Undifferentiated Sarcomas in Children Harbor Clinically Relevant Oncogenic Fusions and Gene Copy-Number Alterations: A Report from the Children's Oncology Group

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Abstract

Purpose: A comprehensive analysis of the genomics of undifferentiated sarcomas (UDS) is lacking. We analyzed copy-number alterations and fusion status in patients with UDS prospectively treated on Children’s Oncology Group protocol ARST0332.

Experimental Design: Copy-number alterations were assessed by OncoScan FFPE Express on 32 UDS. Whole-exome and transcriptome libraries from eight tumors with sufficient archived material were sequenced on HiSeq (2 × 100 bp). Targeted RNA-sequencing using Archer chemistry was performed on two additional cases.

Results: Five-year overall survival for patients with UDS was 83% (95% CI, 69%–97%) with risk-adapted therapy (surgery, chemotherapy, and radiotherapy). Both focal and arm-level copy-number alterations were common including gain of 1q (8/32, 25%) and loss of 1p (7/32, 22%), both of which occurred more often in clinically defined high-risk tumors. Tumors with both loss of 1p and gain of 1q carried an especially poor prognosis with a 5-year event-free survival of 20%. GISTIC analysis identified recurrent amplification of FGF1 on 5q31.3 (q = 0.03) and loss of CDKN2A and CDKN2B on 9p21.3 (q = 0.07). Known oncogenic fusions were identified in eight of 10 cases analyzed by next-generation sequencing.

Conclusions: Pediatric UDS generally has a good outcome with risk-adapted therapy. A high-risk subset of patients whose tumors have copy-number loss of 1p and gain of 1q was identified with only 20% survival. Oncogenic fusions are common in UDS, and next-generation sequencing should be considered for children with UDS to refine the diagnosis and identify potentially targetable drivers. Clin Cancer Res; 1–10. ©2018 AACR.

Introduction

Undifferentiated sarcoma is defined in the most recent 2013 World Health Organization (WHO) classification as soft-tissue sarcoma with no identifiable line of differentiation when analyzed by available technology, which may include immunophenotypic and molecular genetic evaluation in addition to standard histomorphology (1, 2). These tumors comprise a heterogeneous group that has been subclassified by the WHO system into morphologic subtypes of spindle cell, pleomorphic, round cell, and epithelioid tumor variants (1). At the time of publication of the 2013 WHO classification of soft-tissue tumors, information was beginning to emerge about potential genetic and prognostic subtypes of undifferentiated sarcomas in addition to the morphologic categories.

Understanding the biology of undifferentiated sarcoma in children may help standardize and improve clinical care. For example, the former Intergroup Rhabdomyosarcoma Study Group (IRSG) included children with undifferentiated sarcoma on clinical trials designed for children with rhabdomyosarcoma, and that group showed outcome for those with localized undifferentiated sarcoma was worse than for those with rhabdomyosarcoma (3). However, subsequent publications suggested that undifferentiated sarcomas in young patients might separate into subsets with more favorable or less favorable outcomes (4–6). Some CD99-staining round cell sarcomas lacking an EWS–FLI1 fusion have been considered to be Ewing-like sarcoma and treated on Ewing sarcoma protocols, while other patients were treated on protocols for nonrhabdomyosarcoma soft-tissue sarcoma (NRSTS; ref. 7). Previous Children’s Oncology Group (COG) Ewing sarcoma protocols did not require confirmation of an EWSR1–FLI1 fusion or any of the less commonly recognized Ewing sarcoma variant fusions (e.g., fusion of EWSR1 with another ETS family member or involvement of FUS rather than EWSR1) for eligibility, and thus these studies likely enrolled some
patients with Ewing-like or round cell undifferentiated sarcoma (8). However, these patients have not been separately analyzed. The recent COG ARST0332 study (NCT00346164) enrolled children with NRSTS, including undifferentiable soft-tissue sarcomas, a subset of which were undifferentiated sarcomas (9, 10). ARST0332 attempted to standardize therapy in a risk-based design using histologic grade (11), tumor size, extent of resection, and presence or absence of metastases; notably, morphologic features of undifferentiated sarcoma were not utilized in treatment assignment. Because of the standardized treatment approaches, this recently completed study provides a valuable opportunity to better understand clinical and biological factors that could predict outcome.

