Establishment and Characterization of the First Pediatric Adrenocortical Carcinoma Xenograft Model Identifies Topotecan as a Potential Chemotherapeutic Agent

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Abstract

Purpose: Pediatric adrenocortical carcinoma (ACC) is a rare and highly aggressive malignancy. Conventional chemotherapeutic agents have shown limited utility and are largely ineffective in treating children with advanced ACC. The lack of cell lines and animal models of pediatric ACC has hampered the development of new therapies. Here we report the establishment of the first pediatric ACC xenograft model and the characterization of its sensitivity to selected chemotherapeutic agents.

Experimental Design: A tumor from an 11-year-old boy with previously untreated ACC was established as a subcutaneous xenograft in immunocompromised CB17 scid/c0/c0 mice. The patient harbored a germline TP53 G245C mutation, and the primary tumor showed loss of heterozygosity with retention of the mutated TP53 allele. Histopathology, DNA fingerprinting, gene expression profiling, and biochemical analyses of the xenograft were conducted and compared with the primary tumor and normal adrenal cortex. The second endpoint was to assess the preliminary antitumor activity of selected chemotherapeutic agents.

Results: The xenograft maintained the histopathologic and molecular features of the primary tumor. Screening the xenograft for drug responsiveness showed that cisplatin had a potent antitumor effect. However, etoposide, doxorubicin, and a panel of other common cancer drugs had little or no antitumor activity, with the exception of topotecan, which was found to significantly inhibit tumor growth. Consistent with these preclinical findings, topotecan as a single agent in a child with relapsed ACC resulted in disease stabilization.

Conclusion: Our study established a novel TP53-associated pediatric ACC xenograft and identified topotecan as a potentially effective agent for treating children with this disease.

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Introduction

Pediatric adrenocortical tumors (ACTs) are rare, with an annual incidence of 0.34 cases per million children younger than 15 years (1–3). ACT is more common among children who harbor germline TP53 mutations (e.g., Li-Fraumeni syndrome) or who have other tumor-prone constitutional syndromes (4). Children with ACT usually develop symptoms related to increased production of androgens, corticosteroids, aldosterone, and estrogens, and approximately 80% present with virilization.

Complete tumor resection is the mainstay of effective treatment for pediatric ACT. Patients with local residual or metastatic disease have a dismal prognosis with mortality rates, around 50%, an outcome that has not significantly improved in the last 30 years (5–7). Children with advanced adrenocortical carcinoma (ACC) are typically treated with mitotane plus etoposide, doxorubicin, and cisplatin (EDP). Remarkably, the inclusion of EDP was originally based on a clinical trial of adult gastric carcinoma (8) and then adapted to the treatment of adults with ACC (9–11). Two large prospective trials of EDP in adults with advanced ACC showed 49% and 23% overall response rates (10, 12). The contribution of each individual agent in the EDP regimen to the overall disease response is controversial (13, 14). Moreover, the acute and long-term complications of EDP are of concern for children with ACC. In particular, with the use of...
transient high. The xenograft model described here provides a unique opportunity to begin exploring these biological and clinical issues. A panel of commonly used chemotherapeutic agents was tested in this model. Cisplatin was showed to be cytotoxic, consistent with its use in frontline ACC therapy. Moreover, topotecan, which has not been previously tested in the context of adrenocortical tumors, was found to be cytostatic, efficiently blocking the growth of the xenograft tumor. Similar results were obtained in xenograft tumors derived from the adult ACC H295RW cell line. In support of these findings, a child with refractory ACC responded to topotecan as a single agent. Our data suggest that topotecan may be effective against ACC, warranting further clinical investigation.

