Multifaceted Dysregulation of the Epidermal Growth Factor Receptor Pathway in Clear Cell Sarcoma of the Kidney

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Abstract

Purpose: Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase overexpressed in a variety of human malignancies, against which targeted therapies have shown efficacy in lung and brain tumors. Clinical responses to EGFR inhibitors have been found to be highly dependent on the presence of activating mutations, whereas gene amplification, downstream activation of Akt, and abnormalities in PTEN are also reported predictive factors. We sought to evaluate these variables in pediatric renal tumors.

Experimental Design: We screened a series of 307 pediatric renal tumors for EGFR expression by immunohistochemistry and gene amplification by chromogenic in situ hybridization. In identifying a striking predilection for certain tumor types, we further analyzed the clear cell sarcomas of the kidney (CCSK) for mutations in EGFR and PTEN.

Results: Although only 23 of 177 (13.0%) nonanaplastic Wilms’ tumors were EGFR positive, 4 of 11 (36.4%) anaplastic tumors showed receptor overexpression. In addition, 5 of 9 (55.6%) mesoblastic nephromas and 12 of 12 (100%) CCSKs were strongly immunoreactive for EGFR. In studying the CCSKs in more detail, we identified gene amplification in 1 of 12 (8.3%) cases and a somatic T790M EGFR mutation in a further case. These two samples additionally harbored mutations in PTEN. Downstream pathway activation, as assayed by phosphorylated Akt expression, was observed in 8 of 12 (66.7%) cases.

Conclusions: Together, these data show dysregulation of the EGFR pathway at multiple levels in CCSKs. Identification of factors predictive of poor response to targeted therapy, including the drug resistance T790M mutation, may provide a rationale for upfront trials with irreversible inhibitors of EGFR in children with these tumors.

The transmembrane receptor tyrosine kinase epidermal growth factor receptor (EGFR; HER1) is expressed in several human tumors, and increased levels of EGFR, its ligands, or both have been correlated with poor prognosis in several solid tumor types in adults (1). Ligand binding induces EGFR homodimerization and heterodimerization with other HER family members, activating the catalytic activity of the enzyme and leading to the autophosphorylation in one or more of the five tyrosine residues in the COOH-terminal tail, where adaptor and docking molecules bind. EGFR signaling ultimately increases proliferation, angiogenesis, and metastasis and decreases apoptosis (2).

Recently, small-molecule and antibody-based inhibitors against EGFR have shown efficacy in clinical trials of human cancer (3). Notably, the small-molecule EGFR tyrosine kinase inhibitors gefitinib (Iressa, AstraZeneca) and erlotinib (Tarceva, OSI Pharmaceuticals) have been shown to cause partial responses in 10% to 20% of all non–small cell lung cancer patients, with clinical responses to gefitinib and erlotinib found to be highly dependent on the presence of activating mutations (4, 5). Somatic EGFR mutations predictive for inhibitor response affect critical amino acids in the ATP-binding cleft of the tyrosine kinase domain of the receptor and include substitutions for G719 in the nucleotide binding loop of exon 18, in-frame deletions within exon 19, in-frame insertions within exon 20, and substitution for L858 or L861 in the activation loop in exon 21. Additional markers reported as predictive for anti-EGFR therapy in different tumor types include gene amplification (6) and activation of downstream signaling, as measured by expression of phosphorylated Akt (p-Akt; 7) or the negative pathway regulator PTEN (8).

In pediatric tumors, the expression of EGFR has not been comprehensively evaluated. Nonetheless, early-phase clinical trials are under way in refractory pediatric solid tumors based on small series describing expression in neuroblastoma, rhabdomyosarcoma, osteosarcoma, and glioma (9). The
presence of anti-EGFR predictive factors in these and other childhood malignancies are unknown. We sought to investigate the prevalence of EGFR overexpression in pediatric renal tumors, to determine whether therapeutic strategies targeting the receptor may be a worthwhile area of development. A recent expression profiling study highlighted the differential expression of EGFR in clear cell sarcoma of the kidney (CCSK) compared with Wilms’ tumor (nephroblastoma; ref. 10). We confirm this association with our receptor screening and further identified a multifaceted dysregulation of the EGFR/Akt pathway, including gene amplification and somatic mutation. These data provide valuable insights into the potential for novel targeted therapies in children with these aggressive tumors.

