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ORIGINAL ARTICLE

KIT, *PDGFRα* and *EGFR* analysis in nephroblastoma

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Abstract Nephroblastoma prognosis has dramatically improved, but an unfavourable prognostic subgroup warrants development of novel therapeutic strategies. Selective KIT, PDGFRα and epidermal growth factor receptor (EGFR) tyrosine kinase inhibition evolved as powerful targeted therapy for gastrointestinal stromal tumours and non-small-cell lung cancer. To investigate a potential role for tyrosine kinase inhibition, we analyzed 209 nephroblastomas for immunohistochemical KIT and EGFR expression, 63 nephroblastomas for mutations in *KIT* exons 9, 11, 13, *EGFR* exons 18, 19, 20 and 21, and all 209 nephroblastomas for *PDGFRα* exons 12, 14 and 18. Twenty-two tumours (10.5%) expressed KIT, 31 (14.8%) EGFR, and 10 (4.8%) both KIT and EGFR, respectively. KIT expression was relatively more common among high-risk tumours (6/27; 22.3%) compared to low-/intermediate-risk tumours (26/181; 14.4%). Nine patients deceased, four of which had high-risk tumours with KIT expression in two of four and EGFR expression in one of four. There were no *KIT*, *PDGFRα* or *EGFR* mutations. Our results suggest no significant contribution of *KIT*, *EGFR* or *PDGFRα* mutations to nephroblastoma pathogenesis. Despite a trend towards association of immunohistochemical KIT and EGFR expression with poor outcome in high-risk nephroblastomas, statistical analysis did not yield significant correlations in this subgroup. Therefore, it remains open if KIT, PDGFRα or EGFR tyrosine kinase inhibition constitute a therapeutic target in nephroblastoma in the absence of *KIT, PDGFRα* or *EGFR* mutations.

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Introduction

Nephroblastoma is the most common malignant renal tumour in childhood and affects 1 in 8,000 children [11]. Over the past three decades, multimodal nephroblastoma therapy has been a story of success. Nephroblastoma prognosis has improved dramatically, and overall 10-year survival now approaches 85% [15]. Nevertheless, a poor prognostic subgroup of nephroblastomas remains. Diffuse anaplasia, persistent blastema after chemotherapy, allelic loss at 1p or 16q, *TP53* mutations and advanced tumour stage have been identified as adverse prognostic factors [3, 10, 11, 31, 32]. Therefore, development of novel therapeutic strategies is mandatory.

Over recent years, receptor tyrosine kinases have evolved as focus of research for potential targeted tumour therapy [26]. Transmembrane receptor tyrosine kinases play an important role in the modulation of growth factor signalling [33]. Receptor tyrosine kinases KIT (v-kit Hardy-Zuckermann 4 feline sarcoma viral oncogene homologue), PDGFRα (platelet-derived growth factor receptor α) and EGFR (epidermal growth factor receptor) participate in cell growth and are implicated in malignant transformation and tumour progression. KIT is claimed to play an important role in kidney organogenesis. It is expressed during metanephrogenesis in blastema and immature epithelium and in the mature kidney in proximal and distal tubules [27]. KIT expression has been demonstrated in a subset of renal cell carcinomas, oncocytomas, mesoblastic nephromas, angiomyolipomas and a small series of nephroblastomas [22, 30]. The KIT internal tyrosine kinase component is structurally related to the platelet-derived growth factor receptor α [40]. PDGFRα overexpression has been described in carcinomas of colon, breast, lung, ovary and pancreas. Expression of activated PDGFRα in stromal cells of colon carcinomas has been associated with metastatic potential [23]. EGFR is expressed in most human tissues and plays an important role in normal cell biology. In the kidney, the human epidermal growth factor family members play a significant role in the mesenchymal to epithelial transition during renal tubulogenesis [41]. EGFR expression is found in a number of tumours, particularly squamous cell carcinomas, sarcomas, gliomas, breast, bladder and lung tumours. In nephroblastoma, EGFR expression has been described in a subset of tumours in one small series [14].

Selective inhibition of receptor tyrosine kinases has evolved as a powerful, targeted therapeutic tool in a number of tumours, and the spectrum of small molecule receptor tyrosine kinase inhibitors is rapidly expanding [26]. Imatinib/Gleevec® and Gefitinib/Iressa® are now well established as therapeutic inhibitors of KIT and EGFR, respectively. In particular, gastrointestinal stromal tumours and leukemias have been shown to be amenable to receptor tyrosine kinase inhibitor therapy [16, 17, 21, 34]. *KIT* iuxtamembranous exon 9, 11 and 13 mutations correspond with susceptibility to tyrosine kinase inhibition and improved survival in gastrointestinal stromal tumours and myeloic leukemia [8, 9, 13, 36, 38]. Up to 90% of gastrointestinal stromal tumours are *KIT*-mutated, and exon 11 mutations are most common [20, 25]. Approximately 7–12% of gastrointestinal stromal tumours without *KIT* mutations show *PDGFRα* mutations instead and are susceptible, though to a lesser degree, to imatinib treatment [7, 20, 38]. Interestingly, *KIT* and *PDGFRα* are located in the same chromosomal region 4q12 [35]. *PDGFRα* mutations are most often located in exon 18, and rarely in exons 12 and 14. In clinical phase 3 studies, a new generation of KIT/PDGFR receptor tyrosine kinase inhibitors for resistant tumours, e.g. sunitinib/Sutent®, are currently being tested [6]. Furthermore, activating *EGFR* gene mutations of exons 18, 19, 20 or 21 in non-small-cell lung carcinoma result in inhibition of apoptosis, in tumour proliferation, angiogenesis, invasion and metastasis [29, 33]. *EGFR* gene mutations confer susceptibility to gefitinib/Iressa® EGFR receptor tyrosine kinase inhibition [19].

In nephroblastomas, immunohistochemical KIT and EGFR expression has only been investigated in a small number of tumours [14], and neither *KIT*, *PDGFRα* nor *EGFR* mutations have been reported in these tumours so far. In a separate small set of 12 KIT-positive nephroblastomas, we detected mutations of *KIT* exon 11 in two tumours (Bruder et al., manuscript submitted). Moreover, no information is available so far with regard to tyrosine kinase expression and outcome. To provide a rationale for potential targeted small molecule receptor tyrosine kinase inhibition in nephroblastomas, we screened 209 nephroblastomas for immunohistochemical KIT and EGFR expression. Tumours with KIT expression were analysed for mutations of *KIT* exons 9, 11, 13. Similarly, tumours with EGFR expression were submitted to sequence analysis of *EGFR* exons 18, 19, 20 and 21. All 209 tumours were analysed for *PDGFRα* exons 12, 14 and 18 mutations.

Materials and methods

Nephroblastomas

From the archives of the Kiel Paediatric Tumour Registry, 209 nephroblastomas were compiled (1993–2002). Patient data were available on gender, age and stage at diagnosis,

tumour subtype, therapy mode and patient outcome. Male to female ratio was 1:1.27. Median patient age was 4.3 years. Preoperative chemotherapy had been administered to 181 patients according to the current International Paediatric Oncology Society (SIOP) protocol, and 28 patients underwent primary tumour resection. All nephroblastomas were classified according to the current SIOP classification [39] (Fig. 1). This current nephroblastoma classification accounts for the differential prognostic impact of blastema before and after chemotherapy and newly categorises residual blastema after chemotherapy in tumours with more than one third viable tumour tissue and more than two thirds of the viable tumour tissue of blastemal type (i.e. blastema predominant) as high-risk histology [39]. Tumours were reviewed by IL, DH, EB and SCW as well as by the SIOP nephroblastoma panel.

