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Differential expression of SHP2, a protein-tyrosine phosphatase with SRC homology-2 domains, in various types of renal tumour

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Abstract SHP2, a widely distributed protein-tyrosine phosphatase with src homology-2 (SH2) domains, is highly expressed in the brain and may play a role in synaptic communications or cellular proliferation. In this study, we examined SHP2 protein expression in 110 renal cell tumours of various histological subtypes, including clear, granular, papillary, chromophobe, collecting duct, and sarcomatoid-type renal cell carcinoma (RCC), and oncocytoma. SHP2 was expressed predominantly in normal distal tubules and collecting ducts, and positivity in various types of renal tumours was as follows: clear cell RCC, 0% (0/77 cases); granular, 7.7% (1/13); papillary, 50% (3/6); sarcomatoid, 0% (0/1); chromophobe, 85.7% (6/7); collecting duct carcinoma, 0% (0/2); oncocytoma, 100% (4/4). Clear and granular-type RCCs showed a very low but positive expression of SHP2. Chromophobe RCC and oncocytoma showed the highest rates and strongest intensities of SHP2 protein on immunostaining. SHP2 may serve as a powerful marker in detecting rare tumours. Estimates of its expression may be useful in histological diagnosis.

Key words Chromophobe renal cell carcinoma · SHP2 · Protein tyrosine phosphatase · Renal oncocytoma

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Introduction

Renal epithelial cell tumours display cells of greatly varying cytoplasmic differentiation. Clear/light (hypernephroid) cells are a feature of the majority of renal cell carcinomas (RCC) [60]. Masson and Apitz have also characterized various other cells [5, 35], and oncocytes are encountered in renal cell tumours [10, 24, 34, 55, 59]. In nitrosomorpholine (NNM)-induced renal cell tumours, Bannasch et al. have described "chromophobe adenomas" as a rare form [7]. Chromophobe cells have been characterized as voluminous and polygonal, with a lighly opaque, finely reticular cytoplasm and round, oval, or in some cases rather condensed nuclei [52]. In contrast to clear cells, the cytoplasm of chromophobe cells shows a distinct positive reaction with Hale's colloidal iron method and a weak positive with alcian blue [52]. Electron microscopy (EM), which shows innumerable cytoplasmic microvesicles in chromophobe cells, is necessary to distinguish chromophobe cells from clear cells [11, 22, 52, 53]. Chromophobe cell type RCC is an uncommon subtype of renal carcinoma that is characterized by cells containing abundant translucent and reticulated cytoplasm and with distinct prominent cell borders [52, 54]. Some cells have eosinophilic cytoplasm (eosinophilic variant) and others have pale cytoplasm (typical vari-

ant) [54]. The lesion closely resembles some of the RCC found in rats exposed to nitrosomorpholine, and it is significant that this subtype can be confused with renal oncocytoma, a benign neoplasm with an excellent prognosis [7, 54]. On EM, Hale's colloidal iron stain and cytoplasmic microvesicles reveal the important differences between chromophobe cell type RCC and oncocytoma [7, 52]. It has been suggested that the prognosis of chromophobe cell carcinoma may be somewhat better than that of the other types, although this suggestion has not yet been substantiated by additional studies [3].

SHP2 is a human protein-tyrosine phosphatase (PTPase) that possesses two SH2 domains [1, 2, 8, 9, 20, 37, 56]. It binds directly to growth factor receptors, such as platelet-derived growth factor (PDGF) and epidermal growth factor receptors, in response to stimulation with ligand and undergoes tyrosine phosphorylation [28, 32]. SHP2 also binds via its SH2 domains to insulin receptor substrate-1 in response to insulin [31, 38]. Although the precise role of SHP2 in signal transduction remains unclear, it may act as a positive signalling molecule by dephosphorylating phosphotyrosines that negatively regulate signalling mediators such as the Src family kinases [32]. In support of a positive role in growth regulation, the *Drosophila* homologue of SHP2, which is encoded by the *Corkscrew* gene, functions in conjunction with D-*Raf* kinase in a positive manner downstream of the *torso*<~>-encoded receptor tyrosine kinase [18, 20, 32, 44]. Moreover, we and others have recently shown that SHP2 mediates insulin-stimulated Ras activation and subsequent MAP kinase, thereby stimulating DNA synthesis in Chinese hamster ovary cells overexpressing insulin receptors [39, 51]. A previous study has shown that the expression of SHP2 mRNA is ubiquitously observed and highly expressed in the synaptic membrane in the rat brain [50].

