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Original Article

Relationship of PCNA, C-erbB2 and CD44s expression with tumor grade and stage in urothelial carcinomas of the bladder

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Abstract: In the present study, the intention was to reveal the relationship of histological grade and stage with c-erbB2, CD44s, and PCNA immunoreactivity in bladder urothelial carcinomas (UC). In our study, we evaluated 46 items of transurethral resection material of patients submitted by YYU Faculty of Medicine, Main Department of Pathology, with a mass revealed in their bladder after clinical and radiological studies at our laboratories and who were diagnosed with urothelial carcinomas. PCNA, c-erbB2, and CD44s were applied in an immunohistochemical manner comprised from nine low-malignant potential papillary urothelial neoplasia, 23 low-grade papillary urothelial carcinoma, and 14 high-grade papillary urothelial carcinoma. Immunostaining was scored according to the percentage of positive cells. The immunohistochemical study demonstrated that the c-erbB2 and PCNA staining ratio increased when an increase occurred in stage and grade. The CD44s staining ratio decreased. C-erbB2, PCNA, and CD44s appear to be a useful marker in the assessment of the prognosis and treatment options in urothelial carcinomas.

Keywords: Urothelial carcinomas, PCNA, CD44, C-erbB2, immunohistochemical study

Introduction

Nowadays, histologic grade and stage are considered to be basic parameters that determine the prognosis of bladder urothelial carcinomas (UCs) [1, 2]. However, histological grades of bladder UCs have a significant role in the biological behavior and tendency for recurrence. Tumors with a high histological grade are predisposed to have a higher stage and a worse prognosis at the very first diagnostic step when compared to tumors with a low histological grade [1, 3].

The c-erbB2 gene is localized on chromosome $17q^{12}$-q^{21-32}, and c-erbB2 protein is a 185 kDa transmembrane tyrosine kinase receptor, coded by this gene, which is a member of a family that consists of four receptors, which are necessary to be present in signal transmission routes that coordinate the differentiation of cellular growing [4, 5]. Due to the increased expression of this protein and gene amplification, it is the most frequently encountered c-erbB2 abnormality in cancer [6]. An increase in c-erbB2 expression is a very rare condition; however, when determined, it is closely related with the progression of bladder cancer [7]. PCNA is a 36 kDa nucleic-acidic protein essential for nuclear proliferation [8]. In miscellaneous studies carried out on bladder UCs, PCNA appeared to increase gradually when the grade and stage of the tumor escalated [7-9]. Regarding bladder UCs, there are several studies on the histological features of the tumor and the expressions of CD44s and its isoforms [10-13]. The CD44 family in human beings is coded by a single gene localized on chromosome

Table 1. Comparisons of the histopathological characteristics among subgroups of the histological grade

		Total (n = 46)	Histologic Grade			p
			LG-PUN (n = 23)	HG-PUN (n = 14)	PUNLMP (n = 9)	
Age		59.80 ± 12.57	59.08 ± 2.60	63.50 ± 10.94	55.88 ± 14.63	0.481
Gender	Male	41 (89.1)	20 (87.0)	12 (85.7)	9 (100)	0.502
	Female	5 (10.9)	3 (13.0)	2 (14.3)	0	
Stage	pT1	17 (37)	15 (65.2)	2 (14.3)	0	< 0.001[a,b,c]
	pT2	12 (26.1)	0 (0)	12 (85.7)	0	
	pTa	17 (37)	8 (34.8)	0	9 (100)	
CD44	(-)	2 (4.3)	0	2 (14.3)	0	0.011[c]
	(+)	8 (17.4)	3 (13)	5 (35.7)	0	
	(++)	21 (45.7)	11 (47.8)	5 (35.7)	5 (55.6)	
	(+++)	11 (23.9)	8 (34.8)	2 (14.3)	1 (11.1)	
	(++++)	4 (8.7)	1 (4.3)	0	3 (33.3)	
CerbB2	(-)	9 (19.6)	7 (30.4)	0	2 (22.2)	< 0.001[a,c]
	(+)	10 (21.7)	2 (8.7)	5 (35.7)	3 (33.3)	
	(++)	17 (37)	12 (52.2)	1 (7.1)	4 (44.4)	
	(+++)	10 (21.7)	2 (8.7)	8 (57.1)	0 (0)	
PCNA	(+)	13 (28.3)	7 (30.4)	1 (7.1)	5 (55.6)	0.001[a,c]
	(++)	17 (37)	12 (52.2)	2 (14.3)	3 (33.3)	
	(+++)	16 (34.8)	4 (17.4)	11 (78.6)	1 (11.1)	