Materials and Methods

Tissue microarrays. Primary Wilms’ tumor samples were obtained after approval by local and multicenter Ethical Review Committees. Pediatric renal tumor tissue microarrays (TMA) were constructed containing replicate representative cores (n = 885) from all available cellular components from 274 Wilms’ tumors, 13 CCSKs, 10 mesoblastic nephromas, and 7 rhabdoid tumors of the kidney. Tumors were treated by either immediate nephrectomy or delayed surgery following preoperative chemotherapy. There was a slight enrichment of tumors that relapsed. The presence of tumor tissue on the arrayed samples was verified on a H&E-stained section. Tumor cell positivity and cellular distribution were assessed independently by three pathologists (M.R.-P., I.S.R.-F., and G.M.V.).

Immunohistochemistry. Immunohistochemistry was done on 4-μm formalin-fixed, paraffin-embedded sections both from the TMA and from the whole sections for the CCSK cases. EGFR overexpression was assessed using the mouse monoclonal antibody 31G7 (Zymed) at a dilution of 1:50 using the Envision-horseradish peroxidase system (K4006, DAKO) as described previously (11). EGFR immunostaining was analyzed according to the Herceptest (DAKO) scoring system; negative, no membrane staining or ≤10% of cells stained; 1+, incomplete membrane staining in >10% of cells; 2+, >10% of cells with weak to moderate complete membrane staining; and 3+, strong and complete membrane staining in >10% of cells. Cases with scores of 2+/3+ were considered positive for EGFR expression. p-Akt was analyzed using a rabbit monoclonal antibody raised against the protein phosphorylated at Ser473 (clone 736E11, Cell Signaling), at a dilution of 1:50, also using the Envision-horseradish peroxidase system (K4006) as described previously (7). antigen retrieval enabled by boiling in citrate buffer for 10 min. Assessment of tumor cell positivity was carried out compared with an isotype control experiment.

Chromogenic in situ hybridization. Chromogenic in situ hybridization was done using Spot-Light amplification probes for EGFR (Zymed), according to the manufacturer’s protocol and as described previously (11). As the interpretation guidelines for the Spot-Light EGFR amplification probe have been validated previously, we did not use an α-satellite probe for chromosome 7. Only unequivocal signals were counted. Signals were evaluated at ≥400, and ≥630 and at least 60 cells were counted for the presence of the EGFR probe. A given area was considered to be amplified for EGFR when >50% of the neoplastic cells harbored (a) more than five signals per nuclei or (b) large gene copy clusters.

Mutation analysis. Genomic DNA was isolated from 10-μm thick unstained tissue sections containing >85% tumor cells, as determined from a serial H&E-stained section, using the QIAamp DNA mini kit (Qiagen) according to the manufacturer’s instructions. Exons 18 to 21 of the EGFR gene, coding for the tyrosine kinase domain, and exons 1 to 9 of PTEN, encompassing the whole gene, were amplified by PCR (11, 12). Products were purified using the QIAquick PCR purification kit and subjected to bidirectional sequencing using BigDye Terminator Mix 3.1 (Applied Biosystems), according to the manufacturer’s instructions. Capillary sequencing was done on an ABI 3100 genetic analyzer (Applied Biosystems), and sequences were evaluated for the presence of mutations using Mutation Surveyor software (SoftGenetics LLC). All reported mutations were scored as reproducible, having been identified in at least two independent PCR amplifications.

Statistical analysis. All statistical tests were done in R2.3.4. Correlations between categorical values were done using the χ² and Fisher’s exact tests. Correlations between continuous and categorical variables were done using ANOVA, when continuous values showed a normal distribution. Cumulative survival probabilities were calculated using the Kaplan-Meier method, with differences between survival rates analyzed with the log-rank test. All tests were two tailed, with a confidence interval of 95%.

