A comprehensive circulating tumor DNA assay for detection of translocations and copy number changes in pediatric sarcomas

Avanthi Tayi Shah¹, Tej D. Azad², Marcus R. Breese¹, Jacob J. Chabon³, Emily G. Hamilton⁴, Krystal Straessler¹,⁶, David M. Kurtz⁵, Stanley G. Leung¹, Aviv Spillinger¹, Heng-Yi Liu¹, Inge H. Behroozfard¹, Frederick M. Wittber⁷, Florette K. Hazard⁸, Soo-Jin Cho⁹, Heike E. Daldrup-Link⁷, Kieuhoa T. Vo¹, Arun Rangaswami¹, Allison Pribnow¹⁰, Sheri L. Spunt ³,¹⁰, Norman J. Lacayo ³,¹⁰, Maximilian Diehn¹¹, Ash A. Alizadeh⁵, E. Alejandro Sweet-Cordero¹,#

¹ Division of Hematology and Oncology, Department of Pediatrics, University of California San Francisco, CA. USA
² Stanford University School of Medicine, Stanford University, CA. USA
³ Stanford Cancer Institute, Stanford University, CA. USA
⁴ Stanford Cancer Biology, Stanford University, CA. USA
⁵ Division of Oncology, Department of Medicine, Stanford University School of Medicine, Stanford University, CA. USA
⁶ University of Utah School of Medicine, Salt Lake City, Utah
⁷ Department of Radiology, Stanford University School of Medicine, Stanford University, CA. USA
⁸ Department of Pathology, Stanford University School of Medicine, Stanford University, CA. USA
⁹ Department of Pathology, University of California, San Francisco, CA. USA
¹⁰ Division of Hematology and Oncology, Department of Pediatrics, Stanford University School of Medicine, Stanford University, CA. USA
¹¹ Division of Radiation Therapy, Department of Radiation Oncology, Stanford University School of Medicine, Stanford University, CA. USA.

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# Corresponding author:
E. Alejandro Sweet-Cordero
1550 4th St.
Rock Hall Room #384
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ABSTRACT

Most circulating tumor DNA (ctDNA) assays are designed to detect recurrent mutations. Pediatric sarcomas share few recurrent mutations but rather are characterized by translocations and copy number changes. We applied CAncer Personalized Profiling by deep Sequencing (CAPP-Seq) for detection of translocations found in the most common pediatric sarcomas. We also applied ichorCNA to the combined off-target reads from our hybrid capture to simultaneously detect copy number alterations. We analyzed 64 prospectively collected plasma samples from 17 pediatric sarcoma patients. Translocations were detected in the pre-treatment plasma of 13 patients and were confirmed by tumor sequencing in 12 patients. Two of these patients had evidence of complex chromosomal rearrangements in their ctDNA. We also detected copy number changes in the pre-treatment plasma of 7 patients. We found that ctDNA levels correlated with metastatic status and clinical response. Furthermore, we detected rising ctDNA levels before relapse was clinically apparent, demonstrating the high sensitivity of our assay. This assay can be utilized for simultaneous detection of translocations and copy number alterations in the plasma of pediatric sarcoma patients. While we describe our experience in pediatric sarcomas, this approach can be applied to other tumors that are driven by structural variants.
INTRODUCTION

Pediatric sarcomas consist of diverse subtypes that are rare and often difficult to diagnose. Diagnosis is currently performed using a combination of radiologic features, histologic analysis, and identification of molecular markers, including in some subtypes disease-defining translocations (1). In routine clinical practice, methods for translocation detection rely primarily on fluorescent in-situ hybridization (FISH) of the tumor specimen. Most often, these are “break apart” probes that identify an alteration in one translocation partner, but do not definitively identify the second partner, a practice that can sometimes lead to an erroneous diagnosis.

While some translocations are relatively common, many rare variants exist, often making diagnosis difficult using standard approaches. For example, in Ewing sarcoma, EWSR1-FLI1 is the most common translocation and occurs in 85% of tumors, while EWSR1-ERG occurs in 10% of tumors (2,3). However, a small subset of Ewing sarcoma tumors do not have a translocation involving EWSR1. FISH break apart probes, which target EWSR1, fail to identify these non-EWSR1 rearranged tumors. Many other tumor subtypes are also characterized by EWSR1 translocations (4) and have distinct clinical courses and treatment regimens, necessitating accurate diagnosis. Although FISH may aid in the diagnosis of EWSR1 translocated tumors in some cases, the failure to identify the translocation partner can lead to inappropriate management.

