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ErbB4 is Downregulated in Renal Cell Carcinoma

A Quantitative RT-PCR and Immunohistochemical Analysis of the Epidermal Growth Factor Receptor Family

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In the present study the authors evaluated the expression of the EGFR family members ErbB2-4 in renal cell carcinoma (RCC). Thirty-one RCCs were examined for gene expression of ErbB2-4 mRNA by quantitative real-time RT-PCR. For eight of the patients samples of non-neoplastic kidney cortex were also evaluated. Expression of ErbB4 mRNA was analysed in the eight matched tumour and kidney cortex samples by isoform-specific real-time RT-PCR analysis. ErbB4 protein expression was analysed by immunohistochemistry. In summary the results showed that ErbB2 mRNA was downregulated in conventional (clear cell) RCC; ErbB3 mRNA levels were low and heterogeneous in both tumours and kidney cortex; ErbB4 mRNA and protein were strongly downregulated in conventional and papillary RCC. Thus, ErbB2 and ErbB4 are not likely to be oncogenes in the majority of RCCs; instead, the observed downregulations suggest that these receptors might function as tumour suppressors in RCC.

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Renal cell carcinoma (RCC) represents 2–3% of all cancer. Conventional (clear cell) RCC accounts for approximately 75% of these tumours, papillary RCCs for 10–15%, chromophobe RCCs for about 5%, and collecting duct carcinomas for less than 1%. There are specific differences in the genetic alterations (1) and gene expression profiles (2) between the different RCC subtypes but so far this has not been translated into differential treatments of RCC subtypes. Despite many efforts to develop effective therapies, including immunotherapy, hormone manipulation, and chemotherapy, the survival of patients with advanced RCC still remains poor (3, 4).

The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases includes four members, EGFR (ErbB1), ErbB2, ErbB3, and ErbB4, which are involved in the regulation of cellular proliferation, differentiation and migration (5). The binding of receptor-specific ligands to the extracellular domains of the receptors results in the formation of homo- and heterodimeric receptor complexes and subsequent activation of intracellular signalling pathways (6).

EGFR over-expression is common in many human tumours and of prognostic value in, for example, non-small cell lung cancer and gliomas (7). Pharmacological inhibition of the EGFR tyrosine kinase has in early clinical trials produced promising results in several cancer types (8, 9). Previous studies have shown EGFR over-expression in RCC but its prognostic significance remains to be established (10–13). The EGFR ligands EGF and TGFα are also over-expressed in RCC and may be involved in an autocrine loop (14). These observations indicate that EGFR and its ligands may play important roles in the genesis and development of RCC. Although ErbB2 over-expression seems to be a powerful prognostic factor in many cancers (7), the expression of ErbB2 in RCC seems to be lower than in normal kidney tissue (15). The expression of ErbB3 and ErbB4 has

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not been thoroughly studied in RCC. The ErbB4 receptor has at least four isoforms with two variations in the extracellular juxtamembrane region (JM-a and JM-b) and two variants in the cytoplasmic region (CYT-1 and CYT-2) (16). The juxtamembrane variants differ in their susceptibility to metalloproteinase cleavage: JM-a is cleaved by tumour necrosis factor alpha converting enzyme (TACE), whereas JM-b is not (17, 18). Cleavage of ErbB4 may be involved in receptor degradation in response to ligand binding (19). It has also been suggested that metalloprotease cleavage followed by γ-secretase cleavage mediates nuclear translocation of part of the receptor, which then mediates growth inhibition signalling (20). The cytoplasmic variants differ in their ability to couple to phosphoinositide 3-kinase (PI3-K) signalling and to stimulate cell survival and chemotaxis (21).

In the present study, we evaluated the expression of the EGFR family members ErbB2–4 with special emphasis on ErbB4 in RCC. This report provides for the first time a quantitative evaluation of ErbB3 and ErbB4 expression in renal malignancy and presents evidence for a downregulation of ErbB4 in RCC.