If the undifferentiated sarcomas as defined in the 2013 WHO classification represent a heterogeneous group of mesenchymal tumors, then recently emerged next-generation DNA and RNA sequencing analyses hold the potential to clarify the underlying biology of individual undifferentiated sarcomas, although this potential has not yet been realized. Indeed, what is known comes from small series in which limited sets of genes are interrogated and, from isolated case reports. For example, chromosome 8 gains, identified by fluorescence in situ hybridization (FISH), have been observed in a series of 11 patients with undifferentiated sarcoma (12). Recently, BCOR–CCNB3 fusions and fusions of CIC with either DUX4 or its paralog DUX4L10 have been found in Ewing-like and undifferentiated round cell sarcomas (7, 13, 14). The same fusions have also been identified in undifferentiated sarcomas with spindled and epithelioid cells (15, 16). In individual case reports and small series of undifferentiated sarcoma, a number of other fusion genes have been described, including PRDM10 (17), MLL–GPS2 (18), BCOR (19, 20), YWHAE–NUTM2B (19), and CIC with genes other than DUX4 (21).

Recognizing limitations inherent in isolated case reports or narrowly focused analyses, we have undertaken a larger analysis of ARST0332 study subjects with undifferentiated sarcoma. We focused on the morphologic characteristics and DNA copy-number in all of those cases, and we performed next-generation DNA and RNA sequencing on a subset with available material. To our knowledge, this is the largest multifaceted clinical, pathologic, and molecular analysis of undifferentiated sarcoma in children. Although we report a favorable outcome for the majority of these patients, we also identify poor outcomes for those with copy-number changes involving chromosome 1, and very frequent oncogenic fusions in the subset that were analyzed by next-generation sequencing.

Materials and Methods
Specimens
ARST0332 was a prospective nonrandomized therapeutic protocol that enrolled children and young adults up to 30 years of age with newly diagnosed NRSTS. All subjects enrolled on ARST0332 provided written informed consent prior to enrollment including consent for research into molecular factors correlating with outcome, and the protocol was approved by the institutional review board at all participating institutions. The clinical trial was conducted in accordance with the Declaration of Helsinki. On this study, two pediatric soft-tissue pathologists conducted rapid central review and determined the diagnosis using the 2002 WHO classification system for soft-tissue tumors (22) and Pediatric Oncology Group (POG) grade based on available slides and pathology reports from the originating institutions (9, 11). In ARST0332, the central pathology review classification included a category of “undifferentiated soft-tissue sarcoma,” which comprised a total of 94 cases. For the present report, two pathologists (J.O. Black and C.M. Coffin) re-reviewed all available H&E slides of the undifferentiated soft-tissue sarcoma specimens to distinguish truly undifferentiated sarcomas that were extensively investigated from tumors that simply could not be classified further due to insufficient materials or information. Pleomorphic undifferentiated sarcomas were categorized separately in ARST0332 and are not included in the present analysis. Following the publication of the 2013 WHO classification system, the same two pathologists (J.O. Black and C.M. Coffin) re-reviewed all available H&E slides of the undifferentiated sarcomas to divide these into morphologic subtypes per WHO 2013 criteria. Spindle cell tumors were defined by elongated tumor cells with a fasiccular pattern and tapered nuclei. Round cell tumors were relatively uniform, with rounded to ovoid cells showing minimal cytoplasm, resembling Ewing sarcoma. Epithelioid tumors contained polygonal cells with moderate cytoplasmic volume, vesicular nuclei, prominent cell borders, and variably prominent nucleoli. Tumors that were undifferentiated but contained multiple patterns, or otherwise precluded categorization within a single subtype, were classified as undifferentiated sarcoma, not otherwise specified (NOS), as designated in the 2013 WHO classification.

Treatment
Risk-based treatment on ARST0332 was stratified into three groups, as follows: (i) Those with a localized low-grade tumor and a gross total resection (GTR), or a localized high-grade tumor less than 5 cm in diameter with a complete resection and negative margins were observed. (ii) Those with a high-grade, nonmetastatic tumor less than 5 cm in diameter with microscopic positive margins were treated with 55.8 Gy adjuvant radiation (RT). (iii) All others, including those with high-grade tumor > 5 cm, unresectable primary tumor, or metastatic disease, received ifosfamide- and doxorubicin-based chemoradiotherapy (55.8 Gy adjuvant RT for upfront resection or 45 Gy neoadjuvant RT with a boost to 55.8 or 64.8 Gy depending on surgical margins at the time of delayed resection). Metastatic sites were treated surgically whenever possible, and with RT when surgery was not possible. Clinical data used in this analysis were current as of September 20, 2017. The overall results of ARST0332 will be reported elsewhere.

Copy-number alteration analysis
DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) specimens in 25 cases (23 cases with five 4- to 6-mm-thick slides and two cases with two 10-µm scrolls) and from frozen specimens embedded in optimal cutting temperature (OCT) compound in seven cases (each with one 60-µm scroll) using the DNeasy Blood and Tissue Kit (Qiagen). Purified DNA was quantified by PicoGreen (Thermo Fisher) and submitted for OncoScan FFPE Express service at Affymetrix.

