CANCER GENETICS

Germline Sequencing Improves Tumor-Only Sequencing Interpretation in a Precision Genomic Study of Patients With Pediatric Solid Tumor

Jaclyn Schienda, ScM[1]; Alanna J. Church, MD[2]; Laura B. Corson, PhD[1]; Brennan Decker, MD, PhD[2]; Catherine M. Clinton, MS[1]; Danielle K. Manning, PhD[3]; Alma Imamovic-Tuco, MS[1]; Deirdre Reidy, BA[1]; Gianna R. Strand, MS[1]; Mark A. Applebaum, MD[4]; Rochelle Bagatell, MD[5]; Steven G. DuBois, MD, MS[1]; Julia L. Glade-Bender, MD[6]; Wenjun Kang, MS[7]; AeRang Kim, MD, PhD[8]; Theodore W. Laetsch, MD[5]; Margaret E. Macy, MD[9]; Luke Maese, DO[10]; Navin Pinto, MD[11]; Amit J. Sabnis, MD[12]; Joshua D. Schiffman, MD[10]; Susan I. Colace, MD[13]; Samuel L. Volchenboum, MD, PhD[4]; Daniel A. Weiser, MD[14]; Jonathan A. Nowak, MD, PhD[3]; Neal I. Lindeman, MD[3]; Katherine A. Janeway, MD, MMSc[1]; Brian D. Crompton, MD[1,15]; and Junne Kamihara, MD, PhD[1]

PURPOSE Molecular tumor profiling is becoming a routine part of clinical cancer care, typically involving tumor-only panel testing without matched germline. We hypothesized that integrated germline sequencing could improve clinical interpretation and enhance the identification of germline variants with significant hereditary risks.

MATERIALS AND METHODS Tumors from pediatric patients with high-risk, extracranial solid malignancies were sequenced with a targeted panel of cancer-associated genes. Later, germline DNA was analyzed for a subset of these genes. We performed a post hoc analysis to identify how an integrated analysis of tumor and germline data would improve clinical interpretation.

RESULTS One hundred sixty participants with both tumor-only and germline sequencing reports were eligible for this analysis. Germline sequencing identified 38 pathogenic or likely pathogenic variants among 35 (22%) patients. Twenty-five (66%) of these were included in the tumor sequencing report. The remaining germline pathogenic or likely pathogenic variants were single-nucleotide variants filtered out of tumor-only analysis because of population frequency or copy-number variation masked by additional copy-number changes in the tumor. In tumor-only sequencing, 308 of 434 (71%) single-nucleotide variants reported were present in the germline, including 31% with suggested clinical utility. Finally, we provide further evidence that the variant allele fraction from tumor-only sequencing is insufficient to differentiate somatic from germline events.

CONCLUSION A paired approach to analyzing tumor and germline sequencing data would be expected to improve the efficiency and accuracy of distinguishing somatic mutations and germline variants, thereby facilitating the process of variant curation and therapeutic interpretation for somatic reports, as well as the identification of variants associated with germline cancer predisposition.

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ASSOCIATED CONTENT

Appendix

Author affiliations and support information (if applicable) appear at the end of this article.

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BACKGROUND

Precision medicine among oncology patients has been accelerated by the widespread use of molecular tumor profiling. In pediatric oncology, tumor profiling can refine a histologic diagnosis, inform prognosis, and identify variants that predict response to targeted therapies.[1,2] Many academic and commercial assays are designed to detect variants in a panel of genes commonly associated with cancer. Although some groups use matched paired tumor-normal sequencing,[3-6] the College of American Pathologists reported that 90% of clinical next-generation sequencing (NGS) laboratories perform tumor-only testing.[7]

For tumor-only assays, analytic pipelines aim to reduce the number of germline variants in the output, often by filtering out variants recurrently reported in genomic population databases. Rare germline variants, however, can still be included in reports.[6,8-10] By focusing on somatic events, these computational filters may also mask germline variants associated with cancer predisposition, as demonstrated in prior studies.[6,10,11] To further refine tumor variant calls, sequencing data from tumor-only panels are often

CONTEXT

Key Objective

Does the addition of germline sequencing affect the interpretation of tumor-only sequencing data in a therapeutic study of pediatric high-risk solid tumors?

