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Expression of MUC1, Thomsen-Friedenreich-related antigens, and cytokeratin 19 in human renal cell carcinomas and tubular clear cell lesions

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Abstract The expression of MUC1, MUC2, mucin-associated Thomsen-Friedenreich-related antigens (TF, sialosyl-TF, Tn, and sialosyl-Tn), and cytokeratin 19 (CK19) was systematically investigated in situ in 58 resected human kidney tumours, surrounding tissue of normal appearance, and two normal kidneys obtained at autopsy, using monoclonal antibodies. In kidney tissues of normal appearance, TF, s-TF, MUC1 and CK19 were positive in distal tubules and collecting ducts but negative in proximal tubules. In contrast, MUC2, Tn, and s-Tn were negative throughout the normal renal tubular system. Almost all renal cell carcinomas (RCCs) showed strong immunoreactivity for MUC1, but all were negative for MUC2. Some RCCs expressed TF, Tn, s-Tn, and CK19. In addition, the immunomorphological characteristics of the majority of clear-cell RCCs and clear/granular RCCs with anti-MUC1 and anti-CK 19 closely resembled those of the collecting duct and the distal tubule rather than the proximal tubule. In the renal tissue of otherwise normal appearance adjacent to clear-cell RCCs and clear/granular RCCs, clear cells with excessive storage of glycogen were often found in the collecting duct system, but only rarely in the proximal tubules. These results suggest that the majority of clear-cell RCCs and clear/granular RCCs may originate from the collecting duct system.

Key words Renal cell carcinomas · Preneoplastic lesions · Mucin · Thomsen-Friedenreich-related antigens · Cytokeratin

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Introduction

Renal cell carcinoma (RCC) is the major type of kidney cancer. Stage is the most important factor predicting survival [12]. However, early diagnosis is very difficult, since RCC usually runs a silent course in its early stages and the clinical presentation is often diverse. Extensive studies of other types of carcinoma suggest that assays for tumour-associated antigens, particularly serum assays, are helpful in early diagnosis of cancer, although both false-negative and false-positive results do occur. However, to the best of our knowledge, no tumour-associated antigens are available for the diagnosis of RCC. With other carcinomas, dramatic alterations of mucins and mucin-associated carbohydrate antigens were observed during malignant transformation, and these have been used as serum markers for carcinoma diagnosis [14, 40]. Of special interest is the overexpression of the so-called Thomsen-Friedenreich-related antigens [TFRAs, TF/Tn system, including TF, Galβ1–3GalNAcα-*O*-; sialosyl-TF, NeuAcα2–3Galβ1–3GalNAcα-*O*- and Galβ1–3(NeuAcα2–6)GalNAcα-*O*-; Tn, GalNAcα-*O*-; sialosyl-Tn, NeuAcα2–6GalNAcα-*O*-]. Thus, a systematic and comprehensive analysis of mucin and mucin-associated TFRAs in RCCs and normal kidney was considered pertinent, and in the present study we used monoclonal antibodies (mAbs) to examine MUC1, MUC2, and mucin-associated TFRs (TF, sialosyl-TF, Tn, and sialosyl-Tn) in RCCs, surrounding tissue of normal appearance, and two normal kidneys obtained at autopsy. Our main objective was to obtain basic information about the distribution of this group of antigens in RCCs, in order to determine whether they could be used as diagnostic markers.

A second aim was to analyse whether biosynthetically linked structures are expressed simultaneously. Beyond this goal, we considered histogenetic aspects. The origin of clear-cell RCCs and clear/granular RCCs is controversial. Three different hypotheses have been proposed: (1) that clear-cell RCCs and clear/granular RCCs originate from the proximal tubule [13, 15, 16, 30]; (2) that all RCCs arise from primitive cells [9, 10, 23];

and (3) that clear-cell RCCs and clear/granular RCCs develop from the distal tubule and the collecting duct [3, 28]. MUC1 and cytokeratin 19 (CK19) are present in the distal tubule and collecting duct but absent from the proximal tubule [25, 27, 45]. Peptide chains of MUC1 and intermediate filament proteins have been shown to be stable and reliable markers for cells of various histogenetic origins and have been extensively used to study the origin of tumours. Therefore, we examined the expression of both antigens in normal adult kidney tissues and in RCCs to gain some insight into their histogenesis.

