Somatic TP53 Mutations Are Detectable in Circulating Tumor DNA from Children with Anaplastic Wilms Tumors1,2

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Abstract

BACKGROUND: Diffuse anaplastic Wilms tumor (DAWT) is a rare, high-risk subtype that is often missed on diagnostic needle biopsy. Somatic mutations in TP53 are associated with the development of anaplasia and with poorer survival, particularly in advanced-stage disease. Early identification of DAWT harboring TP53 abnormalities could improve risk stratification of initial therapy and monitoring for recurrence. METHODS: Droplet digital polymerase chain reaction (ddPCR) was used to evaluate 21 samples from 4 patients with DAWT. For each patient, we assessed TP53 status in frozen tumor, matched germline DNA, and circulating tumor DNA (ctDNA) from plasma, serum, and urine collected throughout treatment. RESULTS: Mutant TP53 was detectable in ctDNA from plasma and serum in all patients. We did not detect variant TP53 in the same volume (200 μl) of urine. One patient displayed heterogeneity of TP53 in the tumor despite both histological sections displaying anaplasia. Concentration of ctDNA from plasma/serum taken prenephrectomy varied significantly between patients, ranging from 0.44 (0.05-0.90) to 125.25 (109.75-140.25) copies/μl. We observed variation in ctDNA throughout treatment, and in all but one patient, ctDNA levels fell significantly following nephrectomy. CONCLUSION: We demonstrate for the first time that ddPCR is an effective method for detection of mutant TP53 in ctDNA from children with DAWT even when there is intratumoral somatic heterogeneity. This should be further explored in a larger cohort of patients, as early detection of circulating variant TP53 may have significant clinical impact on future risk stratification and surveillance.
Introduction
Wilms tumor (WT) or nephroblastoma is the most common childhood renal cancer, with 1 in 100,000 children diagnosed annually [1]. In Europe, children are treated with neoadjuvant chemotherapy prior to surgery as per The International Society of Pediatric Oncology Renal Tumors Study Group (SIOP RTSG) guidelines [2]. Conversely, in North America, children undergo immediate surgery prior to chemotherapy in accordance with the Children’s Oncology Group (COG). For both groups, tumor histology and stage dictate the intensity of postoperative treatment, with chemotherapy and sometimes radiotherapy. Regardless of the protocol used, overall survival approaches 90% [3].

Despite this excellent prognosis, approximately 15% of patients relapse, and for the subgroup displaying high-risk histology, more than one in four patients need intensified treatment for disease recurrence [4]. Diffuse anaplasia (DAWT) is classified as high risk by both SIOP RTSG and COG. Needle biopsy rarely captures anaplasia, but it is found in 5%-10% of cases following surgical resection [5]. Approximately 60% of DAWTs have somatic mutations in the tumor suppressor gene TP53 and/or 17p loss. Although these mutations likely confer an increased risk of both relapse and mortality, particularly in advanced tumor stage, genetic testing of surgically resected tumors is not routinely performed [6,7].

At diagnosis, there are no radiological findings to clearly differentiate a WT from other renal tumors or to differentiate the subtype of WT. In most countries that follow the SIOP approach, preoperative chemotherapy is commenced without a confirmatory biopsy. Furthermore, biopsy provides limited diagnostic information in part due to the high degree of genetic intratumoral heterogeneity (ITH) found in WT [8,9]. In order to circumvent the issues of ITH and the limitations and risks related to the biopsy procedure, minimally invasive molecular biomarkers are urgently needed.

