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ORIGINAL ARTICLE

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Expression of CD44 isoforms in human renal cell carcinomas

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Abstract A series of 27 renal cell carcinomas 4 oncocytomas and 7 samples of tumour free kidney parenchyma were analysed immunohistochemically using eight different CD44 isoform-specific monoclonal antibodies. In normal kidney expression of CD44 isoforms (containing variant exons v6, v7/8 and v10) was found predominantly at the distal tubules. The majority of clear cell carcinomas investigated showed expression of variant exons v5, v7/8 and v10, but not v6. Lack of CD44v6 expression was confirmed by reverse transcription-polymerase chain reaction analysis. Carcinomas of the chromophilic cell type were almost completely devoid of CD44 expression, including the standard form CD44s. This study shows that there are statistically significant differences in the CD44 expression pattern of the two major histological subtypes of renal cell carcinomas (clear cell and chromophilic carcinomas). Moreover, the almost complete lack of CD44 expression in chromophilic carcinomas contrasts with carcinomas of other histogenetic origin investigated including stomach, breast and lung which express various CD44 isoforms abundantly.

Key words CD44 isoforms · Immunohistochemistry · Renal clear cell carcinomas · Chromophilic carcinomas · Normal kidney

Introduction

CD44 glycoproteins form a family of structurally related cell surface proteins which differ in their degree of glycosylation and their extracellular amino acid composition. The smallest CD44 isoform, CD44s or the haematopoietic form, is expressed ubiquitously and mediates different functions including lymphocyte homing, binding to extracellular matrix components or lymphocyte activation (for review see [8]). Larger CD44 isoforms, also called CD44 variants, differ from CD44s by the insertion of additional amino acids into the extracellular part of the molecule. The existence of a variety of protein isoforms is based on the structure of the CD44 gene, which consists of at least 20 exons, 10 of which can be subject to differential splicing [16, 22].

In a rat model system, it has been shown that CD44 variants play an important role in the process of tumour cell dissemination. Overexpression of certain variant isoforms of rat CD44 in an originally non-metastasizing rat pancreatic adenocarcinoma cell line leads to spontaneous metastatic behaviour of these cells [7, 17]. Within the last few years numerous studies concerning CD44 isoform expression in human tumours have been published, suggesting that certain isoforms of CD44 also play a role in tumour progression and dissemination in man, at least in some types of cancer (breast, colon, stomach carcinomas, non Hodgkin's lymphomas) [12–15, 19, 23].

If one attempts to draw a general conclusion, it appears that there is a high level of CD44 variant expression in most tumours derived from epithelial tissues, whereas many non-epithelial tumours only express the standard form of CD44 or show a different isoform expression pattern to epithelial tumours. In order to see whether renal cell carcinomas behave in a similar way to other carcinomas with respect to CD44 isoform expression, we examined 31 cases of different histological types, using a panel of eight different CD44-specific monoclonal antibodies (mAbs).

Materials and methods

Thirty-one tumours and samples from seven non-tumour bearing kidneys were selected from the files of the Department of Patholo-

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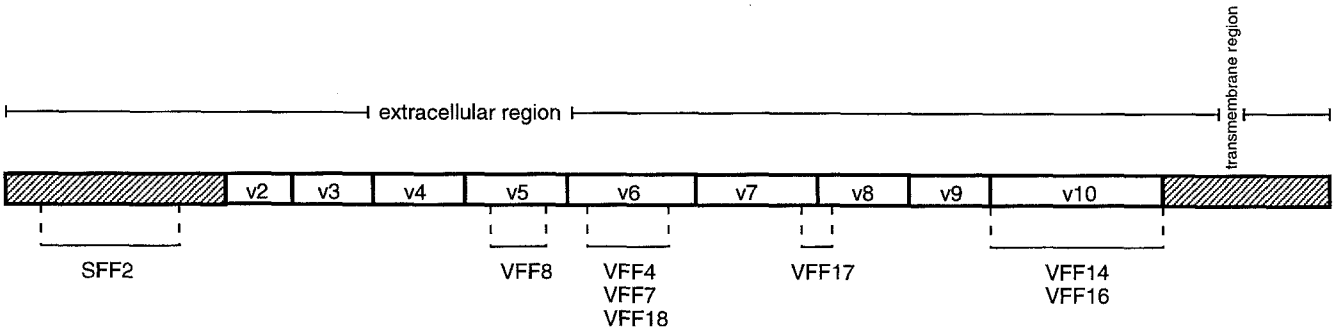