Materials and methods

Tissue

Human samples were taken from a total of 58 resected kidneys. They included 53 RCCs and adjacent normal kidney tissues (obtained from sporadic cases), and 5 oncocytomas. In addition, two normal adult kidneys obtained at autopsy were studied. All specimens were fixed in Carnoy's solution or in 10% buffered formalin and embedded in paraffin. Renal cell tumours were classified as clear-cell RCC, clear/granular RCC, chromophobic RCC, and oncocytoma according to the most recent international classification [26] on the basis of findings in haematoxylin and eosin- and periodic acid-Schiff (PAS)-stained sections.

Immunohistochemistry

The monoclonal antibodies employed were A53-B/A2 (anti-CK19, [17]), A78-G/A7 (anti-TF, [18]), and A-76A/C7 (anti-MUC1, which recognises the peptide epitope APDTRP of the MUC1 tandem repeat [5]), which were described previously; CCP58 (anti-MUC2) was kindly provided by Dr. P-X. Xing (Heidelberg, Victoria, Australia, [42]); HBTn (anti-Tn) and B72.3 (anti-s-Tn) were purchased from DAKO (Copenhagen, Denmark) and Biogenesis (Bournemouth, UK), respectively.

Paraffin sections 4 µm thick were deparaffinised. Nonspecific binding sites were blocked with normal rabbit serum. After washing with Tris·HCl-buffered saline (TBS), sections were incubated with mAbs overnight at 4°C. The thoroughly washed sections were treated with rabbit anti-mouse immunoglobulin antiserum for 30 min at room temperature (RT), and thereafter with the alkaline phosphatase anti-alkaline phosphatase (APAAP) complex (DAKO). Colour development was accomplished with the DAKO Fast Red Substrate System (DAKO). Counterstaining was achieved with haematoxylin. Positive controls were performed with normal colon (for MUC2) and colon carcinoma sections. Negative controls were incubated with a comparable dilution of an IgM and IgG from a mouse plasmocytoma (Sigma, Deisenhofen, Germany) instead of the mAb.

For the immunohistochemical detection of sialosyl-TF, sections were incubated with neuraminidase from *Vibrio cholerae* (Serva, Heidelberg, Germany) at a concentration of 0.02 U/ml in PBS containing 0.01 M Ca^{2+} for 1 h at RT to remove NeuAc, washed, and reacted with anti-TF mAb. Erythrocytes present in vessels in the tissue sections functioned as an internal positive control for sialosyl-TF.

Periodate oxidation was performed on sections of normal kidney before incubation with anti-MUC1 or anti-MUC2 as described in our previous report [6].

Scoring

Scoring of immunohistochemical staining was performed as follows: - all cells negative; + <30% positive cells; 2+ 30–60% positive cells; 3+ >60% positive cells. The percentage of positive cells was estimated in several optical fields (10× lens).

Data were analysed with Fisher's exact probability test. Correlation analysis of numerical data was also performed [35]. The significance level (α) was 0.05.