Knowledge Generated

Clinical reports with therapeutic recommendations on the basis of tumor-only sequencing analyses can include a significant number of genetic variants that cannot be definitively classified as germline or somatic on the basis of variant allele fraction or expert curation. In addition, tumor sequencing alone may not adequately identify all germline variants with clinical significance.

Relevance

Integrated tumor-germline genetic profiling would improve the classification of somatic mutations in cancer, decrease the amount of time and resources spent curating variants, and increase the identification of germline variants with clinical significance. This has particular relevance for the clinical care of individuals and families with cancer predisposition.

reviewed and interpreted by molecular pathologists or geneticists who may identify the possibility that certain variants are germline in their reports. Together, these approaches are designed to improve the accuracy of variant reporting to refine diagnoses, aid in treatment decisions, and refer patients and families to genetic counseling when indicated. However, direct assessment of the effectiveness of computational filters, expert review, and the potential added value of comparative or paired tumor and germline analysis has not been extensively examined in pediatric cancer cohorts.

In this study, we analyzed a subset of patients with childhood solid malignancies enrolled in a genomic precision medicine trial, Genomic Assessment Improves Novel Therapy (GAIN) consortium study (ClinicalTrials.gov Identifier: NCT02520713). Tumor samples underwent tumor-only profiling followed by germline profiling, with separate reports provided to clinicians. Given the sequential analysis of tumor followed by germline sequencing, data from this cohort provide a unique opportunity for a post hoc analysis to compare how the addition of germline sequencing may affect clinical variant interpretation and clinical recommendations. We hypothesized that tumor-only sequencing could result in the inability to definitively distinguish germline from somatic variants, potentially complicating treatment decisions and missing opportunities to identify hereditary cancer risk in patients and their families.

MATERIALS AND METHODS

GAIN Trial Enrollment and Selection of Patients for Study

Patients were identified among those enrolled on the GAIN Consortium trial approved by the Institutional Review Boards at Dana-Farber Cancer Institute and participating sites. Eligible patients had a high-risk (defined as an expected 2-year progression free survival of 50% or less) newly diagnosed or relapsed or refractory solid malignancy outside of the central nervous system with an age at initial diagnosis of 30 years or younger. Written informed consent was obtained for all study participants. Tumor samples were acquired from archival material collected as part of routine clinical care, and peripheral blood was obtained during a clinical blood draw. Physicians and research staff provided pathologic diagnosis and demographic information, including participant-reported race and ethnicity. Individuals from the GAIN trial were eligible for inclusion in this analysis if both tumor and germline profiling results, as well as completed clinical interpretation and reports, were available for analysis by April 2019.

Tumor Sequencing and Variant Curation

Tumor samples were sequenced by the Center for Advanced Molecular Diagnostics at Brigham and Women's Hospital using a custom hybrid capture sequencing assay, OncoPanel, targeting 300 genes (version POPv2) or 447 genes (version POPv3).[12] Briefly, extracted DNA underwent next-generation sequencing of targeted genes using the TruSeq LT library preparation kit (Illumina, San Diego, CA), a custom RNA bait set (Agilent SureSelect, Agilent, Santa Clara, CA), and the Illumina HiSeq2500. Analysis included the detection of sequence variants, copy-number alterations, and structural variants, as previously described.[13-16] The lower limit of detection for sequence variants was 10% allelic fraction at 50× coverage. Likely polymorphisms and artifacts were filtered by comparing variant calls to both a panel of normal samples and in-batch normal controls, as well as those found in the NHLBI Exome Sequencing Project (ESP) and/or gnomAD databases at $> 0.1\%$ frequency in any subpopulation. Variants flagged for filtering that were present in Cosmic at least twice were subsequently rescued. Variants were interpreted and reported by a molecular pathologist according to guidelines recommended by the Association for Molecular Pathology, College of American Pathologists, and American Society of Clinical Oncology[17] within a 5-tier schema: Variants in tiers

1-2 associated with strong evidence, tier 3 with weak evidence, tier 4 with uncertain significance of clinical impact, and tier 5 unlikely to have impact. The potential germline etiology was commented upon in the report, at the discretion of the molecular pathologist.