Similarly, the diagnosis and management of rhabdomyosarcoma requires assessment for the presence or absence of a PAX3- or PAX7-FOXO1 translocation. Molecular analysis has shown that a subset of rhabdomyosarcoma tumors are characterized by fusions involving PAX3 or PAX7 and FOXO1 and the presence of
these fusions portends a worse prognosis (5). Proper identification of fusion status and specific fusion partners is therefore paramount, as it can have therapeutic and prognostic implications (6–9).

Even for patients in whom the diagnosis can be accurately made using current approaches, there is a lack of sensitive and specific methods for monitoring disease burden during and after treatment. Imaging modalities can be misleading because tumor size does not always accurately reflect the presence of viable tumor, as some sarcomas can demonstrate a persistent mass despite adequate tumor necrosis (10–13). Furthermore, imaging approaches are expensive, may require sedation in young children, often lack the sensitivity to detect early relapse, and may contribute to the risk of future malignancies due to exposure to ionizing radiation (14).

One approach for non-invasive, sensitive, and specific monitoring of cancer is to evaluate circulating tumor DNA (ctDNA)(15–17). However, ctDNA assays have primarily been optimized to detect single nucleotide variants. While detection of pediatric sarcoma translocations in ctDNA has been described, this has primarily relied on PCR-based amplification (18–20). Such methods require prior sequencing analysis of a tumor specimen for identification of the patient-specific translocation breakpoint followed by design of a customized assay for that patient. Thus, their clinically utility is limited, particularly for cases where the tumor is not available for analysis to define the translocation breakpoint. Additionally, PCR-based assays cannot simultaneously detect copy number alterations (CNAs), which are also highly prevalent in many pediatric sarcomas. While copy number changes are not typically used for diagnostic purposes, they have increasingly significant prognostic and therapeutic implications (21–23).
Recognizing these limitations, some groups have utilized next-generation sequencing approaches for identification of translocations or CNAs in pediatric sarcomas (24,25), however, a single assay that simultaneously detects these alterations may be advantageous. To develop a sarcoma-specific ctDNA assay capable of concurrently detecting translocations and copy number changes, we used the CAncer Personalized Profiling by deep-Sequencing (CAPP-Seq) (26,27) approach to design a “pan-sarcoma” ctDNA assay that could be used “off-the-shelf”, without requiring a primary tumor specimen for analysis. Here we describe the use of this assay to diagnose and characterize pediatric sarcomas non-invasively and to monitor disease during the course of therapy.

MATERIALS AND METHODS

Patient selection

Written informed consent for participation in this study was obtained from newly diagnosed or newly relapsed patients treated at Lucile Packard Children’s Hospital Stanford or UCSF Benioff Children’s Hospital San Francisco. The study was approved by the institutional IRB in accordance with established ethical guidelines (Belmont Report). Patients with a pathology-confirmed diagnosis of Ewing sarcoma, osteosarcoma, rhabdomyosarcoma, or synovial sarcoma were included if a plasma sample was available for collection prior to initiation of any curative intervention, including excisional biopsy. Clinical data, including histologic diagnosis, metastatic status at diagnosis, fluorescence in situ hybridization (FISH) results, clinical gene panel testing results through Foundation Medicine (Cambridge, MA, USA) or UCSF500 (San Francisco, CA, USA), treatments received, and response to therapy was...
retrospectively collected (Supplementary Table S1). All radiographic examinations and laboratory analyses, including FISH, RT-PCR for translocations, and clinical gene panel testing, were obtained as part of routine clinical care and at the discretion of the treating physician. Standardized tumor volumes from imaging studies were calculated using TeraRecon Aquarius reconstruction using manual circumscription, free region of interest demarcation, and volumetric exclusion (28).