Data processing and analysis of the SNP array data were performed using Nexus Express Software for OncoScan (Biodiscovery) with reference to human genome assembly GRCh37/hg19. The quality of SNP array results for analysis was evaluated by Nexus sample Quality score (the Quality score is based on measurements of the noise level of the data, percent aberrant cells, and overall ploidy, as well as copy-number events and percent loss of...
heterozygosity [LOH] for each sample). The lower the QC score, the better quality the sample is. A total of 28 samples showed good quality for analysis (quality score <1); 3 specimens had a quality score between 1 and 2, and 1 had a quality score of >2. The default average parameter setting of Nexus was used for analysis. In addition, active annotation tracks integrated within Nexus or hyperlinked to a web-based resource were selected to provide additional information in the analysis [e.g., Sanger Cancer Gene Census list, Database of Genomic Variants (DGV) in Toronto, and Decipher database information, NCBI Gene, among others].

**GISTIC analysis**

The significance of copy-number variation was examined by the Genomic Identification of Significant Targets in Cancer (GISTIC) method as described previously (23). A custom script written in PERL programming language was integrated into the original GISTIC scripts to allow GISTIC to adapt to OncoScan data format. The standard \( q \) value cutoff of 0.25 was used to define significant regions of recurrent copy-number variation (23).

**Whole-exome sequencing**

Whole-exome sequencing (WES) was performed on specimens with available paired germline specimens as described previously (24). Briefly, WES libraries were enriched using a NimbleGen SeqCap EZ HGSC whole-exome VCRome Kit v2.1 probe set designed at the Human Genome Sequencing Center at Baylor College of Medicine (25) and available commercially (Roche Inc.). The VCRome probe set covers 23,585 genes and 189,028 nonoverlapping exons of the human hg19 exome (July 2014 Ensembl annotation) and is used for enrichment of the protein coding exome. Following enrichment, pooled libraries were sequenced in paired-end mode (2 \( \times \) 100-bp reads) on a HiSeq 2500 platform (Illumina Inc.). Sequencing runs generated approximately 300 to 400 million reads on each flow cell lane, yielding a range of 6 to 11 Gb. For cancer exomes, expected average coverage is \( >200 \times \). Data analysis including somatic variant calling was done using the HGSC Mercury pipeline v.3 (26), with variant calls made at a variant allele fraction (VAF) of 0.05 with a minimum depth of coverage at 50 \( \times \).

**Transcriptome sequencing**

Transcriptome sequencing was performed for fusion gene detection using three different protocols based on specimen type and RNA quality. For high-quality total RNA (RIN \( > 7 \)) from fresh-frozen tissue, whole-transcriptome RNA sequencing (RNA-seq) was performed using a strand-specific, poly-A\(^+\) RNA-seq protocol as described previously (7). Briefly, poly-A\(^+\) mRNA was extracted from 1 \( \mu \)g total RNA, followed by fragmentation, first-strand cDNA synthesis, end-repair, A-tailing and ligation and sequenced in paired-end mode (2 \( \times \) 100-bp reads) on a HiSeq 2500 platform (Illumina Inc.). For RNA of lower quality extracted from FFPE specimens, a modified exome-capture targeted transcriptome sequencing (CTS) protocol was used. Using the CTS protocol, 40 ng of total RNA was processed for stranded RNA-seq library preparation as before but without any further poly-A\(^+\) selection or fragmentation. CTS libraries then underwent capture-based target enrichment using the same SeqCap EZ HGSC whole-exome VCRome Kit v2.1 probe set as used for WES. Two independent fusion calling algorithms, deFuse and SOAPfuse, were used to analyze the RNAseq data, and only fusions identified by both algorithms were considered confirmed. For two cases with extremely low quality of RNA that failed CTS, an amplicon-based targeted RNA-seq approach, Archer FusionPlex Sarcoma Kit (ArcherDx Inc.), was used to generate libraries following the manufacturer's protocol and analyzed using Archer Analysis pipeline for fusion calling.

**Statistical analysis of clinical cohort**

Outcome analyses of event-free and overall survival were performed based on the Kaplan–Meier method (27). The log rank test was used to make comparisons (28). Statistical significance was considered at the 0.05 level. Statistical analyses were completed using SAS 9.4 (SAS Institute Inc.). The September 30, 2017, data freeze was used.