Clinical Interpretation

The GAIN study aims to evaluate whether tumor sequencing increases the use of molecularly targeted therapies and whether those therapies are associated with treatment responses. Such individualized cancer therapy (iCat) recommendations were made on the basis of peer-reviewed literature and, in cases with weak or conflicting evidence, consensus opinion from an expert panel. Treatment recommendations were tiered (separate from the variant tiers described above) on the basis of the strength of the evidence supporting potential response to a specific therapy.[1] The iCat recommendation, including a comment about possible germline origin if appropriate, was communicated in a written report.

Germline Sequencing and Variant Curation

Germline profiling was performed on DNA extracted from peripheral blood samples using OncoPanel version POPv3. Analysis was informatically restricted to 147 genes known to be associated with increased cancer risk, as previously described.[18] Germline single-nucleotide variants (SNVs) and copy-number variants (CNVs) were classified as pathogenic (P), likely pathogenic (LP), variant of uncertain significance (VUS), likely benign (LB), or benign (B) by a molecular pathologist according to guidelines for the interpretation of germline variants, as recommended by the American College of Medical Genetics and the Association for Molecular Pathology.[19] Although the B/LB variants were not included in the pathologists' reports, these were available for review for the purposes of this study.

Comparison of Tumor-Only and Germline-Only Reports With Integrated Data Analysis

We compared the variant lists from tumor-only reports to those of germline reports and the B/LB germline variant list. We assessed the tumor-only and germline-only sequencing results for (1) the percentage of variants in the tumor-only report that were determined to be germline (including P/LP, VUS, and B/LB), (2) the proportion of P/LP germline variants present in the tumor-only report, and (3) the variant allele fractions (VAFs) of true somatic events compared with germline variants in the tumor-only report and germline-only report using descriptive analyses.

For each germline P/LP variant identified, tumor sequencing data were examined for the presence of a second alteration in the same gene within the tumor, including loss-of-function SNVs, deletions, or copy-neutral loss of heterozygosity (CN-LOH). In addition, for the P/LP variants not present on the tumor-only sequencing report, we reviewed the tumor sequencing data, including filtered variants, within the laboratory variant database.

TABLE 1. Baseline Characteristics for the Cohort

Variable	Mean (SD) or No. (%)
Age at diagnosis mean (SD), years	12 (5.8)
Age at enrollment mean (SD), years	14 (6.3)
Sex, No. (%)	
Female	75 (47)
Male	85 (53)
Race, No. (%)	
White	113 (71)
Black or African American	14 (9)
Asian	9 (6)
More than one race	1 (1)
Native Hawaiian/Other Pacific Islander	1 (1)
Unknown	3 (2)
Other	19 (12)
Ethnicity, No. (%)	
Hispanic or Latino	17 (11)
Non-Hispanic	127 (79)
Unknown	16 (10)
Cancer diagnosis type, No. (%)	
Osteosarcoma	58 (36)
Rhabdomyosarcoma	18 (11)
Ewing sarcoma	13 (8)
Other sarcoma	29 (18)
Renal tumor	11 (7)
Neuroblastoma	12 (8)
Liver tumor	4 (3)
Carcinoma	4 (3)
Other	11 (7)

Abbreviation: SD, standard deviation.

RESULTS

Germline Variants Identified in the Study Cohort

One hundred and sixty participants enrolled in the GAIN trial met the eligibility criteria for this study (Fig 1). In this cohort, the average age at diagnosis was 12 years (range, 1 month to 27 years). There were 42 different cancer diagnoses, with osteosarcoma being the most frequent (Table 1).