Sample collection and processing

Pre-treatment peripheral blood samples were prospectively collected from all patients prior to initiation of treatment for newly diagnosed or newly relapsed pediatric sarcoma patients. When feasible, on-therapy samples of peripheral blood were also collected over the course of disease treatment. These time points generally coincided with surveillance scans and before/after surgical resection or radiation therapy courses. For a subset of patients, serial samples were also collected after completion of therapy, during surveillance visits. Plasma samples were also collected if there was concern for relapse. Peripheral blood (5-15 mL) was collected in K$_2$EDTA tubes and processed within 2 hours of collection. Plasma was isolated by centrifugation at 1800 x g for 10 minutes and aliquoted into 2 mL cryovial tubes. The remaining plasma-depleted whole blood was mixed and aliquoted into 2 mL cryovial tubes. Plasma and whole blood samples were stored at -80°C. Cell-free DNA (cfDNA) was extracted from plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, cat. no. 55114) according to the manufacturer’s instructions. Germline DNA was extracted from peripheral blood mononuclear cells using the DNeasy Blood & Tissue Kit (Qiagen, cat. no. 69504) according to the manufacturer’s instructions.
When fresh, unfixed tumor samples were available for analysis, samples were snap frozen, embedded in OCT, and sectioned. Cryosections were cut with a section depth of 5 μm on a cryostat and stained with hematoxylin and eosin (H&E). Sections were evaluated by a pediatric pathologist (F.K.H. or S.J.C.) and samples with >70% tumor content were macro-dissected from the OCT block to a depth of up to 5mm. Using a mortar and pestle under liquid nitrogen, fresh frozen tumor samples were disrupted and homogenized with a QIAshredder (QIAgen, cat. no. 79654), and DNA was extracted using the AllPrep kit (QIAgen, cat. no. 80204). For FFPE tumor tissue, 5 μm slides were obtained and a representative slide was stained with H&E and evaluated. Areas high in tumor content were scraped off adjacent slides for extraction. DNA from FFPE samples was extracted using the AllPrep DNA/RNA FFPE kit (Qiagen, cat. no. 80234).

cfDNA, tumor, and germline DNA were quantified using the Qubit High-Sensitivity dsDNA assay (Thermo Fisher). Tumor and germline DNA were also quantified using the Nanodrop 2000c (Thermo Fisher). Tumor and germline nucleic acid quality was determined using the Genomic DNA Analysis ScreenTape (Agilent, cat. no. 5067-5365) on the TapeStation 4200 (Agilent). Tumor genomic DNA used for CAPP-Seq library preparation was sonicated to ~170 bp fragments using the S2 ultrasonicator (Covaris). Sheared DNA and cfDNA fragment size was determined using the 2100 Bioanalyzer (Agilent) or the Fragment Analyzer (AATI).

CAPP-Seq selector design

The CAPP-Seq targeted gene panel was designed by reviewing the Catalog of Somatic Mutations in Cancer (COSMIC) (29) and the literature (30–37) to identify
intrinsic regions involved in translocation breakpoints. For some translocations, both gene partners were not included in the selector design because the intrinsic regions were too large and would saturate the selector size. For example, the intrinsic regions of FOXO1 involved in PAX-FOXO1 translocations span ~130 kb. The final selector covers 150 kb and includes 25 intrinsic regions from 11 genes (Table 1). These intrinsic regions were used as input for the NimbleDesign software (Roche) to design a custom SeqCap EZ Choice capture panel (Supplementary Table S2).

CAPP-Seq library preparation, hybrid capture, and sequencing

A minimum of 32 ng of DNA was used as standard input for cfDNA library preparation, with up to 60 ng used if significant genomic DNA contamination was present. Genomic contamination was considered significant if >50% of DNA fragments were derived from high-molecular weight DNA as determined by Bioanalyzer or Fragment Analyzer. If less than 32 ng of cfDNA was obtained, the maximum amount available was used for library preparation (Supplementary Table S3). Plasma DNA was not amplified prior to library generation. A maximum of 100 ng of sheared genomic DNA was used as input for CAPP-Seq tumor DNA library preparation. Libraries were prepared using the KAPA Hyper Prep Kit (Kapa Biosystems, cat. no. KK8502) and custom unique molecular identifiers, as described previously (26,27). Pooled sequencing libraries subsequently underwent hybridization with the CAPP-Seq selector. Post-hybridization pooled libraries were sequenced on an Illumina HiSeq4000 instrument using 2 x 150 paired-end reads with an 8-base indexing read. Median sequencing depth for cfDNA and tumor were 1,744X and 2,729X, respectively.