Data were shown as mean ± standard deviation and n (%). According to pairwise comparison of chi square test; [a]: Statistically significant difference between t LG-PUN and HG-PUN groups, [b]: Statistically significant difference between t LG-PUN and DMP-PUK groups, [c]: Statistically significant difference between t HG-PUN and DMPPUK groups.

$11p^{13}$, and consists of a minimum 20 exons. Standard human CD44 isoform (CD44s) is a type 1 transmembrane molecule and consists of 341 amino acids [14].

In the study, we investigated the importance of c-erbB2, PCNA, and CD44s expression, and their relationship with the histopathological grade and stage of UCs.

Materials and methods

Study subject

Forty-six patients who were diagnosed with UC and had undergone a transurethral resection (TUR) to their bladder during the five-year period at the Pathology Main Scientific Department Laboratory were enrolled into the study. The entire cross-sections dyed with hematoxylin-eosin (H&E) extracted from the glass slide were re-reviewed. The histological grade was re-evaluated according to WHO/ISUP (World Health Organization and International Society of Urologic Pathology) consensus classification. Staging was determined using the TNM system of AJCC/UICC (American Joint Committee on Cancer Union for International Cancer Control). Then, paraffin blocks with a single item of H&E-stained glass slide, which illustrated the tumor best in 46 patients, were selected to be stained with c-erbB2, CD44s, and PCNA. Cross-sections with a thickness of four microns were deparaffinized in xylene, dehydrated in ethanol series, and incubated for 10 minutes in 3% H_2O_2.

Methods and immunohistochemical study

Afterwards, the glass slide was washed thoroughly with distillated water for 20 minutes and exposed to antigen for a total period of 20 minutes, with a one-minute break at five-minute intervals, in a target retrieval solution, which was diluted by 1/10. The solution was left for twenty minutes in room temperature and then washed with distillated water. It was then kept for five minutes in phosphate-buffered saline (PBS). Next, it was incubated for one hour with 1/200 diluted c-erbB2 antibody (polyclonal rabbit anti-human c-erbB2, DAKO, Glostrup, Denmark), 1/20 diluted ready-for-use PCNA antibody (monoclonal mouse anti-PCNA, clone code PC10, Dako, Glostrup, Denmark), and

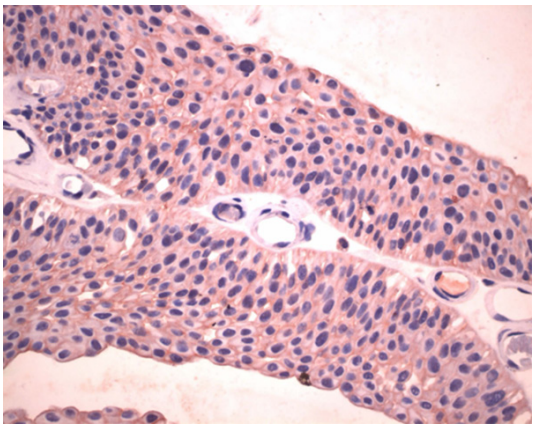

Figure 1. ++ staining with C-erbB2 (Immunperoxidase × 400) in patient with PUNLMP.