Translational Relevance

This study reports the establishment and characterization of the first experimental model of childhood adrenocortical carcinoma (ACC). Standard chemotherapeutic regimens for treating this aggressive malignancy are usually not effective and disease-associated mortality remains high. The xenograft model described here provides a unique opportunity to begin exploring these biological and clinical issues. A panel of commonly used chemotherapeutic agents was tested in this model. Cisplatin was showed to be cytotoxic, consistent with its use in frontline ACC therapy. Moreover, topotecan, which has not been previously tested in the context of adrenocortical tumors, was found to be cytostatic, efficiently blocking the growth of the xenograft tumor. Similar results were obtained in xenograft tumors derived from the adult ACC H295RW cell line. In support of these findings, a child with refractory ACC responded to topotecan as a single agent. Our data suggest that topotecan may be effective against ACC, warranting further clinical investigation.

Immunohistochemical analysis

Immunohistochemical analysis was done on 4 μm sections of formalin-fixed, paraffin-embedded tumor tissue using Benchmark XT (Ventana Medical) and BondMax (Leica Microsystems) automated stainers with the reagents supplied by the manufacturers. The primary antibodies for CK8, p53, Ki-67, S-100, antihuman melanosome, inhibin, synaptophysin, and chromogranin A were used according to the recommendations of the suppliers. Appropriate positive and negative controls were included.

DNA fingerprinting

Genomic DNA was extracted from the primary and xenograft tumors, eluted in TE buffer (1 mmol/L Tris and 0.1 mmol/L EDTA, pH 8), and amplified for 16 genetic loci by the PowerPlex 16 System kit (Promega Corp.) following the manufacturer’s recommendations. PCR-amplified products were analyzed on a 3730 x/l DNA Analyzer (Applied Biosystems) and resolved according to size (100–300 bases), giving an overall profile of short tandem repeat sizes (alleles).

Mutational screening for TP53 gene

Mutational screening was done for the entire coding region (exons 2–11) and intron–exon boundaries of the TP53 gene by PCR and direct DNA sequencing. The primer sequences and program conditions for PCR analysis are available upon request.

RNA/protein extraction

Total RNA was extracted using the Qiagen RNeasy Midi kit (Qiagen). Agilent BioAnalyzer 2100 (Agilent) was used to assess the integrity of the total RNAs extracted from all of the samples. Tumor tissue lysates and whole-cell extracts were prepared using T-PER lysis buffer (Pierce Chemical) containing a complete protease-inhibitor cocktail (Roche Diagnostics Corporation). Homogenates were incubated for 1 hour at 4°C and centrifuged at 15,000 × g for 30 minutes at 4°C. The supernatant was collected and frozen at −80°C. Aliquots of supernatant were collected for protein quantification by the Bradford method (Bio-Rad protein assay, Bio-Rad Laboratories).

RNA expression profile

Affymetrix gene expression analyses were done by the St. Jude Children’s Research Hospital Hartwell Center for Bioinformatics and Biotechnology Core Facility using the Gene Chip U133v2 platform according to the manufacturer’s recommendations (Affymetrix). Results from the primary and xenograft tumors were compared with normal adrenal cortex and a cohort of previously characterized ACTs using hierarchical clustering analysis as previously described (18).

Protein analysis

Total protein (50 μg) was separated on 12% (w/v) polyacrylamide gel using the Novex NuPAGE system (Invitrogen), transferred to nitrocellulose membranes (Whatman GmbH), and blocked with 5% nonfat milk in Tris-buffered.

Materials and Methods

Patients

Institutional Review Board approval and informed consents for establishing the xenograft were obtained. The conditions were in compliance with NIH Policies and Guidance for human subjects.

Establishment of xenograft tumor model

A primary sample of fresh human pediatric ACC was transplanted subcutaneously onto the flanks of male CB17 scid−/− mice (6–8 weeks old; Taconic Farms) as previously described (17). The initial tissue transplant grew within 2 months and was maintained in vivo by subsequent serial passages into healthy mice. Xenograft tumor tissue was snap frozen in liquid nitrogen for molecular studies, and a fragment was fixed in 10% neutral buffered formalin for histologic studies.
saline (pH 7.4) containing 0.1% Tween 20 for 1 hour at room temperature. Membranes were probed with antibodies against human IGF-2 (1:500; Sigma-Aldrich Chemical), human TP53 (1:2,500; Oncogene), and β-actin (1:2000; Sigma-Aldrich Chemical). Corresponding horse-radish peroxidase-labeled anti-rabbit and anti-mouse antibodies were used as secondary antibodies (Cell Signaling Technology). The immune complexes were detected using Supersignal West Dura chemiluminescence reagent (Pierce) according to the manufacturer’s protocol.