Fig. 1 Epitope localization of the CD44-specific monoclonal antibodies (mAbs). SFF2 mAb recognizes an epitope in the N-terminal constant region of the CD44 molecule (*hatched box*). Antibodies VFF4, VFF7 and VFF18 recognize different, but overlapping epitopes in variant exon v6; mAbs VFF14 and VFF16 recognize epitopes encoded by exon v10. VFF17 mAb recognizes an epitope formed by the transition of exons v7 and v8, and mAb VFF8 recognizes an epitope encoded by exon v5. The constant part of the molecule (*hatched boxes*) is not drawn to scale

gy, University of Graz, Austria. All samples were snap-frozen in liquid nitrogen immediately after surgical removal and stored in liquid nitrogen until analysis. In parallel, paraffin-embedded samples were used for histopathological evaluation. The following tumours were investigated for the expression of CD44 isoforms: 21 cases of the clear cell type, 5 cases of the chromophilic and 1 case of the chromophobic type (according to the classification of Thoenes et al. [21]). In addition, four oncocytomas were analysed. The mean age of the patients was 61.2 years (23–74 years) and the male to female ratio was 20:11.

The generation of anti-variant CD44 mAbs has been described in detail elsewhere [13]. The exon specificity of the mAbs was determined by enzyme linked immunosorbent assay and western blot assays using glutathione S-transferase fusion proteins encoded by CD44 exons v3–v10 (see Fig. 1). For further characterization of certain epitopes, overlapping peptides were synthesized and the exact epitope sequence was determined. SFF2 mAb recognizes an epitope common to all CD44 isoforms. VFF4, VFF7 and VFF18 mAbs recognize different, but overlapping epitopes encoded by exon v6. The mAb VFF17 reacts with an epitope formed by the transition of exon v7 to v8. VFF8 mAb and mAbs VFF14 and VFF16 react with an epitope encoded by exon v5 and v10, respectively.

For immunohistochemistry, cryostat tissue sections (6 µm) were fixed in methanol/acetone (1:1) at –20° C for 10 min. Sections were washed in phosphate buffered saline (PBS) and preincubated with normal goat serum (10% of PBS). After three washes in PBS the sections were incubated with the primary antibody [SFF2, VFF14, 16: 20 µg/ml; VFF4, 7, 8, 17: 10 µg/ml; VFF18: 5 µg/ml; EBI-1 (isotype-matched negative control; [1]) 10 µg/ml in PBS/1% bovine serum albumin for 1 h. As positive control for the staining reaction normal human skin sections were used, since keratinocytes express a v3–v10-containing CD44 isoform [11]. Endogenous peroxidases were blocked with 0.3% hydrogen peroxide in methanol and the sections incubated with the secondary biotinylated antibody for 30 min (anti-mouse IgG-F(ab')$_2$, DAKO). For colour development the sections were further incubated (30 min) with horseradish peroxidase that had been coupled to biotin as a streptavidin-biotin-peroxidase complex (DAKO). The sections were then incubated in 3,3-amino-9-ethyl carbazole substrate (Sigma Immunochemicals) for 5–10 min, the reaction was stopped in water and the sections were counterstained with haematoxylin. The staining reactions were evaluated as follows: +++ more than 80% of the tumour cells positive; ++ 50%–80% of the tumour cells positive; + 10%–50% of the tumour cells positive; – less than 10% of the tumour cells positive. Only tumour cells which exhibited membrane staining were scored positive.

CD44 expression patterns were analysed by applying Pearson chi-square calculation and Mantel-Haenszel test for linear association using the program SPSS for Windows. *P* values equal to or less than 0.05 were considered significant.