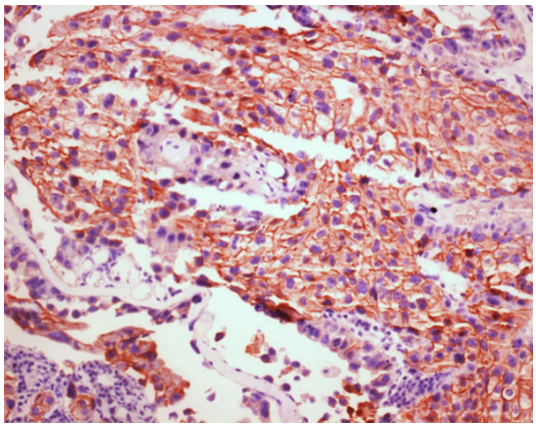

Figure 2. +++ staining with C-erbB2 (Immunperoxidase × 200) in patient with HG-PUN.

1/50 diluted CD44s antibody (mouse anti-human CD44s, clone code 156-3C11, Dako, Glostrup, Denmark). Then, it was kept for 10 minutes in PBS. After it was incubated for about 15 minutes in a biotin solution, it was kept for 10 minutes in PBS. Then, it was incubated for about 15 minutes in a streptavidin peroxidase solution. It was kept for five minutes in AEC (3 amino-9-etylcarbazole) chromogen after it was kept for 10 minutes in PBS. It was washed with distillated water. Cross-sections were then kept for five minutes in Mayer's hematoxylin for opposite staining. Afterwards, it was flushed with tap water and enclosed with a mounting medium (Entellan, Merck Millipore, Darmstadt, Germany).

The absolute membranous staining was mainly considered for c-erbB2 positive staining in

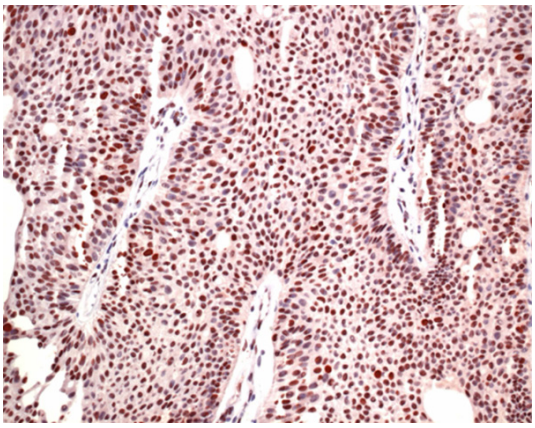

Figure 3. +++ staining with PCNA (Immunperoxidase × 200) in patient with LG-PUN.

preparations stained with c-erbB2 antibody, and cytoplasmic staining was assessed as negative. The entire cross-sectional tumor areas were considered as a whole during assessment of the staining process. The patient was considered to be c-erbB2 negative when there was no staining in any of the tumor cells. C-erbB2 immunoreactivity was considered (+) when incomplete membranous immunoreactivity was present in more than 10% of the tumor cells. C-erbB2 immunoreactivity was considered (++) when weak-moderate degree circumferential complete membranous immunoreactivity was present in more than 10% of the tumor cells. C-erbB2 immunoreactivity was considered (+++) when severe complete membranous immunoreactivity was present in more than 10% of the tumor cells [15]. The preparations stained with PCNA antibody were assessed after nuclear staining in tumor cells was considered. Low nuclear activity (staining in less than 25% of the tumor cells) was considered (+), moderate nuclear activity (staining between 25-75% of the tumor cells) was (++), and high nuclear activity (staining in more than 75% of the tumor cells) was (+++) [16]. In preparations stained with CD44s antibody, the staining was considered positive or negative according to the intensity and the number of stained cells. Non-staining was accepted as (-), weak-patching type staining as (+), moderate degree patching type staining as (++) (less than 50% of the cells), moderate degree diffused staining as (+++) (more than 50% of the cells), and strong diffuse staining as (++++) (more than 50% of the cells) [17].