Germline sequencing identified a P/LP variant among 22% of participants (n = 35). Thirty-eight P/LP variants in 22 genes were identified, including 35 SNVs and three CNVs (Fig 2 and Appendix Table A1). All variants were heterozygous, with 18 variants identified in 12 genes known to have autosomal dominant inheritance (Fig 2A), including one oncogene *MITF*, and 20 variants identified in 10 autosomal recessive genes (Fig 2B). There were 17 different cancer diagnoses for these 35 patients, with osteosarcoma

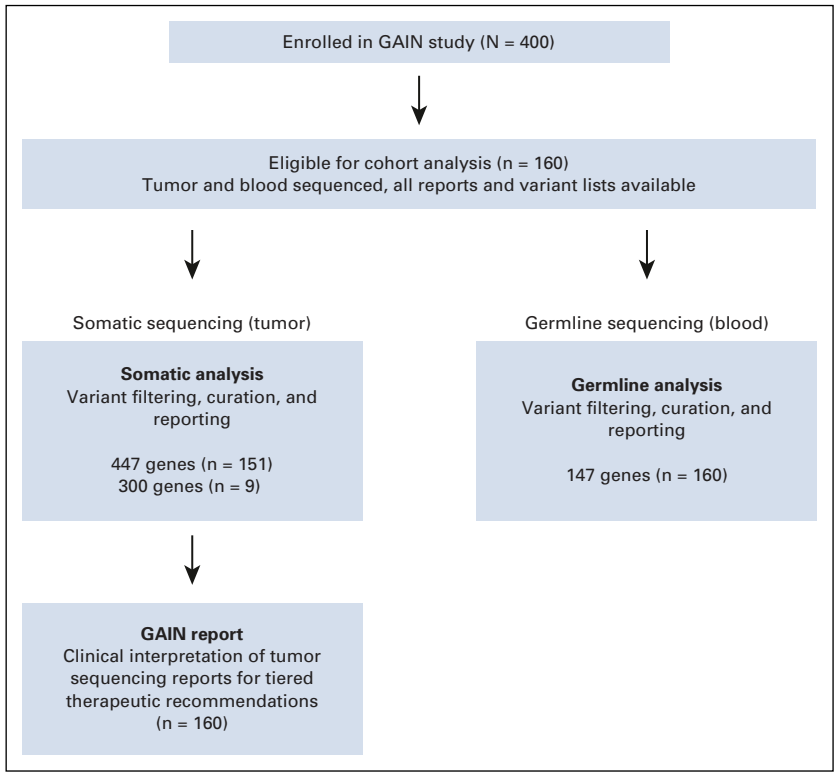

FIG 1. Post hoc comparison of tumor and matched germline sequencing. Of the 400 patients enrolled in GAIN at the time of this study, 160 had tumor sequencing reports and separate germline sequencing reports available. Each tumor-only sequencing report also had a separate clinical interpretation report (GAIN report) that conveyed therapeutic recommendations on the basis of the variants present in tumor-only sequencing. GAIN, Genomic Assessment Improves Novel Therapy.

being the most common (n = 12, Appendix Table A1). Ten (26%) of the P/LP variants were in genes known to be associated with the patient's cancer diagnosis. Pathogenic variants in genes not known to be clearly associated with the patient's diagnosis included *BRCA1* in a child with neuroblastoma, *MITF* in a child with Ewing sarcoma, and *FAM175A* variant in a child with osteosarcoma (Appendix Table A1).

Of the 17 germline P/LP variants in autosomal dominant cancer risk genes, we identified a second alteration within the same gene in 9 (53%) of the associated tumors (Fig 2A, Appendix Table A1). Four patients with *DICER1* variants had a second *DICER1* hotspot SNV in the tumor, three patients had a somatic deletion of the other allele, and two had CN-LOH associated with overrepresentation of the variant allele. None of the tumors from patients who were carriers of an autosomal recessive cancer risk gene had a second alteration identified.