**Results**

**Clinical cohort**

Of the 94 patients with unclassified soft-tissue sarcoma enrolled on ARST0332, 48 had tumors that met criteria for undifferentiated sarcoma, of which 44 had H&E slides available for re-review and classification according to WHO 2013. There was sufficient tumor tissue available for molecular analysis for 32 of these subjects who form the cohort analyzed here (Fig. 1). For these 32 patients, the median age at enrollment was 13.6 years (range, 0.6–29.8 years) and 21 (66%) were male (Table 1). Metastatic disease was present in five of 32 patients (16%) at study entry. The 5-year event-free and overall survival for this cohort were 71% (95% CI, 54%–87%) and 83% (95% CI, 69%–97%), respectively (Fig. 2A).

Nine children (28%) had low-risk disease that had been grossly excised and were observed \((n = 8)\) or treated with radiotherapy alone \((n = 1)\). With a median of 5.2 years of follow-up in this cohort, the 5-year event-free survival was 65% (95% CI, 27%–100%; Fig. 2B). However, the 5-year overall survival was 100%, suggesting that those patients with recurrent low risk disease were salvageable with additional therapy. The remaining 23 study subjects had either high-grade tumors >5 cm in diameter, unresectable tumor, or metastatic disease. They were classified as intermediate- or high-risk based on the presence or absence of metastases and were treated with chemoradiotherapy, with delayed surgery when feasible for those with gross disease at study entry. At a median of 5.7 years of follow-up in this cohort, the 5-year event-free survival was 73% (95% CI, 55%–92%) with an overall survival of 77% (95% CI, 60%–95%; Fig. 2C).

**Histologic classification of cases**

Among the 32 samples with tumor available for molecular analysis, 5 tumors were classified as epithelioid, 13 as round cell, 12 as spindle cell, and two as undifferentiated sarcoma, NOS, according to the 2013 WHO criteria (Supplementary Fig. S1). This distribution of morphology was similar to the entire cohort of patients with undifferentiated sarcoma who had slides available for classification according to WHO 2013. Of these 44 tumors, 10 were epithelioid, 20 were round cell, 12 were spindle cell, and two were classified as undifferentiated sarcoma, NOS. The morphologic subtype was assigned according to the dominant pattern, but notably, many tumors showed a mixture of patterns. All but two patients had POG grade 3 (high grade) tumors (11). There was a nonstatistically significant trend toward lower event-free and overall survival in patients with spindle cell morphology \((P > 0.16; \text{Fig. 2D and E)}\).
Chromosome and arm-level copy-number gains/losses are common, and 1p loss is associated with poor outcomes

SNP array data on the 32 cases with archival tissue available were analyzed with Nexus Express to identify recurrent copy-number alterations (CNA). CNAs were common across the cohort of tumors, with a mean of 120 CNAs per tumor (range, 6–601). The most common whole chromosome or chromosome arm-level CNAs were loss of 1p (8/32 cases, 25%), gain of 1q (8/32 cases, 25%), gain of chromosome 8 (8/32 cases, 25%), and gain of chromosome 2 (5/32 cases, 16%; Fig. 3). Each of these alterations was more common in patients classified as intermediate or high risk: 1p loss occurred in one of nine (11%) low-risk patients versus six of 23 (23%) high-risk patients, 1q gain occurred in 1 of 9 (11%) low-risk patients versus seven of 23 (30%) high-risk patients, gain of chromosome 2 occurred in 1 of 9 (11%) low-risk patients versus four of 23 (17%) high-risk patients, and gain of chromosome 8 occurred in no low-risk patients versus eight of 23 (35%) high-risk patients. Separating tumors by the primary morphology, we found no significant difference in the frequency of these alterations between the cases with round cell (n = 13) and spindle cell (n = 12) morphology (P > 0.05). The epithelioid undifferentiated sarcomas were not separately analyzed due to the small number of cases (n = 5).

There were strong associations between loss of chromosome 1p or gain of chromosome 1q and outcome. Individuals with loss of 1p had a trend toward worse event-free survival (P = 0.07) and significantly worse overall survival (P < 0.001) than those without (Fig. 4A). Similarly, patients with gain of 1q had worse event-free survival (P = 0.02) and overall survival (P = 0.003) than those without (Fig. 4B). Loss of chromosome 1p and gain of chromosome 1q frequently co-occurred, and those with both alterations had markedly worse event-free (20% at 5 years, P = 0.004) and overall survival (20% at 5 years, P < 0.0001) than those without both alterations (Fig. 4E). While all of the individuals with 1p loss and 1q gain were prospectively classified as intermediate or high risk by clinical features, only one of the four who died of disease had metastatic disease at enrollment, suggesting that these alterations may further refine risk classification in intermediate-risk patients. While patients with gain of chromosome 8 had worse overall