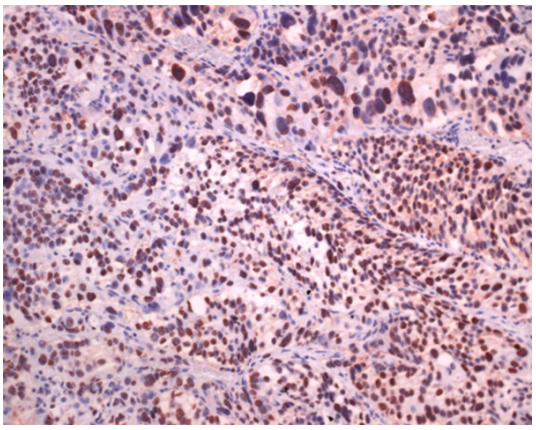

Figure 4. +++ staining with PCNA (Immunperoxidase × 200) in patient with HG-PUN.

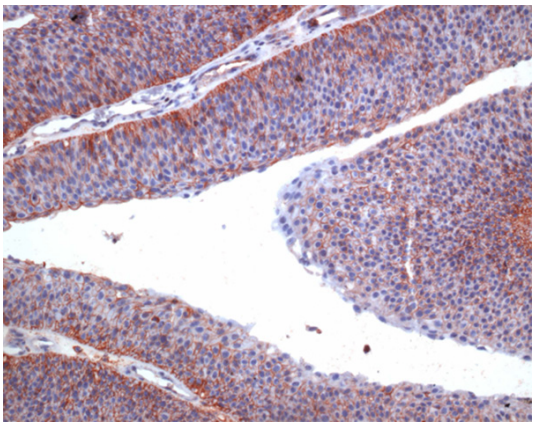

Figure 5. ++++ staining with CD44s (Immunperoxidase × 200) in patient with PUNLMP.

Statistical analysis

A chi-square test was used to compare the categorical variables. Categorical variables are shown as counts (n) and percentages (%). Spearman's correlation coefficients were used for the determination of the correlations between c-erbB2, CD44s, and PCNA immunoreactivity indexes WHO/ISUP histologic grade and stage. p values of < 0.05 were considered statistically significant. Analyses were performed using statistical software (IBM SPSS Statistics 20, SPSS Inc. An IBM Corp., Armonk, NY).

Results

The 46 patients who were included within the scope of the study consisted of 41 males (89.1%) and five females (10.9%). Comparisons of the histopathological characteristics among subgroups of the histological grade were shown at **Table 1**. During the staining process with c-erbB2, the staining observed in two patients out of nine with PUNLMP was (-) (22.2%). In three patients out of nine with PUNLMP, (+) (33.3%) staining was observed. In four patients out of nine with PUNLMP, (++) (44.5%) staining was observed (**Figure 1**). In seven patients out of 23 with LG-PUN, (-) (30%) staining was observed. In two patients out of 23 with LG-PUN, (+) (9%) staining was observed. In 12 patients out of 23 with LG-PUN, (++) (52%) staining was observed. In two patients out of 23 with LG-PUN, (+++) (9%) staining was observed. In five patients out of 14 with HG-PUN, (+) (36%) staining was observed. In one patient out of 13 with HG-PUN, (++) (7%) staining was observed. In eight patients out of 14, the staining observed was (+++) (57%) (**Figure 2**).

During the staining process with PCNA, the nuclear staining observed in five patients out of nine with PUNLMP was (+) (56%). In four patients out of nine with PUNLMP, (++) (44%) nuclear staining was observed. In seven patients out of 23 with LG-PUN, (+) (31%) nuclear staining was observed. In 12 of the 23 patients, (++) (52%) nuclear staining was observed. In four of the patients, (+++) (17%) nuclear staining was observed (**Figure 3**). In one patient out of fourteen with HG-PUN, (+) (7%) nuclear staining was observed. In two of the patients, (++) (14%) nuclear staining was observed. In 11 of the patients, (+++) (79%) nuclear staining was observed (**Figure 4**).