The distribution and role of SHP2 in human renal tissue are unknown. The aim of this study was to elucidate the role of SHP2 in human renal tissue by reviewing and comparing the pathological findings and immunohistochemical data with polyclonal and monoclonal antibodies to SHP2 in a series of various histological types of RCC and oncocytoma.

Materials and methods

We reviewed specimens taken from 110 cases of renal cell carcinoma (RCC) during nephrectomy at Kobe University Hospital, Kobe Central Municipal Hospital, Hyogo Medical Center for Adults, Nishi-Kobe Medical Center and Takatsuki Hospital from 1985 to 1995. Formalin-fixed and paraffin-embedded tissues were prepared for each case. Sections 4 μm thick were cut and stained with haematoxylin-eosin, Hale's colloidal iron and immunohistochemical stains against anti-SHP2 antigens. Haematoxylin-eosin and Hale's (1946) colloidal iron-stained slides were reviewed by three of the authors (N.K., Y.H. and K.H.) and classified into seven categories: clear cell-type RCC (77 cases), granular cell-type RCC (not including carcinomas showing a chiefly papillary proliferative pattern; 13 cases), papillary-type RCC (6 cases), sarcomatoid-type RCC (1 case), chromophobe cell-type RCC (7 cases), collecting duct carcinoma (2 cases) and oncocytoma (4 cases). Immunohistochemical studies of SHP2 protein were carried out in all renal tumour cases, normal adult kidney and kidney of a 21-week fetus. In situ hybridization was also carried out, to detect SHP2 in normal kidney. Western blot analysis of SHP2 was carried out in normal adult kidney and in chromophobe- and clear-type RCC. The polyclonal antibodies to SHP2 were generated as previously described, with glutathione S-transferase (GST) fusion protein containing the COOH-terminal region of SHP2 as an antigen [39]. The monoclonal antibodies to SHP2 were also generated by immunizing mice with a GST fusion protein containing a full-length SHP2 as previously described [40].

The distribution of the SHP2 protein expression in tissue sections was examined immunohistochemically with monoclonal and polyclonal antibodies against the human SHP2 antigen. Immunohistochemical staining was carried out with the large-volume DAKO LSAB kit, peroxidase (Dakopatts, Copenhagen, Denmark) according to the manufacturer's instructios. We used 5-μm formalin-fixed sections, which were deparaffinized in xylene (5 min, three times), then dehydrated in ethanol, incubated with 3% hydrogen peroxide for 15 min, with blocking solution for 30 min and with monoclonal and polyclonal anti-SHP2 antibodies diluted 1:100 in 0.01% PBS for 2 h at room temperature. After washing with Tris buffer, the sections were visualized with aminoethylcarbazole chromogen containing hydrogen peroxide. The sections were counterstained with haematoxylin and mounted. The percentage of tumours reacting with SHP2 proteins was determined.

Digoxigenin-labelled single-strand RNA probes were prepared with a DIG RNA-labelling kit (Boehringer Mannheim Biochemica, Mannheim, Germany) according to the manufacturer's instructions. For generation of human SHP2 probe, the 785 bp *Pst*I–*Eco*RI fragment encoding the COOH-terminal region of the SHP2 cDNA clone was subcloned into the Vector Bluescript SK(+) (Stratagene, Calif.). These plasmids were either linearized with *Pst*I and transcribed with T7 RNA polymerase to generate an antisense (cRNA) probe or linearized with *Eco*RI and transcribed with T3 RNA polymerase to generate a sense probe.

