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Genetic Characterization of Pediatric Sarcomas by Targeted RNA Sequencing

Matthew R. Avenarius,[*] Cecelia R. Miller,[*†] Michael A. Arnold,[†‡] Selene Koo,[†‡] Ryan Roberts,[§] Martin Hobby,[*] Thomas Grossman,[*] Yvonne Moyer,[*] Richard K. Wilson,[*¶] Elaine R. Mardis,[*¶] Julie M. Gastier-Foster,[*¶] and Ruthann B. Pfau[*†]

From the Institute for Genomic Medicine and the Departments of Pathology[‡] and Hematology and Oncology,[§] Nationwide Children's Hospital, Columbus; and the Departments of Pathology[†] and Pediatrics,[¶] Wexner Medical Center, The Ohio State University, Columbus, Ohio*

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Address correspondence to Ruthann B. Pfau, Ph.D., Nationwide Children's Hospital, Institute for Genomic Medicine, 575 Children's Crossroad, Columbus, OH 43205. E-mail: ruthann.pfau@nationwidechildrens.org.

Somatic variants, primarily fusion genes and single-nucleotide variants (SNVs) or insertions/deletions (indels), are prevalent among sarcomas. In many cases, accurate diagnosis of these tumors incorporates genetic findings that may also carry prognostic or therapeutic significance. Using the anchored multiplex PCR–based FusionPlex system, a custom RNA sequencing panel was developed that simultaneously detects fusion genes, SNVs, and indels in 112 genes found to be recurrently mutated in solid tumors. Using this assay, a retrospective analysis was conducted to identify somatic variants that may have assisted with classifying a cohort of 90 previously uncharacterized primarily pediatric sarcoma specimens. In total, somatic variants were identified in 45.5% (41/90) of the samples tested, including 22 cases with fusion genes and 19 cases with SNVs or indels. In addition, two of these findings represent novel alterations: a *WHSC1L1/NCOA2* fusion and a novel in-frame deletion in the *NRAS* gene (NM_002524: c.174_176delAGC p.Ala59del). These sequencing results, taken in context with the available clinical data, indicate a potential change in the initial diagnosis, prognosis, or management in 27 of the 90 cases. This study presents a custom RNA sequencing assay that detects fusion genes and SNVs in tandem and has the ability to identify novel fusion partners. These features highlight the advantages associated with utilizing anchored multiplex PCR technology for the rapid and highly sensitive detection of somatic variants. *(J Mol Diagn 2020, 22: 1238–1245; https://doi.org/10.1016/j.jmoldx.2020.07.004)*

Sarcomas are a heterogeneous group of neoplasms derived from mesenchymal tissues, with >100 subtypes reported to date.[1] These tumors are broadly categorized as malignancies of the bone or soft tissue and typically manifest in muscle, adipose tissue, or cartilage. Although sarcomas account for approximately 1% of adult solid tumors, epidemiologic studies demonstrate that sarcomas represent approximately 21% of solid tumors in the pediatric population, revealing a clearly disproportionate distribution between the two populations.[2]

The diagnostic workup of sarcomas routinely relies on integrating patient history and physical examination findings with the characterization of biopsied material and imaging studies. The findings from these studies are evaluated and managed by a multidisciplinary team of experts to establish a diagnosis, evaluate for malignancy, and stage the disease. Ancillary techniques, such as immunohistochemistry, cytogenetics, and molecular genetic testing, may also be incorporated into the workup as adjunctive or confirmatory testing to further assist with the classification of tumors and to guide clinical management.[3,4]

The genetic characterization of sarcomas was established by early studies that identified recurrent abnormalities, primarily fusion genes, in association with some sarcoma subtypes.[5] For example, the *PAX/FOXO1* fusion is highly

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Current address of M.R.A., Department of Pathology, Wexner Medical Center, The Ohio State University, Columbus, OH; of M.A.A., Department of Pathology and Laboratory Medicine, Children's Hospital Colorado, Aurora, CO; of Y.M., Biopathology Center, Nationwide Children's Hospital, Columbus, OH; of J.M.G.-F., Department of Pediatrics, Baylor College of Medicine, Houston, TX.