Table 1. Characteristics of the 32 undifferentiated sarcoma cases evaluated, including the predominant morphologic pattern as determined by central re-review per WHO 2013 criteria

<table>
<thead>
<tr>
<th>Total</th>
<th>N = 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.6–29.8 years</td>
</tr>
<tr>
<td></td>
<td>Median</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>21 (66%)</td>
</tr>
<tr>
<td>Female</td>
<td>11 (34%)</td>
</tr>
<tr>
<td>Site</td>
<td></td>
</tr>
<tr>
<td>Paraspinal</td>
<td>8 (25%)</td>
</tr>
<tr>
<td>Extremity</td>
<td>7 (22%)</td>
</tr>
<tr>
<td>Abdominal wall</td>
<td>3 (9%)</td>
</tr>
<tr>
<td>Head/neck</td>
<td>3 (9%)</td>
</tr>
<tr>
<td>Pelvis</td>
<td>3 (9%)</td>
</tr>
<tr>
<td>Chest wall</td>
<td>2 (9%)</td>
</tr>
<tr>
<td>Intraabdominal</td>
<td>2 (6%)</td>
</tr>
<tr>
<td>Intrathoracic</td>
<td>2 (6%)</td>
</tr>
<tr>
<td>Retroperitoneal</td>
<td>2 (6%)</td>
</tr>
<tr>
<td>Extent of disease at study entry</td>
<td></td>
</tr>
<tr>
<td>Localized</td>
<td>27 (84%)</td>
</tr>
<tr>
<td>Metastatic</td>
<td>5 (16%)</td>
</tr>
<tr>
<td>ARST0332 risk group</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>9 (28%)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>18 (56%)</td>
</tr>
<tr>
<td>High</td>
<td>5 (16%)</td>
</tr>
<tr>
<td>Predominant histology</td>
<td></td>
</tr>
<tr>
<td>Round cell</td>
<td>13 (41%)</td>
</tr>
<tr>
<td>Spindle cell</td>
<td>12 (38%)</td>
</tr>
<tr>
<td>Epithelioid</td>
<td>5 (16%)</td>
</tr>
<tr>
<td>NOS</td>
<td>2 (6%)</td>
</tr>
</tbody>
</table>

Figure 1. Consort flow diagram. The 32 patients with undifferentiated sarcoma enrolled on ARST0332 with slides available for copy-number analysis were analyzed here.
survival \((P = 0.04)\), there were no other associations with outcome in patients with gain of chromosome 2 or 8 (Fig. 4C and D).

**GISTIC analysis identifies recurrent focal CNAs**

Next, we performed a GISTIC analysis to investigate recurrent focal CNAs. Sixteen regions of recurrent copy-number gain and 13 regions of recurrent copy-number loss were identified with \(q < 0.25\) (Supplementary Fig. S2). They included recurrent amplification of \(FGF1\) on 5q31.3 \((q = 0.03)\) and loss of \(CDKN2A\) and \(CDKN2B\) on 9p21.3 \((q = 0.07)\). However, most events were not associated with classic oncogenes. Due to limited statistical power, the morphologic subsets were not separately analyzed.

![Figure 2.](image)

Event-free and overall survival for all 32 patients with undifferentiated sarcoma (A), event-free survival by risk group (B), and overall survival by risk group (C). Event-free (D) and overall survival (E) by morphologic subtype. Int, intermediate.

![Figure 3.](image)

Recurrent copy-number gains and losses in all tumors and in tumors classified as primarily round cell or spindle cell variants.
Potential oncogenic fusions are observed in the CNA data

Given prior reports of oncogenic fusions in sarcomas (7, 13–21), we sought to identify oncogenic fusions from the copy-number data. Other groups have identified oncogenic fusions by evaluating copy-number breakpoints within known fusion genes (29). The Nexus Express software was used to generate a list of copy-number breakpoints occurring within a gene. This list was manually reviewed along with the OncoScan data by an experienced molecular pathologist (J.A. Bridge) to generate a list of potential fusion partners. 

Four of the 14 events, Table 2. Of these, potential fusion partners were identified in two of nine cases and three of 14 events (21%), while no evidence of a classic fusion partner was detected in the remaining seven cases (11 events, 78%). Notably, in patient 4, SNP array analysis provided evidence for a potential oncogenic fusion involving the COL1A1 and PDGFRB genes, a fusion gene event characteristic of dermatofibrosarcoma protuberans (DFSP). After this potential fusion event was identified, re-review of an H&E slide from this case showed that this tumor was a spindle cell lesion with a storiform pattern similar to DFSP. However, it had been classified by central pathology review as an undifferentiated sarcoma prior to molecular testing. Also, a potential breakpoint in BCOR was identified in sample 27, and possible NTRK3 breakpoints were identified in samples 30 and 31.