Results

Histopathology

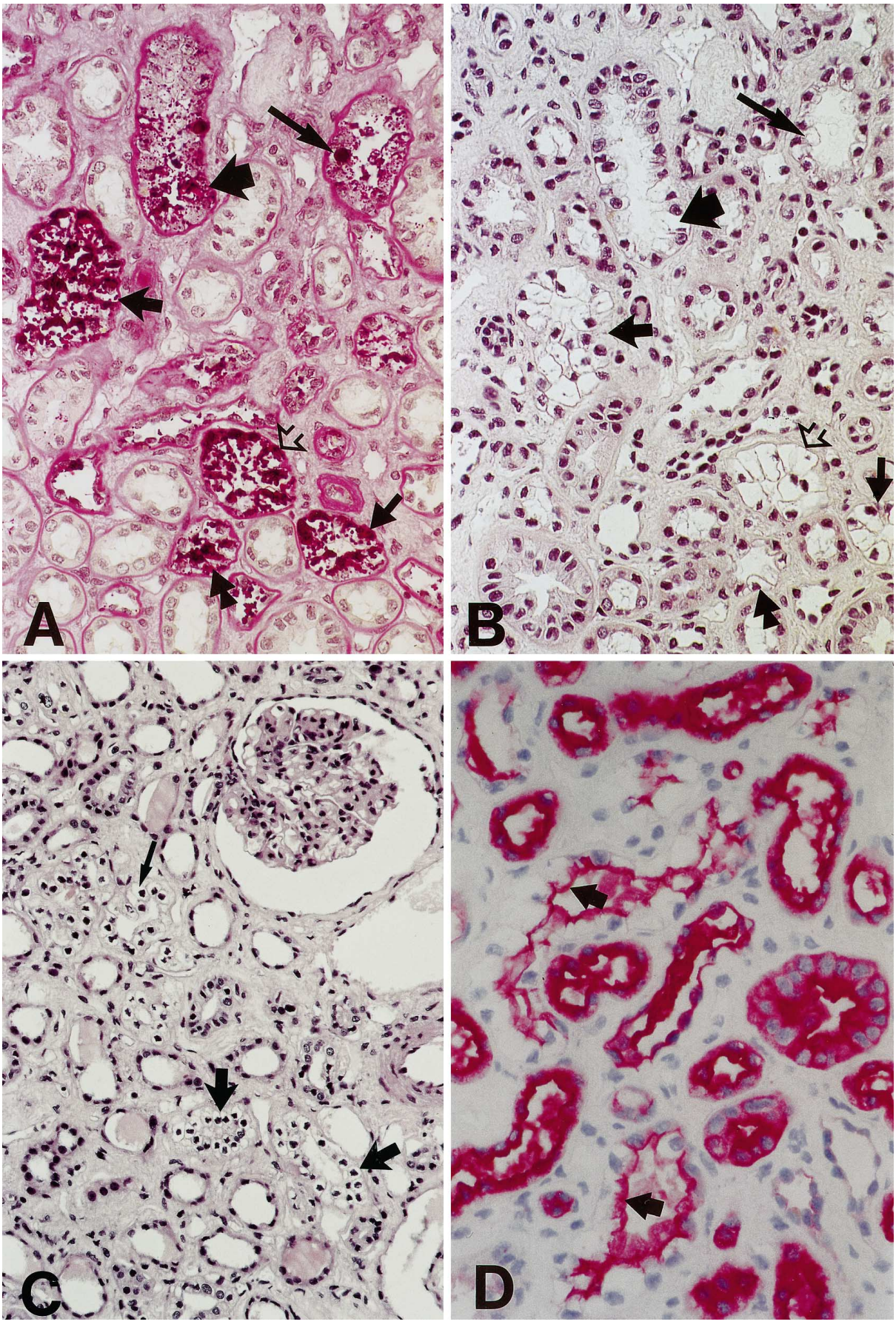
Of the RCCs, 25 were of the clear cell type, 22 of the clear/granular cell type, 4 of the chromophobic cell type, and 2 of the sarcomatoid carcinoma type. In addition, 5 oncocytomas were examined.

Clear-cell RCCs were predominantly composed of clear cells, but often contained variable proportions of more acidophilic granular cells. The tumour cells were arranged in a solid or alveolar fashion, or as sheets, trabeculae, tubules, and papillary processes. Some otherwise normal appearing collecting ducts in kidney at the border between cortex and medulla and at the cortex adjacent to tumours showed positive staining for the PAS reagent; in haematoxylin and eosin-stained serial sections these glycogen storage cells appeared as clear cells; in 12% (3/26) of cases of clear-cell RCC distinct clear cell tubules were found in the collecting duct system; single clear cells were observed in the collecting ducts in an additional 38% (10/26) of cases. Their nuclei were uniform and small, and mitotic figures were not apparent (Fig. 1). Occasionally, clear cells were observed in proximal tubules.