For tumour diagnosis, at least one block per centimeter tumour diameter was embedded. A mean of five blocks were available for tumour classification at the Kiel Paediatric Tumour Registry.

A paraffin tumour tissue microarray was constructed for efficient expression screening as previously described [24] (Fig. 2). For adult tumours, like urothelial carcinoma, one single punch cylinder per tumour was previously shown to be representative of each tumour [28]. For the present study of nephroblastoma, the paraffin tissue microarray construction technique was modified and specifically adapted to account for nephroblastoma as a heterogeneous embryonal tumour. Therefore, each histological tumour component of all 209 nephroblastomas was sampled, the respective tumour area circled on an H&E slide, and the corresponding paraffin block area marked before punching, resulting in five to six punch cylinders (mean 5.4, median 5) for each tumour on a total of three recipient blocks with a total of 1,178 punch cylinders. Normal renal parenchyma was included whenever present.

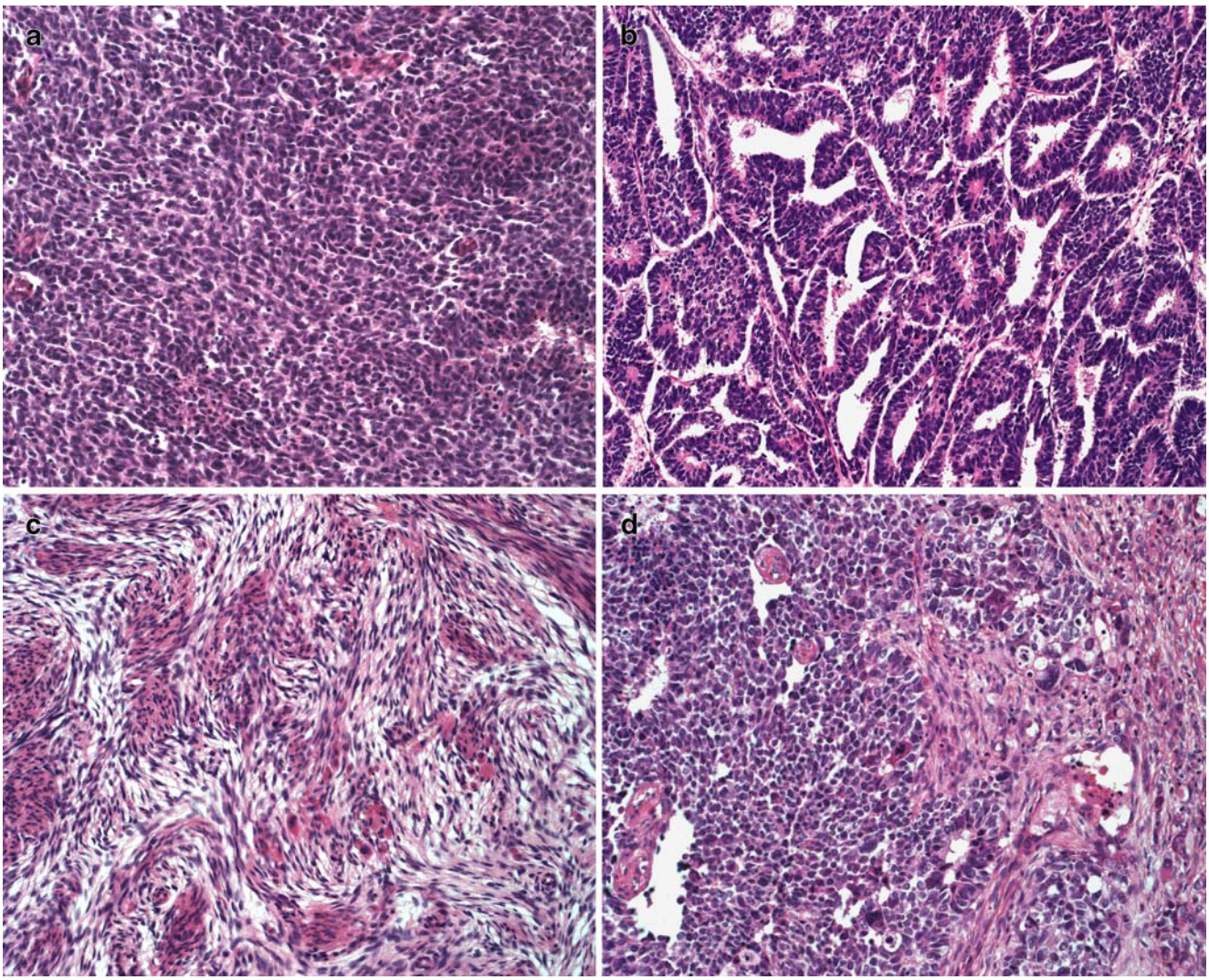

Fig. 1 Conventional histology: **a** Blastemal nephroblastoma (H&E×200). **b** Epithelial nephroblastoma (H&E×200). **c** Stromal nephroblastoma (H&E×200). **d** Diffuse anaplastic nephroblastoma (H&E×200)

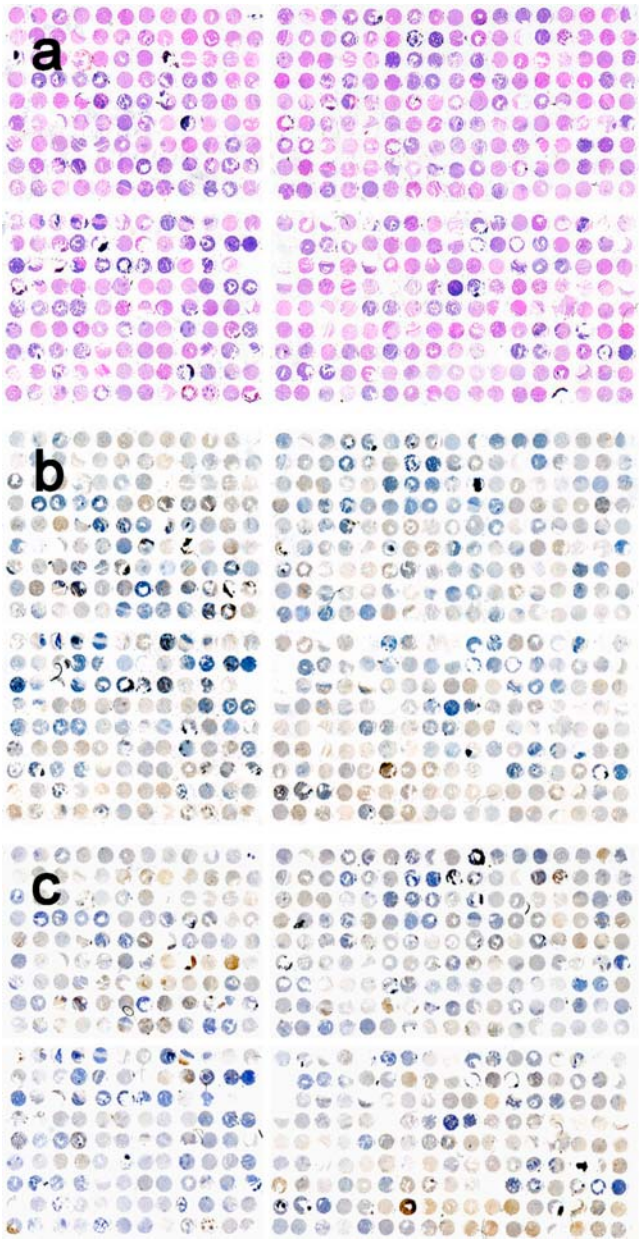

Fig. 2 Nephroblastoma array: **a**–**c** Overview of one paraffin block of the constructed nephroblastoma array (**a** H&E, **b** CD117/ KIT immunohistochemistry, **c** EGFR immunohistochemistry slide scans)