NGS identifies frequent oncogenic fusions

Given the potential oncogenic fusions we identified using the CNA data, as well as recent published reports of other fusions identified in undifferentiated sarcomas (7, 13–21), we turned to next-generation sequencing approaches to more robustly investigate oncogenic fusions as well as point mutations/small indels that were not evaluable from the CNA data. Of the 32 cases, only eight yielded sufficient DNA/RNA for RNAseq and WES, while two additional tumors were analyzed for fusions using Archer chemistry.

NGS identified fusions classically associated with round cell sarcomas expressing CD99 (“Ewing-like”) in five of 10 cases (BCOR–CCNB3, n = 3; CIC–DUX4, n = 2). Highlighting the challenge of identifying potential fusions by morphologic subtype, of the five tumors harboring fusions that pediatric oncologists currently label “Ewing-like,” only two had round cell morphology, while two had spindle cell and one epithelioid morphology. Four of the five patients whose tumors harbored BCOR–CCNB3 or CIC–DUX4 fusions were classified as intermediate- or high-risk and treated with ifosfamide, doxorubicin, and radiation. Three of these four patients had localized disease and were alive 5 years after enrollment, although one of these individuals with a BCOR–CCNB3 fusion had a pulmonary recurrence. The fourth patient, with a CIC–DUX4 fusion, had metastatic tumor to the lungs at enrollment and died of disease. Importantly, one patient with a 3.7-cm pelvic tumor harboring a BCOR–CCNB3 fusion that was completely resected was observed on the

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Event-free survival (top) and overall survival (bottom) of patients with and without loss of 1p (A), gain of 1q (B), gain of chromosome 2 (C), gain of chromosome 8 (D), and both loss of 1p and gain of 1q (E).

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**Table 2.** Samples with potential oncogenic fusions identified by either SNP data or RNAseq

<table>
<thead>
<tr>
<th>Sample</th>
<th>Potential breakpoints identified in CNA data</th>
<th>Fusions identified by RNAseq/Archer</th>
<th>Mutations in known cancer genes by WES</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>COL1A1–PDGFRB; ALK–TPR</td>
<td>COL1A1–PDGFRB</td>
<td>None</td>
</tr>
<tr>
<td>8</td>
<td>PLAGL1–LYN</td>
<td>Not done</td>
<td>Not done</td>
</tr>
<tr>
<td>13</td>
<td>ETV6</td>
<td>Not done</td>
<td>Not done</td>
</tr>
<tr>
<td>23</td>
<td>None</td>
<td>EWSR1–COL1A1</td>
<td>Not done</td>
</tr>
<tr>
<td>24</td>
<td>None</td>
<td>CIC–DUX4</td>
<td>Not done</td>
</tr>
<tr>
<td>25</td>
<td>FUS–GRK5; CRYBB2; ZNF138</td>
<td>BCOR–CCNB3</td>
<td>None</td>
</tr>
<tr>
<td>26</td>
<td>FAM76B</td>
<td>BCOR–CCNB3</td>
<td>None</td>
</tr>
<tr>
<td>27</td>
<td>BCOR</td>
<td>BCOR–CCNB3</td>
<td>None</td>
</tr>
<tr>
<td>28</td>
<td>PLSCR4</td>
<td>CIC–DUX4</td>
<td>None</td>
</tr>
<tr>
<td>30</td>
<td>NTRK3</td>
<td>KIAA1549–BRAF</td>
<td>None</td>
</tr>
<tr>
<td>31</td>
<td>NTRK3</td>
<td>DCCER1 p.E1B33D; PDGFRB p.D842V; TP53 p.A138V</td>
<td>None</td>
</tr>
<tr>
<td>32</td>
<td>None</td>
<td>SAMDS–SASH1</td>
<td>None</td>
</tr>
</tbody>
</table>

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low-risk arm without chemotherapy or radiation and is alive without disease recurrence 8 years later.

In addition to these five fusions, NGS analysis confirmed the COL1A1–PDGFB fusion in sample 4 suggested by the CNA analysis, and identified a KIAA1549–BRAF fusion common in pilocytic astrocytomas (30), and an SDHD–SASH1 fusion associated with skull base chordoma (31) in one case each (Table 2). However, none of the other potential fusions identified using the CNA data were confirmed by RNAseq, suggesting either false positives in the CNA-based analysis or the presence of true translocations that are simply not detectably expressed. Thus, in total, eight of the 10 (80%) samples tested had recognized oncogenic fusions, of which only two (25%) were suggested by CNA analysis.

