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Expression of CD44V2 in transitional cell carcinoma of the urinary bladder and in urine

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Abstract CD44 is the principal cell surface receptor for hyaluronate. Variant forms of the receptor, produced by alternative splicing, have been found to be associated with tumor progression in a variety of cancers. Based on investigations at the RNA level, it has recently been proposed that expression of CD44 variant V2 was present in urothelial cancer but not in normal urothelium. Since a distinctive marker for urothelial cancer would be extremely useful, frozen sections of normal urothelium and urothelial cancer were examined for expression of standard CD44 and CD44V2. Frozen sections of specimens of 35 patients with transitional cell carcinoma of the bladder, 16 specimens of normal bladder and 5 ureters were examined. Immunohistochemical staining was performed using a polyclonal antibody to CD44V2 (PAB CD44V2), a monoclonal antibody to CD44V2 (MAB CD44V2) and a monoclonal antibody to CD44S (MAB CD44S). CD44V2 and CD44S were also measured in lysates of urine sediments from 21 patients by enzyme-linked immunoabsorbent assay (ELISA). All investigated transitional cell carcinomas expressed CD44V2. There was no differentiation between invasive and non-invasive carcinoma. CD44V2 was also expressed in normal urothelium. Standard CD44 was expressed by the transitional cell carcinoma, normal urothelium, musculature and interstitial tissue. The amount of CD44V2 and CD44S in lysates of urine sediments is not correlated to diagnosis. In contrast to investigations at the RNA level, CD44V2 on the protein level seems not to be a distinctive marker for urothelial cancer. Therefore, CD44V2 will not be a useful diagnostic marker for detection of transitional cell carcinoma.

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Introduction

CD44, the principal cell surface receptor for hyaluronate, is a glycoprotein which plays an important role in the adhesion and migration of cells. Standard CD44 (CD44S) is found nearly ubiquitously in different tissues [5, 6]. Alternative splicing gives rise to so-called splice variants of CD44 (CD44V). To date, ten different variant exons are known in men, of which nine are expressed at least at the RNA level in varying degrees and compositions in malignant tumors [19, 22]. The coding sequence of the CD44 family is restricted to a single gene found on chromosome 11p13 [19]. In contrast to the standard variant, the exact function of the splice variants is not yet clearly understood [4]. The variants of CD44 attracted particular attention in 1991 when Günthert et al. showed that transfection of a rat pancreas carcinoma cell line with CD44V6 led to development of a metastatic phenotype [8].

In colon carcinoma, a stage-dependent expression of variant V6 has been reported [23]. Whether the expression of CD44 variants in various tumors can serve as a prognostic marker is currently the subject of intense debate [1, 3, 7, 9, 10, 11, 13, 14, 18, 20]. Most of these studies on different tumors were carried out by immunohistochemistry with antibodies to V5, V6 and V9. Molecular biologic investigations have furnished evidence that particularly variant CD44V2 may play an important role in malignant tissue [15, 16, 21, 22]. At the RNA level, Tarin demonstrated the presence of CD44V2 in bladder carcinoma but not in normal urothelium. To date, no marker has yet been found which can, with high sensitivity and specificity, differentiate between normal urothelium and urothelial carcinoma. A marker differentiating between bladder carcinoma cells and normal urothelium would open new doors especially in the

diagnosis and follow-up of patients with urothelial carcinoma. The objective of the present study was to investigate immunohistochemically the expression of CD44V2 and CD44S in carcinoma of the bladder and normal urothelium using the first available antibodies against CD44V2 [2].

Material and methods

Tissue specimens

Thirty-five tissue samples of urothelial carcinoma of the urinary bladder were obtained from operative specimens collected in the Department of Urology, Universitätsklinikum Benjamin Franklin, Freie Universität, Berlin, from May 1994 to December 1995. The specimens were immediately shock frozen. Histologic classification showed 17 invasive urothelial carcinomas and 18 superficial bladder carcinomas. In addition, 16 tissue samples from normal bladder mucosa and 5 ureters were obtained. After shock freezing, tissue samples were stored at –80°C. For the immunohistochemical examination, 5-µm frozen sections were prepared using a Leitz cryostat.