Criteria for tumour components for inclusion in the paraffin tissue microarray comprised all known histological nephroblastoma components, i.e. blastemal, epithelial, stromal and anaplastic tumour areas [11]. To carefully account for tumour heterogeneity and regional tumour variability, each histological tumour component was diligently selected for punching and was represented on the paraffin tissue microarray. Tumour punch samples originated from one or multiple blocks (median 3 blocks, mean 2.7 blocks). Punch core needle diameter was 0.6 mm as described previously [24, 28].

Immunohistochemistry

Paraffin tissue microarray sections were cut at 4 μm according to a protocol standardised in our laboratory. Immunohistochemistry for KIT receptor CD117 protein was performed on a Bond™ Immunostainer (Bond Max, Vision BioSystems; Figs. 3 and 4). For KIT/ CD117 immunohistochemistry, sections were pretreated in the manufacturer's ' Epitope Retrieval buffer 2' (Tris–EDTA buffer at pH 8.8) for 20 min at 100°C, incubated with polyclonal antibody Anti CD117 primary antibody (DAKO, 1:1000), followed by Bond polymer refine detection kit according to the manufacturer's protocol. A gastrointestinal tumour served as positive control. As it is known that KIT immunohistochemistry is prone to false positive staining results [20], the immunohistochemical reaction for KIT was carefully tested and set up in our laboratory with particular attention to negative controls so as to avoid false positive immunohistochemical staining results. For EGFR, immunohistochemistry was performed according to the EGFR pharmDx™, Code K1492 (Dako Cytomation) kit protocol. A cytospin of an EGFR-positive cell line contained in the kit served as positive control. A test array slide, containing a combination of normal and neoplastic human tissue cores, was included as negative control for each KIT and EGFR immunohistochemical reaction. Immunohistochemistry for PDGFRα was not performed because in our experience, PDGFRα immunohistochemistry is currently not reliable. Instead, all tumours were submitted to PDGFRα sequence analysis (see below).

Immunohistochemical staining for KIT/CD117 and EGFR was evaluated for each individual array punch tissue sample and recorded according to tumour tissue component and intensity of staining. Staining intensity was graded from + to +++, with + denoting faint cytoplasmatic staining, ++ intermediate cytoplasmatic staining and +++ exclusively only awarded to those tumours with distinct membranous in addition to cytoplasmic staining (Table 1). Immunohistochemical scoring was performed by SCW and EB independent of further tumour and patient information. Tumours were classified as KIT- or EGFR-positive, respectively, only in the presence of membranous KIT or EGFR expression and selected for subsequent DNA sequence analysis. Immunohistochemical scoring was amended to include the percentage of KIT- or EGFR-positive cells. In Table 1, the percentage of immunohistochemically positive tumour cells is provided.

KIT, PDGFRα and EGFR sequence analysis

For *KIT*, *PDGFRα* and *EGFR* sequence analysis, those tumours with strong immunohistochemical KIT and/or EGFR expression were selected. As in gastrointestinal

