Clinical Use of Tumor Markers for the Detection and Prognosis of Bladder Carcinoma: A Comparison of CD44, Cytokeratin 20 and Survivin

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Purpose: To investigate the role of CD44, cytokeratin 20 (CK20) and survivin for the detection and prognosis of patients with urothelial carcinoma of the bladder.

Materials and Methods: The study included 82 patients who underwent transurethral resection of bladder tumors between 2009 and 2014. The patient and tumor characteristics with relevance to age, tumor size and focality, grade and stage, recurrence and progression were noted. Patients with carcinoma in situ, those who had at more than 3 sites of lesions and greater than 3 cm tumors were excluded. All cases were ex-smokers. All histological samples stained with hematoxylin and eosin were re-evaluated according to the 2004 World Health Organization/International Society of Urological Pathology (WHO/ISUP) classification system and immunohistochemically stained for CD44, CK20 and survivin.

Results: The study group comprised 57 (69.5%) males and 25 (30.5%) females with a mean age of 60 years (range, 26-87 years). All were newly-diagnosed patients with bladder tumors. Immunohistochemical evaluation revealed that there was a statistically significant correlation between the grade and stage of the tumor with CK20 and survivin positivity ($P < .05$). As the grade and stage increased CD44 immunoreactivity significantly decreased ($P = .002$, $P = .0001$, respectively). However, relationship of protein expressions with recurrence and progression remained insignificant ($P > .05$).

Conclusion: In cases of bladder urothelial carcinoma positivity for CD44, CK20, and survivin has significant relation with the tumor grade and stage while no significant relationship was determined in terms of recurrence and progression

Keywords: biomarkers, tumor/analysis; carcinoma, transitional cell/pathology; disease progression; ımmunohistochemistry; neoplasm grading; neoplasm invasiveness; prognosis; urinary bladder neoplasms/mortality.

INTRODUCTION

Bladder carcinoma is the 9th most common malignant neoplasm among all cancers and the most common cancer of the urinary tract.[1] Approximately 75% of patients have non-muscle-invasive disease while 20% have invasive and 5% have metastatic tumors at first presentation.[2] Of non-muscle-invasive patients, more than 60% will eventually experience disease recurrence and approximately 42% will experience disease progression in ten years.[3] Markers suitable for diagnosis and prediction of prognosis have always been a major concern in management of these patients. Cystoscopy has been standing as the gold standard diagnostic and follow-up measure. Yet, development of urine-based markers such as fluorescent in situ hybridization (FISH), nuclear matrix protein 22 (NMP22), bladder tumor antigen (BTA) and ImmunoCyt® has increased the accuracy of diagnosis.

CD44 is a widely distributed cell surface adhesion molecule that has a crucial role in tumor-endothelial interaction, cell migration, adhesion, tumor progression, and metastasis. In particular, CD44 is expressed in many cancers including urothelial carcinoma and has prognostic value.[4-8]

Cytokeratins (CK) are intermediate filament proteins expressed in epithelial cells with more than 20 known subtypes compiled in two groups as CK9 to 20, type 1; and CK1 to 8, type 2.[9] Along with colon, gastric, pancreatic and mucinous ovarian tumors CK20 is expressed in bladder transitional cell carcinoma (TCC). CK20 expression is associated with cell differentiation and CK20 is essentially expressed on the surface of um-

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brella cells. This surface restriction is lost in bladder tumor cells and CK20 expression can be observed in all layers.[10,11]

Survivin is an intracellular protein comprising 142 amino acids and is a member of the inhibitors of apoptosis gene (IAP) family. It is responsible for the inhibition of apoptosis by blocking caspase 3, 7 and 9 pathways.[12] It is expressed at G2/M phase in the cell cycle. Survivin expression has been determined in many types of cancer.

The aim of this study was to assess the relationship of CD44, CK20 and survivin expressions as probable prognostic factors with relevance to tumor grade and stage in urothelial carcinoma of the bladder.