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specific for alveolar rhabdomyosarcomas, whereas the *EWSR1/FLI1* fusion is found in Ewing sarcomas.[6,7] Most commonly, detection of fusion genes is performed by fluorescent *in situ* hybridization or RT-PCR.[8] These techniques are able to rapidly identify common translocations with a high degree of specificity and sensitivity, which makes them the currently favored method for fusion gene detection.[9] However, these techniques are limited in their scalability, they may require knowledge of both fusion partners (RT-PCR and locus-specific fluorescent *in situ* hybridization), and formalin-fixed, paraffin-embedded material is more challenging to test.[10] Therefore, certain cases may require alternative strategies, including next-generation sequencing–based panel testing.

In addition to technical limitations, other genetic factors, including alternative breakpoints, promiscuity with noncanonical fusion partners, or the presence of a less common fusion (eg, *FUS/ERG* or *FUS/FEV* in Ewing sarcoma), may also elude standard approaches, such as RT-PCR. In these atypical cases, massively parallel sequencing strategies, such as anchored multiplex PCR, an amplicon-based massively parallel sequencing strategy that enriches for a series of user-specified gene targets to identify fusion genes, single-nucleotide variants (SNVs), and insertions/deletions (indels), have been successfully used to identify a host of recurrent abnormalities in both clinical and research settings (Supplemental Figure S1).[11–15]

The present study aimed to retrospectively evaluate the clinical utility of a 112-gene custom FusionPlex RNA sequencing panel in sarcoma samples that were negative for the clinically ordered RT-PCR fusion gene testing. When available, clinical data were used in combination with the targeted sequencing results to infer diagnoses and assess whether the genetic results obtained may have had a clinical impact.

Materials and Methods

Specimen Selection and Institutional Review Board Study Approval

A cohort of 90 diagnostic samples was selected from a pool of 511 cases originally submitted between 2002 and 2017 for clinical RT-PCR fusion testing. The RT-PCR test menu included sarcoma-related gene fusions with consensus primers detecting common breakpoints for five types of primarily pediatric sarcomas: Ewing sarcoma (*EWSR1/FLI1*, *EWSR1/ERG*), rhabdomyosarcoma (*PAX3/FOXO1*, *PAX7/FOXO1*), desmoplastic small round cell tumor (*EWSR1/WT1*), synovial sarcoma (*SS18/SSX1*, *SS18/SSX2*), and infantile fibrosarcoma and congenital mesoblastic nephroma (*ETV6/NTRK3*). Of the 511 samples, the selection criteria considered only samples that tested negative for the clinically ordered RT-PCR fusion gene(s) and had a sufficient amount of residual RNA to permit up to two library preparations plus potential orthogonal RT-PCR confirmation of any newly detected somatic variants. To ensure a breadth of samples, further inclusion criteria were stipulated on the basis of the number of clinically ordered RT-PCR fusion gene(s) ordered (ie, single fusion, a subset of two to four fusions, or the entire sarcoma fusion panel). In total, 90 samples were chosen for targeted sequencing.

Clinical information, submitted at the time the sample was originally accessioned for clinical RT-PCR fusion gene testing, was curated. This may have included patient age, sex, RT-PCR fusion testing ordered, indication for study/referring diagnosis, pathologic report, including histologic description, site of resection, and any additional information that may have accompanied these internally and externally referred specimens. Before screening, the samples and the accompanying clinical data were de-identified such that the results from the study could not be traced back to the identity of the patient.

This study was approved by the Nationwide Children's Hospital Institutional Review Board as exempt research based on the definition of human subjects research defined by the US Department of Health and Human Services (45 CFR part 46 or 21 CFR part 50).

Targeted Detection of Fusion Genes

At the time of clinical testing, samples were accessioned into the molecular diagnostic laboratory and RNA was extracted from the tumor specimens (snap frozen or optimal cutting temperature embedding compound embedded). Using 250 ng of the originally extracted RNA, cDNA libraries were generated using the customized FusionPlex Solid Tumor Kit (catalog number dSA09260; Archer, Boulder, CO). Furthermore, the libraries were repaired, adenylated, and ligated with Archer adapters for Illumina (San Diego, CA) sequencing, according to the manufacturer's specifications. Two rounds of PCR were performed using universal and gene-specific primers to generate the targeted enriched libraries for Illumina sequencing (Supplemental Table S1).