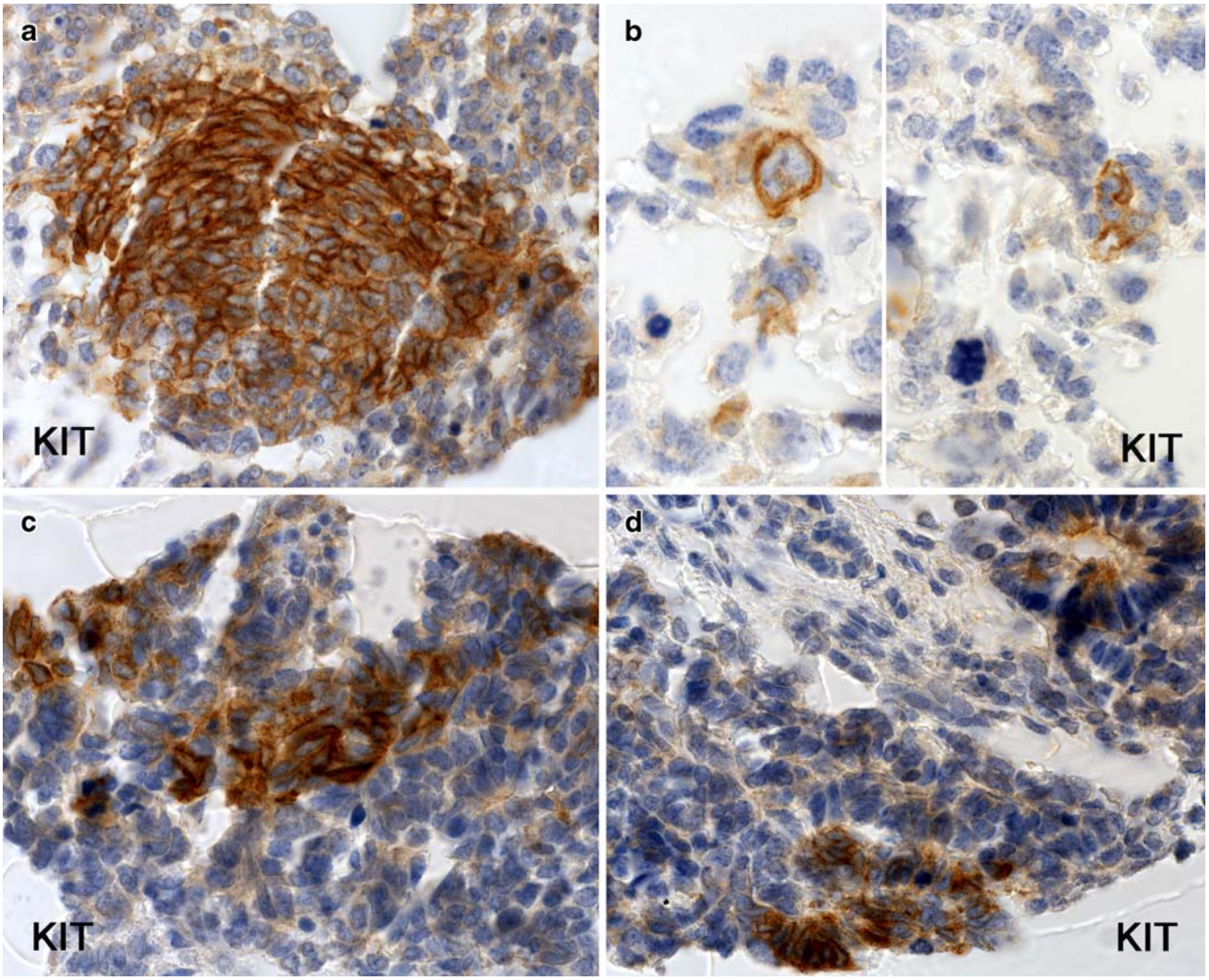

Fig. 3 KIT immunohistochemistry: **a** Membranous KIT expression in a blastemal nephroblastoma (KIT immunohistochemistry×630). **b** Focal membranous KIT expression in a nephroblastoma with diffuse anaplasia (KIT immunohistochemistry×630). **c**, **d** Focal membranous KIT expression in a nephroblastoma of mixed subtype (KIT immunohistochemistry×630). **c** Represents a blastemal component of this tumour, whereas **d** depicts a focus of KIT expression in immature epithelium in addition to a group of blastemal cells (KIT immunohistochemistry×630)

stromal tumours the presence of either *KIT* or *PDGFRα* mutations is regarded as predictive of tumour response to receptor tyrosine kinase inhibition [2], we proceeded to submit all KIT expressing nephroblastomas not only to *KIT* but also to *PDGFRα* sequence analysis. *KIT* and *PDGFRα* sequence analysis was performed on 22 of 209 tumours with strong +++ membranous and cytoplasmic CD117 expression. *EGFR* sequencing was performed on 31 of 209 tumours with strong +++ membranous and cytoplasmic EGFR expression. Ten nephroblastomas revealed both EGFR and KIT expression and were submitted to *KIT*, *PDGFRα* as well as *EGFR* sequencing analysis. Consequently, *KIT* and *EGFR* DNA sequence analysis was performed on a total of 63 nephroblastomas. In addition, in the absence of an immunohistochemical PDGFRα screening test, all 177 CD117 negative nephroblastomas were submitted to *PDGFRα* sequence analysis, resulting in *PDGFRα* sequence analysis of all 209 nephroblastomas included in this study.

For DNA extraction, one to three punch cylinders 0.6 mm in diameter were removed from identical matched tumour regions of the original paraffin tissue blocks of each selected nephroblastoma. DNA was extracted according to the SIOP 1 and the QIAamp® DNA mini kit DNA extraction protocols (Qiagen AG, Basel, Switzerland) [37]. The extracted DNA was submitted to a semi-nested or nested multiplex polymerase chain reaction (PCR) for *KIT* exons 9, 11, 13, *PDGFRα* exons 12, 14 and 18 and *EGFR* exons

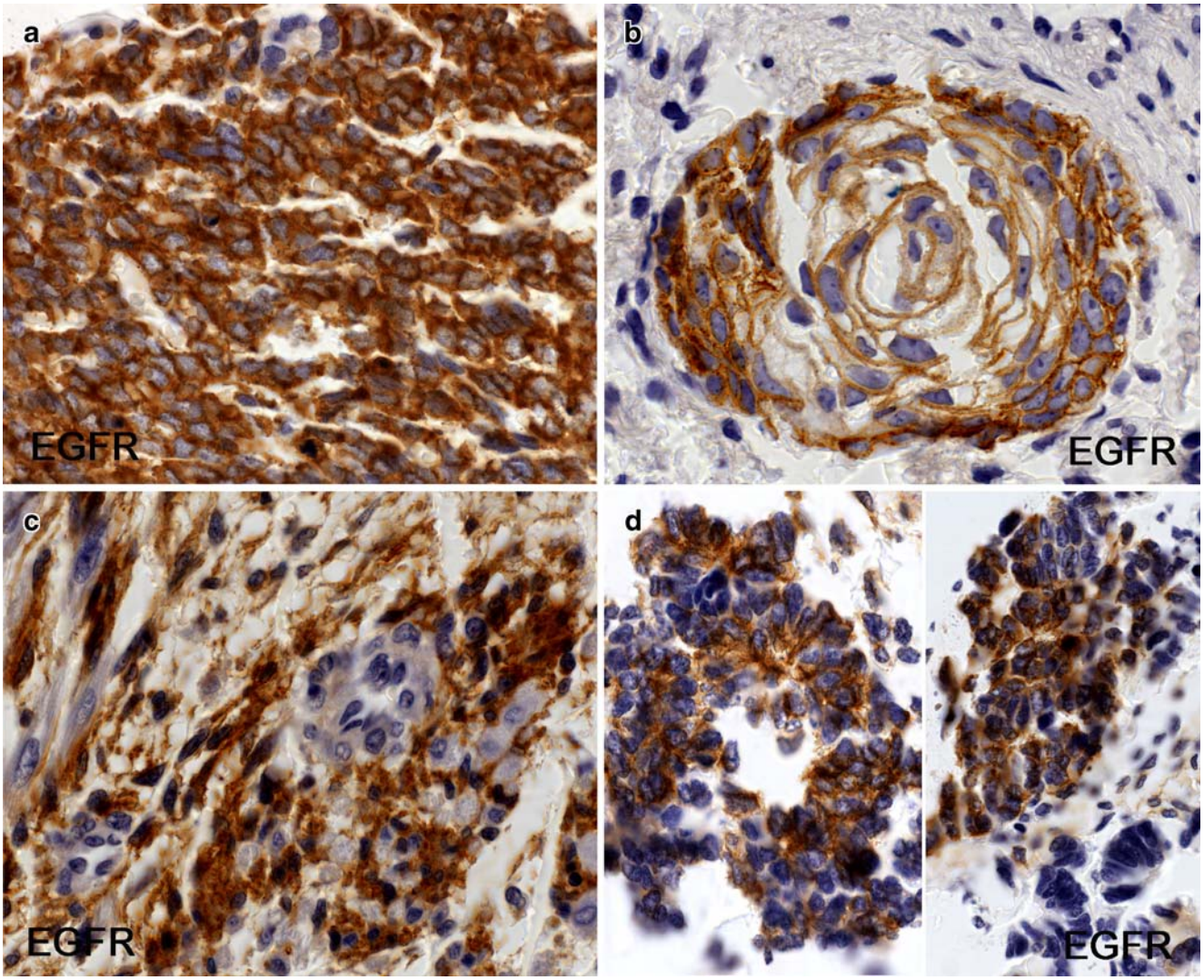

Fig. 4 EGFR immunohistochemistry: **a** Membranous EGFR expression in a blastemal predominant nephroblastoma. At the *top border* of the figure is an EGFR-negative tubule (EGFR immunohistochemistry×630). **b** Membranous EGFR expression in a focus of squamous epithelium. Surrounding stroma is negative for EGFR (EGFR immunohistochemistry×630). **c** EGFR expression in a stromal predominant nephroblastoma. Foci of rhabdomyoblastic differentiation are negative for EGFR (EGFR immunohistochemistry×630). **d** EGFR expression in blastemal foci and immature epithelium in a mixed type nephroblastoma (EGFR immunohistochemistry×630)

18, 19, 20 and 21. DNA sequencing was performed on the PCR products on an ABI PRISM 310xl Genetic Analyzer (Applied BioSystems). Sequencing primers were identical to initial PCR primers. Primer sequences are given in Table 2.