MATERIALS AND METHODS

Study Population

The study included 82 patients who underwent transurethral resection of bladder tumors between 2009 and 2014. There were 57 male (69.5%) and 25 female (30.5%) cases with a mean age of 60 years (range; 26-87 years). Patients with carcinoma in situ, those with tumors at more than three sites and greater than 3 centimeters and those who had incomplete resection were excluded. Staging was done by using the Tumor/Node/Metastasis (TNM) system of the American Joint Committee on Cancer / Union Internationale Contre l'Cancer (AJCC/UICC).

Procedures

Histopathological specimens of the patients were re-evaluated by single pathologist (E.S.A.) to determine the appropriate blocks for immunohistochemical (IHC) staining. And these findings were validated by another pathologist. The hematoxylin-eosin (H & E) sections were re-evaluated to verify tumor grades according to the 2004 World Health Organization/International Society of Urological Pathology (WHO/ISUP) classification system. Sections for IHC staining for CD44, CK20 and survivin were selected from best representative specimen blocks.

The IHC staining was done using the avidin-biotin peroxidase method with a diaminobenzidine (DAB) chromatogen and counterstained with hematoxylin. Tissue sections were deparaffinized, rehydrated, and microwave antigen retrieval in ethylenediaminetetraacetic acid (EDTA) buffer was performed. For 20 minutes pH 7.6 was maintained. The sections were then incubated with polyclonal survivin (Survivin-Thermo- RTV- B9245R1); CD44 (Biocare Medical Concentered- CM318A); and CK20 (Biocare Medical-RTV- PM062AA) at a dilution of 1: 200 for 60 minutes.

Table 1. Patient and tumor characteristics.

Clinicopathological Characteristics		Values
Age, (year)		60 (26-87)
Sex, no (%)	Male	57 (69.5)
	Female	25 (30.5)
Tumor grade, no (%)	Low grade	44 (53.7)
	High grade	38 (46.3)
Tumor stage, no (%)	Ta	45 (54.9)
	T1	28 (34.1)
	T2	9 (11)
Recurrence, no (%)	Present	49 (59.8)
	Absent	33 (40.2)
Progression, no (%)		12 (14.6)

Evaluations

CD44 is often applied as membranous staining in our study. Survivin was considered positive for cytoplasmic staining. Cytoplasmic staining was evaluated for cytokeratin 20.

A common scoring system was determined for all staining based evaluations and scoring was noted from 0 to 4 as, no staining (-) = 1; patchy weak staining (+) = 1; moderate patchy staining (in less than 50% of the cells) (++) = 2; moderate diffuse staining (more than 50% of the cells) (+++) = 3; and strong diffuse staining (more than 50% of the cells) (++++) = 4.

Statistical Analysis

All statistical analyses were performed with Statistical Package for the Social Science (SPSS Inc, Chicago, Illinois, USA) version 17.0. Categorical data were analyzed using Fisher's exact test and the chi-square test. The relationship between two categorical variables was assessed with Spearman's correlation coefficient. Differences were considered significant when $P < .05$.

RESULTS

The WHO/ISUP classification revealed 44 cases (53.7%) of low-grade papillary urothelial carcinoma and 38 cases (46.3%) of high-grade papillary urothelial carcinoma among all newly diagnosed patients with bladder carcinoma. Staging with the TNM system disclosed 45 pTa patients (54.9%); 28 pT1 patients (34.1%); and 9 pT2 cases (11%). Recurrences occurred in 49 of 82 patients (59.8%). A new tumor detection was described as recurrence within 2 years. During a mean follow-up of 23 months 7 patients progressed from Ta to T1 disease and 5 patients progressed form

Table 2. Comparison of WHO/ISUP grade and stage with the expression of CD44, CK20 and survivin.