MATERIAL AND METHODS

Patients

Specimens from RCC were obtained from 31 patients (Table 1) who underwent nephrectomy at the Department of Urology, Umeå University Hospital between 1986 and 1998. Of these tumours, 18 were conventional RCC, 10 papillary, and 3 chromophobe RCC. In 8 of the patients (5 with conventional and 3 with papillary RCC) we analysed matched specimens of corresponding kidney cortex.

Quantitative RNA analysis

RNA was prepared from tissue samples by mechanical disruption in TRIzol reagent (Gibco-BRL, Rockville, MD), followed by chloroform extraction and ethanol precipitation according to the manufacturer's instructions as described in (22). Real-time quantitative reverse transcription-(RT-) PCR of ErbB receptors was performed as described (22, 23) using an iCycler (Bio-Rad, Hercules, CA). Primers and probes were synthesized by Scandinavian Gene Synthesis AB (Köping, Sweden) (Table 2). RNA samples were run in triplicate using 20 ng of RNA per reaction. Relative quantification was performed by comparing the threshold cycle values (C_T) for the samples, with standard curves generated with cloned cDNAs of respective genes (22). As negative control we used ultra-pure water (Gibco-BRL). To correct for differences in RNA quality and quantity, the apparent levels of 18S rRNA were used to normalize the ErbB values in respective RNA samples.

Table 1

Characteristics of the patients and tumours included in the study

Total no of patients	31
Sex (male/female)	17/14
Age median (range)	64 (36–85)
Tumour diameter median (range)	80 (30–250)
TNM Stage I	7
TNM Stage II	5
TNM Stage III	10
TNM Stage IV	9
WHO Tumour grade 2	5
WHO Tumour grade 3	21
WHO Tumour grade 4	5
Known metastasis at time of nephrectomy	9
Survival time in months median (range)	44 (1–139)

Analysis of the ErbB4 isoforms was performed by synthesizing cDNA using random hexamer primers (Promega, Madison, WI) and M-MLV RNase H minus reverse transcriptase (Promega), followed by PCR (Taqman, PE Biosystems) using oligonucleotide primers and probes previously demonstrated to specifically detect the human ErbB4 isoforms (24). Isoform mRNA expression was normalized against β-actin mRNA expression from parallel samples. Measurements of ErbB4 isoform and β-actin mRNA expression were done in duplicate. The range between the two parallel C_T values was less than 5% of the mean in each case. Expression of each ErbB transcript was presented as the percentage of ErbB4 isoform mRNA expression relative to the expression of the control β-actin mRNA.

Statistical analyses of matched samples were conducted with the Wilcoxon signed-rank test.

Immunohistochemisry

From the formalin-fixed and paraffin-embedded tissue blocks 5 μm-thick sections were cut and mounted with samples of kidney cortex and tumour tissue on the same slide. The sections were deparaffinized in xylene, and rehydrated in absolute ethanol, and 95% and 70% ethanol for 2×5 min each. Endogenous peroxidase activity was blocked in 3% peroxide in methanol for 20 min followed by washing in PBS (0.15 M phosphate buffer pH 7.4) 3×5 min. The samples were emerged in 0.01 M citrate buffer pH 6.8 and treated with microwaves, 1400 W for 2×5 min. Samples were then incubated with 10% goat serum in PBS for 30 min and with polyclonal rabbit antibody against ErbB4 (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:300, overnight. Specimens were washed in PBS 3×5 min before application of biotinylated secondary goat antibody (Vector Laboratories, Burlingame, CA) diluted 1:200 in PBS for 40 min. Thereafter specimens were washed for 3×5 min in PBS and incubated with a DAB substrate kit (Vector Laboratories), according to the manufacturer's instructions. The sections were rinsed in water, counterstained with Mayer staining, again rinsed in water, and mounted. Negative controls with a primary polyclonal