The clear/granular RCCs consisted of clear and granular cells, which were usually acidophilic but sometimes basophilic cells. Acidophilic and basophilic cells were poor in glycogen. The arrangement of tumour cells was similar to that of clear-cell RCCs. Single clear cells in collecting ducts were detected in 23% (5/22) of cases of otherwise normal appearing tissue adjacent to the tumour.

The chromophobic RCCs were composed of cells with a finely reticular, pale cytoplasm, forming solid sheets or alveolar structures. Neither clear-cell nor chromophobic tubular alterations were found in the 4 cases evaluated.

Fig. 1A, **B** Serial sections of kidney tissue adjacent to a clear-cell renal carcinoma (RCC). Note that some epithelial cells of the collecting ducts at the outer medulla are stained with periodic acid–Schiff (**A**, *arrows*), these being seen as clear-cell type (*arrows*) in the haematoxylin and eosin-stained section (**B**). **C** Clear-cell segments in collecting ducts (*arrows*) at the cortex adjacent to a clear-cell RCC. **D** Kidney tissue adjacent to clear-cell RCC, A76-A/C7 (anti-MUC1) stains the luminal surface of clear-cell tubules (*arrows*) ▶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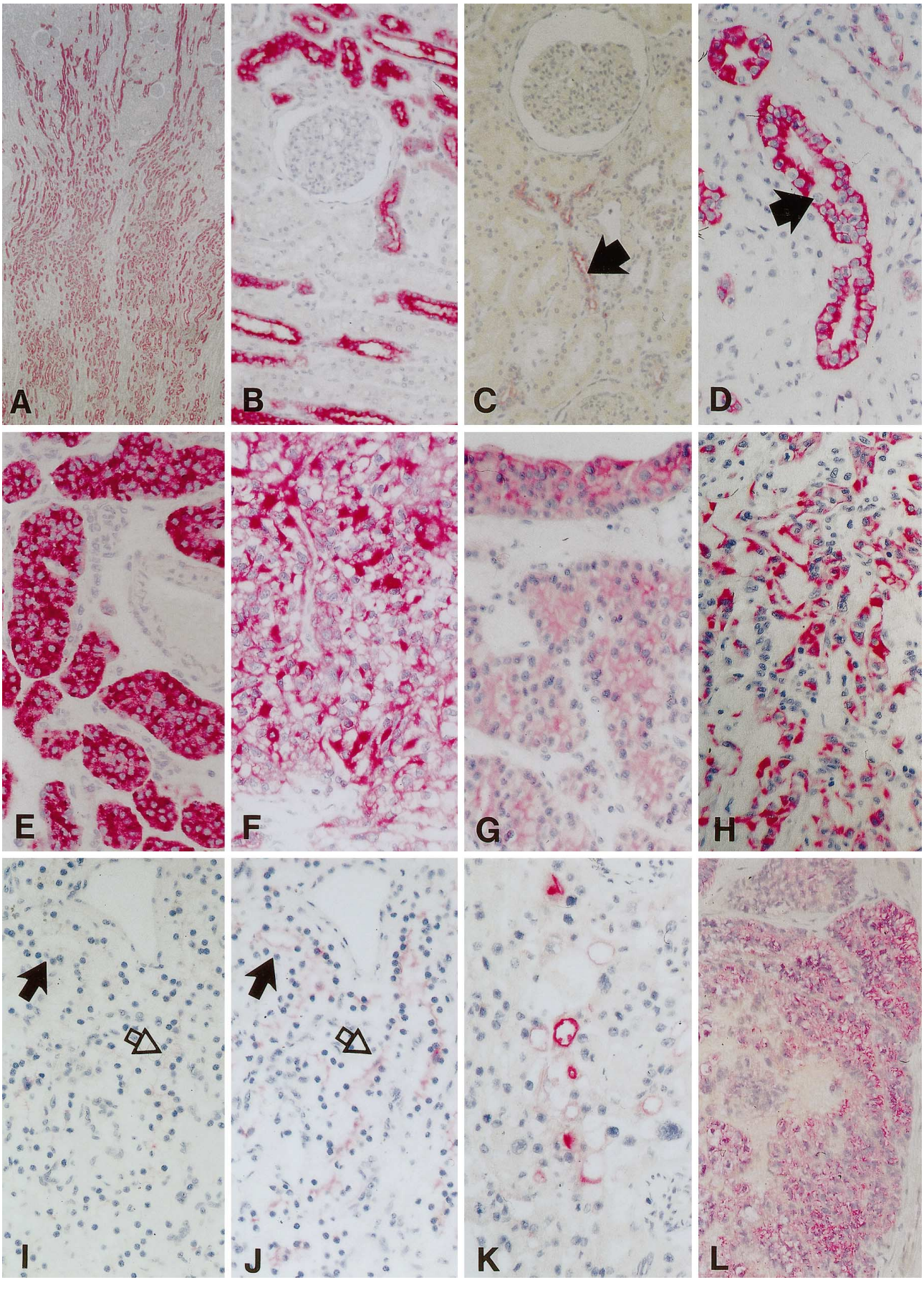
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Table 1 Expression of MUC1, Thomsen-Friedenreich-related antigens, and cytokeratin 19 in renal cell carcinomas (RCCs) and oncocytomas (total numbers of tumours in parentheses)