Variants Identified in Tumor-Only Sequencing Are Frequently Germline in Origin

To study the potential germline etiology of variants included in tumor profiling reports, we compared tumor-only and germline-only sequencing reports from the 160 patients included in this study (Fig 1). For 9 cases using OncoPanel version POPv2, our analysis was restricted to 86 genes analyzed by both tumor and germline pipelines, whereas our analysis included 147 genes for 151 cases profiled with POPv3. Four hundred thirty-four tumor SNVs (48 tier 1-2, 43 tier 3, 343 tier 4), 492 germline P/LP/VUS SNVs, and 332 germline B/LB SNVs were identified.

Of the 434 SNVs identified by tumor sequencing, 285 (66%) were reported as P/LP/VUS in the germline sequencing report and 23 (5%) were classified as B/LB variants by the molecular pathologist. Only 126 variants (29%) were present solely in the tumor sequencing data, and thus determined to be of somatic origin (Fig 3A). Fifteen of 48 (31%) variants within tier 1-2, 23 of 43 (53%) in tier 3, and 270 (79%) in tier 4 were determined to be of germline origin (Fig 3B).

Next, we investigated how many of the P/LP germline variants had been noted as potentially germline in the tumor sequencing report. Of the 285 P/LP/VUS germline SNVs present in the tumor-only sequencing report, 23 were P/LP germline variants occurring in nine autosomal dominant genes (*APC*, *CHEK2*, *DICER1*, *FAM175A*, *RB1*, *SDHA*, *SMARCA4*, *TP53*, and *UROD*) and seven autosomal recessive genes (*BLM*, *DOCK8*, *GBA*, *MUTYH*, *SERPINA1*, *WRN*, and *XPA*). All 23 variants, except one autosomal dominant LP variant in *UROD*, were noted as possibly germline. In 18 of 23 cases, the tumor-only sequencing reports also emphasized that the sequencing assay could not distinguish between germline or somatic variants, with 15 of these adding that genetic counseling may be helpful, clinically indicated, or specifically recommended. Two referenced previous clinical germline testing that had identified the same variant.