During the staining process with CD44s, the membranous staining observed in five out of nine patients with PUNLMP was (++) (56%). In one patient, the membranous staining observed was (+++) (11%). In three of the patients, the membranous staining was (++++) (33%) (**Figure 5**). In three out of 23 patients with LG-PUN, the membranous staining was (+) (13%). In 12 of the patients, the membranous staining was (++) (52%). In seven of the patients, the membranous staining was (+++) (31%). In one patient, the membranous staining was (++++) (4%). In two patients out of fourteen with HG-PUN, (-) (14.3%) membranous staining was observed. In five of the patients, the membranous staining was (+) (35.7%). In two of the

Table 2. Comparisons of immunstaining among subgroups of the histological stages

		Histologic Stage			p
		pT1 (n = 17)	pT2 (n = 12)	pTa (n = 17)	
CD44	(-)	1 (5.9)	1 (8.3)	0 (0)	0.062
	(+)	2 (11.8)	5 (41.7)	1 (5.9)	
	(++)	9 (52.9)	4 (33.3)	8 (47.1)	
	(+++)	5 (29.4)	2 (16.7)	4 (23.5)	
	(++++)	0 (0)	0 (0)	4 (23.5)	
CerbB2	(-)	5 (29.4)	0 (0)	4 (23.5)	0.001[a,b]
	(+)	2 (11.8)	3 (25)	5 (29.4)	
	(++)	9 (52.9)	1 (8.3)	7 (41.2)	
	(+++)	1 (5.9)	8 (66.7)	1 (5.9)	
PCNA	(+)	5 (29.4)	1 (8.3)	7 (41.2)	< 0.001[a,b]
	(++)	10 (58.8)	0 (0)	7 (41.2)	
	(+++)	2 (11.8)	11 (91.7)	3 (17.6)	

Data were shown as n (%). According to pairwise comparison of chi square test; [a]: Statistically significant difference between t pT1 and pT2 groups, [b]: Statistically significant difference between t pT2 and pT3 groups.

Table 3. Correlations between histological grade and stagewith immunstaining

	Histologic Grade		Histologic Stage	
	r	p	r	p
Histologic Stage	0.854	**< 0.001**	-	-
CD44	-0.449	**0.002**	-0.422	**0.004**
CerbB2	0.372	**0.011**	0.403	**0.006**
PCNA	0.550	**< 0.001**	0.493	**0.001**

r: Spearman's correlation coefficients. Statistically significant *p* values were shown as bold.

patients, the observed membranous staining was (+++) (14.3%). Comparisons of the histopathological characteristics among subgroups of the histological stage were shown at **Table 2**.

When stage was compared with the WHO/ISUP grading system, nine of the entire patients with PUNLMP displayed limited invasion at the mucosa (pTa), eight patients out of 23 with LG-PUN displayed limited invasion at the mucosa (pTa), 16 of the patients displayed submucosal invasion (pT1), two patients out of 14 with HG-PUN displayed submucosal invasion (pT1), and 12 of the patients displayed muscle invasion.

Areas with focal squamous differentiation was present in four of our patients with HG-PUN, and focal necrosis areas were observed in six patients. Vessel invasion was present in three patients. All the patients with a vein invasion were diagnosed with HG-PUN and were at stage pT2. In the pT2 stage (25%), (+) (25%) staining was observed in three patients, (++) (8%) staining was observed in one patient, and (+++) (67%) staining was observed in eight patients.