Xenograft therapeutic assays

Animal studies were done in accordance with a protocol approved by the St. Jude Institutional Animal Care and Use Committee. Tumor-bearing mice were treated with doxorubicin (Bedford Labs), cyclophosphamide (Baxter), rapamycin (LC Laboratories), vincristine (Sicor), actinomycin D (Ovation Pharmaceuticals), topotecan (GlaxoSmithKline), etoposide (Bedford Labs), cisplatin (APP Pharmaceuticals), melphalan (Sigma-Aldrich), temozolomide (Selleck Chemicals), CPT-11 (Pharmacia & Upjohn), or 5-fluorouracil (Sigma-Aldrich), or a combination of the above (Supplemental Table S2). Mice received drug when tumors reached between 200 and 500 mm³ as previously described (17). Mice were randomized to groups of 10. Tumor volumes were measured for each tumor at the initiation of the study and weekly for up to 84 days after study initiation. Assuming tumors to be spherical, tumor volumes were calculated from the formula (π/6) × d³, where d represents the mean diameter. Tumor status (e.g., progressive disease, relative tumor volumes, and event-free survival) was determined as previously reported (19).

Results

Clinical features of the pediatric ACC patient

Adrenocortical tumor tissue was obtained from an 11-year-old boy with a right adrenal mass incidentally found during the work-up for abdominal trauma. Magnetic resonance imaging revealed a 9.7 × 8.6 cm right suprarenal mass that was resected and confirmed to be ACC. Clinical signs and symptoms of virilization or hypercorticism were not remarkable, although pubic hair (Tanner stage II) was noted. On admission, the patient was 43.1 kg (percentile 80) and 157.4 cm (percentile 96). Laboratory tests revealed levels of serum Cortisol (pre- and postsurgery, 9.2 and 6.8 μg/dL), Testosterone (presurgery, 194 ng/dL), dihydroepiandrosterone-sulfate (pre- and postsurgery, 84 and <3 μg/dL), dehydroepiandrosterone (pre- and postsurgery, 933 and 97 ng/dL), and androstenedione (pre- and post-surgery, 1,085 and 36 ng/dL). These data suggest that the tumor was functional although without remarkable signs and symptoms of virilization. The patient developed metastatic disease in the lungs and mediastinum 24 months after the initial surgical procedure and died 33 months after diagnosis. Family history of cancer revealed a paternal uncle with a brain tumor, an aunt with a breast tumor diagnosed at the age of 40 years, and a grandmother with lung carcinoma. On the maternal side, a grandmother had uterine carcinoma and a great-grandmother had a diagnosis of pancreatic carcinoma at the age of 86 years.

Establishment and characterization of the primary and xenograft tumors

Pediatric ACC tissue was obtained after surgery and transplanted subcutaneously into CB17 scid −/− mice. Histopathologic analysis of the primary ACC and the xenograft tumor, referred to as SJ-ACC3, was done. The right pediatric adrenocortical mass weighed 672 g, measured 16 cm in greatest dimension, and was grossly necrotic (80%). Microscopically, the viable tumor showed significant cellular pleomorphism, with predominantly large polygonal cells with eosinophilic cytoplasm and round to oval nuclei (Fig. 1A). Frequent mitoses (18/20 HPF), including atypical mitoses, were present. Foci of anaplastic and polyhucleated tumor cells were seen. The tumor focally invaded through the capsule to periadrenal fat; invasion of small capsular veins was also seen. Immunohistochemical analysis showed that the tumor cells were positive for inhibin A (Fig. 1B), keratin 8, and synaptophysin, and negative for chromogranin, HMB-45, and S-100 (data not shown). p53 protein showed strong nuclear staining in more than 90% of tumor cells (Fig. 1D). Ki-67 proliferative index was approximately 60%. Pathomorphology of the tumor xenograft at passage 1 was similar to that of the primary tumor (Fig. 1C), with similar almost universal p53 immunoreactivity.