Statistical analysis

For statistical analysis, associations between follow-up data, histological nephroblastoma subtype and immunohistochemical expression of KIT and EGFR were analysed, respectively. The association between survival, recurrence and metastasis with KIT and EGFR expression was evaluated using the chi-square test or Fisher's exact test. P values<0.05 were considered statistically significant. All analyses were carried out using SAS (version 9.0, The SAS Institute, NC, USA).

Results

In this study, we analyzed 209 nephroblastomas for immunohistochemical KIT and EGFR expression, a total of 63 tumours for mutations in *KIT* exons 9, 11, 13, *EGFR* exons 18, 19, 20 and 21, as well as *PDGFRα* exons 12, 14 and 18 in the total series of 209 nephroblastomas. Tumour localisation was equally distributed between both kidneys, with 108 tumours localised in the right and 101 in the left kidney.

Table 1 Clinicopathologic characteristics and immunohistochemistry (*N*=209): risk group, nephroblastoma subtype, membranous KIT and EGFR expression, preoperative chemotherapy, adverse events, follow-up interval

Risk group	Histology	KIT expression	EGFR expression	KIT and EGFR expression	Local recurrence	Metastasis	Death of disease
Low 2 (1%)	CPDN 2 (100%)	0	0	0	0	0	0
Intermediate 179 (86%)	Blastemal (pretreatment) 9 (5%)	2	0	1	0	1	0
	Epithelial 6 (3.4%)	0	0	0	1	1	0
	Stromal 29 (16.2%)	7	7	2	1	2	0
	Mixed 91 (50.8%)	12	23	4	6	10	2
	Regressive 42 (23.5%)	4	7	2	3	10	3
	Focal anaplasia 2 (1.1%)	0	0	0	0	0	0
High 27 (12.5%)							
	Blastemal (post-treatment) 21 (77.8%)	4	2	1	4	2	3
	Diffuse anaplasia 6 (22.2%)	1	1	0	1	1	1
Unclassified (0.5%)	Unclassified 1 (0.5%)	1	0	0	0	0	0
Total 209 (100%)		22 (10.6%)	31 (14.8%)	10 (4.8%)	16 (7.6%)	27 (12.9%)	9 (4.3%)

CPDN cystic partially differentiated nephroblastoma

The 209 tumours were classified according to the SIOP classification (Table 1, Fig. 1): two low-risk tumours (1%; CPDN: cystic partially differentiated nephroblastoma), 179 intermediate-risk tumours (86%), 27 high-risk tumours (12.5%) and one non-classifiable tumour (0.5%). Tumour group details are provided in Table 1. The 179 nephroblastomas of intermediate risk were: 91 mixed (52%), 42 regressive (23%), 29 stromal (16%), nine pre-chemotherapy blastemal (5%), six epithelial (3%) and two with focal anaplasia (1%). The 27 nephroblastomas of high-risk histology were: 21 of 27 (78%) post-chemotherapy blastemal and 6 of 27 diffuse anaplastic nephroblastomas (22.3%). The nephroblastomas classified as post-chemotherapy blastemal showed more than one third viable tissue of which more than two thirds of the viable tumour tissue was of blastemal type (i.e. blastema predominant).

Follow-up between 7 and 132 months (mean 66 months) was available for all 209 patients. Of 209 patients, nine (4%) died of tumour: five intermediate-risk tumours (56%; three regressive and two mixed), four high-risk tumours (44%; three post-chemotherapy blastemal, one diffuse anaplastic nephroblastoma; Fig. 1). Of the 27 patients with a high-risk histology tumour, four died (14.8%). Thus, our study confirms the association of high-risk nephroblastomas with poor outcome. Of the nine patients deceased, four had metastases, predominantly pulmonary: two patients with intermediate-risk tumours (regressive) and two high-risk tumours (one post-chemotherapy blastemal, one diffuse anaplastic nephroblastoma). A total of 27 (13%) tumours metastasised: 24 intermediate-risk tumours [89%; ten mixed (37%), two stromal (7%), ten regressive (37%; two patients died], one epithelial (4%), one pre-chemotherapy blastemal nephroblastoma (4%) and three high-risk tumours (11%; two post-chemotherapy blastemal (7%; one patient died), one with diffuse anaplasia (4%; this patient died).

Immunohistochemistry for KIT and EGFR

Tumours with membranous immunohistochemical KIT or EGFR expression were graded +++ and classified as positive. Immunohistochemical results are provided in Table 1. In most tumours, KIT and EGFR expression was focal and observed in blastemal and immature epithelial tumour components (Figs. 3 and 4). For more precise analysis, the percentage of KIT- and EGFR-expressing tumour cells per punch was estimated and is indicated in Table 1. Assuming that each KIT- or EGFR-expressing tumour area might be of potential biological relevance, all tumours with membranous immunohistochemical KIT or EGFR expression were classified as positive irrespective of the percentage of positive tumour cells. Sixty-three (30.1%) nephroblastomas demonstrated expression of KIT, EGFR or both: 22 (10.6%) nephroblastomas were KIT-positive, 31 (14.8%) nephroblastomas were EGFR-positive, and 10 (4.8%) tumours expressed both KIT and EGFR. Consequently, a total of 32 of 209 nephroblastomas (15.3%) showed immunohistochemical KIT expression. Of these, 26 were intermediate-risk tumours (81.3%) and one was not classifiable (3.1%). KIT expression was observed in 12 (37.5%) mixed nephroblastomas, seven (21.9%) stromal, four (12.5%) regressive and three (9.4%) pre-chemotherapy blastemal nephroblastomas. Of the 27 high-risk histology