Antibodies

As antibody against CD44 Standard, we used the monoclonal mouse antibody MAB CD44S (F10-44-2, Boehringer Mannheim, Germany, No. 1441272). The antibodies against CD44V2 were a polyclonal sheep antibody PAB CD44V2 (6127) and a monoclonal mouse antibody MAB CD44V2 (M 23.6.1). Both antibodies were kindly provided by Boehringer Mannheim, Tutzing, Germany. Production, isolation and specificity of the v2-antibodies is described by Borgya et al. [2].

Immunohistochemical staining

Frozen sections were subsequently fixed in acetone for 10 min at room temperature and then dried for 5 min at room temperature. Rehydration was carried out with wash buffer (0.05 M TRIS/HCl; 0.15 M CaCl, pH 7.6) over 5 min. Samples were preincubated for 10 min with 5% normal rabbit serum in order to block nonspecific protein binding. Thereafter, the slices were incubated for 30 min with the first antibody (see above). PAB CD44V2 was applied at a concentration of 6.25 µg/ml, MAB CD44V2 at 10 µg/ml and MAB CD44S at 10 µg/ml. The samples were then rinsed with wash buffer for 5 min, followed by incubation with the second antibody. The slices with PAB CD44V2 were incubated for 30 min with a biotinylated rabbit anti-sheep antibody (Dianova, Hamburg, Germany, No. 313-065-045) at a concentration of 1:500 in wash buffer. The slices with MAB CD44V2 and MAB CD44S were incubated for 30 min with a biotinylated rabbit anti-mouse antibody (Dianova, Hamburg, Germany, No. 315-065-045) at a concentration of 1:500 in wash buffer. Slices were then rinsed again for 5 min with wash buffer. This was followed by incubation for 30 min in alkaline phosphatase-conjugated streptavidine (Dako, Glostrup Denmark, No. DO396) at a concentration of 1:100 in wash buffer as detection reagent. Samples were then again rinsed with wash buffer for 5 min, after which the slices were finally incubated for 15 min in substrate solution. Hematoxylin was used as a counterstain. Control samples were incubated with inactivated sheep serum or inactivated mouse serum. Staining of more than 30% of the cells was classified as a positive staining. Classification of the samples was performed by two independent investigators.

Urine sediment samples

Fifty-milliliter samples of first voided urine were obtained from nine patients with confirmed bladder carcinoma, three patients with inflammatory diseases of the urinary tract (cystitis or pyelonephritis), seven patients with normal urinary tracts and two patients with dysplasia of the urothelium. The urine was immediately suffused with protease inhibitors (1 tablet of protease inhibitor cocktail/50 ml urine, Boehringer Mannheim, Germany, No. 1697498) and then centrifuged at 4°C and 500 *g*. The supernatant was decanted. Thereafter, pellet weight was determined and the equivalent weight of TRIS buffer (20 mM, pH 8.0) was added. Samples were then stored at –20°C.