The reverse transcription-polymerase chain reaction (PCR) was performed as follows. One microgram of total RNA was prepared and reverse-transcribed as previously described [7]. Five microlitres of first-strand cDNA were amplified by Taq polymerase (Promega, Madison, USA) in a volume of 50 µl using the buffer conditions recommended by the manufacturer. For testing quality and abundance of cDNA-synthesis, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-PCR with oligonucleotides homologous to positions 8–29 and 362–339 of the published GAPDH-cDNA sequence [2] was performed. After 25 rounds of amplification (95° C for 1 min, 62° C for 1.5 min) 10 µl of the reaction were analysed on a 2% agarose gel and the amplification product was visualized under UV-light after staining the gel with ethidium bromide. For amplification of CD44v6-containing cDNAs, primers homologous to the 5'-end of exon v6 (position 357–386; [11]) and the 3'-constant region of CD44 (positions 934–958; [18]) were used. After 35 cycles of amplification (94° C for 1 min, 62° C for 1.5 min) 10 µl of the reaction mixture were analysed on a 1.2% ethidium bromide-stained agarose gel.

Results

Twenty-seven specimens of cryostat tumour sections of renal cell carcinomas and 4 of oncocytomas were analysed immunohistochemically with a panel of seven different CD44 variant-specific antibodies and one anti-pan CD44 mAb (see Fig. 1). The results are summarized in Table 1. An example of the typical staining pattern of clear cell carcinomas is shown in Fig. 2. SFF2 mAb, which recognizes an epitope in the extracellular constant region of CD44 (CD44s), reacted with all but one of the clear cell carcinomas, but stained only one of five chromophilic tumours. The variant-specific mAbs VFF8 (exon v5), VFF14, VFF16 (both exon v10) and VFF17 (exons v7/8) showed a similar staining pattern as mAb SFF2, with reactivity to the majority of the clear cell tumours and the chromophobic tumour but almost no reactivity with the chromophilic tumours. Three mAbs specific for the variant exon v6 (VFF4, VFF7, VFF18) did not stain the tumour cells, except for three weak and one strong reactions of mAb VFF7 and VFF18 with clear cell carcinomas and one moderate reaction of VFF18 in one chromophilic carcinoma, respectively. The four investigated oncocytomas stained constantly with the mAb specific for exons v7/8 (VFF17), all other antibodies showed various reactivities.