In situ hybridization was carried out as previously described [27]. Hybridization of SHP2 mRNAs was done at 50° C for 16 h, and the signals were detected with a nucleic acid detection kit (Boehringer Mannheim Biochemical). The slides were then counterstained with haematoxylin. The controls included hybridization with the sense probes, RNase treatment before hybridization and use of neither the antisense RNA probe nor anti-digoxigenin antibody. The three experiments showed no detectable signals.

Eigteen micrograms of protein samples were subjected to 12.5% SDS-PAGE, then electrotransferred onto nitrocellulose filters (Hybond-ECL, Amersham, Bucks, UK) for western blot analysis. The monoclonal antibody against human SHP2 (PY-20) was used. The immunocomplex was detected with the enhanced chemiluminescence western blotting detection system (Amersham). Finally, the membrane was exposed to X-ray film (Hyperfilm-ECL).

Results

The distribution of SHP2 in human adult normal kidney is shown in Table 1. Both monoclonal and polyclonal anti-SHP2 antibodies reacted similarly with distal tubules

Table 1 Distribution of SHP2 proteins in human adult normal kidney

Location	Expression
Capillary loop	–
Mesangium	–
Bowman's capsule	–
Proximal tubule	–
Distal tubule	+
Collecting duct	+
Blood vessel	–

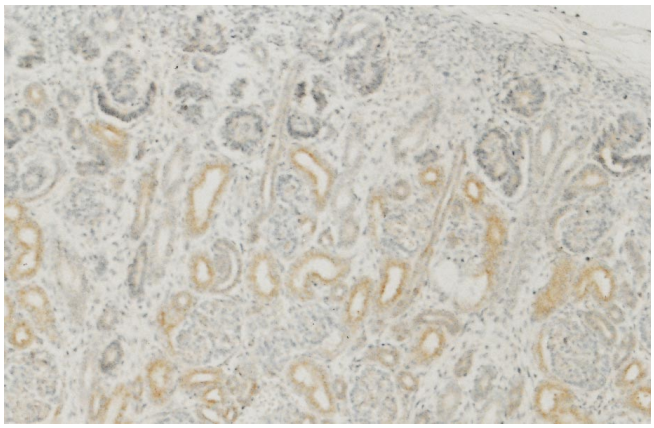


Fig. 1 Expression of SHP2 in fetal kidney (21 weeks of gestations). SHP2 reacts with ureteric buds and collecting ducts in the medulla

Table 2 Expression of SHP2 proteins in various subtypes of renal tumours

Subtype	No.	Positive	Rate (%)
Clear	77	0	0
Granular	13	1	7.7
Papillary	6	3	50
Sarcomatoid	1	0	0
Chromophobe	7	6	85.7
Collecting duct	2	0	0
Oncocytoma	4	4	100

and collecting ducts in normal kidney, but they did not react with other cells, including proximal tubules, glomeruli and endothelial cells of blood vessels. In fetal kidney, SHP2 was present in the cytoplasm of cells of ureteric buds, S-shaped body and collecting ducts of the medulla (Fig. 1). No SHP2 was detected in the undifferentiated blastema, vesicle, or mature glomerulus. Six of seven chromophobe cell-type RCC manifest expression of SHP2 in cellular membranes and the cytoplasm of cancer cells; some of them demonstrated diffusely positive signals of SHP2, and others were positive in scattered cells. Positive signals appeared similar in both types of chromophobe cell-type RCC, eosinophilic cytoplasm (eosinophilic variant) and pale cytoplasm (typical variant). Furthermore, all cases of oncocytoma expressed SHP2 proteins, although none of the clear cell-type RCC demonstrated signals. In granular cell-type RCC, only 1 of 13 cases stained positive; however, the positive signals were located exclusively in its minor papillary structure. One sarcomatoid-type RCC did not react to SHP2 antibodies. Staining intensities of 3 of 6 papillary-type RCC were much weaker than those of chromophobe cell-type RCC. Neither of the 2 collecting duct carcinomas expressed SHP2 proteins (Fig. 2). The positivity rate for each histological type of RCC to SHP2 is shown in Table 2. The clinico-pathological data for 14 cases of SHP2-positive renal tumours is shown in Table 3. Patients ranged in age from 38 to 80 years (mean 63.4 years). A nuclear grade was assigned according to the methods of Fuhrman et al. [21]. In addition, a pathological stage was defined for each case using Robson's classification [46]. Follow-up information was available for 11 of 14 patients (78.6%): 2 papillary RCCs, 5 chromophobe RCCs and 4 oncocytomas. All patients were disease free after 30–113 months of follow-up (mean, 71.7 months).