Circulating tumor DNA (ctDNA), shed by tumor cells and detectable in a range of bodily fluids, is a promising candidate as it represents contributions from multiple tumor subclones [10]. However, efforts to characterize ctDNA in pediatric tumors have lagged behind the work achieved in adult cancers. One principal limitation in detection of ctDNA is its variable mutant allele fraction (MAF), among an abundant background of cell-free DNA released by nontumor cells. Droplet digital PCR (ddPCR) is an ideal tool for characterizing ctDNA as it provides a limit of detection comparable to MAF. This technique partitions a sample into 15,000-20,000 droplets, with PCR occurring in each droplet [11]. The number of DNA templates within each droplet is modeled by a Poisson distribution, and by quantifying the fraction of positive droplets, absolute DNA levels can be determined without extrapolation from a standard curve [12]. Due to the relative concentrations of targets and inhibitors in each droplet, ddPCR is more resistant to inhibition and more reproducible at low target concentrations than real-time quantitative PCR, making it attractive for diagnostic applications [13].

The purpose of this study was to test the feasibility of ctDNA detection in children with high-risk DAWT using plasma, serum, and urine collected throughout treatment.

Materials and Methods

Patients
All patients with a diagnosis of WT were enrolled in the UK-wide Improving Population Outcomes for Renal Tumours of Childhood (IMPORT) study. Informed consent to undertake genetic testing of samples was obtained as part of the study, which was approved by the national research ethics committee (London Bridge REC 12/L0/0101). Normal kidney and multisampled tumor tissues were collected at surgery, while blood and urine were collected at up to five treatment time points: diagnosis, midchemotherapy, preoperative, postoperative, and end of treatment. Patients were treated with preoperative chemotherapy regimens according to stage as per SIOP WT 2001. Nonmetastatic cases (stages I-III) received vincristine/actinomycin-D for 4 weeks, with doxorubicin added for metastatic disease (stage IV) for 6 weeks. Following nephrectomy, patients with focal or diffuse anaplasia were risk stratified to intermediate- or high-risk subgroups, respectively, and postoperative treatment was further refined according to stage of disease at surgery.

DNA Extraction
All samples used for tumor DNA extraction were fresh-frozen specimens obtained at nephrectomy and stored at −80°C. Specimens underwent centralized histology review to confirm stage and histology, and those with tumor content of more than 50% were utilized for the study [14]. Tumor DNA and germline DNA from normal tissue were extracted by standard methods using either a standard detergent lysis/phenol-chloroform technique or the DNAeasy Blood and Tissue Kit (Qiagen). Germline DNA from cell fraction was extracted with the QIAamp DNA Blood Midi Kit (Qiagen). DNA was stored at −20°C. Extraction of ctDNA from 200 μl of plasma, serum, and urine supernatant was carried out with the Plasma/ Serum Cell-free Circulating DNA Purification Mini Kit (Norgen) and the Circulating Nucleic Acid Kit (Qiagen) as per protocol. Elution volumes were 50 μl and 75 μl for the Norgen and Qiagen kits, respectively. Quantification and further quality control of tumor and germline DNA were undertaken with a NanoDrop spectrophotometer (Thermo Fisher Scientific), Qubit fluorometer (Thermo Fisher Scientific), and agarose gel.

Sequencing of Tumor and Germline DNA
For TP53 mutational analysis, Sanger sequencing of tumor DNA was carried out by Great Ormond Street Hospital Genetics. Bidirectional sequencing was undertaken for all 11 exons of the gene. For the five patients with TP53 mutations, we performed Sanger sequencing on germline DNA. PCR amplification products were run on an agarose gel prior to clean-up with Illustra ExoStar 1-Step (GE Healthcare Life Sciences). Cleaned PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Sequences were examined both manually and electronically using ApE (v1.8 Mekentosi, Amsterdam) prior to genome alignment to GRCh37 with Blat [13] and functionally annotated with Annovar [14]. Single nucleotide polymorphisms (SNPs) were excluded if present in The Database of Single Nucleotide Polymorphisms (dbSNP) [15].