Table 2

Oligonucleotide primer and probe sequences for quantitative real-time RT-PCR

Templates	Oligonucleotides	Sequences	PCR product size (bp)
ErbB2	Forward primer	5′-GCACCCAAGTGTGCACCGGC-3′	114
	Reverse primer	5′-ATTGGTGGGCAGGTAGGTGAGTTC-3′	
	Probe	5′-[1]fACCCACC[2]QGGACATGCTCCGCCACCTCTACC-3′	
ErbB3	Forward primer	5′-CGAACGACGCTCTGCAGGTGC-3′	109
	Reverse primer	5′-GTCACACTCAGGCCATTCAGAGTC-3′	
	Probe	5′-[1]fCTTTTCAGCC[2]QGGCCCGGGGCTCCGAGGT-3′	
ErbB4	Forward primer	5′-GATTCTCAGTCAGTGTGTGCAGGAAC-3′	126
	Reverse primer	5′-TATCTCCAGGTTGCCCATGACAACC-3′	
	Probe	5′-[1]fCTCTCTCTC[2]QGACCTGGAACAGCAGTACCGAGCCTTG-3′	
18S rRNA	Forward primer	5′-CGGCGACGACCCATTCGAAC-3′	99
	Reverse primer	5′-GAATCGAACCCTGATTCCCCGTC-3′	
	Probe	5′-[1]fCCTATCAAC[2]QTTCGATGGTAGTCGCCGTGCC-3′	

[1]f denotes a 5′-conjugated fluorescein and [2]Q denotes a T with a conjugated Dark Quencher molecule.

rabbit antibody against GFP, a non-human protein, showed no staining. The intensity of the staining of the tumour was compared with that of the corresponding normal tissue and graded as follows: 0 = no staining, + = less staining than in kidney cortex from the same patient, ++ = equal staining to kidney cortex, +++ = more staining than of the corresponding kidney cortex.

RESULTS

Quantitative RNA analysis

In order to examine the expression of the EGFR-receptor family members ErbB2–4 in RCC, 31 tumour and 8 kidney cortex RNA samples were analysed by quantitative RT-PCR. Expression of ErbB2 was in all conventional RCC samples lower than the mean expression in kidney cortex (Fig. 1a). A significant downregulation of ErbB2 mRNA ($p = 0.04$) was observed in conventional RCC compared with kidney cortex, where all five matched samples showed downregulation (Fig. 1b). The levels of ErbB3 mRNA appear to be low in non-neoplastic kidney cortex compared with other tissues such as breast and prostate (unpublished observation). Similarly, the ErbB3 mRNA levels in the 31 RCC samples were low but variable (Fig. 1c). When RCC was compared with corresponding kidney cortex tissue the changes in ErbB3 expression were not significant ($p = 0.16$) (Fig. 1d). ErbB4 was highly expressed in kidney cortex tissue but the expression was low in 17 of 18 conventional and in all 10 papillary RCC. In 14 of the 31 tumours ErbB4 mRNA was not detected at all (Fig. 1e). Compared with conventional and papillary tumours, the chromophobe tumours showed less ErbB4 downregulation. The expression of ErbB4 mRNA in RCC tumour tissue was significantly reduced compared with matched kidney cortex ($p = 0.02$) (Fig. 1f). In the papillary RCC, ErbB4 expression was decreased in 2 of 3 tumours, while it was very low in both tumour and kidney cortex in the in the third case (Fig. 1f). Of the ErbB4 isoforms, the juxtamebrane variant JM-a predominated and JM-b expression was very low or absent, in both kidney cortex and in the tumours that expressed ErbB4. Of the cytoplasmic variants, the expression of CYT-1 was higher than that of CYT-2, in both kidney cortex and in the tumours that expressed ErbB4. The isoform ratio was thus not significantly altered in the few tumours where ErbB4 could be detected (Fig. 2). The ErbB4 expression in the kidney cortex sample from patient P.8 appeared disproportionally low when the ErbB4 isoforms were analysed individually (Fig. 2) compared with when total ErbB4 was analysed (Fig. 1f). The reason for the discrepancy between the assays in this single sample was not further investigated.