CAPP-Seq data analysis
Sequencing data was processed using a previously described custom bioinformatics pipeline (26,27). Briefly, samples were de-multiplexed and paired-end reads were mapped to the hg19 reference genome using BWA 0.6.2 with default parameters (38). Samples were sorted and indexed using SAMtools (39).

Structural variant detection was performed using FACTERA (40), a highly sensitive and specific method for structural variant detection in cfDNA. FACTERA takes as input a .bam alignment file of paired-end reads produced by BWA (38), exon coordinates in .bed format (hg19 RefSeq coordinates utilized), and a .2bit reference genome to enable fast sequence retrieval (hg19). In addition, the analysis was restricted to reads that overlap genomic regions in the CAPP-Seq selector used in this work. FACTERA processes the input in three sequential phases: identification of discordant reads, detection of breakpoints at base pair-resolution, and in silico validation of candidate fusions. For structural variants identified in tumor or in pre-treatment cfDNA, monitoring in subsequent plasma samples was performed using the sequence across the breakpoint and submitting BLAST queries (41) for all reads (properly paired, improperly paired, and not paired) mapping to the genes involved in the fusion region. If even one read detected a structural variant, this was used to make a call, as translocations, unlike mutations, are unlikely to be attributed to sequencing errors. Allelic frequencies (AFs) for structural variants not formally called by FACTERA were computed by dividing the average depth at the positions on each end of the SV by the number of reads containing the templated fusion sequence.
Off-target reads were combined to obtain low depth coverage across the genome (median depth of 6.4X). Copy number detection was performed using ichorCNA (17) and manual inspection.

**WGS library preparation, sequencing, and data analysis**

WGS libraries were made using the TruSeq Nano kit (Illumina) with a 350-bp insert as per the manufacturer's instructions. Tumor libraries were made using 400 ng of input DNA and sequenced to a depth of 60X. Germline libraries were made using 200 ng of input DNA and sequenced to a depth of 30X. Pooled libraries were sequenced on an Illumina HiSeq system with paired-end 2 × 150 bp reads. For 2 patients (042 and 005), germline and tumor were sequenced to a depth of 50X on an Illumina HiSeq system with paired-end 2 × 100 bp reads by Illumina, Inc. Raw DNA FASTQ data were pre-processed with NGSUtils (42) and aligned to the sex-specific GRCh38 reference genomes using BWA. Structural variants and copy number alterations were determined as previously described (23).

**Statistical Analysis**

To test the hypothesis that metastatic patients had higher pre-treatment ctDNA levels than non-metastatic patients, we utilized the two-tailed Mann-Whitney U test. Kaplan-Meier estimates of progression-free survival and overall survival stratified by median pre-treatment ctDNA level were performed using GraphPad Prism, version 8.2.1 for MacOS (GraphPad Software). Hazard ratio and P-value were calculated by the log-rank test.

**RESULTS**
Patients and specimens

Samples were prospectively collected at the time points indicated in Figure 1A. Samples available are summarized in Figure 1B. The CAPP-Seq selector was applied to pre-treatment plasma samples from 17 newly diagnosed or newly relapsed patients. Serial samples over the course of therapy were available from 9 patients. Of these, 7 patients also had plasma samples collected after completion of therapy. Matched tumor samples were available for genomic analysis by CAPP-Seq and/or research-grade whole genome sequencing (WGS) for 13/17 patients (Supplementary Table S4). Sequencing reports from clinical gene panel testing were available for comparison for 4 patients (Supplementary Table S4).

Recurrent sarcoma translocations detected in pre-treatment plasma

We first analyzed the ability of CAPP-Seq to detect translocations in pre-treatment plasma samples. Sixteen patients had tumors with translocations identified by FISH or tumor sequencing. Translocations were identified in ctDNA with 81.3% sensitivity (13/16 patients) at AFs ranging from 0.31 to 22.1% (median 10.7%) (Figure 2A). The same translocations were also identified by sequencing of the tumor for the 12 patients that had tumor available for analysis (Supplementary Table S4). EWSR1-FLI1 translocations were detected in 4 Ewing sarcoma patients, with a median AF of 12.9%. We did not identify the canonical EWSR1 rearrangements in the pre-treatment plasma of 2 Ewing sarcoma patients. Patient 492 had tumor WGS that demonstrated an EWSR1-FLI1 translocation that should have been detectable with our selector. Patient 577 was EWSR1 rearranged by break-apart probe analysis, but did not have
any tumor available for sequencing, thus we were unable to confirm if this $EWSR1$ rearrangement should have been detected using our assay.