Results

Overexpression of EGFR in pediatric renal tumors. EGFR expression in our series of pediatric renal tumors was initially assessed by immunohistochemistry on a TMA. The vast majority of cases on these arrays were Wilms’ tumors, and these were found to be largely EGFR negative (Fig. 1A). In all, 26 of 188 (13.8%) assessable Wilms’ tumors exhibited receptor positivity in one or more cellular components (Fig. 1B). Intriguingly, anaplastic tumors had an increased frequency of receptor overexpression (4 of 11, 36.4%; Fig. 1C) compared with those without evidence of anaplasia (23 of 177, 13.0%), although the small numbers meant that this observation just failed to reach formal statistical significance (P = 0.055, Fisher’s exact test). EGFR expression was observed in equivalent frequencies in blastemal, epithelial, and stromal components (P = 0.798, χ² test). There was no association between EGFR receptor positivity and age at diagnosis, tumor stage, histologic subtype, and exposure to preoperative chemotherapy, nor to 4-year relapse-free or overall survival (data not shown).

When additional pediatric renal tumor entities were analyzed, we observed a striking predilection for certain histologies. All CCSK cases assessable on our TMA (9 of 9, 100%) were EGFR positive (Fig. 1D), a clear difference in comparison with Wilms’ tumor (P < 0.001, Fisher’s exact test). In addition, 5 of 9 (55.6%) of mesoblastic nephromas were also receptor positive (Fig. 1E), significantly greater than for Wilms’ tumor (P = 0.02, Fisher’s exact test). There was no EGFR expression observed in any of five rhabdoid tumors of the kidneys (Fig. 1F) nor in each of a single case of renal cell carcinoma or primitive neuroectodermal tumor.

CCSKs exhibit EGFR overexpression, gene amplification, and downstream pathway activation. Given the strong association of EGFR expression with CCSKs, we chose to study these cases in greater detail using whole sections available from 12 cases, including those not assessable on our TMA. Immunohistochemistry on these sections confirmed the TMA data, with 12 of 12 (100%) cases exhibiting receptor positivity (Fig. 2B and G; Table 1). In addition, we carried out chromogenic in situ hybridization for the EGFR gene to determine whether amplification was the mechanism behind the overexpression in CCSKs. We observed a clearly increased copy number consistent with gene amplification in 1 of 12 (8.3%) of our cases (Fig. 2C). The remaining cases displayed either normal
copy number or copy numbers suggestive of polysomy, but no definite amplification (Table 1). Chromogenic in situ hybridization for EGFR was also carried out on the TMA, where in assessable cores, the results for the CCSKs agreed with the whole section data. In addition, we observed no amplifications in any of the Wilms’ tumors or other pediatric renal tumors present on the TMA. We next used phosphorylation of Ser473 in tumor Akt, determined by immunohistochemistry, as a surrogate for downstream pathway activation via protein kinase B/Akt (7). We observed positive immunoreactivity in 8 of 12 (66.7%) of CCSKs (Table 1), with a strong staining in 4 cases (33.3%; Fig. 2D).

CCSKs harbor mutations in PTEN, as well as a somatic T790M in the kinase domain of EGFR. To determine whether the constitutive Akt activation was a result of PTEN mutation, sequencing for the entire gene was carried out in our CCSK cases. We identified 2 of 12 (16.7%) cases with novel mutations in the coding sequence of PTEN (Table 1). One mutation, a T383I in exon 9 (Fig. 2E), was found in cases RMH0471, which additionally harbored EGFR gene amplification and p-Akt expression. The second PTEN mutation consisted of a cytosine to thymine transition resulting in an amino acid change from threonine to isoleucine at residue 2 (Fig. 2I). It is unknown whether this mutation inactivates PTEN function, as it has not been reported previously, to our knowledge. The observation of negative p-Akt immunostaining, indicative of a lack of protein kinase B/Akt activation, suggests that this may not be the case and is perhaps tolerated due to its location immediately following the start codon.

Furthermore, we screened the sequence of the EGFR gene encoding for the tyrosine kinase domain for activating mutations, as have been reported most notably for non–small cell lung, in particular in relation to response to anti-EGFR therapy. We observed a single mutation, a T790M, in case RMH1226 (Fig. 2H), the sample with the T2I PTEN mutation, and negative p-Akt staining (Fig. 2I). The T790M mutation is strongly associated with drug resistance, acquired secondarily in response to treatment by EGFR inhibitors, although examples have been reported in untreated cases of non–small cell lung and esophageal cancer (13). Additionally, as germ-line T790M mutation has been linked with inherited susceptibility to lung cancer (14), we screened DNA extracted from adjacent normal kidney cells and determined that the T790M observed in our case of CCSK was somatic.