Immunohistochemistry

Immunostaining of ErbB4 in kidney cortex yielded staining of tubules, with a higher staining intensity of the distal tubules than of the proximal tubules. No staining was seen in the glomeruli. This staining pattern is consistent with a previous report (25). Six of eight RCC did not stain for

Table 3

Immunohistochemical staining intensity of ErbB4 in tumours compared with kidney cortex from the same patients

Patient	Type of RCC	ErbB4 staining
C.2	Conventional	0
C.9	Conventional	0
C.11	Conventional	+[1]
C.15	Conventional	0
C.17	Conventional	0
P.6	Papillary	0[2]
P.8	Papillary	+
P.9	Papillary	0

[1]Tumour was weakly and only partially stained.
[2] Normal tissue was very weakly stained.
The tumour staining intensity was graded as: 0 = no staining, + = positive, but less staining than the kidney cortex tissue from the same patient, ++ = equal staining to kidney cortex, +++ = more staining than in the kidney cortex tissue.

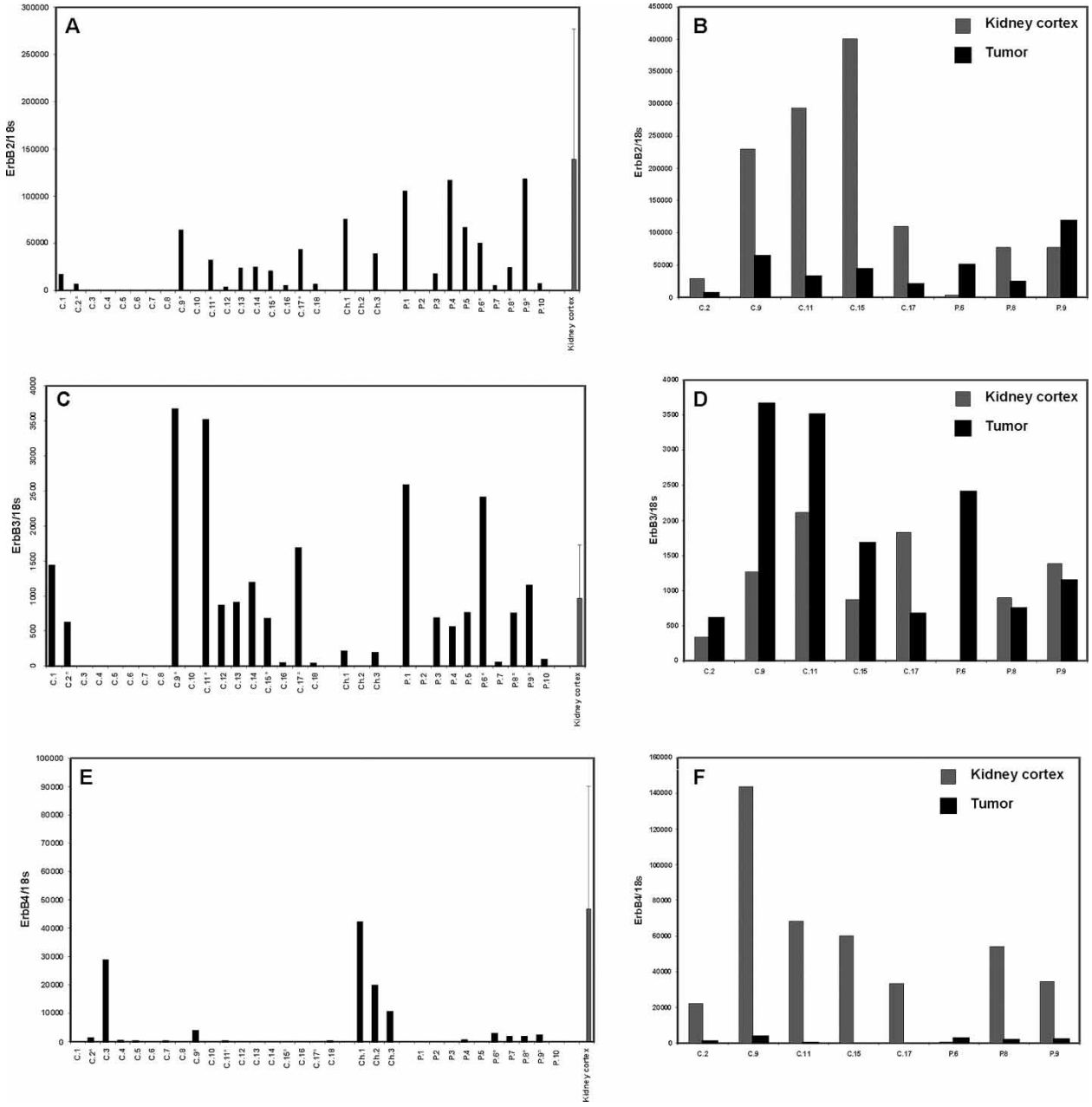

Fig. 1. Expression of ErbB2-4 mRNA in RCC and kidney cortex. Total RNA was analysed for mRNA levels of ErbB2 (A, B), ErbB3 (C, D), and ErbB4 (E, F) by quantitative real-time RT-PCR. ErbB levels were normalized to the 18S rRNA levels in respective samples and shown as apparent ErbB/18S rRNA ratios. A total of 31 RCC (A, C, E) and 8 RCCs with matched kidney cortex tissues (B, D, F) were analysed. Significant decreases were noted for ErbB2 in conventional