomas (Ta)	Invasive carcinomas (T1)
Patients age (years)		
•Mean	64,6	70,9
•Range	33–86	35–92
Gender		
•Male	34 (68%)	41 (82%)
•Female	16 (32%)	9 (18%)
Tumor grade (WHO 2004)		
•Low	37 (74%)	7 (14%)
•High	13 (26%)	43 (86%)
Recurrence	20 (40%)	24 (48%)
•With progression	11 (55%)	11 (45,8%)
•w/o progression	9 (45%)	13 (54,2%)
Follow-up time (months)		
•mean	38,8	57,3
•range	14–107	14–124

Dako, Dennmark; dilution 1:25) for 60 min, primary monoclonal mice antibody against matrix metalloproteinase 2 (code ab3158; Abcam, Cambridge, UK; dilution 1:20) for 30 min or primary mice monoclonal antibody against interleukin 8 for 60 min (code ab18672, Abcam, Cambridge, UK, dilution 1:100). This was followed by incubation with the labeled polymer (EnVision HRP; Dako, Dennmark). Color was developed by incubation with 3,3′-diaminobenzidine tetrahydrochloride and slides were counterstained by hematoxylin. Normal lung tissue (collagen IV), normal placental tissue (MMP-2) and lung adenocarcinoma (IL-8) were used as a positive control.

The presence of basal membrane around tumor glands was determined immunohistochemically with antibodies against collagen IV, scanning the whole slide under medium (×100) and high (×400) magnification.

The results of immunohistochemical analysis were determined semiquantitatively using immunohistochemical staining index (ISI), obtained by multiplying the percentage of positive cells (PPC) and staining intensity (SI). The percentage of positive cells (PPC) was scored as 0 for no positive cells, 1 for up to 10% positive cells, 2 for >10–50% positive cells and 3 for more than 50% positive cells, while SI was scored as 0 for no staining, 1 for weak staining, 2 for moderate staining and 3 for strong staining. The immunohistochemical staining index was labeled as following: 0 = zero; 1–3 = low; 4–6 = moderate and 9 = high.

Statistical Methods

Statistical analysis was done using Kolmogorov-Smirnov, Kruskal-Wallis, $\chi 2$ and Spearman's rank correlation tests. Kaplan-Meier curve and log rank test were used for survival analysis. $P < 0.05$ was considered to be statistically significant. The analysis was made using IBM SPSS Statistics software version 21.0.

Results

Peritumoral retractions were found more often in papillary urothelial tumors of pT1 stage than those of stage pTa ($\chi 2$-test, $p < 0{,}001$), precisely in 16 cases of pT1 tumors but in none of the pTa tumors. Of positive cases, 7 showed peritumoral retractions in <50% of tumor nest circumference, and 9 in >50% of tumor nest circumference (Table 2 and Fig. 1). Peritumoral retractions were seen in additional two cases of pTa tumors and one case of pT1 tumor, but these were regarded as an artifact, since the basement membrane around tumor nests was highlighted by collagen IV staining.

Also, a statistically significant correlation was found between the presence and extent of peritumoral retractions and tumor grade ($\chi 2$-test, $p = 0{,}001$). All 16 cases showing peritumoral retractions (regardless of the extent) were high grade tumors.

Table 2 Presence of peritumoral retractions and immunohistochemical expression of MMP-2 and IL-8 in pTa and pT1 papillary urothelial carcinoma of urinary bladder

		pTa	pT1
Peritumoral retractions	None	50 (100%)	34 (68%)
	<50% circumference	0 (0%)	7 (14%)
	>50% circumference	0 (0%)	9 (18%)
MMP-2 staining index	No reaction	8 (16%)	13 (26%)
	Weak reaction	30 (60%)	31 (62%)
	Intermediate reaction	11 (22%)	6 (12%)
	Strong reaction	1 (2%)	0
IL-8 staining index	No reaction	34 (68%)	35 (70%)
	Weak reaction	16 (32%)	15 (30%)
	Intermediate reaction	0	0
	Strong reaction	0	0

There was no statistically significant correlation between pTa and pT1 group regarding the occurrence of recurrent disease, number of disease recurrences and progression of the disease. Also, there was no statistically significant correlation between tumors with and without peritumoral retractions regarding disease recurrence/progression. The extent of peritumoral retractions did not correlate with disease recurrence (log-rank test, $P = 0{,}796$)(Fig. 2). There was no statistically significant difference between median survival time of patients regarding the presence and extent of peritumoral retractions (Table 3).