Tumour type	Score	MUC1	TF	s-TF[a]	Tn	S-Tn	CK19
Clear-cell RCC (25)	–	0	21	17	23	14	13
	+	2	4	6	2	5	9
	++	5	0	2	0	6	3
	+++	18	0	0	0	0	0
Clear/Granular	–	0	19	15	17	13	12
RCC (22)	+	5	3	6	5	6	8
	++	5	0	1	0	3	1
	+++	12	0	0	0	0	1
Chromophobic RCC (4)	–	0	4	2	3	3	0
	+	0	0	2	1	1	4
	++	1	0	0	0	0	0
	+++	3	0	0	0	0	0
Sarcomatoid RCC (2)	–	0	2	2	1	1	0
	+	0	0	0	0	1	1
	++	0	0	0	1	0	0
	+++	2	0	0	0	0	1
Oncocytoma (5)	-	0	5	5	5	5	5
	+	0	0		0	0	0
	++	1	0	0	0	0	0
	+++	4	0	0	0	0	0

[a] A78-G/A7 after neuraminidase treatment

The 2 sarcomatoid RCCs were made up of spindle cells and multinucleated giant cells. Clear cells in collecting ducts of the surrounding tissue were not observed.

Oncocytomas consisted exclusively of uniform large cells with a finely granular, strongly acidophilic cytoplasm. No conspicuous tubular alterations were present in the surrounding tissue.

Immunohistochemistry

Fifty-three RCCs, 32 cases of normal appearing kidney tissue adjacent to tumours, 5 oncocytomas, and 2 normal adult kidneys were examined immunohistologically, with the following results. In normal adult kidneys from autopsy cases and kidney tissue of normal appearance adjacent to resected neoplasms, s-TF, MUC1, and CK19 were positive in distal tubules and collecting ducts but negative in proximal tubules (Fig. 2). In principle, this also applies to TF; however, the expression of TF is sometimes also negative in distal tubules and collecting ducts. TF and s-TF were localised at the luminal surface of the distal tubules and collecting ducts, whereas MUC1 was found in the cytoplasm and at the luminal surface. CK19 was localised in the cytoplasm of these epithelial cells. In contrast, MUC2, Tn, and s-Tn were negative throughout the normal renal tubular system. Comparison of serial sections revealed different immunoreactions for the intercalated cells (dark cells) and the principal cells (light cells) of the collecting ducts: the intercalated cells expressed MUC1 and s-TF in the cytoplasm, but not CK19; the principal cells showed membranous staining for MUC1 and s-TF, and cytoplasmic reactivity for CK19 (Fig. 2). The staining for MUC1 and MUC2 in normal kidney did not change after periodate oxidation. All clear cell tubules in kidney tissues of normal appearance adjacent to neoplasms showed staining for MUC1 and s-TF at the apical membrane (Fig. 1). Some clear cell tubules were positive for TF at the apical membrane, and others were negative.