tumours ($p = 0.04$), and for ErbB4 in all tumours ($p = 0.02$). The tumour types were: conventional RCCs (C.1–18), chromophobe RCCs (Ch.1–3), and papillary RCCs (P.1–10). In A, C, and E the mean and standard deviations of the ErbB expression in 9 (the 8 matched and 1 additional sample) kidney cortex tissues are shown.

ErbB4 at all (Table 3, Fig. 3); one conventional tumour and one papillary RCC displayed signs of weak staining for ErbB4.

DISCUSSION

In this study, using quantitative RT-PCR and immunohistochemistry, the novel observation was made that ErbB4 was strongly downregulated in RCC. The results also showed downregulation of ErbB2 mRNA in conventional RCC (12, 15). The EGFR has been implicated in the development of RCC and its up-regulation in these tumours is well documented (10–13) and has been confirmed in the material used in this study (26). ErbB2 is an established oncogene in many tumour types, including lung and breast carcinoma, and over-expression is correlated to advanced

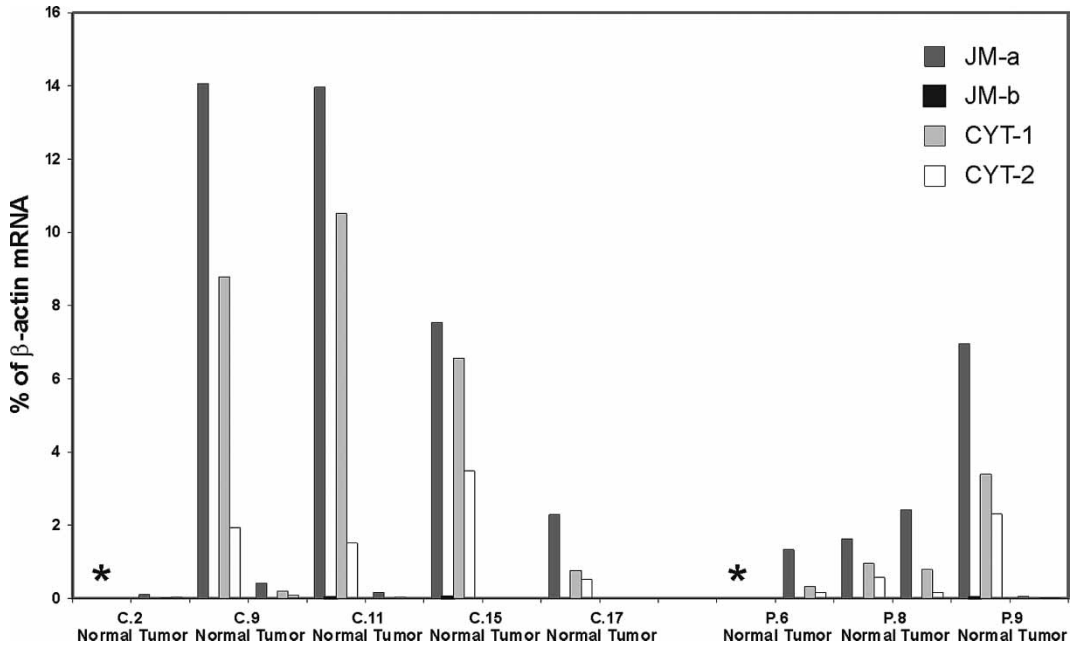

Fig. 2. Expression of ErbB4 isoforms in RCC and kidney cortex tissue. Expression of ErbB4 isoforms in RCC tumours and corresponding kidney cortex tissue were analysed by real-time RT-PCR. Expression levels are presented as percentages of β-actin mRNA levels. Patients C.1–18 had conventional RCC and patients P.1–10 had papillary RCC. * Indicates samples that were not analysed owing to lack of material.