Variables	Grade no (%)			Stage no (%)			
	Low	High	*P* Value	pTa	pT1	pT2	*P* Value
CD44							
0	2 (4)	7 (18)		1 (2)	6 (21)	2 (22)	
1+	5 (12)	17 (45)		6 (13)	11 (39)	5 (56)	
2+	8 (19)	10 (26)	.0001	11 (25)	5 (17)	2 (22)	.002
3+	25 (58)	4 (11)		23 (52)	6 (23)	0 (0)	
4+	3 (7)	0 (0)		3 (8)	0 (0)	0 (0)	
CK20							
0	13 (31)	1 (2)		12 (28)	1 (3)	1 (11)	
1+	22 (52)	4 (10)		21 (49)	5 (18)	0 (0)	
2+	2 (5)	11 (28)	.0001	3 (7)	9 (32)	1 (11)	.001
3+	4 (10)	16 (47)		4 (9)	10 (36)	6 (67)	
4+	1 (2)	6 (13)		3 (7)	3 (11)	1 (11)	
Survivin							
0	14 (32)	0 (0)		14 (31)	0 (0)	0 (0)	
1+	26 (60)	2 (5)		26 (60)	2 (6)	0 (0)	
2+	4 (8)	21 (55)	.000	4 (7)	17 (62)	4 (45)	.000
3+	0 (0)	13 (35)		1 (2)	8 (29)	4 (45)	
4+	0 (0)	2 (5)		0 (0)	1 (3)	1 (10)	

Abbreviation: WHO/ISUP, World Health Organization/International Society of Urological Pathology.

T1 to T2 disease yielding a total progression rate of 14.6% (**Table 1**).

CD44

Only 1 case was excluded because of failure in IHC staining and 81 cases were eligible for evaluation. Loss of CD44 immunoreactivity was significantly associated with increasing tumor grade and stage (P = .0001, P = .002, respectively) (**Table 2**). CD 44 expression was positive in 43 patients (90%) with recurrences and in 29 patients (88%) without recurrence and the difference remained insignificant. On the other hand, 3 of 12 cases (25%) with tumor progression and 6 of 69 cases (9%) without progression showed loss patterns of CD44 protein expression. This difference also did not reach statistical significance (P = .2) (**Figures 1 and 2**).

Cytokeratin 20 (CK20)

The results of the IHC staining for CK20 are summarized in **Table 2**. Since 2 cases failed to show enough tumor cells for IHC staining the results were evaluated for 80 patients. Increased CK20 positivity was significantly associated with increasing tumor grade and stage (P = .0001; P = .001, respectively). In relevance to tumor recurrence and progression CK 20 expression yielded similar results with those obtained for CD44. Although recurrence rate in patients with CK20 staining was higher than CK20 negative cases (93% versus 75%) the difference did not reach statistical significance (P = 0.1). Similarly, CK20 expression did not show significant correlation with tumor progression (P = 0.3) (**Figures 3 and 4**).

Survivin

Increased survivin positivity was significantly associated with increasing tumor grade (P = .000). Diffuse staining more than 50% (score 2) was not evident in low-grade tumors while it was observed in all cases of high grade tumors. Survivin immunoreactivity in low and high grade tumors are shown in **Figures 5 and 6**. Diffuse strong staining (score 3) was not observed in Ta stage tumors, but was present in all patients with T2 tumors. Over-expression of survivin was associated with

Table 3. Comparison of CD44, CK20 and survivin.

Variables		CD44	CK20
CD44	Correlation coefficient (P)		
	n		
CK20	Correlation coefficient (P)	-0.484 (*)	
	n	79	
Survivin	Correlation coefficient (P)	-0.493 (*)	0.646 (*)
	n	81	80

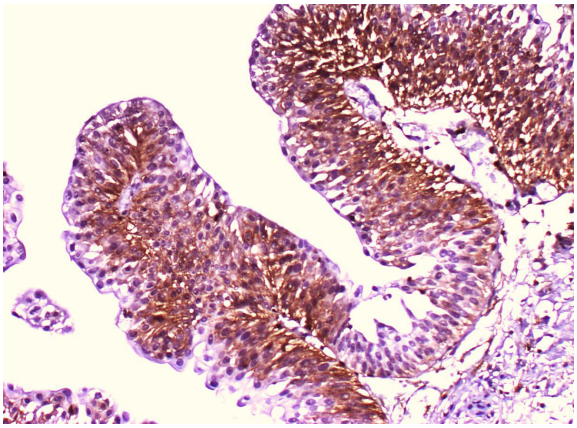

Figure 1. Low grade papillary urothelial carcinoma, with staining CD44 (+++) (×200).