ddPCR
In four out of the five cases with TP53 mutations, assays with sequence-specific primers and TaqMan-based probes for mutant and wild-type alleles were purchased (PrimePCR ddPCR Mutation Assay Bio-Rad for TP53). Primer and probe sequences are not provided by Bio-Rad, but amplicon context sequence and length for each assay are as follows: p.R273C 17:7,577,060-7,577,182 65 nt; p.R337C 17:7,573,957-7,574,079 79 nt; p.R248Q 17:7,577,477-7,577,599 62 nt.
To assess optimum annealing temperature, each assay was run on a temperature gradient with germline DNA and a 50:50 mix of tumor DNA spiked into germline DNA. Each ddPCR mixture was made up of 11 μl 2× ddPCR Supermix (No dUTP; Bio-Rad), 1.1 μl mutant-specific FAM probe, 1.1 μl wild-type–specific HEX probe, 0.5 μl restriction enzyme (MseI or HaeIII; New England BioLabs), 0.5 μl nuclease-free water, and 8.8 μl sample DNA. Twenty microliters of ddPCR mixture was partitioned into droplets using the QX100 Droplet Generator (Bio-Rad). The thermal cycling profile was 95°C for 10 minutes, followed by 40 cycles of 94°C for 30 seconds and 52°C for 1 minute, then 1 cycle of 98°C for 10 minutes. Droplets were read on a QX100 Droplet Reader (Bio-Rad). Three technical replicates were used for each sample, as well as no template and positive controls (tumor DNA with mutant TP53). QuantaSoft software (version 1.3.2.0, Bio-Rad) was used to set thresholds manually by evaluating maximum separation between positive and negative clusters in control wells. Data are presented in accordance with minimum reporting guidelines for digital PCR studies [16]. Raw data were exported into R Studio and plotted with the ggplot2 package (H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2009). MAF is calculated by dividing the concentration of the mutant allele by the sum of the concentrations of the mutant and wild-type alleles. Calculating the MAF normalizes the mutant allele signal against the background of wild-type alleles and represents the data as would be expected in a next-generation sequencing (NGS)–based assay.

Results

Patient Demographics

Ten patients from the IMPORT study were identified with WT displaying focal or diffuse anaplasia (confirmed by central histology review) and with plasma, serum, and urine collected at more than one time point. The clinicopathological information for each case is shown in Table 1. None of the five patients with wild-type TP53 relapsed, while three of five patients with TP53 mutations died of their disease.

Sanger Sequencing and ddPCR of Tumor Tissue

Sanger sequencing of tumor DNA identified TP53 mutations in five patients. Multiple spatially distinct tumor samples from each patient were also analyzed on an NGS panel that included TP53 as part of another experiment. The TP53 mutation in case 10 was subclonal, while all other cases 1, 2, 6, and 9 were homogeneous for mutant TP53 (data not shown). Only specimens with anaplasia were used for analysis. There is no single hotspot locus for mutant TP53 in WT, and each patient had individual mutations as shown in Table 1.

Table 1. Clinicopathological Details for the Included Patients with Wilms Tumor, Including TP53 Status

<table>
<thead>
<tr>
<th>Case</th>
<th>Age at Diagnosis (Months)</th>
<th>Sex</th>
<th>Biopsy</th>
<th>Histology</th>
<th>Pathological Local Stage</th>
<th>Metastatic at Diagnosis</th>
<th>Relapse</th>
<th>Died of disease</th>
<th>TP53 status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>58</td>
<td>F</td>
<td>no anaplasia</td>
<td>DAWT</td>
<td>III</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>p.R248Q</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>F</td>
<td>no anaplasia</td>
<td>DAWT</td>
<td>I</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>p.E285K</td>
</tr>
<tr>
<td>3</td>
<td>47</td>
<td>F</td>
<td>no anaplasia</td>
<td>DAWT</td>
<td>III</td>
<td>1.00</td>
<td>1.00</td>
<td>0.00</td>
<td>wild type</td>
</tr>
<tr>
<td>5</td>
<td>53</td>
<td>F</td>
<td>no anaplasia</td>
<td>focal anaplasia</td>
<td>I</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>wild type</td>
</tr>
<tr>
<td>6</td>
<td>45</td>
<td>F</td>
<td>no anaplasia</td>
<td>DAWT</td>
<td>III</td>
<td>1.00</td>
<td>1.00</td>
<td>0.00</td>
<td>p.R337C</td>
</tr>
<tr>
<td>7</td>
<td>75</td>
<td>M</td>
<td>no anaplasia</td>
<td>DAWT</td>
<td>I</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>wild type</td>
</tr>
<tr>
<td>8</td>
<td>71</td>
<td>M</td>
<td>no anaplasia</td>
<td>DAWT</td>
<td>II</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>wild type</td>
</tr>
<tr>
<td>9</td>
<td>117</td>
<td>F</td>
<td>diffuse anaplasia</td>
<td>DAWT</td>
<td>I</td>
<td>1.00</td>
<td>0.00</td>
<td>0.00</td>
<td>p.R273C</td>
</tr>
<tr>
<td>10</td>
<td>108</td>
<td>F</td>
<td>unknown</td>
<td>DAWT</td>
<td>III</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>p.H179L</td>
</tr>
</tbody>
</table>