Positive immunohistochemical reaction for MMP-2 was found in 42 cases of pTa and 37 cases of pT1 tumors, with most tumors having weak immunohistochemical staining index (Table 2 and Fig. 3). There was no statistically significant correlation between the expression of MMP-2 and the presence of peritumoral retractions, or grade and stage of the tumor, or disease recurrence/progression. A statistically significant but weak correlation was found between MMP-2 and IL-8 expression ($\chi 2$-test, $p = 0{,}015$). The expression of MMP-2 did not correlate with disease recurrence (log-rank test, $P = 0{,}478$)(Fig. 4). There was no statistically significant difference between median survival time of patients regarding the expression of MMP-2 (Table 4).

Positive immunohistochemical reaction for IL-8 was found in 16 cases of pTa and 15 cases of pT1 tumors, with all tumors having weak immunohistochemical staining index. There was no statistically significant correlation between the expression of IL-8 and presence of peritumoral retractions, grade and stage of the tumor, or disease recurrence/progression.

Aberrant expression for collagen IV was noticed in muscularis propria, and for MMP-2 and IL-8 in inflammatory cells.

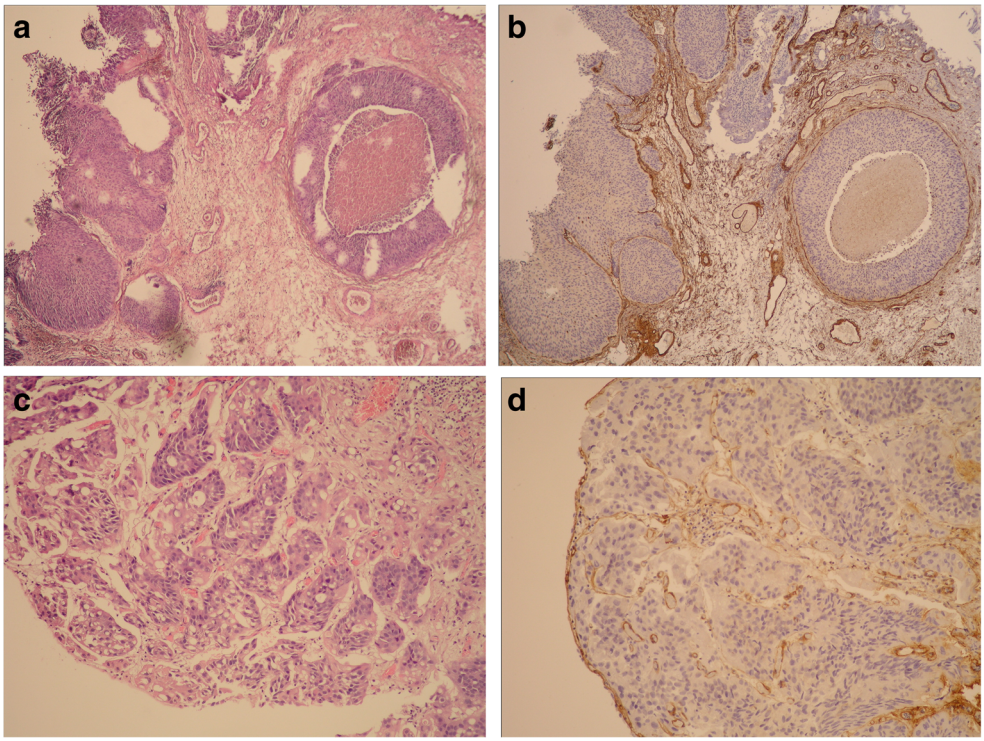
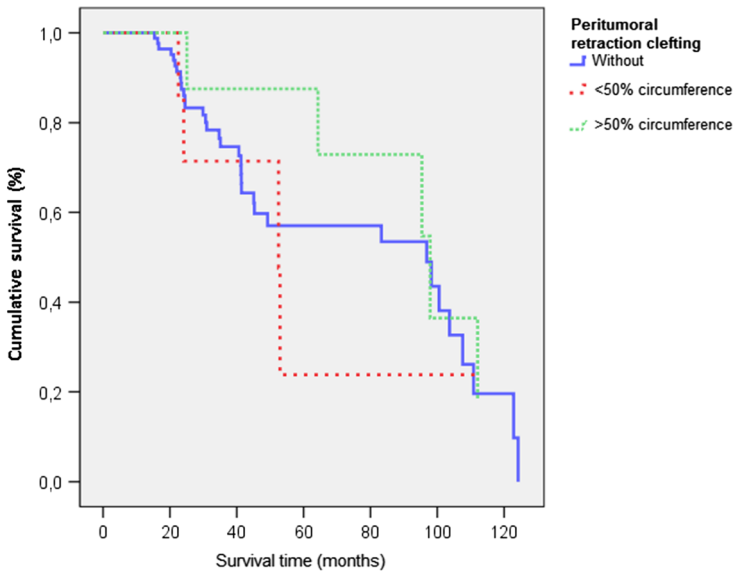