The immunohistological data for MUC1, TF, s-TF, Tn, s-Tn, and CK19 expression in renal cell tumours are summarised in Table 1 and shown in Fig. 2. All RCCs expressed MUC1, but the intensity of the reaction was usually lower than in the positive parts of the normal tubular system. Binding was predominantly localised throughout the plasma membrane of the tumour cells. The frequencies (percentage of positive cases) of TFRAs were: TF, 13%; s-TF, 32%; Tn, 17%; s-Tn, 42%. Fifty-three per cent of all RCC cases were positive for CK19. The majority of TFRA-positive clear cells in RCCs showed only membranous staining, and only some cells exhibited both a membrane-associated and a cytoplasmic reaction. In TFRA-positive granular cells, the antigens were detected predominantly in the cytoplasm. In chro-

◀ **Fig. 2A**, **B** Normal kidney, distal tubules and collecting ducts are stained with A76-A/C7 (anti-MUC1), whereas renal glomeruli and proximal tubules are negative. **C** Normal kidney, A78-G/A7 (anti-TF) stains the luminal surface of the distal tubules and collecting ducts, but not the renal glomeruli and proximal tubules. **D** Normal kidney: cytokeratin 19 is detected with A53-B/A2 in distal tubules and collecting ducts. Note that the intercalated cells (*arrow*) are negative for A53-B/A2. **E** Oncocytoma: A76-A/C7 stains the cytoplasm of tumour cells strongly. **F** Clear-cell RCC: most tumour cells show staining of entire membrane for A76-A/C7, but some also have a cytoplasmic reaction. **G** Chromophobic cell RCCs positive for A76-A/C7. **H** Clear/granular RCC: note A53-B/A2 staining in the cytoplasm. **I, J** Serial sections of a clear-cell RCC, **I** weakly stained with A78-G/A7 (anti-TF; *arrows*) and **J** more strongly stained after neuraminidase treatment (*arrows*). **K** Clear-cell RCC: HBTn (anti-Tn) reacts with some tumour cells. **L** Clear-cell RCC: B72.3 (anti-s-Tn) reacts strongly

mophobic RCCs, diffuse cytoplasmic and membrane-associated reactions were observed. The oncocytomas showed a cytoplasmic staining pattern for MUC1. MUC2 was negative in all RCCs and oncocytomas.

The correlation of expression of MUC1, TF, s-TF, Tn, s-Tn in RCCs was analysed: TF and s-TF, $P<0.05$, r (correlation coefficient) = 0.568; TF and Tn, $P>0.05$; TF and s-Tn, $P>0.05$; s-TF and Tn, $P>0.05$; s-TF and s-Tn, $P>0.05$; Tn and s-Tn, $P<0.05$, $r = 0.435$. MUC1 did not show any correlation with TF, s-TF, Tn, or s-Tn.

Discussion

Mucins are a group of highly glycosylated, high-molecular-weight glycoproteins that can be subdivided into secretory and membrane-bound types. The best-known function of mucins is to provide a barrier between the luminal membranes of epithelial cells and their environment. MUC1 and MUC2 are membrane-bound and secretory mucins, respectively. The protein backbone of mucins (apomucins) is rich in serine and threonine as potential *O*-glycosylation sites. The first step of mucin-type *O*-glycosylation is the addition of *N*-acetylgalactosamine to serine or threonine residues to form the Tn antigen [8]. Tn is a precursor of TF. Sialosyl-TF and sialosyl-Tn are sialylated forms of TF and Tn, respectively. TF, Tn, and s-Tn glycotopes occur in limited amounts in normal adult human tissues [4], but much more frequently and in higher amounts as "pancarcinoma antigens" expressed on cancer cells [19]. MUC1 expression in RCCs has been reported (reviewed in [45]). However, a systematic investigation of the expression of MUC1, MUC2, and mucin-associated TFRAs using mAbs with higher specificity than that of lectins [39] in normal adult human kidneys and kidney tumours has hitherto not been performed.