Libraries were quantified (07960140001; Roche, Basal, Switzerland), pooled at a final concentration of 10 pmol/L with 20% PhiX (catalog number FC-110-3001; Illumina), and denatured with sodium hydroxide. Sequencing was performed on the HiSeq 2500 using on-board clustering (catalog numbers PE-402-4002 and FC-402-4023; Illumina). Sequence data were analyzed using Archer Analysis software version 5.1.3 to process, filter, and identify somatic variants.

Orthogonal Confirmation by RT-PCR and Sanger Sequencing

Fusion-positive samples were orthogonally confirmed by Sanger sequencing. Total RNA (1.5 μg) was used to generate cDNA using the High-Capacity Reverse

Table 1 RT-PCR Primer Sequences Used to Validate Fusion Detected by the FusionPlex Assay

Fusion	Primer sequence
EWSR1/CREB1	F: 5′-GTAAAACGACGGCCAGagtcactgcacctccatcct -3′
	R: 5′-CAGGAAACAGCTATGACaacaactccaggggcaatag-3′
COL1A1/USP6	F: 5′-GTAAAACGACGGCCAGtgttcagctttgtggacctc-3′
	R: 5′-CAGGAAACAGCTATGACaacgatcaatgctgctgttg-3′
EWSR1/FLI1	F: 5′-GTAAAACGACGGCCAGccatggatgaaggaccaga-3′
	R: 5′-CAGGAAACAGCTATGACgaattgccacagctggatct-3′
ETV6/NTRK3	F: 5′-GTAAAACGACGGCCAGgggctgaggttgtagcactc-3′
	R: 5′-CAGGAAACAGCTATGACaagggaagcccatcaacctc-3′
TPM3/ALK	F: 5′-GTAAAACGACGGCCAGaggtggctcgtaagttggtg-3′
	R: 5′-CAGGAAACAGCTATGACtcgtcctgttcagagcacac-3′
EWSR1/ETV1	F: 5′-GTAAAACGACGGCCAGatggcactcagcctgcttat-3′
	R: 5′-CAGGAAACAGCTATGACcaaaaactgccagagctgaa-3′
PDE4DIP/NTRK1	F: 5′-GTAAAACGACGGCCAGgtcaccaaatccctttgagc-3′
	R: 5′-CAGGAAACAGCTATGAgcactcagcaaggaagacct-3′
WHSC1L1/NCOA2	F: 5′-GTAAAACGACGGCCAGaccagcttccattacgatgc-3′
	R: 5′-CAGGAAACAGCTATGAgatggcatagtaggccgaga-3′
PAX3/NCOA1	F: 5′-GTAAAACGACGGCCAGagacctcttaccagcccaca-3′
	R: 5′-CAGGAAACAGCTATGAcgggtggacagagaagctcat-3′
BCOR/CCNB3	F: 5′-GTAAAACGACGGCCAGtgcctatagcgatgtgtttga-3′
	R: 5′-CAGGAAACAGCTATGAcctcctcatgatttgagcact-3′
CDC42BPB/BRAF	F: 5′-GTAAAACGACGGCCAGatccaacccaaccaacttca-3′
	R: 5′-CAGGAAACAGCTATGAcctcgagtcccgtctaccaag-3′
TFG/MET	F: 5′-GTAAAACGACGGCCAGcctttcctttgcaattcagtg-3′
	R: 5′-CAGGAAACAGCTATGAgatgattccctcggtcagaa-3′
KIAA1549/BRAF	F: 5′-GTAAAACGACGGCCAGaagccccaagtcaaagatcc-3′
	R: 5′-CAGGAAACAGCTATGAttttcactgccacatcacca-3′
BRD4/NUTM1	F: 5′-GTAAAACGACGGCCAGctacgccgctactcccttc-3′
	R: 5′-CAGGAAACAGCTATGAtgagggcagtctgagtaagga-3′
CIC/DUX4	F: 5′-GTAAAACGACGGCCAGgaggctcctctccctgtacc-3′
	R: 5′-CAGGAAACAGCTATGAtactcccctgggacgtgggtg-3′
EWSR1/KLF15	F: 5′-GTAAAACGACGGCCAGcatgagtggccctgataacc-3′
	R: 5′-CAGGAAACAGCTATGAttcatcttcagagacgggtga-3′
FUS/TFCP2	F: 5′-GTAAAACGACGGCCAGgtggttacaaccgcagcag-3′
	R: 5′-CAGGAAACAGCTATGAtgggtttcatcatggagtttca -3′

M13 tails are uppercase, and gene-specific sequences are lowercase.
F, forward; R, reverse.