ELISA of CD44 in urine sediment samples

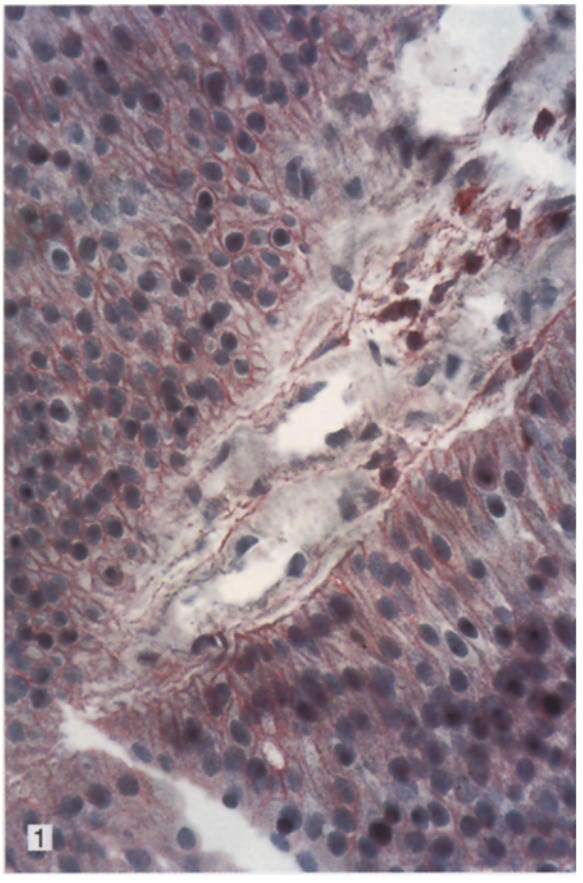
The amounts of CD44S and CD44V2 present in urine sediment samples were measured after lysis of the pellets by repeated freezing and thawing and subsequent centrifugation in the resulting supernatants. For the enzyme-linked immunosorbent assay (ELISA), streptavidin-coated microtiter plates were used in combination with biotin- and digoxigenin-labeled antibodies. CD44V2 was identified using PAB CD44V2, labeled with biotin for ELISA. The digoxigenated antibodies MAB KZ-1-Dig and PAB <dig> S-Fab-POD served as detection antibodies. Determination of CD44S was performed with biotinylated MAB CD44S. MAB KZ-1-Dig (kindly provided by Dr. Anstee, IBGRL, Bristol, UK) and PAB<dig> S-Fab-POD (Boehringer Mannheim, Germany) were again used as detection antibodies. The concentrations of CD44V2 are given in units per milliliter, since no recombinant protein is currently available for calibration of CD44V2. Concentrations of standard CD44 are given in nanograms per milliliter. The results have been standardized to the pellet wet weight. All samples were measured in duplicate without knowledge of the clinical diagnosis. The specificity of the CD44V2 ELISA was tested and described by Borgya et al. [2] using lysates of the cell line RT112 showing high CD44V2 expressions as determined by reverse transcriptase polymerase chain reaction (RT-PCR) and the cell line MDA 231-4A4 with no detectable CD44V2 expression.

Fig. 1 CD44S expression in bladder cancer (histologic classification: pTa G2). The monoclonal antibody MAB CD44S led to a membrane-staining pattern. Urothelial carcinoma cells as well as connective tissue expressed the standard form of CD44

Fig. 2 CD44V2 expression in bladder cancer (same tissue sample as in Fig. 1, histologic classification: pTa G2). PAB CD44V2 led to a cytoplasmatic staining pattern of the urothelial carcinoma with a homogeneous staining of the cell layers of the urothelial carcinoma. The connective tissue showed no expression of CD44V2

Fig. 3 CD44V2 expression in normal urothelium of the ureter. PAB CD44V2 led to a cytoplasmatic staining pattern of the normal urothelium. In contrast to the urothelial carcinomas, in which no cell-layer-dependent differences in staining behavior could be observed, in normal urothelium particularly the basal cell layers stained strongly