The specific signals with SHP2 antisense RNA probes were detected in normal kidney. SHP2 mRNA was localized at the cytoplasm of distal tubules and collecting ducts. This distribution by in situ hybridization was identical to that by immunohistochemical studies. Hybridization for SHP2 sense RNA probe showed no signals (Fig. 3).

On western blot analysis weak signals were detected in normal kidney. Strong signals were detected in chromophobe-type RCC. The clear-type RCC cell line did not show SHP2 antigen signals (Fig. 4).

Discussion

Renal cell carcinoma is classified by its cellular and structural features into clear, granular, papillary, and sarcomatoid subtypes. The chromophobe cell-type RCC

Table 3 Clinico-pathological data of SHP-2 positive renal tumours (*ND* no data; follow-up was not possible in cases 1, 4 and 9)

Case	Age	Sex	Histology	Nuclear grade	Staging	Follow-up (months)	Recurrence or metastasis
1	71	Male	Granular	G2	Stage 2	ND	ND
2	65	Female	Papillary	G2	Stage 2	109	–
3	71	Male	Papillary	G1	Stage 1	52	–
4	62	Female	Papillary	G2	Stage 2	ND	ND
5	49	Female	Chromophobe	G2	Stage 2	30	–
6	75	Male	Chromophobe	G1	Stage 2	113	–
7	73	Male	Chromophobe	G2	Stage 2	53	–
8	38	Female	Chromophobe	G2	Stage 2	101	–
9	80	Male	Chromophobe	G2	Stage 2	ND	ND
10	62	Male	Chromophobe	G2	Stage 2	64	–
11	52	Female	Oncocytoma	–	–	102	–
12	64	Male	Oncocytoma	–	–	79	–
13	62	Female	Oncocytoma	–	–	56	–
14	64	Male	Oncocytoma	–	–	30	–