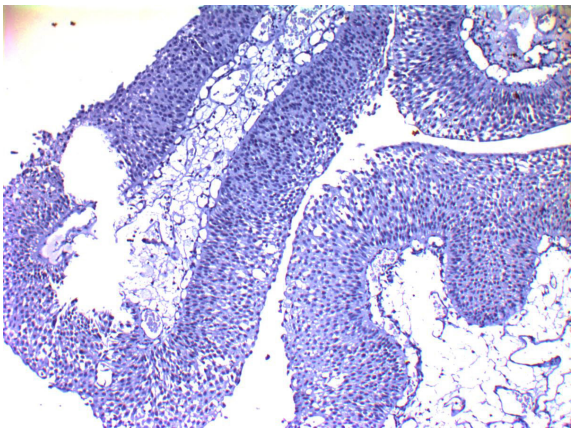

Figure 3. Low grade papillary urothelial carcinoma, with staining CK20 (-) (×100).

a higher tumor stage ($P = .000$) (**Table 2**). Survivin positivity did not show statistically significant correlation with tumor recurrence or progression ($P = .8$ and $P = .5$, respectively). Survivin immunoreactivity was observed in 86% (42/49) of cases without recurrence, and in 80% (26/33) of patients with recurrence.

The results of survivin, CD44 and CK20 evaluated together are summarized in **Table 3**. A statistically significant negative relationship was present between CD44 with survivin and CK20 ($P < .01$; $P < .01$, respectively). A significant positive correlation was found between survivin and CK20 ($P < .01$).

With both standard histopathological and IHC evaluation, tumor grades were found to be significantly higher in patients with solid and mixed patterns, in cases with multiple tumors and in cases with squamous differentiation of the tumor. As the tumor grade increased, tumor progression also increased. The tumor stage was associated with tumor pattern and squamous differentiation but was independent of sex. In all cases survivin, CD44 and CK20 immunoreactivity was associated with tumor grade and stage but did not show significant correlation with recurrence and progression.

DISCUSSION

Biochemical markers are inadequate to predict potentially more aggressive tumors and ensure the right treatment. Molecular studies are particularly considered for this purpose. CD44, CK20, and survivin have been investigated as markers but not reviewed for predicting prognosis.

CD44 is a widely distributed cell surface adhesion molecule. Alterations of CD44 expression patterns are linked to tumor invasion and formation of metastases. [4] In a study by Desai and colleagues CD44 immunoreactivity was reported to be significantly related to grade with papillary pTa and pT1 neoplasms in 120 patients with urothelial carcinoma. Loss of CD44 immunoreactivity and increasing CK20 positivity were also significantly associated with increasing tumor grade and stage. [5] Lipponen and colleagues investigated expression of

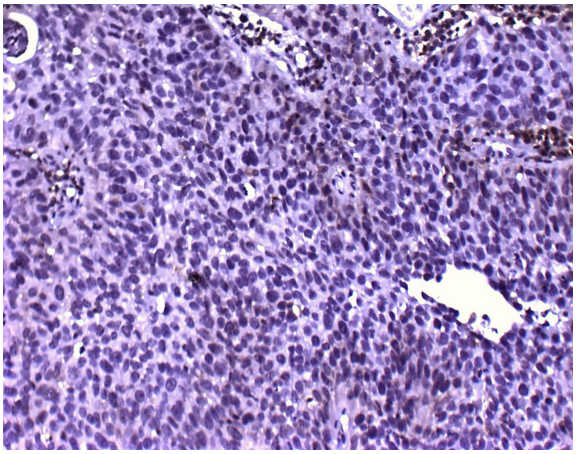

Figure 2. High grade papillary urothelial carcinoma, with staining CD44 (-) (loss of patterns) (×200).