Table 2 Polymerase chain reaction primer sequences

Primer name	Primer direction	Primer sequence 5′→3′
KIT exon 9	Forward_1	TCC TAG AGT AAG CCA GGG CTT
	Forward_2 (nested)	AGC CAG GGC TTT TGT TTT CT
	Reverse	TGG TAG ACA GAG CCT AAA CAT CC
KIT exon 11	Forward	CCA GAG TGC TCT AAT GAC TG
	Reverse_1	AGC CCC TGT TTC ATA CTG AC
	Reverse_2 (nested)	ACT CAG CCT GTT TCT GGG AAA CTC
KIT exon 13	Forward_1	GCT TGA CAT CAG TTT GCC AG
	Forward_2 (nested)	TGA CAT CAG TTT GCC AGT TG
	Reverse	AAA GGC AGC TTG GAC ACG GCT TTA
PDGFRα exon 12	Forward_1	GCT CTG GTG CAC TGG GAC TTT GGT A
	Forward_2nested	TGG TGC ACT GGG ACT TTG GTA ATT CA
	Reverse_1	TTG TGT GCA AGG GAA AAG GGA GTC TT
	Reverse_2nested	GGG AAA AGG GAG TCT TGG GAG GTT AC
PDGFRα exon 14	Forward_1	CTTTCAACAGCCACGGCCAGATC
	Forward_2nested	GGGGATGGAGAGTGGAGGATTTAAGC
	Reverse_1	CAACAGCCACGGCCAGATCCAG
	Reverse_2nested	CAACCACATGTGTCCAGTGAAAATCCTC
PDGFRα exon 18	Forward_1	CAG CCA GTC TTG CAG GGG TGA TG
	Forward_2nested	GAT GGC TTG ATC CTG AGT CAT TTC TTC C
	Reverse_1	GGG AGG ATG AGC CTG TCC AGT GTG
	Reverse_2nested	GAG CCT GTC CAG TGT GGG AAG TGT G
EGFR exon 18	Forward	GCA TGG TGA GGG CTG AGG TGA
	Forward_nested	ACC CTT GTC TCT GTG TTC TTG TCC C
	Reverse	CCC CAC CAG ACC ATG AGA GGC
	Reverse_nested	GCC CAG CCC AGA GGC CTG TG
EGFR exon 19	Forward	TGC CAG TTA ACG TCT TCC TTC
	Forward_seminested	AAC GTC TTC CTT CTC TCT CTG
	Reverse	CCA CAC AGC AAA GCA GAA AC
EGFR exon 20	Forward	CCA CCA TGC GAA GCC ACA CTG A
	Forward_nested	CCA TGC GAA GCC ACA CTG ACG T
	Reverse	TCC TTA TCT CCC CTC CCC GTA TCT C
	Reverse_nested	CCC CTC CCC GTA TCT CCC TTC C
EGFR exon 21	Forward	AGC TTC TTC CCA TGA TGA TCT GTC C
	Forward_nested	TCC CAT GAT GAT CTG TCC CTC ACA
	Reverse	GGC AGC CTG GTC CCT GGT GTC
	Reverse_nested	CAG GAA AAT GCT GGC TGA CCT AAA G

nephroblastomas, six showed KIT expression (22.3%). Five of 21 post-chemotherapy blastemal nephroblastomas and one of six with diffuse anaplasia were KIT-positive. None of the six epithelial, focal anaplastic or cystic partially differentiated nephroblastomas showed KIT expression. EGFR expression was observed in 41 of 209 nephroblastomas (19.6%). Thirty-eight of them were intermediate-risk tumours (92.7%) and three high-risk tumours (7.3%). Similar to KIT, EGFR expression was observed predominantly in mixed nephroblastomas: 23 of 41 tumours (56.1%) were mixed, seven (17.1%) were stromal, seven (17.1%) regressive and one (2.4%) non-pretreated blastemal nephroblastomas. Only 3 of 27 high-risk nephroblastomas expressed EGFR, two (of 21) post-chemotherapy blastemal and one (of 6) nephroblastoma with diffuse anaplasia. None of the six epithelial, focal anaplastic or cystic partially differentiated nephroblastomas showed EGFR expression.

Immunohistochemical scoring was amended to include the percentage of KIT- or EGFR-positive cells in immunohistochemically positive tumour punches. In ESM Table 1, the percentage of immunohistochemically positive tumour cells per positive tumour punch is provided.

KIT exon 9, 11, 13, EGFR exon 18, 19, 20, 21, PDGFRα exon 12, 14 and 18 sequence analysis

Sequence analysis for *KIT* and *EGFR* was performed on a total of 63 tumours and for *PDGFRα* on all 209 tumours from 209 patients. A total of 957 exons were sequenced. Twenty-two nephroblastomas with KIT expression were

selected for *KIT* exon 9, 11, 13 and *PDGFRα* exon 12, 14 and 18 sequence analysis. All 22 tumours contained *KIT* exon 9, 11, 13 and *PDGFRα* exon 12, 14 and 18 wild-type sequences (Fig. 5). The 31 nephroblastomas with immunohistochemical staining for EGFR were selected for *EGFR* exon 18, 19, 20 and 21 sequence analysis. All 31 tumours contained *EGFR* exon 18, 19, 20 and 21 wild-type sequences (Fig. 5). The ten tumours with both KIT and EGFR expression were selected for *KIT* exon 9, 11, 13, *PDGFRα* exon 12, 14, 18 and for *EGFR* exon 18, 19, 20 and 21 sequence analysis. All ten tumours contained wild-type sequences of *KIT* exons 9, 11, 13, *PDGFRα* exons 12, 14, and 18 and for *EGFR* exons 18, 19, 20 and 21. In addition, all 177 immunohistochemically KIT-negative nephroblastomas were submitted to *PDGFRα* sequence analysis. Similar to the KIT immunohistochemically positive tumours, the immunohistochemically KIT-negative nephroblastomas contained *PDGFRα* exon 12, 14 and 18 wild-type sequences.

PDGFRα sequence analysis was performed in double independently in two separate laboratories at the Institute of Pathology at the University Hospital in Basel, Switzerland