In this systematic study, almost all RCCs demonstrated immunoreactivity for MUC1, while all were negative for MUC2. Some RCCs expressed TF, Tn, and s-Tn. In a previous study using another anti-s-Tn mAb (TKH2) no expression of s-Tn in RCCs was detected [43]. This difference may be due to different mAbs. We have observed that the two anti-s-Tn mAbs, B72.3 and TKH2, may lead to different results in immunohistochemistry in normal human adult tissues and carcinomas (Y. Cao et al., unpublished data). MUC1 and TFRAs are widely expressed in carcinomas such as gastrointestinal carcinomas, transitional cell carcinomas, and hepatocellular carcinomas [7, 14, 45]. Therefore, MUC1 and TFRAs are not useful for distinguishing RCCs from other carcinomas of such types. Although Fujita et al. [11] have recently described an inverse correlation between the expression of MUC1 in RCC and the survival of the patients, our results indicate that MUC1 and TF cannot be used as reliable markers of malignancy in the histopathological diagnosis of RCCs. However, MUC1 and TFRAs of RCCs might be released into the circulation and serve as serum tumour markers in a manner reminiscent of the situation for breast and colon cancers [29]. Although normal breast, colon, and other epithelial tissues also express MUC1[14], no such release appears to occur [29]. Thus, it would be of great interest to examine MUC1 and TFRAs in the blood of RCC patients to determine whether these antigens could be used as serum markers for clinical application, for example in the early detection of RCCs during the follow-up of high-risk individuals with mutations or deletion of the von Hippel-Lindau (VHL) gene [22], or for monitoring for recurrence after surgical resection. Another possibility would be the detection of carcinoma cells in the blood or the bone marrow by virtue of their MUC1 expression in order to quantify tumour burden [34]. MUC1 is localised in the cytoplasm and at the luminal side of the normal renal tubular cells, where it is inaccessible to mAbs injected in vivo or to active immunocytes [32] but is strongly and consistently expressed over the entire surfaces of RCC cells and may hence also serve as a target molecule for immunotherapy.

At this point we would like to emphasise an interesting finding from our study. Generally, in normal epithelial cells MUC1 is heavily glycosylated (core region TF masked by peripheral structures such as sialic acid) and is apically localised at the luminal membrane of most epithelial tissues. We have demonstrated in colorectal carcinomas that apolar localisation and incomplete glycosylation of MUC1 are characteristics of malignancy [5, 6]. Compared with other normal epithelial cells from such tissues as mammary gland, salivary gland, pancreas, bile duct, and colon, we have found that MUC1 of the distal tubule and the collecting ducts of the normal kidney reveals some unique, distinguishing features, namely cytoplasmic localisation and exposed TF glycotopes. Therefore, it may be that MUC1 of normal renal cells may differ in its biological function from that of other normal epithelial cells.

Most RCCs expressed MUC1 strongly but TFRAs only sparsely, if at all. This is a surprising finding, because it differs remarkably from the situation in many other carcinomas, such as those of the breast and gastrointestinal tract, in which there is a correlation between MUC1 and TFRAs (tumours strongly positive for MUC1 are also intensely stained for TFRAs and vice versa). This difference points to distinct mechanisms of alteration in MUC1 glycosylation of RCCs and could be a starting point for further biochemical studies.