Fig. 1 Collagen IV expression in pTa papillary urothelial carcinoma of urinary bladder showing one tangentially cut papilla in the stroma, mimicking invasive nest (**a** – HE, × 40; **b** – collagen IV; ×40); and pT1 papillary urothelial carcinoma of urinary bladder showing multiple invasive nests with retractions (**c** – HE, × 100; **d** – collagen IV; ×100)

Discussion

Peritumoral retractions in bladder cancer have mainly been studied as an artifact that may be mistaken for vascular invasion. The studies revealed that lymphovascular invasion is rather rare in T1 bladder cancer and that fibroblasts encircling the retracted tumoral nests are frequently being misinterpreted as endothelial cells [9, 10]. Peritumoral retractions are also characteristic for the micropapillary variant of urothelial bladder cancer, which is usually high grade and muscle invasive and for which the treatment of choice is early cystectomy [20]. In our study, peritumoral retractions around invasive tumoral nests were found in 32% of T1 tumors. Peritumoral retractions have also been studied in in situ lesions, such as prostatic intraepithelial neoplasia, where they appear less frequently and in lesser



	X2 test	df	P
Log Rank (Mantel-Cox)	0,457	2	0,796

Fig. 2 Analysis of recurrent disease regarding the presence of peritumoral retractions. Kaplan-Meier survival curve and log rank test

Table 3 Median survival time in months (until recurrent disease or the end of follow-up) in relation to presence and extent of peritumoral retractions in papillary urothelial carcinoma of urinary bladder

Peritumoral retraction clefting	Median survival time (months)			
	Estimates	Standard error	95% CI	
			Upper	Lower
None	96,83	28,82	40,34	153,32
<50% circumference	52,50	13,62	25,80	79,20
>50% circumference	97,87	18,42	61,76	133,97
Total	95,40	21,22	53,81	136,99

extent than in invasive tumors [21]. In a study by McKenney et al. [22], about 80% of microinvasive bladder cancers (invasion in lamina propria less than 2 mm deep) showed retraction clefting around invasive tumoral nests [22].