Three patients had detectable $PAX3-FOXO1$ translocations, with a median AF of 10.7%. We did not identify any patients with $PAX7-FOXO1$ translocations in their plasma or tumor. Patient 316 had been clinically diagnosed with fusion negative alveolar rhabdomyosarcoma, based on FISH testing of the tumor for the $FOXO1$ gene rearrangement. However, analysis of the plasma by CAPP-Seq revealed a $PAX3-NCOA1$ translocation, a rare translocation that has been described in a subset of rhabdomyosarcoma patients (30). This fusion results in a chimeric transcription factor activator that has potent $PAX3$ transactivation activity, resulting in a gene expression profile similar to that of the canonical fusion (22,43). Identification of the $PAX3-NCOA1$ fusion is of clinical value, as these tumors are associated with a worse prognosis (5).

In contrast to Ewing sarcoma and rhabdomyosarcoma, osteosarcoma is not associated with a canonical gene fusion event. However, approximately 50% of these tumors have structural variants in $TP53$, most commonly in intron 1 (34). Additionally, structural variants in introns 12 and 15 of $ATRX$ are seen in 14% of tumors (34). We detected $TP53$ translocations at a median AF of 1.2% in 3/3 osteosarcoma patients with TP53 rearrangements identified from sequencing of their primary tumor. $ATRX$ translocations were not identified in the tumor or plasma of any patients in this cohort.

Our initial cohort included only one patient with synovial sarcoma. We were unable to detect the canonical $SSX1-SS18$ translocation in the pre-treatment plasma of this patient. The patient had a solitary lung nodule measuring 0.2 cm$^3$ and we were able to detect the $SSX1-SS18$ translocation in this nodule using WGS and CAPP-Seq.
We hypothesize that the small tumor size may have led to very low levels of ctDNA. Despite the three negative cases, our results support the utility of the CAPP-seq selector to identify fusions in the majority of pediatric sarcomas.

**Complex chromosomal rearrangements detected in ctDNA**

Patient 005 was diagnosed with Ewing sarcoma after biopsy revealed a small round blue cell tumor with an *EWSR1* gene rearrangement by FISH. We detected an *EWSR1-PKNOX2* translocation in the patient’s plasma at the time of relapse, with breakpoints at chr11:125215841 and chr22:29684555 (hg19), at an AF of 16.3%. To our knowledge, this translocation has not previously been described. WGS of the matched primary tumor revealed multiple structural variants (Figure 2B), including the same *EWSR1-PKNOX2* translocation with translocation breakpoints identical to those identified in the ctDNA. Additionally, an inversion of *PKNOX2* and *FLI1*, an intronic inversion of *FLI1*, and an *EWSR1-FLI1* translocation, were detected by WGS (Figure 2B and C). Recent findings show that 40% of EWS-ETS fusions are generated via chromoplexy rearrangements (44). These complex “loop” rearrangements are caused by chain-like events that start at *EWSR1* and end with the ETS partner but involve several intervening genes. The *EWSR1-PKNOX2* translocation identified in this case likely represents an example of such a complex, loop-like rearrangement. Because the breakpoint for the *EWSR1-FLI1* rearrangement occurs within the inverted *FLI1* intron, we may have been unable to detect this *EWSR1-FLI1* translocation in the cfDNA (Figure 2D). Tumors harboring chromoplexy are associated with more aggressive disease and may have contributed to this patient’s relapse. Similarly, we detected an *EWSR1-CBLN4* translocation at an AF of 0.31% in the ctDNA of patient 361. Tumor
WGS suggests that this patient may also have a complex loop rearrangement involving *EWSR1, CBLN4, and FLI1* (Figure 2E and F). To our knowledge, these complex chromoplectic events have not been previously identified in plasma and represent a potentially clinically useful aspect of the assay described here.