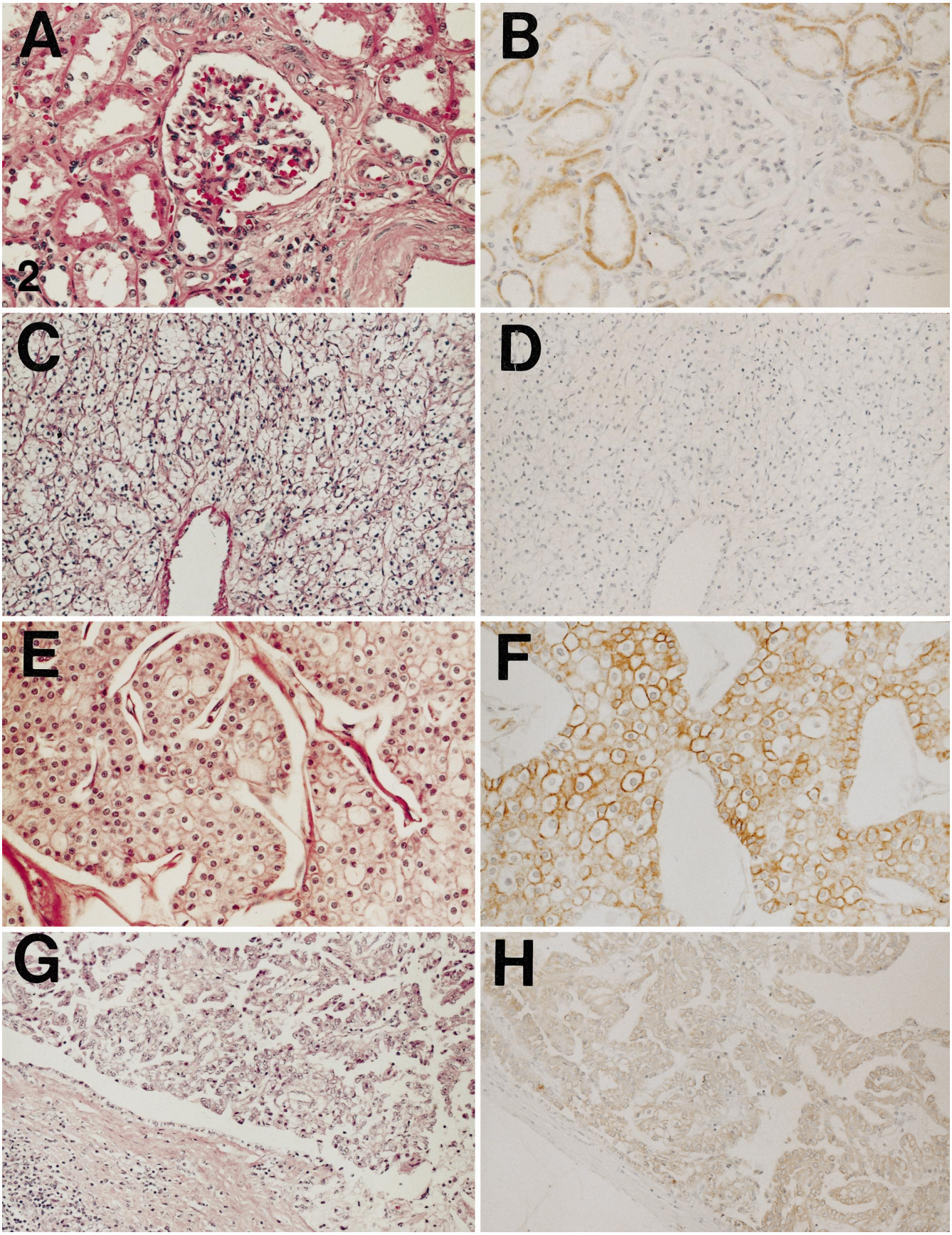
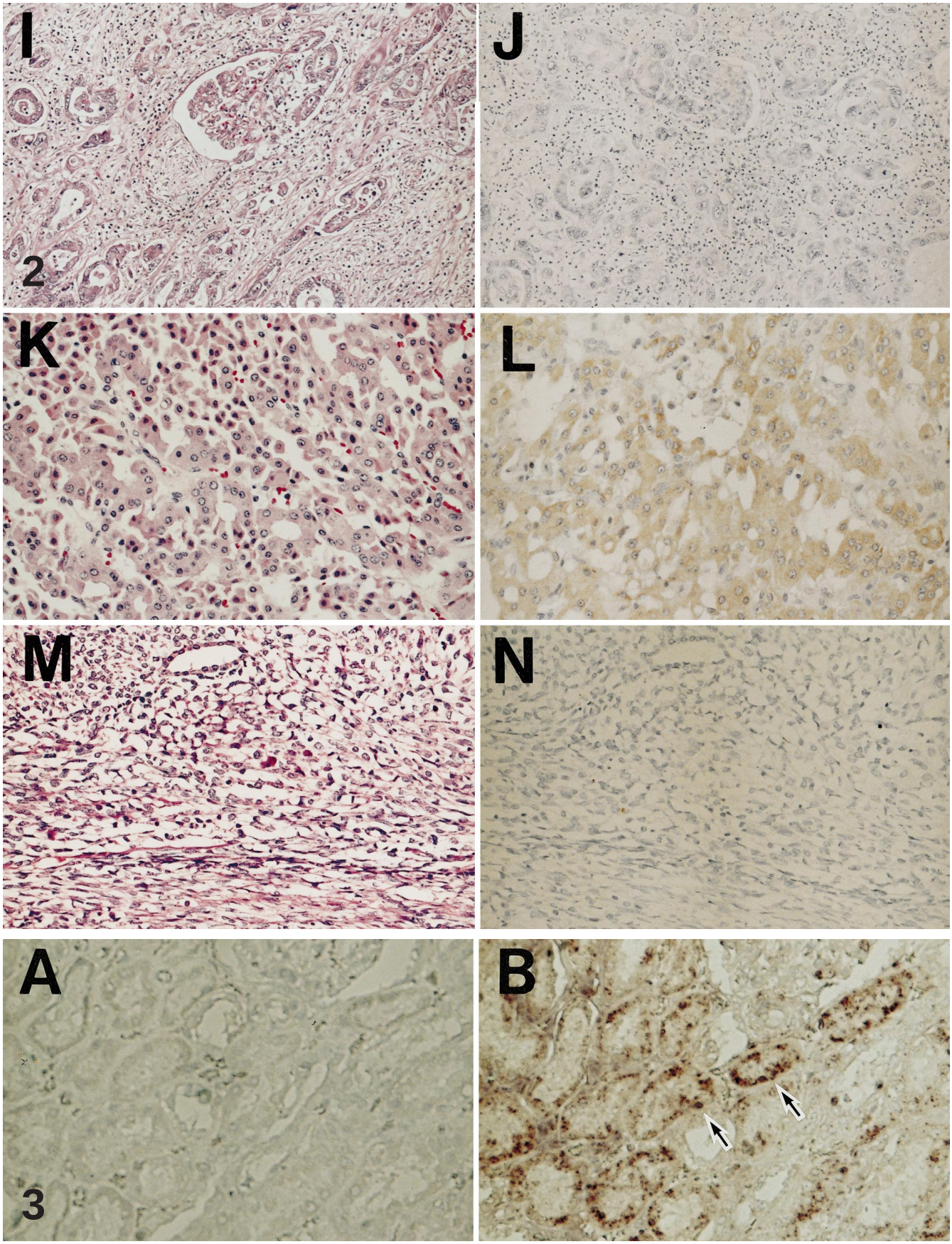