A large number of carcinomas remain at the stage of intraepithelial lesion and never progress to invasive form, although the majority of genetic changes which exist in invasive and metastatic carcinomas is already present in premalignant forms [23, 24]. Even though it was previously thought that changes in tumoral stroma appear secondary to epithelial change, according to some recent studies, alterations in stroma could be a key regulator of tumor biological behaviour, including epithelial-mesenchymal transition [23, 25]. Cancer cells may influence the microenvironment to become more susceptible for its growth and progression, which is achieved by activation of fibroblasts or smooth muscle cells and attraction of endothelial and mesenchymal progenitors and inflammatory cells [26]. Alterations in tumoral stroma are also thought to be the key event in formation of peritumoral retractions. The relation of peritumoral retractions and stromal changes has mostly been analyzed in prostatic adenocarcinoma, where retractions are most extensive in Gleason grade 3, which also shows the most pronounced stromal changes [27]. Favaro et al. [28] found higher expression of MMP-2 in the stroma around tumor glands in prostatic adenocarcinoma, most of which showed retractions, but in our study, we did not find a statistically significant correlation between peritumoral retractions and MMP-2 or IL-8 expression.

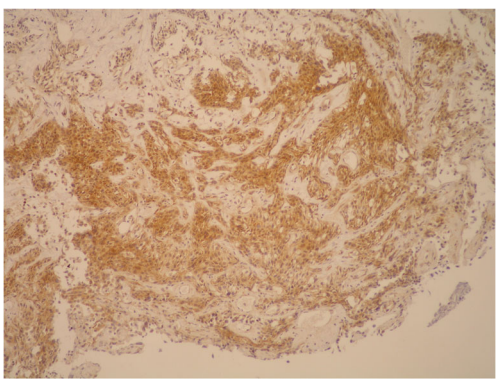
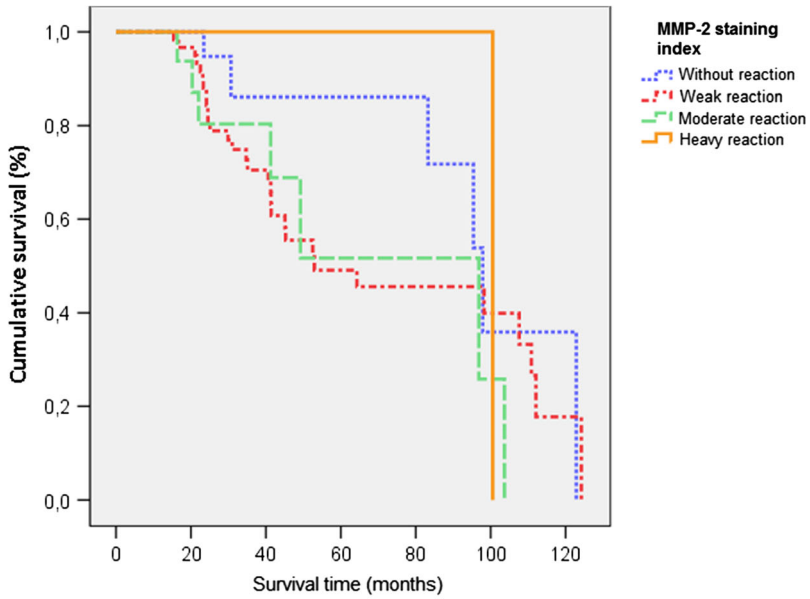


Fig. 3 Immunohistochemical expression of matrix metalloproteinase 2 (MMP-2) in pT1 papillary urothelial carcinoma of urinary bladder. In this case the staining intensity was strong, but the percentage of positive cells was 10–50%, making a moderate immunohistochemical staining index (MMP-2, ×100)

The presence and extent of peritumoral retractions in prostatic adenocarcinoma shows statistically significant correlation with the preoperative PSA value and the shorter biochemical recurrence-free interval [7], while in breast carcinoma, the extensive peritumoral retractions predict poor outcome [29]. Our results did not show a statistically significant correlation between peritumoral retractions in papillary urothelial bladder cancer and disease recurrence or progression, but this could be because of the small sample size of the study.