Transcription Kit (catalog number 4368814; ThermoFisher Scientific, Waltham, MA). To amplify the respective fusions, PCR using gene-specific primers (Table 1) was performed using AmpliTaq Gold DNA Polymerase (catalog number N8080241; ThermoFisher Scientific).

The PCR cycling initiated with a 94°C denaturation step that lasted for 10 minutes and was followed by eight cycles of touchdown PCR that consisted of a 94°C denaturation step (30 seconds), a 65°C annealing step (1 minute) that stepped down 1°C per cycle ending, and a 72°C extension step (1 minute). The remaining cycles included a 94°C denaturation step (30 seconds), a 58°C annealing step (1 minute), and a 72°C extension step (1 minute). The PCR concluded with a final extension of 72°C for 5 minutes. The PCR products underwent gel electrophoresis, were visualized on the UVP BioDoc-IT Imaging System (VWR, Radnor, PA), and were purified using the QiaQuick PCR Purification kit (catalog number 28105; Qiagen, Hilden, Germany).

The purified PCR products were sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (catalog number 4337454; Applied Biosystems, Foster City, CA) and M13F and M13R primers. Following the sequencing reaction, the samples were purified using the Performa DTR V3 Gel Filtration Cartridges (catalog number 42453; Edge Biosystems, San Jose, CA) and were analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems).

Assessment of Pathologic Diagnoses and Changes in Management

An expert panel, consisting of two anatomic pathologists (M.A.A. and S.K.) and a pediatric oncologist (R.R.), reviewed the clinical data before FusionPlex analysis to infer likely diagnoses. Following FusionPlex sequencing, the clinical data were reviewed a second time to derive a proposed post-FusionPlex testing diagnosis. In aggregate,

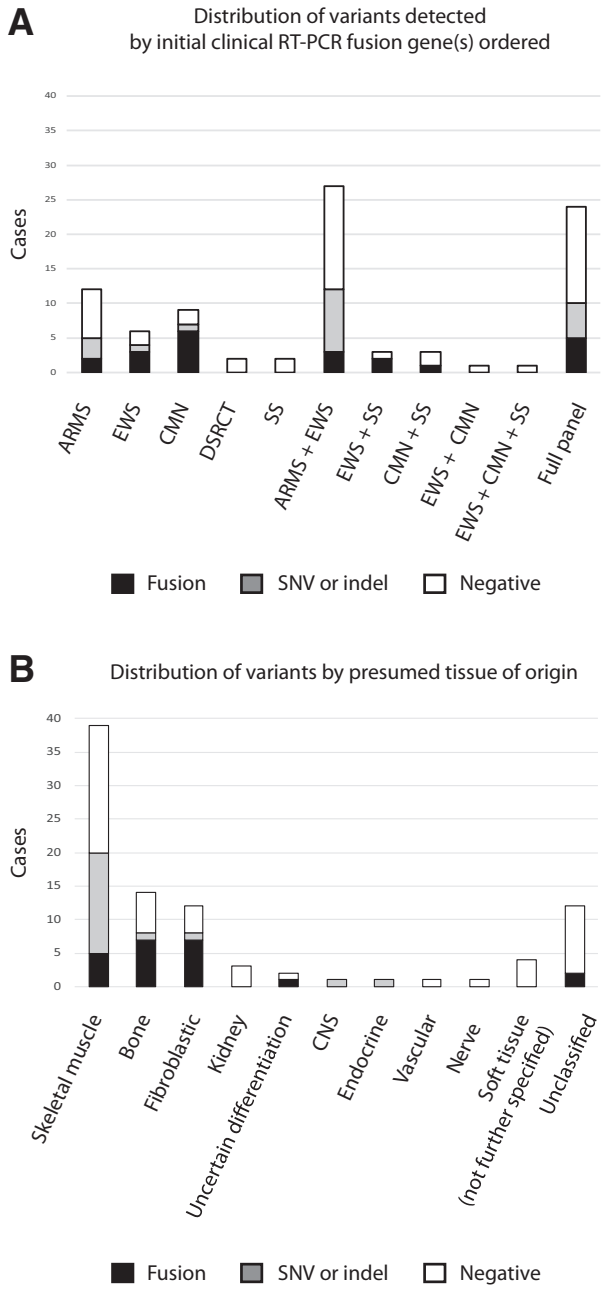

Figure 1 Distribution of FusionPlex variants detected in the 90 primarily pediatric sarcoma specimens tested. FusionPlex results were categorized by the type of variant identified, either fusion or single-nucleotide variant (SNV)/insertion/deletion (indel), and the frequency of each type is displayed by the initial clinical RT-PCR fusion gene test(s) ordered (**A**) and the tissue of origin (**B**). ARMS, alveolar rhabdomyosarcoma; CMN, congenital mesoblastic nephroma; CNS, central nervous system; DSRCT, desmoplastic small round cell tumor; EWS, Ewing sarcoma; SS, synovial sarcoma.