To further characterize the SJ-ACC3 xenograft, a DNA fingerprint analysis using probes for the gender-specific amelogenin and 15 short tandem repeats was done. The
xenograft was found to share all 16 markers with the primary tumor, confirming the derivation of the SJ-ACC3 xenograft tumor (Supplementary Table S1). Sequence analysis of peripheral blood DNA from this patient revealed a germline TP53 mutation in exon 7, corresponding to the DNA binding domain (G245C). The primary ACC and SJ-ACC3 xenograft underwent loss of heterozygosity with loss of the wild-type allele (Supplementary Fig. S1). Single nucleotide polymorphisms and microsatellite markers at the TP53 locus confirmed the same TP53 haplotype in the xenograft as in the primary tumor (data not shown). This mutation has been previously reported in a Li–Fraumeni syndrome family (20).

The gene expression profile of the primary ACC and the SJ-ACC3 xenograft at passage 1 was determined by Affymetrix microarray analysis. No obvious distinction in their expression pattern was observed, suggesting that the xenograft retained the features of the primary tumor. The gene expression profiles of the xenograft and the primary tumor were also compared with a cohort of adrenocortical adenoma and carcinoma samples obtained from the International ACT Registry (http://www.stjude.org/ipactr), and these results were in agreement with the original diagnosis of the primary tumor as an ACC (18). The relative intensity of gene expression in the xenograft and primary tumor was converted to principal components (Fig. 2). Expression of mutant p53-G245C protein in the SJ-ACC3 xenograft was determined by Western blot analysis (Supplementary Fig. S2). High levels of p53 were detected in the xenograft compared with normal adrenal cortex, which is consistent with its strong nuclear staining in both the primary and xenograft tumors (Fig. 1). Furthermore, high molecular weight IGF-2 (8.5–24 kDa), presumably precursor forms, was also found to be strongly expressed in the SJ-ACC3 xenograft (Supplementary Fig. S2), as expected for pediatric ACTs. β-Actin expression was used as a control for protein loading.

**In vivo determination of differential drug sensitivity**

Standard chemotherapeutic agents were evaluated in CB17 scid−/− mice bearing subcutaneous SJ-ACC3 xenografts (Table 1) based on established dosing regimens (Supplementary Table S2). Regression of SJ-ACC3 tumors was observed with the alkylating agent cisplatin at 7 mg/kg on a schedule of Q21D×3 i.v., and complete remission was observed after 6 weeks (Fig. 3). The SJ-ACC3 xenograft model was also sensitive to topotecan administered at 0.6 mg/kg [(D × 5) × 3 i.p., and stable disease was observed throughout the treatment period. The response was considered cytostatic, as tumor progression resumed following cessation of treatment (Fig. 3). CPT-11, administered at 1.25 mg/kg [(D × 5) × 3 i.p., was also tested and resulted in a substantial, but less durable response (Fig. 3). Vincristine, cyclophosphamide, and actinomycin D, as well as melphanal, temozolomide, etoposide, doxorubicin, and 5-fluorouracil were ineffective as single agents (Fig. 3 and data not shown). In parallel, an adult ACC xenograft model established from HAC15RW cells (derived from H295R; ref. 21) displayed similar positive responses to topotecan (Fig. 4).