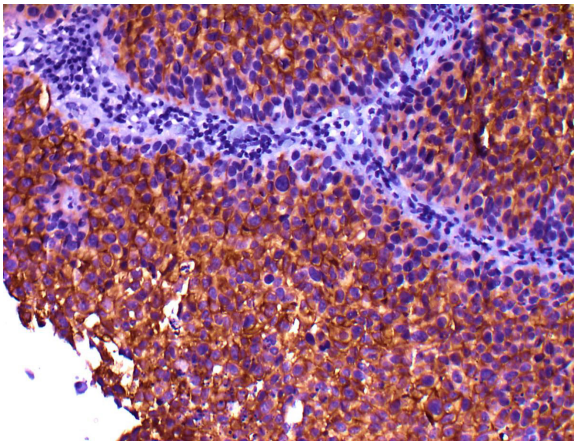

Figure 4. High grade papillary urothelial carcinoma, with staining CK20 (++++) (×200).

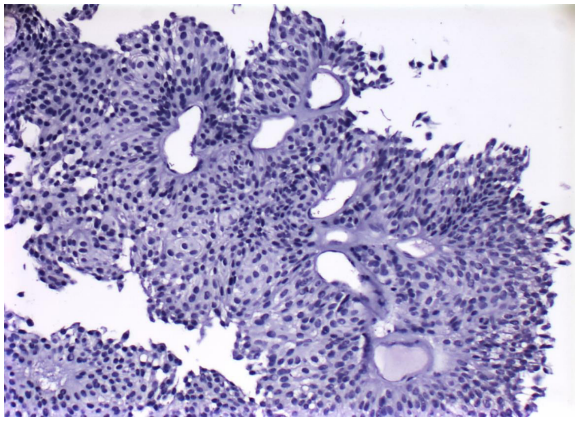

Figure 5. Low grade papillary urothelial carcinoma, with staining survivin (-) (immunoperoxidase ×200).

CD44 and its v6 isoform, CD44v6 in normal bladder and cancer tissues of 173 cases with TCC. Staining was limited to the epithelial basal layer of normal bladder mucosa, and a heterogenous staining pattern with CD44 and CD44v6 was observed on the luminal surface. The expression intensity of CD44 in tumor cells was significantly related to the mitotic index. In addition, these studies estimated the recurrence and progression and CD44 expression was claimed to be an independent prognostic indicator in the determination of survival.[6] In another study, CD44 and CK20 expressions were evaluated using cytology and greater expression was reported in the early stages than those in the advanced stages.[7,13] Omran and colleagues compared the IHC results of CD44 and CD44v6 in 30 cases of TCC and 20 cases of squamous cell carcinoma. The authors reported a statistically significant correlation between CD44 expression and increasing grade and stage. No such correlation between sex and age of the patient; or size of the tumor was documented.[14] In the majority of studies, loss of CD44 has been detected with the increasing stage and grade, however, some studies have claimed the opposite. In the current study, a statistically significant progressive loss of CD44 was determined with increasing grade and stage (P = .0001; P = .002, respectively). The relation between CD44 expression and tumor recurrence or progression remained insignificant (P = 0.2; P = 0.1, respectively). With the exception of the findings by Lipponen and colleagues these results are similar to those of several previous studies.

Cytokeratins are important markers of epithelial differentiation. Although many researchers have suggested the differentiation of CK20 in recurrent disease, especially pTa, pT1 and Cis,[16-18] some studies have also shown mixed or inconsistent results.[19-22] Ogata and colleagues reported CK20 expression in 72% (13/18) of patients with recurrences, in 85% of high-grade tumor cases, and in 34% of low-grade tumor patients in a total of 43 patients with superficial bladder carcinoma. These results indicate significant association of CK20 expression with recurrence (P = .0166) and histological grades (P = .0002) but no correlation with progression.[23] A study by Otto and colleagues revealed CK20 as an independent prognostic factor for recurrence (P = .008).[24] Another study showed no significant correlation CK20 and progression of bladder carcinoma.[25] Data in the literature are generally consistent about the correlation between CK20 expression and the grade, stage and recurrences but not progression of bladder urothelial carcinoma. However, findings of the present study remained insignificant about the relation of CK20 expression with recurrences despite the difference between the recurring and non-recurring groups (93.7% vs. 75%). This can be explained by recurrence due to multifactorial causes.