The histogenesis of some types of renal cell tumours is controversial. The origin of the oncocytoma, a benign tumour of the kidney, from the collecting duct has been well established by several laboratories [31, 37, 44] and has been traced more precisely to the intercalated cells [37]. The observation that oncocytomas showed a strong reaction for MUC1 but no reaction for CK19, like the intercalated cells of the collecting duct, provides further evidence for this view. The expression of MUC1 found in chromophobic RCCs supports the previous suggestion that chromophobic RCCs arise from the collecting duct [31, 38]. An origin of the predominating clear-cell RCCs and clear/granular RCCs from the epithelium of the

proximal tubule has been proposed on the grounds of the observation of certain features resembling the proximal tubule [13, 15, 16, 30]. One of the most convincing arguments for an origin of the clear-cell RCCs and clear/chromophilic (granular) RCCs from the epithelium of the proximal tubule was the demonstration in these tumours of villin that is expressed exclusively in the proximal nephron under physiological conditions [13]. However, villin has also been observed in other tumour types, such as endometrial and pulmonary adenocarcinomas, deriving from tissues in which villin is absent from the normal epithelia [24]. This suggests that villin may be newly expressed during neoplastic development in different tissues.

On the basis of the divergent expression of proximal tubular antigens, distal tubule/collecting duct antigens, and vimentin in clear-cell RCCs and clear/granular RCCs, some authors have argued that these RCCs most probably develop from an early progenitor cell with the potential to differentiate into any type of cell of the tubular system and mesenchyme [9, 10, 23]. However, both interpretations are still in question. The puzzle includes the following aspects:

1. Sufficient evidence for the fundamental steps in the pathogenesis of the human clear-cell RCCs and clear/granular RCCs from the normal proximal tubular epithelium or primitive cell through preneoplastic lesions is so far lacking.
2. Systematic studies of serial sections of a large number rat clear-cell RCCs and clear/granular RCCs usually showed direct connections to the collecting duct system [28, 41], but occasionally a relationship with the proximal nephron was also observed [41]; mutations of the VHL gene were found in some rat clear-cell RCCs and clear/granular RCCs recently, suggesting that the experimental tumour of this type may be an appropriate model of changes in both the genotype and the phenotype of the predominant type of human RCC [36].
3. In the process of neoplastic progression in VHL patients from benign simple renal cysts lined with clear cells through atypical cysts to clear-cell RCCs, which has been confirmed by histopathological observations [33] and direct molecular evidence [22], simple cysts express only distal tubular markers [21].

In our series, 100% and 47% of clear-cell RCCs and clear/granular RCCs expressed MUC1 and CK19, respectively. The immunomorphological characteristics of the majority of clear-cell RCCs and clear/granular RCCs closely resemble those of the distal tubule and the collecting duct, but not those of the proximal tubule, which does not react with mAbs against the peptide backbone of MUC1, even after deglycosylation treatment (periodate oxidation) that would lead to the detection of masked MUC1 [6]. Thus, our data are in line with the hypothesis that clear-cell RCCs and clear/granular RCCs may originate mainly from the collecting duct and/or the distal tubule.

Renal clear cell tubules have been regarded as preneoplastic lesions for rat clear-cell RCCs and clear/granular RCCs [1, 3, 28]. In human VHL disease, renal clear-cell lesions apparently represent the precursors of clear-cell RCCs [33], and this lesion also demonstrates nonrandom allelic loss of the VHL gene [22]. However, to the best of our knowledge, preneoplastic lesions of sporadic human clear-cell RCCs and clear/granular RCCs have not been reported. In this study, clear-cell lesions of similar morphological phenotype were frequently observed in collecting ducts adjacent to clear-cell RCCs and clear/granular RCCs. The immunoreaction of clear-cell tubules for MUC1, s-TF, and TF further resembled that of clear cells of RCCs. These results suggest that clear-cell lesions in collecting ducts might also be precursors of sporadic human clear-cell RCCs and clear/granular RCCs. It should be emphasised that the clear-cell lesions in collecting ducts are easily overlooked in haematoxylin and eosin-stained sections of normal appearing kidney tissue adjacent to RCCs. This may be why these lesions have not been described in earlier reports. PAS staining of alcohol-fixed tissue is necessary for reliable detection of these tubular clear-cell lesions.

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