Table 2. Mean Concentration (copies/μL) of TP53 Mutant ctDNA for Each Sample

<table>
<thead>
<tr>
<th>Case Number</th>
<th>Time Point</th>
<th>Sample Type</th>
<th>Concentration (copies/μL)</th>
<th>Confidence Interval (copies/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.44</td>
<td>Serum</td>
<td>0.05-0.90</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6.63</td>
<td>Serum</td>
<td>4.93-8.15</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.00</td>
<td>Urine</td>
<td>0.00-0.21</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>125.25</td>
<td>Plasma</td>
<td>109.75-140.25</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>121.00</td>
<td>Serum</td>
<td>98.00-140.50</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>20.58</td>
<td>Plasma</td>
<td>16.25-24.33</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>18.98</td>
<td>Serum</td>
<td>10.78-24.65</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>4.53</td>
<td>Plasma</td>
<td>2.18-6.58</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.42</td>
<td>Plasma</td>
<td>0.00-0.92</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.00</td>
<td>Urine</td>
<td>0.00-0.23</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.93</td>
<td>Plasma</td>
<td>0.14-3.23</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1.29</td>
<td>Plasma</td>
<td>0.34-1.56</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.00</td>
<td>Urine</td>
<td>0.00-0.27</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.07</td>
<td>Plasma</td>
<td>0.00-0.36</td>
<td></td>
</tr>
</tbody>
</table>

Time points as follows: Dx, diagnosis; MC, midchemotherapy; PrO, preoperative; PO, postoperative; EOT, end of treatment. Wild-type TP53 from cell free DNA is not shown.
wild-type allele, confirming the mutations as somatic changes. Tumor samples were run as positive controls during the ddPCR experiments, and all samples had mutant TP53 in the tumor (data not shown).

ddPCR of ctDNA from Plasma, Serum, and Urine

Mutations in TP53 from ctDNA were detectable from the four cases with mutation-specific Bio-Rad assays. For case 2, there was no commercially available mutation-specific assay.

Concentration varied significantly between patients (Table 2). The most abundant mutant ctDNA was found in case 6 at diagnosis, both in plasma and in serum, with a concentration of 125.25 (109.75-140.25) copies/μl and 121.00 (98.00-140.50) copies/μl, respectively. Case 6 had lung metastases at diagnosis, with progression of disease and new nodules postoperatively. The absence of detectable ctDNA postoperatively suggests that the high levels were derived from the primary tumor rather than metastatic deposits, as these were not removed during nephrectomy. All patients had detectable ctDNA prenephrectomy; we are unable to comment on chemonaive samples as, for two of the cases, we did not receive samples at diagnosis.

In three out of four cases, MAF decreased following nephrectomy (Figure 1). In case 1, ctDNA concentration increased from 0.44 (0.05-0.90) copies/μl to 6.63 (4.93-8.15) copies/μl after nephrectomy. We cannot rule out that the high concentration postoperatively is due to manipulation of the tumor during surgery. However, owing to the very short half-life of ctDNA, this effect is likely to be negligible. This patient has stage III disease and relapsed 339 days postoperatively. In case 1 and case 10, wild-type TP53 (as assessed by cell-free DNA levels) increased postoperatively.