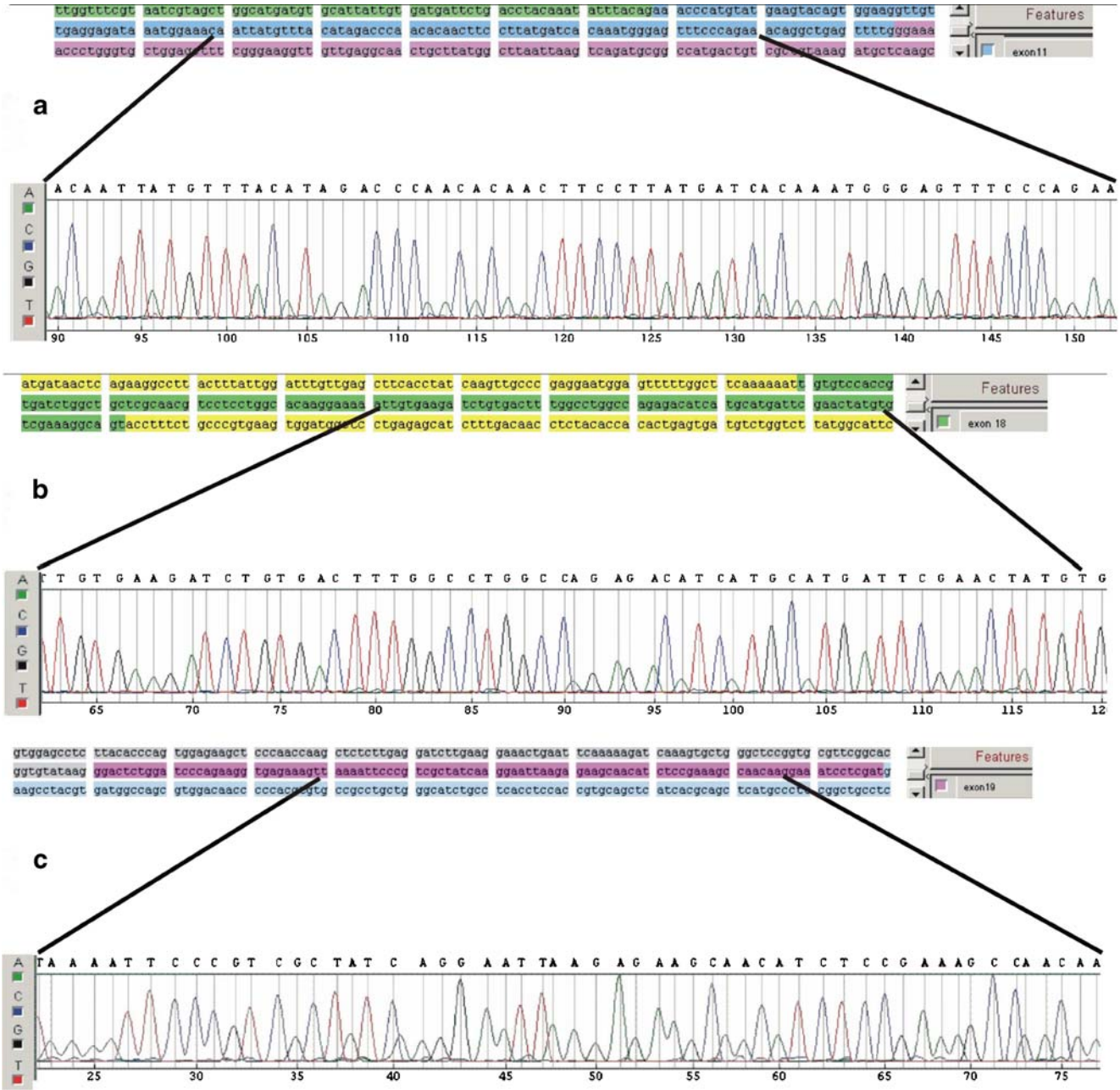

Fig. 5 KIT, PDGFRα and EGFR sequencing results: **a** KIT wild-type sequence (32 nephroblastomas). **b** PDGFRα wild-type sequence (209 nephroblastomas). **c** EGFR wild-type sequence (41 nephroblastomas)

and at the Institute for Clinical Chemistry and Laboratory Medicine at the University of Regensburg, Regensburg, Germany.

All sequencing reactions were repeated twice. Repeat mutation analyses yielded identical results.

Statistical analysis

Correlation of tyrosine kinase receptor expression and outcome

Metastases occurred in 30 patients (14.1%), tumour recurrences were observed in 17 patients (8%), and nine patients died (4.2%). Although we observed a trend for KIT expression in high-risk histology tumours with adverse events, statistically, KIT expression was not significantly associated with outcome, neither in the whole group of nephroblastomas (p=0.18) nor in the group of low- or intermediate-risk histology tumours nor in high-risk histology tumours (p=0.062). KIT expression was independent of immunohistochemical EGFR expression. Similar to KIT, EGFR expression did not correlate with adverse events for the total group of nephroblastomas analysed (p=0.224).

Comparison of treated and untreated nephroblastomas

Preoperative chemotherapy had been administered to 181 patients according to the current SIOP protocol, and 28 patients underwent primary tumour resection without preoperative chemotherapy (see Table 3). In the tumour group with preoperative chemotherapy, there were 27 high-risk histology nephroblastomas, 153 intermediate risk histology nephroblastomas and one unclassified nephroblastoma. In the tumour group without preoperative chemotherapy, there were two low-risk histology tumours and 26 intermediate-risk histology tumours, but no high-risk tumour. None of the high-risk histology tumours were treated with primary resection; all of these had received preoperative chemotherapy.

Comparing the immunohistochemical results of the total group of 209 tumours with those tumours treated preoperatively, there is no statistical difference between the total group and the group treated with preoperative chemotherapy.

Comparison of immunohistochemical expression among the preoperatively treated versus the preoperatively untreated group yields a tendency towards a higher percentage of KIT-expressing tumours in the preoperatively untreated group (p=0.069). Seven of 26 preoperatively untreated nephroblastomas of intermediate-risk histology showed KIT expression, corresponding to 27%, as opposed to 19 of 153 (12%) of intermediate-risk histology nephroblastomas with preoperative chemotherapy. As for EGFR expression, the trend is similar but to a lesser degree (p=0.443): Seven of 26 (27%) preoperatively untreated nephroblastomas of intermediate-risk histology showed EGFR expression versus 31 of 153 (21%) preoperatively treated intermediate-risk histology nephroblastomas.

However, the numbers of individual intermediate-risk histology subgroups without preoperative chemotherapy are small, and analysis of larger numbers is required to confirm such a trend. Similarly, the tumour spectrum within the preoperatively treated versus preoperatively untreated tumour group is different and is therefore likely to introduce a bias into the comparison of these two nephroblastoma groups.

As for outcome analysis among the preoperatively untreated tumours and comparison with the preoperatively treated tumours, a similar bias is likely.

None of the adverse events occurred in these 28 preoperatively untreated tumours except one surviving patient with metastases of a KIT- and EGFR-negative tumour of the intermediate-risk histology group. No patient in the preoperatively untreated group died. All other

Table 3 Comparison of immunohistochemical results in treated and untreated tumours

Chemotherapy	*N*	Histology risk group	*N*	KIT expression	EGFR expression
Yes	181				
		High	27	5 of 27 (19%)	3 of 27 (12%)
		Intermediate	153	19 of 153 (12%)	31 of 153 (21%)
		Unclassified	1	1	0
No	28				
		High	0	0	0
		Intermediate	26	7 of 26 (27%)	7 of 26 (27%)
		Low	2	0	0
Total yes and no	209				
		High	27	5 of 27 (19%)	3 of 27% (12%)
		Intermediate	179	26 of 179 (15%)	38 of 179 (21%)
		Low	2	0	0
		Unclassified	1	1	0

N=209

adverse events occurred in those patients with preoperative chemotherapy. All patients who died were in the group with preoperative chemotherapy.

Discussion

Enhanced understanding of genetic alterations in nephroblastoma and identification of key molecular markers is expected to provide the foundation for novel targeted therapies to address currently poor prognostic nephroblastoma subgroups. Transmembrane receptor tyrosine kinases play a crucial role in growth factor signalling cascades. The role of tyrosine kinases for tumour growth and angiogenesis in nephroblastomas has been described in previous studies [1, 12, 14, 41]. Constitutional protein kinase activation by somatic mutation is a frequent mechanism of tumourigenesis [33]. Targeted small molecule drugs have emerged as elegant and efficient anti-cancer strategies, and selective receptor tyrosine kinase inhibition evolves as focus of research for novel therapies. As receptor tyrosine kinases, KIT and EGFR play a significant role in renal organogenesis and expression has previously been described in a small series of nephroblastomas, we set out to determine the prevalence of KIT and EGFR expression in a large series of 209 nephroblastomas. So far, as known from the experience in gastrointestinal stromal tumours, leukemias and non-small-cell lung cancer, susceptibility to kinase inhibition appears to be linked to constitutionally activating gene mutations, which has held true for KIT as well as for EGFR. Neither KIT nor EGFR mutations have been described in nephroblastomas so far. In a separate small set of 12 KIT-positive nephroblastomas, we detected mutations of KIT exon 11 in two tumours (Bruder et al., manuscript submitted). Therefore, we also determined KIT and EGFR gene sequences of most commonly involved exons in 63 nephroblastomas and PDGFRα of all 209 nephroblastomas to investigate a rationale for potential targeted tyrosine kinase inhibitor therapy in nephroblastoma.