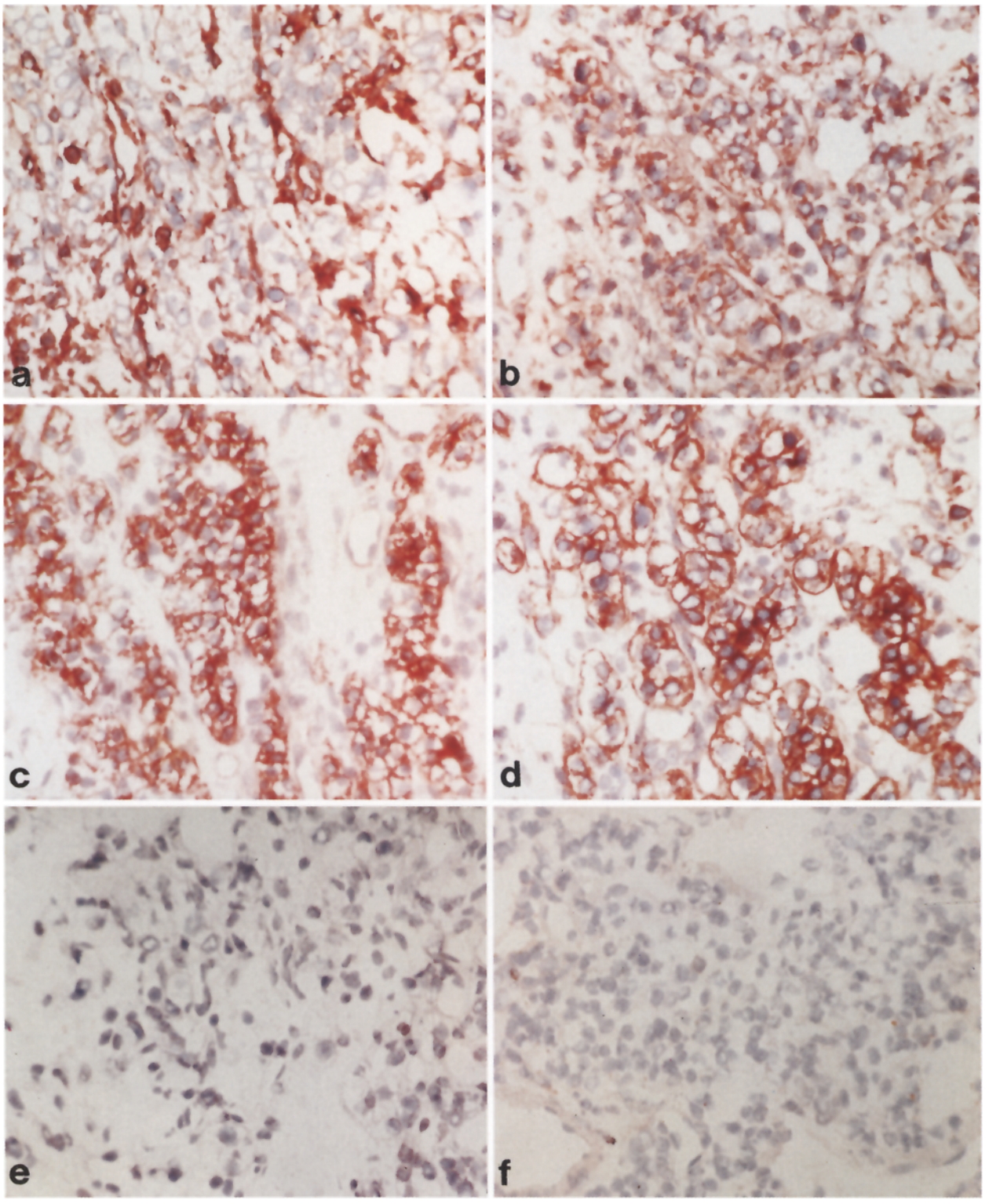


Fig. 2a–f Immunohistochemical detection of CD44 expression in a representative case of clear cell carcinoma. Reaction with mAb SFF2 (CD44 standard; **a**), VFF8 (exon v5; **b**) VFF14 (exon v10; **c**), VFF16 (exon v10; **d**), VFF18 (exon v6; **e**) and the negative control antibody (**f**) are shown. Magnification 280×, counterstained with haematoxylin

To test whether the non-reactivity of the CD44v6-specific antibodies with the majority of tumours is also reflected at the RNA level. RNA from six cases which were negative by immunohistochemistry (cases 4, 14, 15, 16, 24, 25) was prepared and reverse transcribed. The quality and abundance of the cDNA-synthesis was checked via GAPDH-PCR (not shown). For amplification of exon v6-

Table 1 Immunohistochemical analysis of CD44 isoform expression in renal cell tumours. A panel of different isoform-specific mAbs (VFF8, exon v5; VFF4, 7, 18, exon v6; VFF17, exon v7/8, VFF14, 16, exon v10) and a pan-CD44 mAb (SFF2) was used

Tumour	SFF2 (standard)	VFF8 (v5)	VFF4 (v6)	VFF7 (v6)	VFF18 (v6)	VFF17 (v7/8)	VFF14 (v10)	VFF16 (v10)
Clear cell								
1 T1, G1	+++	–	–	–	–	++	+	+++
2 T2, G1	+	++	–	–	–	+++	+++	+
3 T2, G1	–	–	–	–	–	+++	–	++
4 T2, G1	+	++	–	–	–	++	–	++
5 T2, G1	+	++	–	+	+	+++	–	+++
6 T2, G1	++	++	–	+	–	++	+	++
7 T2, G1	++	–	–	–	–	++	–	++
8 T3a, G1	+++	+++	–	–	–	+++	+++	+++
9 Tx, G1	+	++	–	–	–	+++	+	++
10 T2, G2	+	–	–	–	–	+++	++	++
11 T3a, G2	+++	–	–	–	–	+++	++	+
12 T3a, G2	+	+	–	–	–	++	++	+
13 T3a, G2	++	–	–	–	–	+++	–	++
14 T3a, G2	+	+	–	–	–	+++	+++	+++
15 T3b, G2	+	+++	–	–	–	++	+++	+++
16 Tx, G2	+	+	–	–	–	–	–	–
17 T3a, G3	+	+++	–	–	–	–	–	+++
18 T3a, G3	++	++	–	+	+	+++	+	+++
19 T3a, G3	+	++	–	–	–	++	++	+++
20 T3b, G3	+++	+++	+++	+++	+++	+++	++	+++
21 T3b, G3	++	+++	–	–	+	++	+++	+++
Chromophilic								
22 T3a, G2	–	–	–	–	–	–	–	–
23 T3b, G2	–	–	–	–	++	–	–	–
24 T2, G3	–	–	–	–	–	–	–	–
25 T3a, G3	–	–	–	–	–	–	–	–
26 T3b, G3	++	–	–	–	–	–	+	–
Chromophobic								
27 T2, G2	+++	+++	–	–	–	+++	+++	+++
Oncocytoma								
28	–	–	–	–	–	+++	–	–
29	–	+	–	–	–	+	–	–
30	++	–	–	–	+	+	–	++
31	–	+	–	–	–	+	–	–