Fig. 2A–H (Legend see p. 336)



Figs. 2A–I–N, 3A, B (Legend see p. 336)

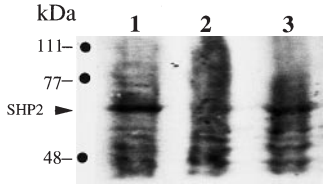


Fig. 4 Western blot analysis of SHP2. *Lane 1* Chromophobe-type RCC, *lane 2* clear-type RCC, *lane 3* adult normal kidney. *Numbers* on the *left* refer to protein markers, whose molecular weights are expressed in kilodaltons. Adult normal kidney and chromophobe-type RCC show signals for SHP2 at 68 kDa

was first discovered in rats by Bannasch et al. [7]. Chromophobe cells have the characteristic of showing slightly opaque or finely reticular cytoplasm on haematoxylin-eosin staining. Later Thoenes et al. described this subtype for the first time in human RCC, reporting that the cytoplasm of human chromophobe cell-type RCC is strongly positive with Hale's colloidal iron and contains a little glycogen [52]. Electron microscopy has revealed many cytoplasmic microvesicles. Thoenes et al. have shown that there are two variants in chromophobe cell-type RCC: the typical and an eosinophilic variant [54].

SHP2 is a protein-tyrosine phosphatase with src homology-2 (SH2) domains and is widely distributed in brain, myocardium, skeletal muscle, liver and other tissues [1, 2, 8, 9, 20, 37, 56]. It has been shown to be highly expressed in the rat brain [33, 48, 50, 58]. Light microscopic immunohistochemistry has shown that SHP2 protein is widely distributed, most abundantly in neuropil and weakly in neuronal somata, and is absent from white matter [50]. Intensely labelled on synapses, concentrated in the pre- and post-synaptic plasma membrane and detected by electron microscopic immunohistochemistry, SHP2 may play a role in synaptic communications in the brain [50]. However, the distribution and function of SHP2 in normal kidneys is unknown. In the present study, SHP2 was mainly expressed in the collecting duct system and in distal tubules, and hardly at all in glomeruli and proximal tubules. Whether or not SHP2 has an important role is unclear, but our study has disclosed that it is a powerful marker for rare renal tumours such as chromophobe RCC or oncocytoma.