Among the 209 nephroblastomas investigated in this study, we found KIT and/or EGFR expression in 63 tumours. Interestingly, KIT expression was more frequent among tumours of high-risk histology (6/27; 22.3%) compared with those of low- and intermediate-risk (26/181; 14.4%). In contrast, EGFR expression was less frequent in high-risk nephroblastomas (3/27; 11%) than in intermediate- and low-risk tumours (38/181; 20%). Did KIT or EGFR expression confer a different prognosis in our set of nephroblastomas? If nephroblastomas with adverse events defined as local recurrence, metastasis or death from disease were regarded separately, adverse events occurred in 37 (17.7%) of the total series of 209 tumours and in five (12.2%) of 41 tumours with EGFR expression. Conversely, adverse events occurred in 32 (19%) of 168 tumours without EGFR expression. Furthermore, adverse events were recorded in three (9.4%) of 32 KIT-expressing tumours, as opposed to adverse events in 34 (19.2%) of 177 KIT-negative tumours. In contrast, in 200 surviving patients, KIT and EGFR were positive in 15% and 19.5%, respectively. It therefore appears that KIT or EGFR expression alone may be associated with slightly better tumour outcome if regarded independently from tumour histology. However, in nine patients who died of their tumour, two (22.3%) tumours showed strong KIT expression and another 2 (22.3%) showed strong EGFR expression, while KIT and EGFR were positive in slightly lower proportions in the 200 surviving patients, 15% and 19.5%, respectively. Moreover, if evaluated in the context of tumour histology, four of the nine patients who died of disease had tumours of high-risk histology, and among these four tumours, two strongly expressed KIT, and one was EGFR-positive ($p=0.371$). Although these findings suggest a tendency that KIT expression, and to a minor degree also EGFR expression, may potentially be associated with poor outcome in the context of unfavourable histology, we were unable to statistically prove such a correlation. This is also true for tumours of low- and intermediate-risk histology associated with adverse events in relation to KIT and EGFR expression: Of 182 tumours with low- and intermediate-risk histology, only one (3.8%) of 26 KIT-expressing tumours and four (10.5%) of 38 EGFR expressing tumours ($p=0.206$) were associated with adverse events, whereas, conversely, 31 of 156 (19.9%) KIT-negative and 28 of 144 (19.4%) EGFR-negative tumours were associated with adverse events. Even if these results might suggest a tendency that low- and intermediate-risk histology nephroblastomas with KIT and EGFR expression appear to be associated with a better prognosis, this correlation is statistically not significant. As numbers of subgroups are small, this issue requires to be addressed in a still larger study. Nevertheless, these results suggest that KIT and EGFR expression should be evaluated in the context of tumour histology and outcome. The potential correlation of KIT and EGFR expression with better prognosis in low-/intermediate-risk histology nephroblastomas in this series is an interesting and important observation.

Could a subset of nephroblastomas merit a trial of selective tyrosine receptor kinase inhibition and be susceptible to this therapeutic approach? As the presence of either KIT or PDGFRα mutations is predictive of tumour response to selective receptor tyrosine kinase inhibition with imatinib in gastrointestinal stromal tumours [2, 18, 20], though to a lesser degree for PDGFRα mutations and no KIT mutations were detected in the subset of nephroblastomas investigated in this study, we therefore proceeded to determine also *PDGFRα* sequences in those nephro-

blastomas with KIT expression, and in the absence of an immunohistochemical screening test for PDGFRα, also in those tumours without KIT expression. Moreover, we investigated EGFR-expressing nephroblastomas for EGFR sequence alterations. None of the 63 nephroblastomas investigated showed any sequence alteration in any receptor tyrosine kinase analysed. Our present study of mutation analysis of 63 and 209 nephroblastomas, respectively, suggests that neither KIT, EGFR nor PDGFRα mutations appear to play a significant role in tumour pathogenesis. KIT and EGFR expression therefore do not appear to result from constitutionally activating mutations in the investigated exons in these nephroblastomas. Why do these tumours express KIT and/or EGFR in the absence of respective gene mutations? Importantly, KIT and PDGFRα receptor tyrosine kinases may be activated by mechanisms other than gene mutation, such as gene fusion and amplification, autocrine and paracrine stimulation of the receptor by its ligand, loss of phosphatase activity, cross-activation by other kinases and epigenetic promoter activation via methylation status alteration. Therefore, absence of KIT or PDGFRα mutations does not exclude selective tyrosine kinase inhibition with imatinib as targeted therapeutic strategy in the KIT-expressing nephroblastomas [35]. Importantly, imatinib has been shown to also effectively inhibit wild-type KIT and PDGFR receptors [2]. Similarly, in a previous study correlating KIT immuno-phenotype with response to tyrosine kinase inhibitor, imatinib even showed a response in gastrointestinal stromal tumours with only weak immunohistochemical staining [4]. Of note, in non-small-cell lung cancer, EGFR receptor tyrosine kinase inhibition with erlotinib/Tarceva® has recently been found to be effective in non-small-cell lung cancer with wild-type EGFR [5]. Therefore, as numbers in this current study are small, further investigations are required to evaluate a potential benefit, and future clinical therapeutic studies might be considered for the limited poor prognostic subgroup of nephroblastomas with high-risk histology also in the absence of *KIT*, *PDGFRα* or *EGFR* mutations. Nevertheless, at present, the question remains open whether the small group of poor prognostic nephroblastomas will benefit from additional receptor tyrosine kinase inhibition.

Addressing the question of preoperative chemotherapy and evaluation of the patient outcome in relation to expression of KIT and EGFR, comparing with patients without preoperative chemotherapy, such a comparison is likely to be biased for the following reasons. According to the current SIOP protocol, most nephroblastoma patients are treated by preoperative chemotherapy [15]. Only a minority of very young patients are judged not to require chemotherapy because of a high likelihood of congenital mesoblastic nephroma. Therefore, the criteria of patient inclusion will render the preoperatively treated versus the preoperatively untreated nephroblastoma groups incongruent.

It has been argued that preoperative chemotherapy may unpredictably alter tumour protein expression and genetics; therefore, only analysis of untreated tumour tissue should be regarded as reliable. However, it is now being recognised that surviving blastemal tumour tissue after chemotherapy constitutes an indicator towards poor chemotherapy response and prognosis, and therefore, nephroblastomas of post-chemotherapy blastemal predominant histology are currently classified among the high-risk histology group accordingly, in contrast to those blastemal predominant nephroblastomas without preoperative chemotherapy. Analysis of a study population of nephroblastomas with available pre-chemotherapy pre-surgical biopsy as well as post-chemotherapy specimens of identical tumours should be undertaken in the future to further contribute to resolve this issue.

In summary, a minor proportion of nephroblastomas express KIT and EGFR. However, KIT, PDGFRα and EGFR mutations do not appear to significantly contribute to nephroblastoma pathogenesis. Hence, unlike in gastrointestinal stromal tumours, leukemia and non-small-cell lung cancer, there appears to be no comparable molecular basis for potential selective tyrosine kinase inhibition in nephroblastoma. In nephroblastomas, immunohistochemical KIT and EGFR expression does not appear to bear a direct association with outcome. Nevertheless, as numbers of high-risk histology nephroblastomas are small, further clinical evaluation studies might be considered to elucidate a potential therapeutic benefit for this poor prognostic subgroup of patients.

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Conflict of interest statement We declare that we have no conflict of interest.

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