**Response to topotecan in a relapsed ACC patient**

An adolescent girl presented with evidence of virilization and hypercortisolism at 15 years of age. She was found to have a left adrenal mass with metastatic deposits in the liver and lung. She underwent left adrenalectomy with removal of the liver metastases and was subsequently treated with EDP plus mitotane. At the end of eight cycles of therapy, there was no definitive evidence of residual disease, and therapy was discontinued. Approximately 3 months later, an enlarged epicardial lymph node was detected, consistent with recurrent, metastatic disease. After two courses of cyclophosphamide and topotecan following the Children’s Oncology Group regimen (22), the node significantly increased in size. Subsequent treatment with sorafenib also
had little effect, and palliative care was considered. However, based on the topotecan schedule used in the preclinical xenograft model, intravenous topotecan targeted to an area under the curve of 100 ± 20 ng h/mL for 5 days in 2 consecutive weeks (23) was administered every 4 weeks. This single-agent regimen produced disease stabilization for the 4 months that it was administered. Because of severe myelosuppression and sepsis requiring intensive care unit support, treatment was discontinued. The patient survived an additional 8 months before succumbing to ACC progression.

Discussion

Here we report the establishment and characterization of the first pediatric adrenocortical tumor xenograft model and evaluation of its response to selected chemotherapeutic drugs.

The pediatric SJ-ACC3 xenograft closely recapitulates the morphologic and biological characteristics of the primary tumor based on histopathology, DNA fingerprinting, gene expression profiling, and Western blot analyses. The tumor was derived from a child who carried a germline TP53 G245C mutation, which expresses a structurally altered, inactive tumor suppressor protein (24–26). Loss of heterozygosity with selection against the wild-type TP53 allele is common in pediatric ACC (4, 27) and was observed in both the primary and xenograft tumors. Affymetrix analysis also revealed similar patterns of gene expression between the primary and xenograft tumors, including the overexpression of IGF-2, and these profiles are consistent with ACC (Fig. 2 and Supplementary Fig. S2; ref. 18). Elevated levels of IGF-2 are usually caused by genetic or epigenetic alterations at chromosome 11p15 and occur in approximately 90% of pediatric ACT (28). Overexpression of IGF-2 presumably drives ACC proliferation and survival, and therefore this signaling pathway may be a rational target for developing new drug therapies. The xenograft tumor could serve as a preclinical model for testing these agents.

Treatment of ACC relies on a variety of chemotherapeutic agents with diverse mechanisms, including 5-fluorouracil, etoposide, cisplatin, carboplatin, cyclophosphamide, doxorubicin, and streptozocin (29). The most common combination used in both childhood and adult ACC consists of cisplatin and etoposide with or without doxorubicin and mitotane (10, 30, 31). However, the efficacy of doxorubicin and etoposide has been questioned. Previous studies have shown similar outcomes in patients treated with cisplatin alone and those treated with EDP (32). Consistent with these findings, the pediatric SJ-ACC3 xenograft robustly responded to cisplatin but not to etoposide or doxorubicin. Notably, both the pediatric and adult ACC xenografts responded well to topotecan given on the daily × 5 × 2 regimen. In support of these preclinical findings, topotecan administration on this protracted schedule to a patient with recurrent ACC induced a significant delay in tumor growth. To our knowledge, there are no published studies using topotecan in the context of adult or childhood ACC. Our findings implicate topotecan as a potentially new chemotherapeutic agent for treating this tumor type and show that the dosing schedule may be important.

It is clear that current protocols are not effective for treating pediatric ACC. Our xenograft model provides the