Classification of renal tumours has so far been based on traditional concepts of histogenesis and differentiation. In the ongoing process of malignant transformation and progression, however, neoplasms may alter patterns of differentiation but do not necessarily maintain the phenotype of their presumed cell of origin, and molecular cytogenetics has a major impact on the classification of renal tumours [30]. Here we have shown strong expression of SHP2 at the protein level in almost all cases of chromophobe cell-type RCC and renal oncocytoma. On the basis of immunohistochemical findings, Storkel et al. have suggested that chromophobe-type RCC, like renal oncocytoma, shows the phenotype of the intercalated cells of the collecting duct system [41, 49]. There is also evidence that oncocytoma has a distal tubular monology [16]. Chromophobe cell-type RCC is a distinct variant of RCC with unique morphological, histochemical and ultrastructural features, and the prognosis for patients with chromophobe RCC almost corresponds to that of clear-cell RCC of low grade and stage [13, 54]. Chromophobe cell-type RCC may have been confused with renal oncocytoma and variants of RCC in the past. The presence of numerous cytoplasmic vesicles containing the enzyme carbonic anhydrase C is the most important finding to differentiate chromophobe cell-type RCC from renal oncocytoma [49]. However, an oncocytic variant of chromophobe RCC that is devoid of cytoplasmic microvesicles has been reported [17]. SHP2 is expressed in both chromophobe cell-type RCC and renal oncocytoma, demonstrating that it has no relation to cytoplasmic vesicles or the enzyme carbonic anhydrase C and may be a key protein in the development of collecting ducts or distal tubules. It is considered that tyrosine phosphatase may play some part, through tyrosine kinase, in the control of cell proliferation and carcinogenesis.

SHP2 contains two Src homology 2 (SH2) domains and binds to insulin receptor substrate 1 (IRS-1) via these domains in response to insulin [31]. It also binds directly to growth factor receptors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors, in response to stimulation with ligand, undergoes tyrosine phosphorylation, and is implicated in their positive signalling and Ras activation [28, 32, 39]. It is well known that Ras activation plays an important part in the development of the adenoma–carcinoma sequence in the colon [57]. Insulin-like growth factors 1 and 2 (IGF1 and IGF2) and PDGF can induce neuronal differentiation of human neuroblastoma cells [25, 42, 43], and SHP2 may be implicated in this differentiation, as we have observed overexpression of SHP2 in ganglion-differentiated cells in neuroblastomas on immunostaining (unpublished data). SHP2 may show obvious expressional change in oncogenesis and/or differentiation in various organs; we detected no change in expression of SHP2 in embryogenesis and oncogenesis of the kidney. Examination of the expression of its protein product level revealed that ureteric buds, parts of the S-shaped body and

Fig. 2A–N HE staining and SHP2 expression of various renal tumours. ×50 **A**, **B** Normal kidney. SHP2-stained collecting ducts and distal tubules. **C**, **D** Clear cell carcinoma show negative reaction. **E**, **F** Chromophobe renal cell carcinoma (RCC). Tumour cells exhibit strong staining in the cytoplasm and plasma membrane. **G**, **H** Papillary carcinoma. This type imparted positive expression; its intensity in this type was not so strong as that in chromophobe type. **I**, **J** Collecting duct carcinoma. Tumour cells show no expression of SHP2. **K**, **L** Oncocytoma. The tumour shows a positive reaction in the cytoplasm. **M**, **N** Sarcomatoid carcinoma. Tumour cells have not reacted with SHP2. **A**, **C**, **E**, **G**, **I**, **K**, **M** HE staining **B**, **D**, **F**, **H**, **J**, **L**, **N** SHP2 immunostaining

Fig. 3A, B Expression of SHP2 mRNA in normal kidney on in situ hybridization. ×50 **A** Normal kidney with sense probe. **B** Normal kidney with antisense probe. Although SHP2 mRNA is expressed in distal tubules and collecting ducts (*arrow*), it has not reacted with proximal tubules

collecting ducts in fetal kidney, distal tubules and collecting ducts in normal adult kidney and RCC showing similar phenotypes were almost all positive for SHP2. The protein may be implicated in some specific function or development in human renal tissues, as SHP2 may be involved in synaptic communications in the rat brain.