**Discussion**

The identification of EGFR overexpression in a significant proportion of anaplastic Wilms’ tumors and mesoblastic nephromas highlights the potential for targeted therapy in a histologically defined subset of children with renal malignancies. In particular, there was a striking association with CCSK, the second most common renal tumor in children following Wilms’ tumor, which has a significantly reduced survival (15), and for which the molecular genetics underlying the disease are largely unknown. We have observed an up-regulation of the EGFR pathway in all cases of CCSK examined, as suggested by a recent expression profiling study (10). This involved a combination of overexpression, gene amplification, p-Akt up-regulation, and mutations in both EGFR and PTEN.

Of particular interest is the discovery of a somatic T790M mutation in EGFR in an untreated case of CCSK. Despite the
initial success of compounds targeting the receptor in patients with activating EGFR mutations, resistance seems to emerge over time in the majority of patients. A single base change leading to a threonine to methionine (T790M) alteration in the ATP-binding pocket of the receptor was discovered as a likely mechanism of acquired resistance to both gefitinib and erlotinib in patients with secondary resistance to EGFR inhibitor therapy (16, 17). However, a recent report identified a T790M mutation in the germline of a family with inherited susceptibility to bronchoalveolar lung cancer (14), and previously untreated cases of non–small cell lung (18), as well as a case of Barrett’s esophagus and the corresponding adenocarcinoma (13), have also been shown to harbor the mutation. Thus, the T790M mutation may mediate altered functional properties of the receptor in addition to conferring drug resistance.

The case with the T790M was also observed to have a mutation in PTEN, a negative regulator of the EGFR/Akt/phosphatidylinositol 3-kinase pathway, suggesting a coordinated activation of signaling. This was also evident in a second case, which had a PTEN mutation in concert with EGFR amplification. The mechanism by which the EGFR is overexpressed in human cancer is frequently through gene amplification, with increased gene copy number itself implicated as a predictive factor for response to anti-EGFR therapies (6). Our identification of a single case of CCSK with EGFR amplification reveals that although this mechanism may be infrequent, it is applicable to at least a subset of such tumors. Few DNA copy number changes in CCSKs have been reported in the literature, with a single study identifying quantitative abnormalities identified in only 4 of 30 CCSKs by metaphase comparative genomic hybridization (19). Intriguingly, of the very few copy number changes reported in CCSK, loss of 10q was identified in a single case (19). Our finding of mutations in PTEN (located at 10q23) in 2 of 12 (16.7%) CCSKs is consistent with inactivation at this locus in a classic tumor suppressor gene manner.

<table>
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<th>Case no.</th>
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<th>Treatment</th>
<th>Age (mo)</th>
<th>Stage</th>
<th>EGFR IHC</th>
<th>EGFR CISH</th>
<th>EGFR mutation</th>
<th>PTEN mutation</th>
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<td>2</td>
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<td>+++</td>
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Abbreviations: IHC, immunohistochemistry; CISH, chromogenic in situ hybridization.
It is notable that although there is a clear association of EGFR activation and CCSK, several indicators predictive of poor response to targeted therapies are observed. As well as the T790M mutation, we also report an activation of signaling pathways downstream of EGFR, by means of p-Akt expression, in 8 of 12 (66.7%) of tumors. In glioblastoma, patients with high levels of p-Akt were found less likely to respond to erlotinib (7). In future clinical trials aimed at testing EGFR inhibitors against CCSK, consideration must be given to the use of irreversible inhibitors such as CI-387,785 (20), potentially in an upfront setting, to maximize efficacy. The lack of CCSK cell lines to robustly test these compounds in a preclinical setting, as well as to elucidate the biological implications of abrogated EGFR signaling, needs urgently to be addressed, however ought not to preclude early-phase clinical evaluation in children with refractory tumors.

Acknowledgments

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References

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