the expert panel of clinicians and pathologists considered the cases before and after FusionPlex testing to determine whether the targeted sequencing may have impacted the clinical management (therapeutic strategy, risk stratification, or diagnostic classification).

Results

Targeted RNA sequencing was performed on 90 primarily pediatric sarcoma specimens from a patient cohort consisting of 39 females and 51 males with average age of 7.34 years (range, 9 days to 63 years). On the basis of available clinical information, the range of initial inferred diagnoses included skeletal muscle tumors [rhabdomyosarcoma (n = 39)]; bone tumors [Ewing sarcoma (n = 9), bone tumor not further specified (n = 5)]; fibroblastic tumors [infantile fibrosarcoma (n = 8), myofibroblastic tumor (n = 1), fibroblastic/myofibroblastic tumor not further specified (n = 2), nodular fasciitis (n = 1)]; kidney tumors [congenital mesoblastic nephroma (n = 2), kidney tumor not further specified (n = 1)]; tumors of uncertain differentiation [desmoplastic small round cell tumor (n = 1), myoepithelial carcinoma (n = 1)]; central nervous system tumors [anaplastic medulloblastoma (n = 1)]; endocrine [poorly differentiated neuroblastoma with pleomorphic features (n = 1)]; vascular tumors [atypical infantile hemangioma (n = 1)]; tumors of the nerve [peripheral nerve sheath tumor (n = 1)]; soft tissue tumors with no further specification (n = 4); and unclassified (n = 12) (Supplemental Table S2).

Among the patients sequenced, all samples and controls demonstrated high sequence quality metrics (Supplemental Tables S3 and S4). Somatic variants were detected in 41 cases (41/90; 45.5%), including 22 fusion-positive cases and 19 SNV- or indel-positive cases. Two novel findings were identified, a *WHSC1L1/NCOA2* fusion identified in case 22 and an indel variant in the *NRAS* gene (*https://www.ncbi.nlm.nih.gov/nuccore*, NM_002524: c.174_176delAGC p.Ala59del) in case 33; all other variants have been previously described.

The proportion of cases identified with a variant was different depending on whether the case was initially screened for a single fusion, multiple fusions, or all fusions on the RT-PCR panel. Cases initially screened for a single fusion were more likely to yield a FusionPlex result than those screened for multiple or all fusions on the RT-PCR panel (Supplemental Figure S2). These results were further broken down by the specific RT-PCR fusion(s) ordered (Figure 1A). Samples initially tested for the *ETV6/NTRK3* fusion found in infantile fibrosarcoma and congenital mesoblastic nephroma were the highest yielding fusion-positive samples (6 cases of 9), whereas those initially tested for the Ewing and alveolar rhabdomyosarcoma fusions yielded the highest number of SNV- or indel-positive cases (9 cases of 27). When parsed by presumed tissue of origin, fibroblastic/myofibroblastic tumors were the highest yielding fusion-positive samples (7/12), whereas rhabdomyosarcomas had the highest number of SNVs or indels (15/39) (Figure 1B). Of note, these 15 SNV- or indel-positive rhabdomyosarcoma cases were not concurrently found to be fusion positive.