Fig. 4 CD44V2 expression in normal urothelium of the bladder. PAB CD44V2 led to a cytoplasmatic staining pattern of the normal urothelium. In normal urothelium particularly the basal cell layers stained strongly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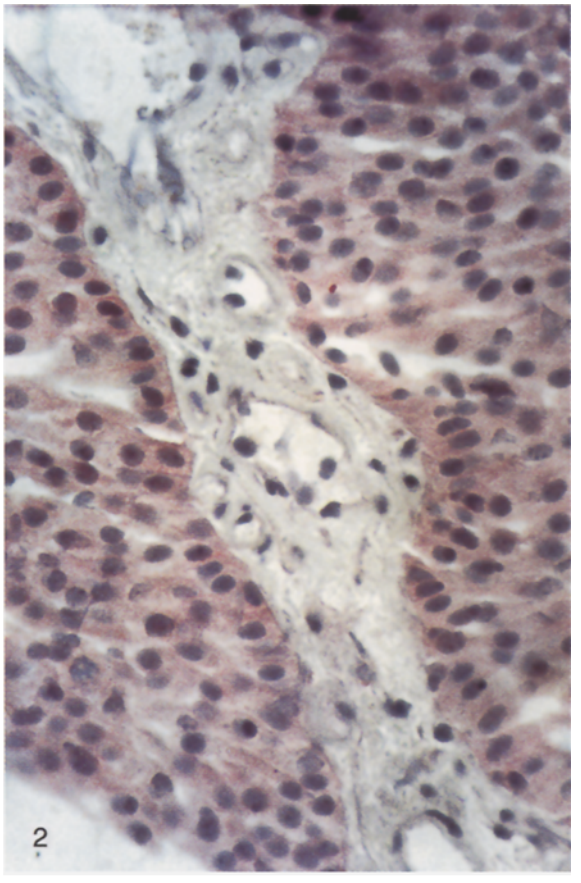
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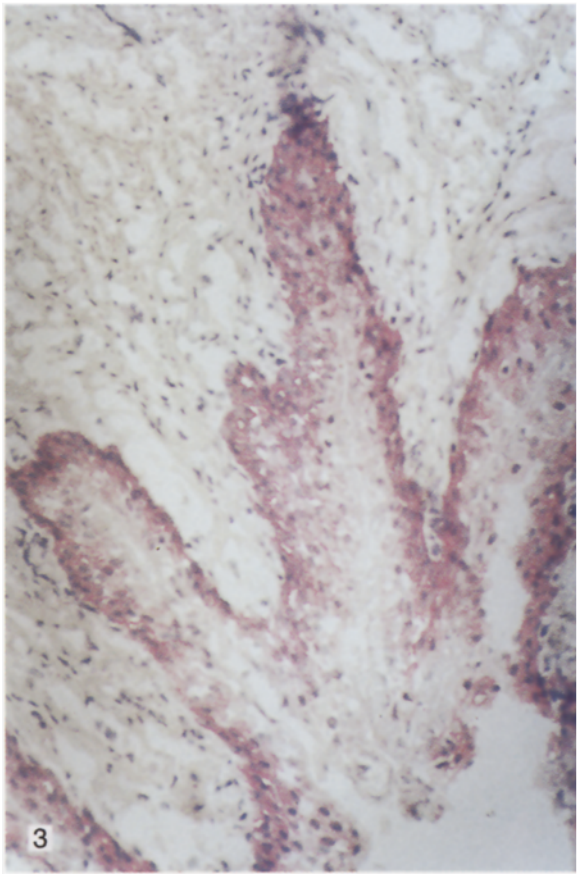
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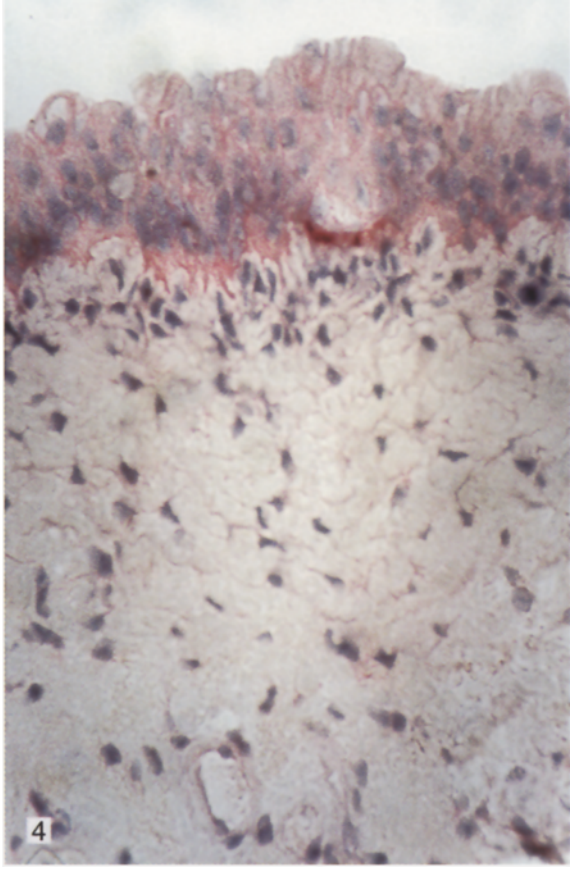
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Results

Immunohistochemistry of CD44V2 in bladder cancer and normal urothelium

The monoclonal antibody MAB CD44S against standard CD44 showed a membrane-staining pattern. Both urothelial carcinoma cells and normal urothelium, as well as connective tissue and, in most cases, muscle cells expressed the standard form of CD44 (Fig. 1).