Discussion

Bladder UC can be seen at any age, including childhood. However, UC is recognized as a condition commonly seen in individuals of advanced age, and it is more likely in men [18]. The disease is somehow well differentiated in adolescents between 30-40 years of age and usually displays a silent behavior. Generic and molecular variations that can be observed in tumors of the bladder, which can be seen in this population, may not be compatible with the clinical behavior and histological grade regarding bladder tumors of individuals of mid and advanced age. The prognosis in young individuals is much better, as these tumors can be more superficial, low-graded tumors [19]. Our study group consisted of 41 males (89.1%) and five females (10.9%) out of 46 patients, and the male/female ratio was 8:1. It was also observed that histological grade showed a tendency to increase by age.

Generally, the grade of a tumor is recognized as a significant prognostic factor in urothelial tumors. Nevertheless, there is a strong relationship between the grade and stage of the tumor and in many studies tumor grade was assessed as a prognostic factor in mixed groups with non-invasive (Ta) and invasive (T1 and higher) tumors [1-3]. In our study, one can easily monitor how the histological grade of tumor has increased while staging increased (P = 0.00). Correlations between histological grade and stage and other parameters of the present study were shown at **Table 3**. Furthermore, patients with a vein invasion and squamous differentiation had high-graded tumors. After all, in some other studies, grade

could not be shown as a significant prognostic parameter [20-22].

In some studies, c-erbB2 expression was shown to increase in UCs [1, 23, 24]. The c-erbB2 ratio in UCs may vary between 2-74% in various studies [1, 25-28]. Yet, the prognostic importance of c-erbB2 is controversial due to the results included in the literature. Therefore, even though some studies demonstrated high c-erbB2 expression, which were somehow related with more aggressive clinical behavior in patients with UC, no prognostic relationship was found in other studies [1, 25-30]. In our study, we observed that c-erbB2 expression increased once the histological grade and stage of UCs increased, and the relationship between them was found statistically significant (P = 0.011) and (P = 0.006).

In our study, we determined that the PCNA immunostaining ration increased as the histological grade and stage of UCs showed a tendency to increase, and consequently the relationship between histological grade and stage of UCs was found statistically significant (P = 0.000) and (P = 0.001). PCNA expression was suggested to be an indicator of malignancy potential and the proliferation capacity of the tumor in tumors of the bladder. In various studies carried out on bladder UCs, it was demonstrated that PCNA gradually increased with an increase in the grade and stage of the tumor. Present studies have revealed the fact that PCNA scores of high-grade tumors were higher. It was also revealed that there was a significant relationship between superficial and invasive bladder tumors and high expressions of PNCA [7-9].

There are several studies that investigate the relationship between the histological characteristics of the tumor and the expressions of CD44s and its isoforms in bladder UCs [10-13]. There are also many studies that have demonstrated that standard CD44s and its isoforms are a prognostic factor in urothelial tumors [11, 31-33]. In a study carried out by Desia et al [31] on 35 patients with urothelial neoplasia, it was reported that CD44s immunoreactivity progressively decreased when the grading in papillary urothelial neoplasia increased during pTa and pT1 stages. Concurrently, they revealed the relationship between cytokeratin 20 and CD44s protein expression, pTa and pT1 papillary urothelial neoplasia, and WHO/ISO grading. In our study, we observed that CD44s staining ratio and severity decreased while the stage and histological grade increased in patients with UC. There was a statistically significant relationship between CD44s expression and stage and histological grade (P = 0.002) and (P = 0.004).

C-erbB2s and PCNAs immunostaining ratio tends to increase as the grade and stage increases in patients with a bladder UC, and there is a statistically significant relationship between them. The high positivism of these identifiers can be assessed as an indicator for aggressiveness. However, there is a reverse relationship between CD44s high expression and the histological grade and stage of the tumor. CD44s expression shows a tendency to decrease as grade and stage increase. C-erbB2, PCNA, and CD44s, which are used as immune markers, are considered to be a useful identifier in the evaluation of the prognosis regarding UCs and their treatment options.

Disclosure of conflict of interest

None.

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