stage and decreased survival (27, 28). The downregulation of ErbB2 in RCC of this oncogene suggests, however, that ErbB2 does not function as an oncogene in most RCCs.

ErbB3 showed very weak and heterogeneous expression in both tumour and kidney cortex tissues. This is consistent with a previous report (29) in which ErbB3 was not detectable by immunohistochemistry in 6 of 6 RCCs studied.

ErbB4 displayed low or no expression in conventional and papillary RCC, as assayed both by RT-PCR and immunohistochemistry. Kidney cortex expressed only the JM-a CYT-1 and the JM-a CYT-2 isoforms, which is in line with earlier findings (17, 30). Total ErbB4 expression was strongly downregulated in RCCs but no change in the isoform ratios was observed. Apparently contradictory reports exist concerning the role of ErbB4 in cancer. ErbB4 activation has been shown to inhibit apoptosis in PC 12 rat pheochromocytoma cells (31) and ErbB4 expression seems to correlate with decreased survival time in childhood medulloblastoma and colon cancer when co-expressed with ErbB2 (32, 33). In contrast, ErbB4 expression has been shown to correlate with increased survival time in breast cancer (28). This latter observation suggests a tumour-suppressing effect of ErbB4 in breast cancer that counteracts the effects of EGFR and ErbB2. Likewise, it is possible that ErbB4 has tumour-suppressing effects in RCC. There are several possible explanations why ErbB4 seems to have opposite effects in different types of cancer. This could for example be due to effects of co-expression of other EGFR family members, or to the expression of functionally

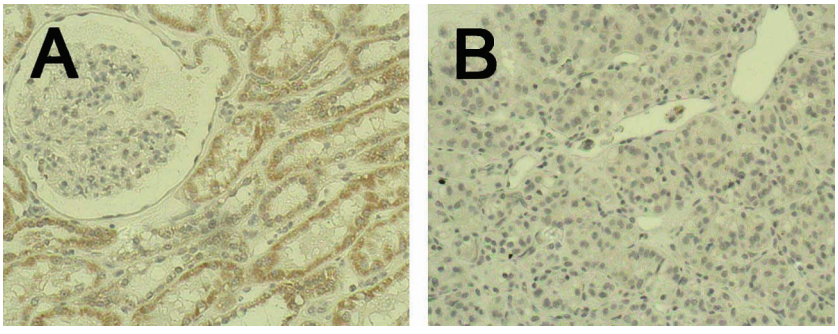

Fig. 3. ErbB4 immunohistochemical staining of tissue sections using a polyclonal antibody against ErbB4. Samples were from the patient referred to as C.9. A: normal kidney. B: conventional RCC. Original magnification 200 times.

distinct ErbB4 isoforms in the different tumour types. Another possibility is that ErbB4 plays no role in oncogenesis and that the different levels of ErbB4 expression in different cancers are merely secondary to other molecular changes in the tumours. Nevertheless, in the present study it was firmly demonstrated, by real-time RT-PCR and by immunohistochemistry, that ErbB4 is downregulated in RCC. The molecular mechanisms behind the changed expressions of ErbB2 and ErbB4 in RCC compared with normal kidney are not known. An investigation of the molecular basis for these changes could be of interest but was beyond the scope of this study.

In conclusion, the downregulation of ErbB4demonstrated in this report indicates that this receptor might function as a tumour suppressor in RCC. We do not know, however, all the functions of ErbB4 in normal and neoplastic kidney and further studies are warranted to elucidate the explicit role of ErbB4 and its isoforms in the oncogenesis of RCC.

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