The use of the polyclonal antibody PAB CD44V2 allowed immunohistochemical identification of the splice variant CD44V2 expressed in all 35 studied cases of urothelial carcinoma (Table 1). PAB CD44V2 led to a cytoplasmatic staining pattern (Fig. 2). The monoclonal antibody MAB CD44V2 showed a significantly lower staining and stained in only 21 of the 35 cases (60%) of urothelial carcinoma (Table 1). MAB CD44V2 showed a slight cytoplasmatic and a membrane staining. Both antibodies showed a homogeneous staining of the cell layers of tumor tissue. Stage-dependent differences in expression between invasive and superficial urothelial carcinoma were not observed (Table 1). Similarly, we were unable to identify differences in the expression of CD44V2 related to the grading of the tumor cells.

In 8 of 16 cases (50%) of normal bladder urothelium examined, polyclonal antibody PAB CD44V2 demonstrated expression of CD44V2 (Fig. 4), while the urothelium of all five ureter specimens examined expressed CD44V2 Fig. 3). PAB CD44V2 led to a cytoplasmatic staining pattern. In normal urothelium, as well, the monoclonal antibody MAB CD44V2 showed a significantly lower staining. MAB CD44V2 showed a slight cytoplasmatic and a membrane staining. But in contrast to the urothelial carcinomas, in which no cell-layer-dependent differences in staining behavior could be observed, in normal urothelium particularly the basal cell layers stained strongly (Figs. 3, 4).

Detection of CD44 in urine sediment lysates

The enzyme-linked immunosorbent assay (ELISA) of the urine pellets yielded for standard CD44 concentrations between 76 and 846 ng/ml and for CD44V2 con-

Table 1 Histologic classification of the tumor tissue and expression of CD44V2 and CD44S in 35 cases of bladder carcinoma using the antibodies PABCD44V2 (polyclonal antibody against CD44V2), MABCD44V2 (monoclonal antibody against CD44V2) and MABCD44S (monoclonal antibody against CD44 standard). Staining of more than 30% of the cells was classified as positive (+). Staining of less than 30% of the cells was classified as negative (−)

No.	Staging	Grading	PABCD44V2	MABCD44V2	MABCD44S
1	pTa	G1	+	+	+
2	pTa	G2	+	+	+
3	pTa	G2	+	−	+
4	pTa	G2	+	+	+
5	pTa	G2	+	+	+
6	pTa	G2	+	+	+
7	pTa	G2	+	+	+
8	pTa	G2	+	+	+
9	pTa	G2	+	−	+
10	pTa	G2	+	+	+
11	pTa	G2	+	+	+
12	pTa	G2	+	−	+
13	pTa	G2	+	+	+
14	pTa	G2	+	+	+
15	pTa	G3	+	+	+
16	pTa	G3	+	−	+
17	pTa	G3	+	+	+
18	pTa	G3	+	+	+
19	pT1	G2	+	−	+
20	pT1	G2	+	+	+
21	pT1	G2	+	+	+
22	pT1	G3	+	−	+
23	pT1	G3	+	−	+
24	pT1	G3	+	+	+
25	pT1	G3	+	−	+
26	pT1	G3	+	−	+
27	pT1	G3	+	+	+
28	pT2	G2	+	−	+
29	pT2	G2	+	+	+
30	pT2	G2	+	−	+
31	pT2	G2	+	+	+
32	pT2	G3	+	−	+
33	pT3a	G3	+	+	+
34	pT3b	G3	+	−	+
35	pT4a	G3	+	−	+

Table 2 Quantitative determination of CD44V2 and CD44S in urine sediment lysates of patients with bladder cancer, benign diseases and without urinary tract diseases using ELISA. Correlations with the clinical diagnosis were not observed for either CD44S or CD44V2. (*these samples could not be measured in each ELISA because of insufficient sample volume)