**ctDNA levels correlate with clinical features**

We sought to determine whether pre-treatment ctDNA levels were correlated with clinical features. Patients with lymph node involvement or distant metastatic disease had higher absolute pre-treatment ctDNA levels (mean AF= 10.6%, median AF = 11.95%) compared to patients with localized disease (mean AF = 2.2%, median AF = 0.45%) (Figure 3A). We also evaluated pre-treatment ctDNA levels to determine their potential prognostic value. We defined a “ctDNA low” and “ctDNA high” group, with the cutoff determined by the median pre-treatment ctDNA level of 4.85% across our cohort of patients. Pre-treatment ctDNA levels did not correlate with risk of relapse (Supplementary Figure 1A). However, patients in the “ctDNA high” group had a significantly increased risk of disease-related death (HR 7.2, P = 0.0245) (Supplementary Figure 1B), though this may be attributable to metastatic status, as all patients in the “ctDNA high” group had metastatic disease.

**Utility of ctDNA for longitudinal analysis in sarcomas**

For the subset of patients with longitudinal samples, ctDNA levels were analyzed and compared to clinical status and imaging studies. Although pre-treatment ctDNA levels did not correlate with primary tumor volume at presentation (Supplementary Figure 1C), we observed a decline in ctDNA levels after initiation of therapy in all patients, in concordance with their clinical response to therapy (Supplementary Table
S5). One patient (Figure 3E) presented with relapsed alveolar rhabdomyosarcoma and multiple distant metastases. We detected a $PAX3$-$FOXO1$ translocation at an AF of 22%. After surgical excision of a lower extremity tumor, systemic chemotherapy, and radiation therapy to a metastatic site, ctDNA levels fell and were no longer detectable within 4 months, in concordance with radiologic findings. ctDNA remained undetectable and the patient remains in remission at last follow-up. Similar sustained declines in ctDNA levels after initiation of treatment were seen in Ewing sarcoma and osteosarcoma (Figure 3C, Figure 3D, and Supplementary Table S5).

cDNA can be detected by CAPP-Seq prior to clinically evident relapse

cDNA was measurable at AFs as low as 0.003%, demonstrating the ultra-sensitive detection capabilities of CAPP-Seq. Patient 311 had an $EWSR1$-$FLI1$ translocation detected in the pre-treatment plasma at an AF of 1.9%. The patient underwent surgical resection with positive margins and had detectable ctDNA post-operatively. He received radiation therapy at his positive margins and was considered to be in remission, however, his ctDNA levels remained elevated throughout his treatment. Plasma samples were obtained during off-therapy surveillance visits and ctDNA levels persisted (Figure 4A). The patient ultimately presented with pulmonary relapse 6 months after completing therapy. The patient with a $PAX3$-$NCOA1$ translocation had ctDNA detected at a plasma AF of 13.2% at diagnosis. ctDNA levels initially declined to undetectable with chemotherapy, but rose to 0.008% 118 days after the pre-treatment blood draw (Figure 4B). The patient experienced clinical relapse 176 days later and ultimately died of disease.

**Copy number alterations detected using CAPP-Seq**
Seven patients had copy number changes that were detectable in the plasma using off-target reads from CAPP-Seq (Figure 5A-G). Four of these patients had tumor specimen available for copy number analysis with CAPP-Seq (Figure 5A-D) and 3 of the 4 patients also had tumor available for copy number analysis by WGS (Supplementary Figure S2). Copy number analysis of the tumor using off-target reads was concordant with WGS and copy number changes detected in the plasma were concordant with those found in the tumor (Supplementary Figure S4). Two Ewing sarcoma patients had gains in chromosomes 8 and 12 identified in their plasma. These CNAs have been previously described in Ewing sarcoma, with chromosome 8 gains being the most common (45,46). These copy number changes have also been shown to be co-associated, but do not necessarily bear a prognostic significance (46). Gain of 1q, which has been associated with poor survival (45,46), was identified in a relapsed Ewing sarcoma patient. CNAs were also identified in the plasma of 2 rhabdomyosarcoma patients. Widespread copy number changes were identified in 2 osteosarcoma patients, including a gain in chromosome 8, which harbors MYC. It has previously been reported that MYC-amplified osteosarcoma is sensitive to targeted treatment with AT7519. While 6 of the patients with detectable copy number changes also had translocations identified in their plasma, 1 of the osteosarcoma patients (387) did not have a TP53 rearrangement detected by sequencing of the tumor or plasma.