Matrix metalloproteinases may be expressed in both epithelial and stromal tumor components and can have adverse effect dependent on which substrates they act on, hence their actual role is hard to predict [30]. The difference between results of studies analyzing MMP expression in cancer is a consequence of, beside the aforementioned, the analysis of different tissues (blood, urin, cancer tissue of various localization, grade and stage), different methodology (mRNA, proenzyme or active protein measurement), different quantification of results and the use of different antibodies or protocols. Certain studies showed a potential role of MMP-2 and MMP-9 as urinary biomarkers for bladder cancer, but since MMPs expression is raised in both neoplastic and inflammatory processes, these tests can not offer a definite diagnosis, but can be used for screening of patients at risk and selecting them for more specific diagnostic work-up [30–32]. Davies et al. [33] found that the activity of MMP-2 and MMP-9, measured by zymography, shows a positive correlation with histologic grade of the tumor and invasion, with mRNA of both markers being localized mainly in stroma, at the border with the epithelial tumor component (in situ hybridization) [33]. The study by Vasala et al. [34] showed a statistically significant but weak correlation between MMP-2 expression and tumor stage, but not grade. Also, patients with MMP-2 positive tumors had an earlier recurrence [34]. Szarvas T et al. [35, 36] analyzed MMP-7 levels in patients with metastatic urothelial bladder cancer using PCR and immunohistochemistry for tissue sample analysis and ELISA for serum and plasma analysis [35, 36]. They found elevated tissue expression and elevated serum/plasma concentration levels of MMP-7 in patients with metastatic urothelial bladder cancer and showed these were a stage and



	X2 test	df	P
Log Rank (Mantel-Cox)	2,484	3	0,478

Fig. 4 Analysis of recurrent disease regarding the expression of matrix metalloproteinase 2 (MMP-2). Kaplan-Meier survival curve and log rank test

grade-independent risk predictors of both metastasis-free and disease-specific survival [35, 36].

In our study we found positive immunohistochemical expression for MMP-2 in 84% cases of pTa and 74% cases of pT1 urothelial papillary bladder cancer, but there was obviously no statistically significant difference between invasive and noninvasive tumors. There was actually a statistically significant negative correlation between MMP-2 expression and the tumor grade, but only according to 1973 WHO classification, meaning that tumors of lower grade showed stronger MMP-2 expression (Spearman correlation coefficient, rho = −0,241, $P < 0{,}001$). We did not find a statistically significant correlation between MMP-2 expression and tumor recurrence or progression.

Table 4 Median survival time in months (until recurrent disease or the end of follow-up) in relation to MMP-2 expression in papillary urothelial carcinoma of urinary bladder

MMP-2 staining index	Median survival time (months)			
	Estimates	Standard error	95% CI	
			Upper	Lower
No reaction	97,87	7,93	82,32	113,42
Low	52,93	26,44	1,12	104,75
Moderate	96,83	26,32	45,25	148,42
High	100,53	.	.	.
Total	95,40	21,22	53,81	136,99

Interleukin 8 is thought to enhance the level of MMP-2 and MMP-9 in tumor cells, which is believed to be responsible for its angiogenic activity [38]. In colorectal cancer, higher IL-8 expression shows positive correlation with tumor stage and lymph node and liver metastases [37]. In bladder cancer, IL-8 has mainly been investigated as a urinary marker using PCR and ELISA. Zhang et al. [39] investigated the correlation of immunohistochemical expression of several markers in bladder cancer, which were detected in urine. They found a positive correlation for 9 of 10 investigated markers, including MMP-9 and IL-8 [39]. In our study, we found IL-8 expression in 32% of pTa cancers and 30% of pT1 cancers, but the reaction was weak in all cases. However, we found a weak correlation between IL-8 and MMP-2 expression.

Conclusion

Our results show that peritumoral retractions are present in urothelial papillary bladder cancer of pT1 stage, but not in the noninvasive pTa stage. Using immunohistochemical staining for collagen IV, we confirmed that the majority of tumoral nests showing retractions were invasive, and we barred out lymphovascular invasion. All tumors showing peritumoral retractions were high grade. Considering the above we believe that in doubtful cases, when differentiating between pTa and pT1 stages of papillary urothelial carcinoma, the presence of peritumoral retractions could point toward an invasive neoplasm.

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Compliance with Ethical Standards

Conflict of Interest The authors declare they have no conflict of interest.

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