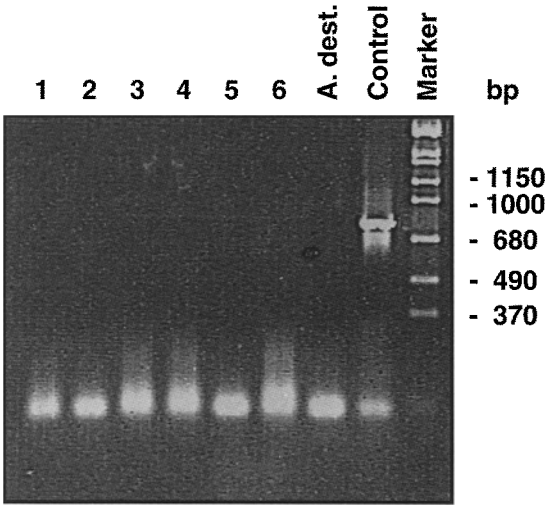
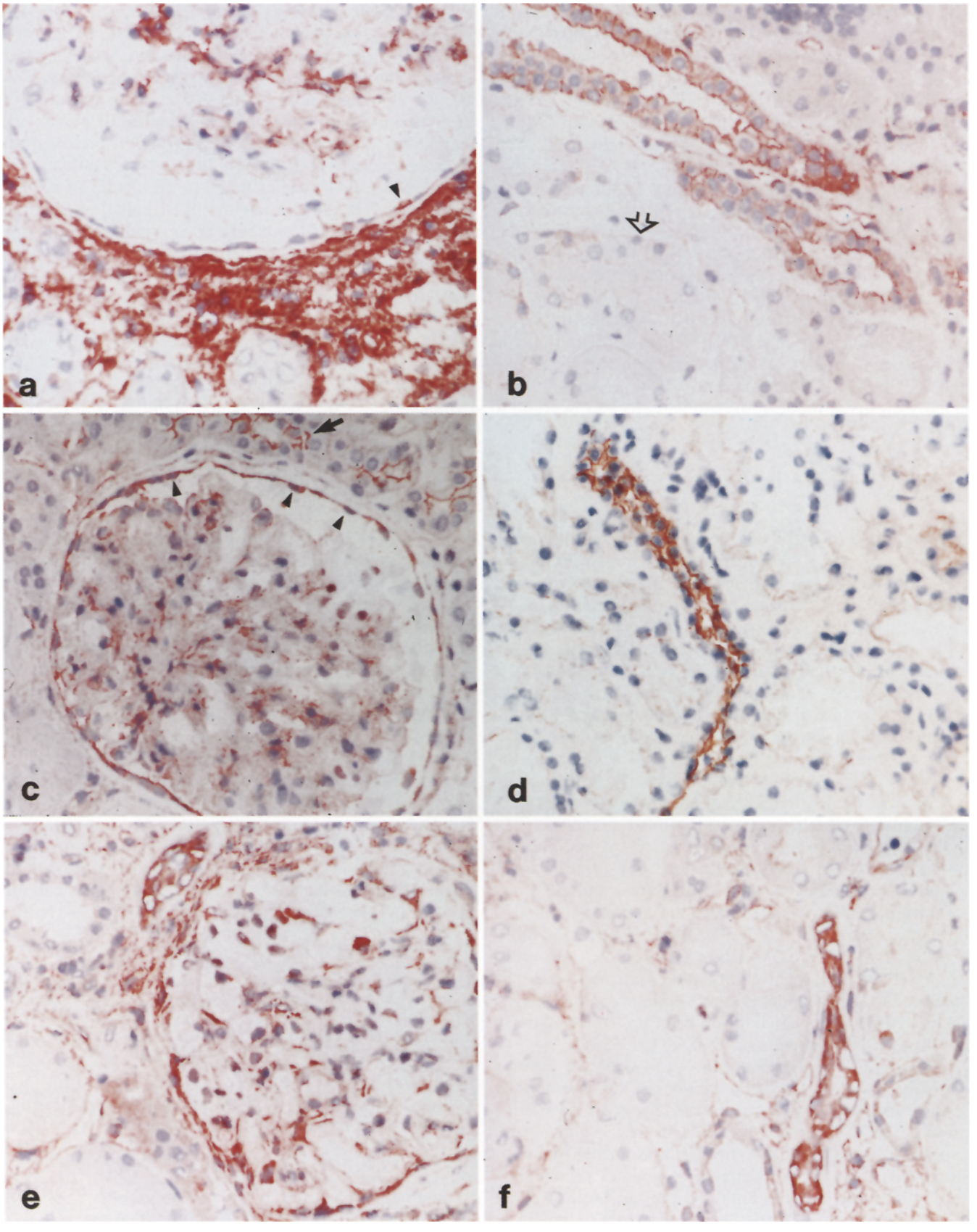