Of the 22 fusions detected in this study, 21 have been previously associated with histologic subclassifications of

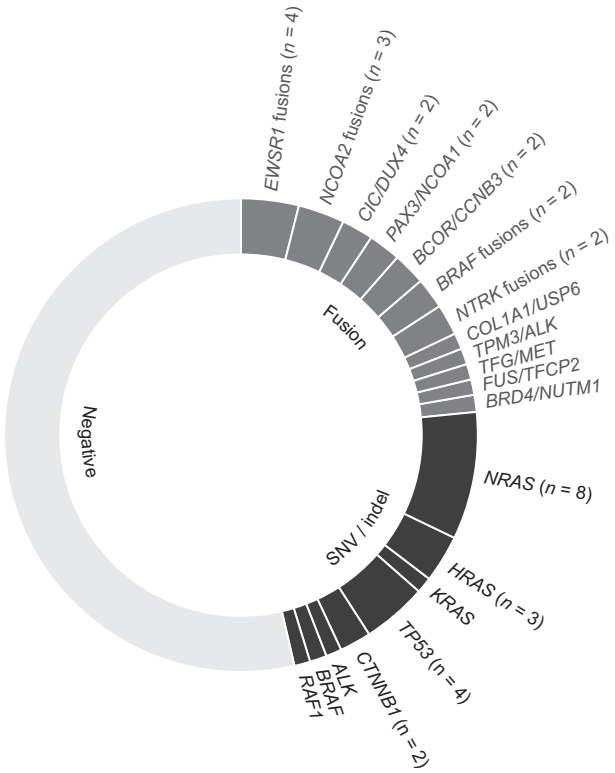

Figure 2 Summary of FusionPlex variants. The frequency of fusion-positive (24.4%; gray), single-nucleotide variant (SNV)/insertion/deletion (indel)—positive (21.1%; dark gray), and variant-negative (54.4%; light gray) cases along with the variants and the number of times they were identified displayed.

sarcoma. The most frequently observed fusion partner was *EWSR1* ($n = 4$); other fusion partners, including *NCOA2* ($n = 3$), *CIC* ($n = 2$), *NCOA1* ($n = 2$), *BCOR* ($n = 2$), *BRAF* ($n = 2$), and *NTRK* ($n = 2$) genes, were also recurrently identified (Figure 2, Supplemental Table S5). To complement the fusion gene analysis, the data set was analyzed for sequence variants in a subset of cancer genes and their known hot spots. A total of 21 SNVs or indels were detected among 19 cases. Alterations were most frequently seen in the *RAS* family members, *TP53*, and *CTNNB1* (Figure 2, Supplemental Table S6). Of particular interest, a novel in-frame deletion of an alanine residue at amino acid position 59, located in the nucleotide-binding domain, of the *NRAS* gene was identified in sample 33. Finally, findings in three other genes, *JAK3* (p.V722I), *RAF1* (p.S257L), and *TP53* (p.Cys135Phe, p.Arg273His, p.His179Arg, and p.Pro301GlnfsTer44), may represent germ-line alterations; however, without paired-normal specimens, the etiologic nature of these variants cannot be fully discerned.

The apparent impact this additional testing may have had on clinical outcomes was evaluated by considering the FusionPlex variant identified in conjunction with the available clinical information. In 25 of the 90 cases (27.8%), a possible impact to clinical management was noted and included changes in chemotherapy regimen, favoring surgical resection over chemotherapy, or integration of a targeted therapy. In 17 of the 90 cases (18.9%), there was a potential change in diagnosis (ie, rhabdomyosarcoma to Ewing sarcoma) or refinement of the diagnosis (ie, Ewing sarcoma to Ewing-like sarcoma). Finally, the expected prognosis in 17 of the 90 cases (18.9%) may have been impacted by these results (Supplemental Tables S5 and S6).

Discussion

In this study, a custom FusionPlex panel with probes targeting 112 genes previously associated with solid tumorigenesis was designed for the simultaneous detection of recurrent fusion genes, SNVs, and indels. This panel was used to perform retrospective sequencing on a cohort of 90 banked diagnostic sarcoma specimens that were negative for the clinically ordered RT-PCR fusion gene testing. Among this cohort, 22 fusion-positive cases and 19 SNV- or indel-positive cases were identified. As highlighted by this study, pediatric sarcomas negative for first-tier fusion gene testing may benefit from reflex testing to methods such as this custom FusionPlex panel.