No.	Diagnosis	Concentration CD44S (ng/ml)	Concentration CD44V2 (U/ml)
1	Carcinoma pTaG2	175.75	44.80
2	Carcinoma CisG3	*	4.60
3	Carcinoma CisG3	209.25	41.75
4	Carcinoma pT1G2	76.00	31.95
5	Carcinoma pT1G2	807.50	52.15
6	Carcinoma pT1G3	*	6.40
7	Carcinoma pT1G3	514.0	102.80
8	Carcinoma pT1G3	911.75	122.05
9	Carcinoma pT2G3	976.0	32.55
10	Dysplasia	708.00	46.00
11	Dysplasia	139.50	8.80
12	Cystitis	175.75	16.70
13	Pyelonephritis	98.75	82.40
14	Pyelonephritis	846.00	5.80
15	Healthy	76.00	13.65
16	Healthy	209.25	73.75
17	Healthy	*	5.20
18	Healthy	*	14.25
19	Healthy	677.25	104.65
20	Healthy	*	*
21	Healthy	*	2.80

centrations between 2.8 and 122 U/ml. Some samples could not be measured in each ELISA because of insufficient sample volume. They are marked with an asterisk in Table 2. Correlations with the clinical diagnosis were not observed for either CD44S or for CD44V2 (Table 2). There was also no correlation between the concentrations of CD44S and CD44V2 in urine sediment lysates.

Discussion

Carcinoma of the urinary bladder is the most common malignant tumor of the urinary tract and, after prostate carcinoma, the second most frequent tumor of the urogenital system [17]. Diagnosis and follow-up of urothelial carcinoma of the bladder, ureter and renal pelvis currently depends on invasive endoscopic procedures, as well as on clinical imaging techniques and urinary cytology.

A marker capable of differentiating between bladder carcinoma cells and normal urothelium would open new doors, particularly in the area of noninvasive diagnostic procedures and patient follow-up. Molecular biologic studies have suggested that the splice variant CD44V2 might be just such a marker [21]. Using an RT-PCR against CD44V2, Matsumara et al. were able to detect bladder tumor cells in urine [16]. The presence of an mRNA, however, does not always correlate with the presence of the respective protein [4]. Furthermore, compared with immunohistochemical examinations, PCR-based methods are more sensitive to contamination and less robust [12]. Therefore, the present study used the first available antibody against CD44V2 to investigate immunohistochemically the expression of CD44V2 in urothelial carcinoma of the bladder and in normal urothelium. Furthermore, we tried to achieve a quantitative measurement of CD44V2 and CD44S in the urine sediment lysates of patients with confirmed urothelial carcinoma and in urine sediment lysates obtained from a control group. Many studies have shown that the standard form of CD44 is nearly ubiquitously expressed [5, 6]. In agreement with the literature, it has been shown that both urothelial carcinoma cells and normal urothelium, as well as connective tissue and in most cases muscle, express the standard form of CD44. The splice variant CD44V2 was detected immunohistochemically in all samples of bladder carcinoma tissue examined. Unlike the expression of other splice variants in other malignant tissues, the expression of CD44V2 is independent of tumor grading and staging [7, 9, 10, 23]. Immunohistochemical studies carried out by Hong et al. and Southgate et al. using antibodies against CD44V3, CD44V4/5 and CD44V6 showed a loss of splice variants in poorly differentiated bladder tumors [11, 20]. The available data, however, are not uniform. The differentiation between malignant and normal tissue based on expression of CD44V2 demonstrated in molecular biologic studies could not be confirmed immunohistochemically, since a large proportion of normal urothelium also expressed CD44V2. Similarly, both Hong et al. and Southgate et al. found the splice variants V3, V4/5 and V6 particularly in the basal cell layers of normal urothelium [11, 20].

The examination of urine specimens using ELISA against CD44V2 and CD44S showed no correlation with clinical diagnosis. Thus, there also seems to be no marker for use in the diagnosis and follow-up of urothelial carcinoma which can differentiate normal urothelium from urothelial carcinoma cells at the protein level. An explanation of the divergent results yielded by molecular biology and immunohistochemistry regarding the expression of CD44V2 and the checking of a possible downregulation of CD44 splice variants at higher tumor stages must await the findings of further molecular biologic and immunohistochemical investigations.

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