WES performed on eight tumors identified no known oncogenic point mutations in any of the fusion positive tumors. Interestingly, in a fusion-negative, undifferentiated sarcoma arising from the kidney (sample 31), WES identified an activating PDGFRA D842V mutation (32), a variant of uncertain significance in TP53 (A138V), and a DICER1 E1813D mutation that has been reported in anaplastic sarcoma of the kidney (Table 2; ref. 33). In summary, while overall numbers are small, a large fraction (90%) of undifferentiated sarcoma samples subjected to next-generation sequencing had either recognized oncogenic fusions or point mutations suggestive of a more specific diagnosis and, potentially, alternative therapeutic approaches.

Discussion

Our analysis of this prospective cohort of children with undifferentiated sarcoma, to our knowledge the largest yet assembled, clarifies elements of undifferentiated sarcoma tumor biology and reveals potential opportunities to improve care. We found no significant differences in clinical outcomes among the morphologic variants, instead identifying prognostic CNAs and a range of oncogenic fusions in the vast majority of cases. Our findings are consistent with the emerging hypothesis that undifferentiated sarcomas are not a single histologic entity, but demonstrate variable morphology that results from an extreme dedifferentiated state that can occur in many distinct tumor types (34). Studies in adult soft-tissue sarcomas have also recently shown a diversity of driver pathways associated with molecular subtypes and outcome (35). In particular, our data support the routine use of molecular profiling for patients with undifferentiated sarcoma, including DNA copy-number analyses and next-generation DNA/RNA sequencing, to increase diagnostic accuracy, identify alternative treatment approaches, including molecularly-targeted therapy, and gauge prognosis. Strengths of our analysis include the relatively large cohort with long follow-up (>5 years) in which all cases were subjected to central review of pathology and children were treated on a prospective protocol with standardized treatments and imaging-based response assessments.

Our first conclusion is that children with undifferentiated sarcoma had a favorable outcome of 83% 5-year overall survival when treated using risk-adapted multimodality therapy as prescribed in ARST0332. Children with clinically defined low-risk disease, who did not receive systemic therapy, had a 5-year event-free survival of 65% and a 100% 5-year overall survival. More specifically, individuals with small, grossly resected nonmetastatic undifferentiated sarcoma were observed without treatment, and adjuvant radiotherapy was only administered to those with high-grade tumors and microscopically positive margins. Our data indicate this to be an appropriate management strategy, sparing the majority of patients exposure to chemotherapy, while being able to salvage those in whom the tumor recurs.

The outcome in patients with intermediate- or high-risk undifferentiated sarcoma, based primarily on tumor size, histologic grade, extent of tumor resection, and presence of metastases was also generally favorable, with a 5-year overall survival of 77% achieved with chemoradiotherapy and surgery when feasible. However, we did identify a subset of patients, those with loss of chromosome 1p and gain of chromosome 1q, for whom outcomes were dismal with only a 20% 5-year overall survival. This finding is similar to that reported in Ewing sarcoma in which a recurrent secondary unbalanced translocation der(16)(1;16) is associated with gain of 1q material and poor outcome (36). Notably, in our series, we did not observe recurrent partial monosomy 16, which would be expected with this translocation, but identification of the nature of the loss of chromosome 1p and gain of chromosome 1q material observed here will be important in future studies.

In neuroblastoma, 1p loss is associated with high-risk features and decreased event-free survival (37). In that disease, 1p loss has been proposed to result in unfavorable outcomes by the loss of multiple tumor suppressors including CASZ1 and CHD5 (38, 39). We concede that the strength of our conclusions related to 1p and 1q in undifferentiated sarcoma is limited by the small size of our study of a rare sarcoma and also the fact that it represents a "convenience cohort," with potential pitfalls (40). Nevertheless, if the poor outcome that we identify for children with undifferentiated sarcoma and 1p loss, 1q gain, or both is confirmed in additional analyses, future clinical trials could include risk-adapted therapy based on these molecular features. Considering such changes in therapy for those molecularly defined groups would best be accomplished in the context of a prospective clinical trial for patients with undifferentiated sarcoma.

In general, the mechanisms by which large copy-number gains and losses contribute to cancer development or progression remain elusive. Gain of chromosome 8, as seen here, is the most common secondary numerical alteration found in fusion gene–driven sarcomas (41–43), as well as a broad spectrum of other cancers (44, 45). However, chromosome 8 contains more than 2,000 genes, and it is unclear which contribute to oncogenesis or unfavorable outcome. Proposed mechanisms have included increased expression of cMYC and FGFR, both located on chromosome 8 (44, 46). Interestingly, our GISTIC analysis finding focal, recurrent amplification of FGFR1, suggests that FGFR1–FGFR signaling may be important in undifferentiated sarcoma. However, further studies, including studies correlating copy-number changes and gene expression, are clearly needed. Obviously, it will also be important to understand how that signaling pathway or other chromosome 8 genes contribute to the activity of the sarcoma-associated fusion genes, which coexist in the majority of tumors.