DISCUSSION

A major challenge in the clinical care of pediatric sarcoma patients is the lack of sensitive, non-invasive methods to accurately diagnose and monitor disease. Currently, biopsy serves as the gold standard for diagnosis, but tumor purity, tumor heterogeneity,
and inadequate sampling can limit detection of somatic variants. Tumor specimens often require sophisticated molecular assays for gene rearrangement detection in order to make a definitive diagnosis. However, there is variability in the sensitivity and specificity of these molecular assays. Identification of these translocations is important for diagnosis and can also be relevant for prognosis. An even larger challenge is finding highly sensitive and specific biomarkers to monitor disease response.

To address these issues, we designed and tested a next-generation sequencing assay for detection of recurrent translocations and CNAs in the plasma of pediatric sarcoma patients. A key goal was to develop an “off the shelf” assay that could be rapidly applied to all patients without requiring primary tumor to be available for analysis. The CAPP-Seq selector tiles across the intronic regions most commonly involved in translocation events. While initially designed to detect a subset of key translocations found in pediatric sarcomas, this assay could easily be expanded to include other less common translocations relevant to the diagnosis and clinical care of pediatric solid tumors. With the current assay, we detected recurrent translocations in the cfDNA of patients with Ewing sarcoma, rhabdomyosarcoma, and osteosarcoma, with an overall sensitivity of 81% for translocation detection. We were able to detect a prognostically important PAX3-NCOA1 translocation that was not detected by standard FISH testing in a rhabdomyosarcoma patient who was presumed to have fusion-negative disease. Furthermore, in two Ewing sarcoma patients, we detected EWSR1 structural variants that may represent components of complex chromosomal loop rearrangements, which are associated with more aggressive disease. Additionally, by leveraging off-target reads from the hybrid capture, we were able to obtain low-depth
coverage across the genome and detect copy number changes in the cfDNA of a subset of Ewing sarcoma, rhabdomyosarcoma, and osteosarcoma patients. One of these patients did not have a detectable translocation in the tumor, demonstrating that the added ability to detect copy number changes can increase our overall sensitivity in detecting ctDNA. Although these copy number changes are not diagnostic, they are of clinical significance, as they can have prognostic and therapeutic relevance. Utilizing a single assay that detects translocations and copy number changes may be beneficial, especially when implementing such assays in the clinical setting, where the costs of specimen preparation, sequencing, and data analysis, as well as turn-around-time, are paramount considerations.

The CAPP-Seq selector used here did not detect expected translocations in the cfDNA of three evaluated patients. For one patient, the SSX1-SS18 translocation was detected by CAPP-seq in the primary tumor, demonstrating that the translocation breakpoint region is detectable with our assay. The inability to detect this translocation in plasma could be due to low tumor burden. We were unable to detect the pre-treatment EWSR1 rearrangements in 2 Ewing sarcoma patients for unclear reasons. Additional studies will be needed to define the sensitivity of our assay in a larger patient population.

Our results suggest that pre-treatment ctDNA levels detected by CAPP-Seq could be used to determine the likelihood of lung metastases in cases deemed indeterminate by imaging alone. ctDNA levels also correlated with clinical response to therapy, demonstrating its utility as a biomarker of disease. Importantly, our assay detected ctDNA several months before relapse was clinically detectable by imaging in
Ewing sarcoma and alveolar rhabdomyosarcoma.

In summary, we have developed an ultra-sensitive, “off the shelf” ctDNA assay that can simultaneously detect a wide variety of pediatric sarcoma translocations and CNAs. This can aid in diagnosis, monitoring, and early detection of relapse. This assay will need to be evaluated in a larger cohort of pediatric cancer patients and, in particular, those with multiple plasma samples available across their disease course.
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REFERENCES


7. Arnold MA, Anderson JR, Gastier-Foster JM, Barr FG, Skapek SX, Hawkins DS,
et al. Histology, Fusion Status, and Outcome in Alveolar Rhabdomyosarcoma
With Low-Risk Clinical Features: A Report From the Children’s Oncology Group.


EWSR1 Fusion-Positive Sarcomas. JCO Precis Oncol. 2017;1–11.