There have been controversial results about survivin in urothelial carcinoma. In one study, nuclear staining for survivin expression was detected in 26 of 45 cases of TCC and in 2 of 14 cases of carcinoma in situ (Cis) and in none of healthy bladder mucosa yielding a significantly higher expression in TCC than both Cis and healthy mucosa (P < .001). The study concludes that nuclear localization of survivin is a marker of TCC but is rarely present in premalignant or benign bladder mucosal specimens.[26] Some studies have determined significant interobserver variability in grading low-grade versus high-grade papillary urothelial carcinoma. Survivin immunohistochemistry can be a useful adjunct tool for the grading of challenging cases.[27] Gonzalez and colleagues reported combined p53 and survivin immunostaining could be helpful in distinguishing patients in high risk of tumor progression.[28] Shariat

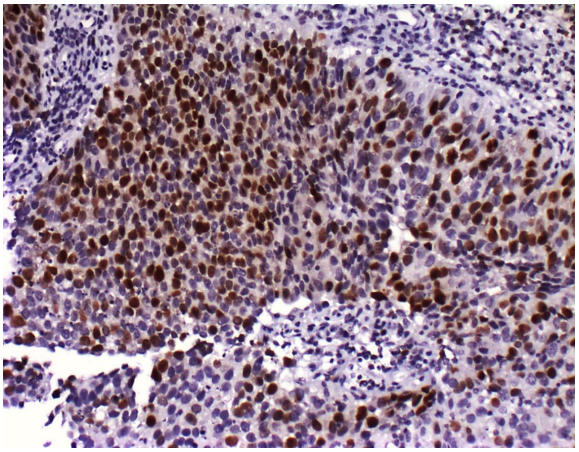

Figure 6. High grade papillary urothelial carcinoma, with staining survivin (+++) (×200).

and colleagues reported that 47 of 50 patients (94%) with lymph node metastasis after radical cystectomy in 222 patients had survivin over-expression. In that study, although nuclear activity was detected in some tumor cells, survivin expression was reported to be predominantly localized to the cytoplasm.[29] Survivin expression was evaluated in a recent study of 125 patients who underwent radical nephroureterectomy due to upper urinary tract tumors. A significant relationship was reported between survivin with histological grade and lymphovascular invasion ($P < .001$ and $P = .022$, respectively). Survivin positivity was determined showing a significant correlation between pathological stage and grade with survivin expression in a polymerase chain reaction (PCR) study in all 32 patients ($P < .001$ and $P < .001$ respectively). Similar results were achieved with Western Blot technique.[26]

On contrary, Ku and colleagues reported greater than 20% survivin expression in 51 of 88 cases (58%) and low expression in the remaining 37 cases (42%) of superficial bladder cancer. There was no significant correlation between survivin expression and tumor grade and stage ($P = .052$ and $P = .131$, respectively).[30]

CONCLUSIONS

In the present study, overall survivin expression was 83% in 82 patients with urothelial carcinoma. Survivin immunoreactivity increased from 68% in low grade disease to 100% in high grade disease indicating significant relation with grade and stage ($P = .000$ and $P = .000$). On the other hand, survivin expression with relevance to recurrence and progression was insignificant in consistence with reports in the literature. In conclusion, CD44, CK20 and survivin expressions in bladder urothelial carcinoma show significant relation with tumor grade and stage. However, prognostic value of these markers has yet to be elucidated in larger series.

CONFLICT OF INTEREST

None.

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