Our report indicates the notable value of next-generation sequencing to reveal a more specific diagnosis and/or a potential targeted therapy when evaluated in the context of other pathologic findings. Of 10 cases analyzed, next-generation sequencing suggested a more specific diagnosis in seven: one with DFSP, three with BCOR–CCNB3 sarcoma, two with CIC–DUX4 sarcoma, and one with DICER1 mutation found in anaplastic sarcoma of the kidney (33). In addition to these seven patients, oncogenic fusions typically associated with other malignancies were found.
in two additional patients: a KIAA1549–BRAF fusion typical of pilocytic astrocytomas in an undifferentiated sarcoma of the neck and a SAMD5–SASH1 associated with skull base chordoma in a retroperitoneal tumor. Recent reports of other BRAF fusions in pediatric spindle cell sarcomas suggest that this was not an isolated finding (47). It is of interest that only two of the 14 potential oncogenic fusions identified by CNA analysis were confirmed by next-generation sequencing. While we cannot definitively consider these false positives, these data highlight the need for complimentary methods to both identify and confirm potential fusions especially as the unconfirmed NTRK3 fusions would have been considered highly actionable.

Next-generation sequencing identified potentially targetable molecular alterations in three cases, which could guide therapeutic choices for primary treatment or in the event of future tumor recurrence. The patient with undifferentiated sarcoma harboring a COL1A1–PDGFB fusion, characteristic of DFSP, died after bony recurrence of her tumor as previously described (48). Imitinib, a multikinase inhibitor that inhibits PDGFRB, is FDA approved for the treatment of DFSP. While this case might be regarded as DFSP with fibrosarcomatous transformation, ARST0332 central review prior to the availability of molecular testing results classified this an undifferentiated sarcoma, highlighting the challenges of classifying these tumors based on morphology. Similarly, second-generation BRAF inhibitors, which unlike first-generation BRAF inhibitors do not activate wild-type BRAF, have shown efficacy against the KIAA1549–BRAF fusion in preclinical studies and a published report highlights a patient with a malignant spindle cell tumor of the chest wall harboring this fusion whose tumor responded to the combination of sorafenib (which has BRAF inhibitory activity), temsirolimus, and bevacizumab (49, 50). Finally, the renal tumor harboring a DICER1 mutation also found to have an activating D842V mutation in PDGFRB. This mutation, found in about 5% of gastrointestinal stromal tumors, is resistant to inhibition by imatinib. However, preclinical studies demonstrate sensitivity to crenolanib, and a clinical trial of this agent in PDGFRB D842V-mutant GIST is ongoing (NCT02847429), further illustrating that careful tumor profiling may uncover potentially useful targeted therapies (32). Recent reports of exceptionally high response rates to TRK inhibitors across a diverse range of tumors harboring TRK fusions, including undifferentiated sarcoma, confirm the value of thorough genomically profiling of pediatric sarcomas (51, 52). Further, because DICER1 and other mutations found in sarcomas may occur in the germ-line, thorough genomic characterization of tumors may uncover underlying cancer predisposition syndromes.

It is notable that the five patients with Ewing-like sarcoma fusions identified here received treatment that is different than that typically used for Ewing sarcoma, including one patient who is in long-term remission following surgery alone without any systemic therapy. Due to the heterogeneity of molecular alterations seen in undifferentiated sarcoma, the small number of patients with each specific molecular alteration limits our ability to draw conclusions about the outcomes of this risk-adapted therapy in patients with specific oncogenic fusions. Other reports have identified worse outcomes in patients with specific alterations such as CIC-rearranged sarcomas (53). It will be important to assess fusion status in future studies of undifferentiated sarcomas and Ewing-like sarcomas to understand how the treatment of patients with these fusions fare with different types of therapy.

In summary, clinical next-generation sequencing should be considered for patients with intermediate-/high-risk or recurrent undifferentiated sarcoma to refine the diagnosis and suggest alternative therapeutic approaches. Future clinical trials should evaluate the use of CNAs to refine risk stratification in these children. The clinical trial from which these samples were obtained was designed in the early 2000s, when WES and RNA sequencing were not available. Our analysis using archived samples from this study highlights the value of prospective clinical trials linked to tumor tissue collection for rare tumors that allow and analysis of the predictive value of different biologic markers after standardized therapy. Ongoing banking of clinically annotated tumor samples using resources including the NCI-funded Translational Science Centers is critical.

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