This study demonstrates the advantages of targeted sequencing using the anchored multiplex PCR technology; however, the retrospective nature of this study has several limitations, including the following: i) incomplete clinical and laboratory (ie, fluorescent *in situ* hybridization, cytogenetics, and immunohistochemistry) data for some samples, which often made inferring the diagnosis or interpreting the clinical significance of the variants challenging; ii) lack of access to the original specimens, which precluded histopathologic review; iii) several fusions could not be confirmed by RT-PCR, which may in part be because of RNA degradation, low tumor percentage, primer placement, or sequence homology; and iv) absent paired-normal specimens made it challenging to determine the origin, germ line or somatic, for some variants. This includes the p.V722I variant in *JAK3*, which seems to have transformative properties but is also relatively frequent in the population (approximately 1%)[16–19]; the p.S257L variant in *RAF1*,[20–23] which has been reported in several malignancies and is associated with Noonan syndrome; and several *TP53* variants that raise concern for an inherited cancer predisposition syndrome.[24,25]

Despite these limitations, the current results highlight several advantages associated with the FusionPlex system. This includes enhanced sensitivity, which, in part, is achieved by deep sequencing and the utilization of multiple primers to target alternative breakpoints of variant transcripts. This was demonstrated by the detection of an *EWSR1/FLI1* fusion in case 1 (Ewing sarcoma) and an *ETV6/NTRK3* fusion in case 14 (infantile fibrosarcoma) by the FusionPlex assay. These cases were initially negative for their respective fusions by clinical RT-PCR analysis. Subsequent RT-PCR confirmation required increasing the

amount of RNA in the reverse transcription reaction and designing primers that were closer to the breakpoints. It is possible that the initial clinical RT-PCR assay may have been unsuccessful in detecting these fusions because of a fusion-positive tumor fraction that fell below the limit of detection or because of a single-nucleotide polymorphism in a primer binding site. In either case, the increased sensitivity of the FusionPlex panel successfully identified the *EWSR1/FLI1* and *ETV6/NTRK3* fusions in these cases, whereas initial clinical RT-PCR testing did not.

Another advantage of the FusionPlex system is that it can detect challenging fusions, such as *CIC/DUX4*, which have variant breakpoints that are mediated by homologous *DUX* and *DUX*-like gene clusters at the terminal end of 4q and 10q.[26] With this technology, probes targeting a single gene in the fusion, in this case targeting *CIC*, are required for detection; therefore, the need for probes targeting the highly homologous *DUX* and *DUX*-like gene clusters is eliminated entirely. The panel identified *CIC/DUX4* fusions in cases 7 (synovial sarcoma or Ewing sarcoma) and 8 (Ewing sarcoma). Although RT-PCR confirmed the *CIC/DUX4* fusion in case 7, the homology between *DUX4* paralogs likely precluded us from confirming this fusion in case 8. Identifying *CIC/DUX4* fusions in these cases demonstrates the ability of the assay to detect challenging fusions when other standard methods, like RT-PCR, may not be reliable.[27,28] These results would have likely clarified the diagnosis as Ewing-like sarcomas in these samples, which would have modified the prognosis.

Another strength of the FusionPlex system is the ability to identify novel variants. For example, a *WHSC1L1/NCOA2* fusion gene was identified in case 22. The differential for this case included Ewing sarcoma and rhabdomyosarcoma and initially tested negative for the associated fusions (*EWSR1/FLI1*, *EWSR1/ERG*, *PAX3/FOXO1*, and *PAX7/FOXO1*) by the clinical RT-PCR assay. Several lines of evidence support the validity of this fusion, including the prior involvement of both partners in fusion events, *WHSC1L1* in the acute myeloid leukemia *WHSC1L1/NUP98* fusion and *NCOA2* in the infantile spindle cell rhabdomyosarcoma *NCOA2/PAX3* fusion.[29,30] In addition, the structural configuration of an N-terminal DNA binding domain fused to a C-terminal transcriptional activation domain has been well described in other *NCOA* fusions.[31] Given this structural arrangement confers transformative properties in other *NCOA* fusions, such as *PAX3/NCOA1* and *PAX3/NCOA2*, it is likely the novel *WHSC1L1/NCOA2* fusion may harbor similar properties. Identification of this fusion in additional samples and functional characterization will be required for establishing the significance of this fusion. The association of *NCOA2* fusions with infantile rhabdomyosarcomas, along with the congenital presentation of this tumor, favors a diagnosis of infantile spindle cell rhabdomyosarcoma, which would have been integral for developing a treatment plan.