Fig. 3 Reverse transcription-polymerase chain reaction amplification analysis of six clear cell carcinomas which did not react with the CD44v6-specific antibodies by immunohistochemistry (cases 4, 14, 15, 16, 24, 25). Primers specific for 5′-exon v6 and 3′-CD44 constant region were used. The ethidium bromide stained agarose gel shows no amplification products with the tumour cDNAs (lanes 1–6), whereas amplification of 5 ng of control DNA yielded the expected band of 830 base pairs

containing cDNAs we used primers homologous to the 5′-end of exon v6 and the 3′-constant region of CD44, respectively (Fig. 3). No amplification products with any of the cDNAs could be detected, whereas amplification of a control plasmid containing variant exons v3–v10 yielded the expected band of 830 base pairs.

Comparison of tumour size and grade with CD44 isoform expression did not reveal any striking correlation between these variables (Table 1). The majority of clear cell carcinomas and one case of chromophobic carcinoma reacted positively with the mAbs SFF2 (CD44s), VFF8 (v5), VFF14 (v10), VFF16 (v10), and VFF17 (v7/8), respectively, whereas chromophilic carcinomas and the oncocytomas were almost completely devoid of

Fig. 4a–f Immunhistochemical analysis of non-tumour kidney parenchyma. Reaction with mAb SFF2 (CD44 standard; **a**), mAb VFF14 (exon v10; **b**), mAb VFF 16 (exon v10; **c**, **d**) and mAb VFF17 (exons v7/8; **e**, **f**). *Arrowheads* (in **a** and **c**) indicate positive CD44 expression in the parietal layer of the Bowman's capsule. *Arrow* (in **b**) indicates a proximal tubule which is negative for CD44 exon v10 whereas a distal tubule in close proximity is positive. *Arrow* (in **c**) indicates a distal tubule with apical CD44 exon v10 expression. Magnification 280×, counterstained with haematoxylin


a
b
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CD44 expression. According to the Pearson chi-square probability calculation the differences in CD44 isoform expression between the clear cell and chromophilic carcinomas yielded the following *P*-values: CD44s (P=0.00145), CD44v5 (P=0.0377), CD44v6 (VFF18; P=0.161), CD44v7/8 (P=0.00022), CD44v10 (VFF16; P=0.00013) demonstrating that the different expression patterns of CD44s, CD44v7/8 and CD44v10 in these two carcinoma types are statistically highly significant.