Three of six cases of papillary type RCC demonstrated weak expression of SHP2 protein. By chromosomal analysis, papillary RCC is considered to be an independent form of RCC, derived from proximal tubules [29]. These RCCs have been and are considered, in some cases, to be associated with atypical hyperplastic changes of collecting tubules and a proliferative pattern like transitional cell carcinoma [19, 23, 36]. This type should now be classified as collecting duct carcinoma [19, 23, 36]. Papillary-type RCC has various phenotypes [26]. We also believe that there is a heterogeneity in its phenotypes, and some forms of this type might indicate not only the phenotype of proximal tubules, but also of distal tubules or collecting duct systems, although there is no obvious difference in the histological appearance and clinical features between SHP2-positive and SHP2-negative papillary-type RCCs. Collecting duct carcinoma is a rare tumour that accounts for between 0.4% and 3% of recorded cases of RCC [12, 19, 47]. This type is assumed to be derived from the ducts of Bellini [47]. Collecting duct carcinoma should be recognized as being different from papillary RCC on the basis of previously described findings, because the former has a poor clinical course compared with the good outcome in the latter [15, 36]. Differential diagnosis between these tumours is difficult because of the lack of definite criteria of collecting duct carcinoma. We encountered two cases of distinct collecting-type RCC that expressed no SHP2 proteins, but cannot infer that this result indicates a collecting duct carcinoma, because such cases, are so rare.

The oncogenesis of sarcomatoid RCC varies from case to case. In the majority of cases, it is taken for granted that this type of RCC arises from a clear or granular RCC [45]. Sarcomatoid tumours derived from papillary, chromophobe, and collecting duct carcinoma have been reported, however, demonstrating that every type of RCC possesses the potential for progression to sarcomatoid carcinoma [4, 6, 45]. The presence of epithelial components would lead to a diagnosis of sarcomatoid carcinoma in tumours with mostly spindle cells before we could consider them to be other renal sarcomas. Determining the origin may not be possible in cases with very few epithelial cells, and in such cases SHP2 may be useful in determining their histogenesis, though this, would not be thought to have great credibility since sarcomatoid components are known to manifest a different immunohistological staining pattern from the epithelial component [14]. When such cases are encountered, AE1/AE3 staining, which is known to be an epithelial marker, should be used in determination of the epithelial origin of sarcomatoid RCC [14].

Differential diagnosis of renal tumours such as clear or granular and chromophobe types of RCC and oncocytoma is often difficult. The distinction between chromophobe-type RCC and oncocytoma is particularly important. By using the immunohistochemical technique for SHP2, clear- or granular-type RCC can be differentiated chromophobe-type RCC and oncocytoma, which have a better prognosis. Chromophobe-type RCC has thick plasma membranes that stain for SHP2, whereas oncocytoma does not have reactive plasma membranes. If Hale's colloidal iron, EM and cytogenetic studies are carried out, chromophobe-type RCC, which has been reported as a malignant oncocytoma, can be recognized as an entity that is distinct from oncocytoma, which is presently considered to be a benign tumour.

SHP2 demonstrated visible signals in normal distal tubules, collecting ducts and renal neoplasms showing similar phenotypes. These results suggest that SHP2 may serve as a useful marker for detection of rare renal cell tumours, particularly chromophobe RCC and oncocytoma. We would like to emphasize that this differential expression of SHP2 may be a supportive tool in the histological diagnosis of renal neoplasms.

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