A novel hot spot mutation, the deletion of an alanine residue at amino acid position 59 (p.Ala59del) in the *NRAS* gene, was detected in case 33 (embryonal rhabdomyosarcoma). Although *NRAS* and its paralogs (*HRAS* and *KRAS*) are recurrently mutated in many cancers, this particular in-frame deletion has not been reported in the literature or in public cancer genomics databases [cBioPortal or Catalogue of Somatic Mutations in Cancer (COSMIC)]. A total of 99.2% of oncogenic *RAS* mutations are localized to codons 12, 13, and 61 and render the RAS proteins in the active GTP-bound state that results in constitutive activation.[32,33] Like the novel p.Ala59del in case 33, other rare alterations clustered around these hot spots have been described.[34] In an exceedingly rare number of cases, the paralogous *KRAS* mutation (p.Ala59del) has been described but has never been identified in the *NRAS* gene.[35–38] Functional characterization of the p.Ala59del mutation in the *KRAS* gene has been shown to impact the rate of guanine nucleotide exchange.[39] Given the high degree of homology between these two *RAS* genes, it is likely that the paralogous *NRAS* mutation may also impact the rate of guanine nucleotide exchange.

The cases included in this cohort all underwent initial RT-PCR fusion gene analysis to confirm a suspected diagnosis or to assist with the classification of atypical cases. Indeed, some cases may represent true fusion-negative samples; however, others may represent uncommon or novel molecular alterations that eluded RT-PCR detection. Several cases in this cohort support reflex testing to the FusionPlex panel, including cases 2 (rhabdomyosarcoma), 4 (myoepithelial carcinoma), 6 (undifferentiated sarcoma), 13 (insufficient clinical information), and 19 (Ewing sarcoma or small cell neuroendocrine carcinoma). These cases were initially screened for the entire clinical RT-PCR fusion panel, suggesting they may have been atypical or difficult to classify. FusionPlex analysis identified *BCOR/CCNB3*, *FUS/TFCP2*, *EWSR1/KLF15*, *EWSR1/ETV1*, and *BRD4/NUTM1* fusions, respectively, among these samples. It is likely these results would have assisted in the initial classification of these diagnostically challenging samples (Supplemental Table S5). In addition, in case 18 (infantile fibrosarcoma), FusionPlex analysis revealed a *TFG/MET* fusion in a tumor resected from the patient's foot. This result carries potential diagnostic and clinical management implications, as it may have assisted with classifying this spindle cell neoplasm and possibly implicates using a targeted therapy (MET inhibitor).[40] Finally, the differential for case 19 included Ewing sarcoma and small cell neuroendocrine carcinoma. FusionPlex analysis identified a *BRD4/NUTM1* fusion, which suggests a diagnosis of a NUT carcinoma and would likely elevate the patient into a higher-risk category.[41] Collectively, these cases highlight the types of variants identified on FusionPlex analysis and illuminate the potential utility they have in classifying specimens and/or guiding clinical management.

Last, this panel was developed to detect variants across a range of solid tumors, including sarcomas. Interestingly, a nearly equal proportion of cases with fusions ($n = 22$) and SNVs or indels ($n = 19$) were identified; however, the

degree of clinical utility varied between the types of alterations. The identification of a fusion may have modified the diagnosis in 14 cases, may have impacted the prognosis in 11 cases, and was consistent with a change in management in 16 cases. This contrasts with the SNV- or indel-positive cases, where only three cases had a likely change in diagnosis, six cases had likely prognostic implications, and nine cases may have had a change in management. The discordance between clinically meaningful variants, in part, may be accounted for by the abundance of diagnostically meaningful fusions among sarcomas. Given the customizable nature of the FusionPlex assay, it may be advantageous to incorporate more genes relevant to inherited predisposition to or diagnosis of sarcomas, such as *NF1*, *CDKN2A*, *SMARCB1*, and *RB1*.

In conclusion, a custom FusionPlex assay was designed to detect an array of fusion genes and SNVs that underlie sarcoma and solid tumorigenesis. Cancer-associated alterations were detected in 45.5% of the study cohort, many of which carry diagnostic (18.9%; 17/90), prognostic (18.9%; 17/90), or clinical management-related implications (27.8%; 25/90). The proportion of positive cases can be attributed to the selection of gene targets and the ability of anchored multiplex PCR technology to simultaneously detect fusions and gene mutations. These results provide further evidence that the FusionPlex system allows for detection of alternative breakpoints, noncanonical fusion partners, and uncommon fusions not typically covered by traditional methods and is a powerful tool available for the diagnostic workup of challenging sarcoma cases.[42]

Acknowledgments

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Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.jmoldx.2020.07.004*.

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