In parallel with the tumours, seven specimens of non-tumor kidney parenchyma were analysed with the same set of mAbs (Fig. 4). With mAbs VFF4 (v6) and VFF8 (v5) no staining was detected, whereas mAbs VFF7 (v6), VFF14, (v10) VFF16 (v10), VFF17 (v7/8) and VFF18 (v6) reacted focally positive with the distal tubules. In addition, a reaction at the apical membrane of the proximal tubules was observed with mAbs VFF14 and VFF16 (both v10). The anti-CD44s mAb SFF2 reacted with distal tubules, mesangial cells and interstitial cells.

Discussion

In this study we analysed 27 renal cell carcinomas and 4 oncocytomas using eight different isoform-specific anti-CD44 antibodies and compared the staining results with histological characteristics of the tumours. All cases of clear cell carcinomas were found to be positive, either with an anti-CD44 standard mAb or mAbs specific for exons v5, v7/8 and v10. This is in agreement with a study from Terpe et al., who also found CD44 standard expression in the majority of investigated clear cell carcinomas, using an anti-CD44 standard antibody [20].

Very surprisingly, all three exon v6-specific antibodies (VFF4, VFF7, VFF18) failed to react with the majority of tumour cells. Since the three mAbs recognize different epitopes of the exon v6 encoded sequence, it is unlikely that the non-reactivity is caused by modification or masking of the epitopes. Reverse transcription PCR analysis with a 5′-exon v6-specific primer and a 3′-constant primer of six selected cases did not reveal any amplification products (Fig. 3). Therefore, we conclude that exon v6-containing CD44 isoforms are only very rarely expressed in renal cell carcinomas. This findings is in contrast to reports from all other types of carcinomas investigated so far, which have been found to express CD44v6 at significant levels [3–6, 9, 10, 12, 14].

In contrast to the abundant expression of several CD44 isoforms in clear cell carcinomas, the chromophilic tumours were almost completely devoid of CD44 expression. This difference in CD44 expression cannot be explained by different selection criteria for the tumours, since our collection of samples contained tumours of similar size and grade in both groups (see Table 1). This to our knowledge is the first report that in a defined histological subgroup of carcinomas the majority of cases lacks CD44 expression, including the standard type of CD44.

Renal cell carcinomas are thought to derive from different tubular epithelial cells of the kidney [21]. The difference in the CD44 expression pattern between clear cell and chromophilic carcinomas does not correlate with the current knowledge of the common histogenesis of these two tumour types. There is evidence that clear cell and chromophilic carcinomas arise from the proximal tubular epithelium [21]. Our findings of predominant expression of CD44 variants in the distal tubules of normal kidneys and carcinomas of the clear cell type indicate that factors unrelated to histogenesis regulate CD44 variant expression in renal cell carcinomas.

In a few cases of the tumours investigated, no staining with the anti-pan CD44 mAb SFF2 was observed, whereas some of the variant-specific mAbs did react with the same specimens. Similar observations were made in breast, colon and stomach carcinomas (K. Zatloukal and K.-H. Heider, unpublished results). One explanation for this phenomenon could be a tissue-specific modification of CD44 molecules, which conceals the SFF2 epitope. This could also explain why the two exon v10-specific mAbs VFF14 and VFF16 in some cases show a completely different staining pattern (for example samples 2 and 5). It is not yet known which type of modification (conformation changes, post-translational modifications such as gycosylation or phosphorylation, or association with other components such as extracellular matrix constituents) influence the binding of the CD44 isoform-specific mAbs to their epitopes. This phenomenon could explain, at least in part, why CD44 analysis with different antibodies of the same exon-specificity yields different results in the same type of tumour (for example, the studies performed in breast carcinoma [6, 12]). It suggests the need to use various antibodies for detailed analysis of CD44 expression.

In summary we have demonstrated that the two major histological subtypes of renal cell carcinoma clearly differ in their CD44 expression pattern. Furthermore they rarely express CD44 variant exon v6. Analysis of larger series of tumours will reveal whether the expression of certain CD44 isoforms (containing exon v10) can be used as a prognostic marker.

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