Genomic contamination, thought to be from lysed blood cells, is greater in serum than in plasma (Supplementary Figure 1).

Figure 1. MAF from serum and plasma for each case varies during treatment. Time points as follows: 1, diagnosis; 2, mid-chemotherapy; 3, pre-operative; 4, post-operative; and 5, end of treatment. MAF is calculated as mutant allele concentration divided by total concentration. Error bars represent the range of MAFs produced within the confidence intervals of the mutant and wild-type allele concentrations. Urine ctDNA is not shown.
Concentration of ctDNA was significantly lower in 200 μl of urine supernatant than those from the same volume of plasma or serum (Table 2). The concentration of mutant ctDNA extracted from 1.8 ml of urine from case 1 postoperatively was 35.50 (32.25-38.75) copies/μl.

Comparison of extraction kits (Qiagen circulating nucleic acid kit versus Norgen Plasma/Serum Cell-free Circulating DNA Purification Mini Kit) was undertaken in two cases with four samples overall. Results are consistent with a previous study and demonstrate comparable ctDNA yields (Supplementary Figure 2) [17].

Discussion
Our explorative study demonstrates that collection and analysis of bodily fluids taken from children with WT are achievable. Further, mutant TP53 is detectable in ctDNA from plasma and serum in patients with DAWT, though not from the same volume of urine.

Although somatic TP53 mutations are infrequent in childhood cancer, pediatric pan-cancer analyses have identified TP53 as the most frequent pathogenic constitutional mutation [18–20]. There are over 200 SNPs in the gene, with some of these variants likely predisposing to cancer [21]. In adults, somatic alterations in TP53 are the commonest genetic event, with six frequently observed hotspot residues [22]. Two of the mutations we identified (p.R248Q and p.R273C) align to data from 25,902 patients showing that these sites are the two most common loci for TP53 mutations [23,24]. Regarding the amino acid residue R248, recurrent TP53 mutations in WT have been observed at this locus [24]. Three mutations (p.R248Q, p.R273C, and p.H179L) are considered to be gain of function (GOF) and are associated with a highly aggressive phenotype, while p.R337C is thought to be a nonfunctional mutant [25].

Mutations in TP53 are seemingly associated with a higher risk of relapse and death for children with advanced-stage anaplastic WT [3,4]. Originally thought to be pathognomonic for DAWT, a recent relapse and death for children with advanced-stage anaplastic WT intensify therapy for children with mutant spectrum from GOF to nonfunctional mutants, any decisions to assessed circulating bodily fluids taken from children with WT are achievable. Further, somatic alterations in TP53 are the commonest genetic event, with six frequently observed hotspot residues [22]. Two of the mutations we identified (p.R248Q and p.R273C) align to data from 25,902 patients showing that these sites are the two most common loci for TP53 mutations [23,24]. Regarding the amino acid residue R248, recurrent TP53 mutations in WT have been observed at this locus [24]. Three mutations (p.R248Q, p.R273C, and p.H179L) are considered to be gain of function (GOF) and are associated with a highly aggressive phenotype, while p.R337C is thought to be a nonfunctional mutant [25].

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A further possible merit in assessment of TP53 status is targeted therapy [27]. Early-phase trials in adult relapsed/refractory solid and hematological malignancies are ongoing to test both negative regulators of p53 [28]. Interestingly, some patients with GOF mutants have demonstrated response to MDM2/MDMX targeted therapy, although there remains the theoretical risk that these inhibitors could increase levels of mutant TP53 and drive cancer progression [28].

Our study was conducted as a proof of principle and hence was targeted therapy [27]. Early-phase trials in adult relapsed/refractory solid and hematological malignancies are ongoing to test both negative regulators of p53 [28]. Interestingly, some patients with GOF mutants have demonstrated response to MDM2/MDMX targeted therapy, although there remains the theoretical risk that these inhibitors could increase levels of mutant TP53 and drive cancer progression [28].

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Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.08.006.

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