TABLES

Table 1. Intronic regions included in the CAPP-Seq selector. EWS – Ewing sarcoma; RMS – Rhabdomyosarcoma; OS – Osteosarcoma; SS – Synovial sarcoma.

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<td>SSX4</td>
<td>1, 4</td>
<td>2,333</td>
<td>SS</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td><strong>150,602</strong></td>
<td></td>
</tr>
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FIGURE LEGENDS

Figure 1. Sample collection schema and samples analyzed. A) All patients had plasma samples collected prior to initiation of treatment. When feasible, additional samples were obtained over the course of a patient’s therapy and during off-therapy surveillance. B) Summary of plasma samples analyzed. Number of samples indicated if more than one sample was available for analysis. A subset of patients also had tumor specimens available for analysis using WGS and/or CAPP-Seq. RMS – Rhabdomyosarcoma; EWS – Ewing sarcoma; OS – Osteosarcoma; SS – Synovial sarcoma.

Figure 2. Translocations detected in pre-treatment ctDNA. A) Oncoprint of pre-treatment ctDNA translocations. Each column represents a specific patient. A bar plot at the top details the allelic frequency (AF) of a given translocation. Key clinical information is depicted below. B) Circos plot for patient 005. Copy number changes are depicted in the outermost circle. Structural alterations are shown in the inner portion of the plot (blue – deletion, red – duplication, black – translocation, purple – inversion) and the weight of the line corresponds to the number of supporting reads. C) Circos plot focusing on rearrangements that form a loop for same sample as in B. D) Schematic showing EWSR1-FLI1 fusion generated from complex loop rearrangement. E) Circos plot for patient 361. F) Circos plot focusing on looped rearrangements for same sample as in E. INV – Inversion.

Figure 3. ctDNA levels correlate with clinical features. A) Metastatic patients (n=10) had higher levels of pre-treatment ctDNA compared to non-metastatic patients (n=6). P-value = 0.0224 (calculated by the Mann-Whitney U test). Serial ctDNA levels
detected by CAPP-Seq reflect response to therapy in B) osteosarcoma, C) Ewing sarcoma, and D) alveolar rhabdomyosarcoma. ND – Not detected; M – Methotrexate; A – Adriamycin; P – Cisplatinum; V – Vincristine; D – Doxorubicin; C – Cyclophosphamide; I – Ifosfamide; E – Etoposide; XRT – Radiation therapy.

**Figure 4.** CAPP-Seq can detect ctDNA before relapse is clinically evident in pediatric sarcoma patients. A) Example of a patient with detectable pre-treatment ctDNA that remained elevated after initiation of therapy. The patient ultimately relapsed 6 months after completion of therapy. B) Example of a patient with detectable pre-treatment ctDNA who experienced an initial decline after starting chemotherapy. ctDNA was detected again 1 month later and remained elevated. The patient ultimately relapsed and died of disease.

**Figure 5.** A-G Copy number alterations can be detected utilizing off-target CAPP-Seq reads in the plasma of Ewing sarcoma, rhabdomyosarcoma, and osteosarcoma. These findings are confirmed by CAPP-Seq of the tumor in a subset of patients (right side of A-D).
Figure 3

A

B

C

D

Patient 364

Patient 361

Patient 042

E

Non-Metastatic Metastatic

Metastatic Status

0 100 200 300 400 500

0.001

0.01

0.1

Tumor Volume (cm³)

Time (Days)

Allelic Frequency (%)

ND

HR = 7.177
P = 0.0245

N = 8

N = 8

Overall survival (%)

ctDNA low
tctDNA high

Resection

Diagnostic Scan

Scan #2 (106 days)

Scan #3 (200 days)

Diagnostic Scan

Scan #2 (93 days)

Resection

nd

EWSR1-CBLN4

Tumor Volume

Time (Days)

Allelic Frequency (%)

ND

PAX3-FOXO1

Tumor Volume (cm³)

Time (Days)

Allelic Frequency (%)

ND

Resection

XRT 50.4 Gy to fibula

XRT 45 Gy to mediastinum

XRT 40 Gy to thigh

Resection

XRT 50.4 Gy to fibula

XRT 45 Gy to mediastinum

XRT 40 Gy to thigh

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Figure 5
A comprehensive circulating tumor DNA assay for detection of translocation and copy number changes in pediatric sarcomas


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