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**Table 1. Determination of differential drug sensitivity in the SJ-ACC3 xenograft model**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>KM estimate of median time to event&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P-value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>EFS T/C&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Median RTV at end of study&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Tumor volume T/C&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Median group response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vincristine</td>
<td>11.9</td>
<td>0.038</td>
<td>1.3</td>
<td>&gt;4</td>
<td>0.69</td>
<td>PD1</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>14.0</td>
<td>0.004</td>
<td>1.6</td>
<td>&gt;4</td>
<td>0.71</td>
<td>PD2</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>12.3</td>
<td>0.038</td>
<td>1.4</td>
<td>&gt;4</td>
<td>0.78</td>
<td>PD1</td>
</tr>
<tr>
<td>Topotecan</td>
<td>&gt;EP&lt;sup&gt;f&lt;/sup&gt;</td>
<td>&lt;0.001</td>
<td>&gt;9.7</td>
<td>2.6</td>
<td>0.28</td>
<td>PD2</td>
</tr>
<tr>
<td>Melphalan</td>
<td>15.0</td>
<td>0.022</td>
<td>1.2</td>
<td>&gt;4</td>
<td>0.60</td>
<td>PD1</td>
</tr>
<tr>
<td>Temozolomide</td>
<td>20.6</td>
<td>&lt;0.001</td>
<td>1.6</td>
<td>&gt;4</td>
<td>0.60</td>
<td>PD2</td>
</tr>
<tr>
<td>CPT-11</td>
<td>42.4</td>
<td>&lt;0.001</td>
<td>3.4</td>
<td>&gt;4</td>
<td>0.72</td>
<td>PD2</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>20.0</td>
<td>&lt;0.001</td>
<td>1.8</td>
<td>&gt;4</td>
<td>0.75</td>
<td>PD2</td>
</tr>
<tr>
<td>Etoposide</td>
<td>11.4</td>
<td>0.335</td>
<td>1.2</td>
<td>&gt;4</td>
<td>0.68</td>
<td>PD1</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>10.9</td>
<td>0.534</td>
<td>1.2</td>
<td>&gt;4</td>
<td>0.77</td>
<td>PD1</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>&gt;EP&lt;sup&gt;f&lt;/sup&gt;</td>
<td>&lt;0.001</td>
<td>&gt;6.8</td>
<td>0.0</td>
<td>0.65</td>
<td>MCR</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>27.4</td>
<td>0.010</td>
<td>2.5</td>
<td>&gt;4</td>
<td>0.96</td>
<td>PD2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Kaplan–Meier estimate of median days to event determined using interpolated days to event.

<sup>b</sup>P-values comparing event-free survival distributions between treated and control groups.

<sup>c</sup>Event-free survival (EFS) T/C is the ratio of the median time to event between treated and control groups.

<sup>d</sup>Median relative tumor volume (RTV) is the ratio of the tumor volume at the end of treatment to the volume at initiation of treatment.

<sup>e</sup>Tumor volume T/C is the ratio of mean tumor volume of treated tumors divided by mean tumor volume of control tumors.

<sup>f</sup>“>EP” indicates that the median EFS for the treated group is greater than the evaluation period (EP).
first opportunity to explore new therapies and has identified topotecan as a possible new drug for treating this tumor type. Future studies will be directed toward: (1) establishing additional pediatric ACC xenografts; (2) testing new drug treatments individually and in combination; and (3) exploiting the xenograft models to challenge the functional relevance of the biological findings (e.g., IGF-2 and FGFR-4 overexpression). Finally, a phase II study will be needed to confirm the efficacy of topotecan against ACT in children and adults.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: E.M. Pinto, C. Morton, L. McGregor, G.P. Zambetti
Development of methodology: E.M. Pinto, C. Morton, A.M. Davidoff, G.P. Zambetti
Acquisition of data (primary animals, acquired and managed patients, provided facilities, etc.): E.M. Pinto, C. Morton, C. Rodriguez-Galindo, A. M. Davidoff, K. Mercer, L. Debelenko, G.P. Zambetti
Analysis and interpretation of data (e.g., statistical analysis, biosatistics, computational analysis): E.M. Pinto, C. Morton, C. Rodriguez-Galindo, L. McGregor, C.A. Billups, R.C. Ribeiro, G.P. Zambetti
Writing, review, and/or revision of the manuscript: E.M. Pinto, C. Morton, C. Rodriguez-Galindo, L. McGregor, A.M. Davidoff, L. Debelenko, C.A. Billups, R.C. Ribeiro, G.P. Zambetti

References

Figure 4. Activity of topotecan in the adult (HAC15RW cells derived from H295R) ACC xenograft model. Kaplan-Meier curves for event-free survival, median relative tumor volume graphs, and individual tumor volume graphs are shown; control (gray lines) and treated (black lines).


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