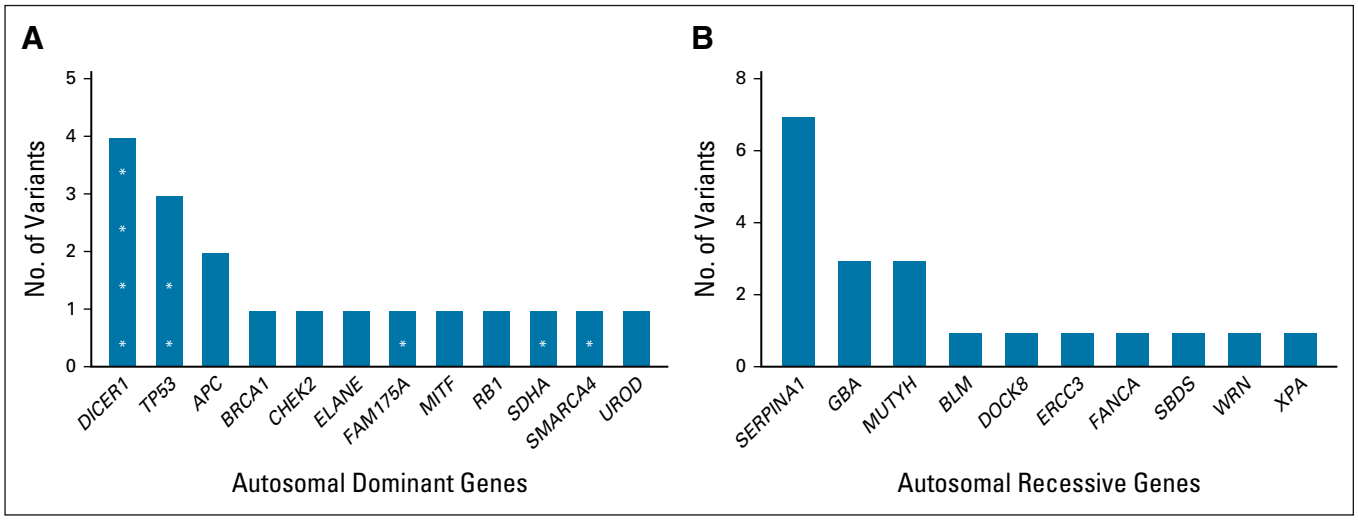

FIG 2. Germline pathogenic and likely pathogenic variants. (A) There were 18 heterozygous P/LP variants detected across 12 different autosomal dominant cancer risk genes. Nine of the tumors had a second mutation within the same gene (*). (B) There were 20 heterozygous P/LP variants detected across 10 different autosomal recessive cancer risk genes. None of the tumors had a second mutation within the same gene. P/LP, pathogenic or likely pathogenic.

Of 148 SNVs reported as possibly germline in the tumor-only report, 52 (35%) were not present in the patient's germline report or B/LB variant list. These variants were also located within all four clinical tiers in the tumor report (Fig 3C).

Finally, we found that two of the three P/LP CNVs identified from germline sequencing were also reported as variants in tumor-only sequencing (*ELANE* and *FANCA*). Neither of these was flagged as possibly germline.

VAF Is Not Sufficient to Clarify Germline or Somatic Origin

We next investigated to what degree the allelic fraction of variants from tumor-only sequencing could help predict which variants were of germline origin. The median VAF for confirmed somatic variants and confirmed germline variants within the tumor-only sequencing was 35.6% (range 3.5%-100%) and 48.3% (range 3.94%-95.3%), respectively (Fig 4). Although the median VAF was significantly different between the two groups ($P < .0001$, Mann-Whitney test), we observed considerable overlap, as 35.1% of true somatic mutations had VAFs inside the informally accepted range of 40%-60% that, in our experience, is frequently discussed as indicating a possible germline origin. Conversely, we found that 30.7% of true germline variants had a VAF that was either $> 60\%$ or below 40% in the tumor-only sequencing data, of which only 35% had a called copy-number variant of the gene (Appendix Fig A1). Interestingly, 94% of germline variants identified from a normal blood sample were found to have VAFs between 40% and 60% (Fig 4).

Impact of Germline Variants on Therapeutic Recommendations

Treatment recommendations were made on the basis of 64 SNVs within tumor-only sequencing for 53 of the 160 cases included in this study, and 46 were made on the basis of genes present on both somatic and germline sequencing panels. Eleven (24%) of these 46 recommendations were on the basis of variants of confirmed germline origin, including seven P/LP SNVs within the genes *CHEK2*, *FAM175A*, *SDHA*, *SMARCA4*, and *TP53*, and four VUS in *AKT1*, *NBN*, *TP53*, and *TSC2* (Table 2). Treatment recommendations noted that seven of these 11 variants may be of germline etiology and identified all four of the VUS alterations as variants of uncertain significance.

Tumor-Only Sequencing May Exclude Significant Germline Variants

Twelve of the 35 germline P/LP SNVs and one of the three germline P/LP CNVs were not reported (Appendix Table A1). Three of these were in autosomal dominant cancer risk genes (*APC*, *BRCA1*, and *MITF*).

In examining the tumor-only BAM files, all 12 SNVs were present in the aligned sequencing data but were filtered out by the somatic analytic pipeline because of their high population allele frequencies. The pathogenic germline CNV in *BRCA1* was masked in the tumor-only sequencing by an overlapping somatic copy-gain in 17q.

DISCUSSION

An important goal of precision medicine in cancer is to improve patient outcomes through a deep understanding of the potential targetable vulnerabilities present in the tumor. At present, tumor-only NGS panels are the most widely used modality to identify biomarkers of response to molecularly targeted therapies. We sought to identify the added benefits of paired tumor-germline sequencing in a pediatric cancer cohort using the availability of sequential tumor and germline sequencing as a proxy. In our high-

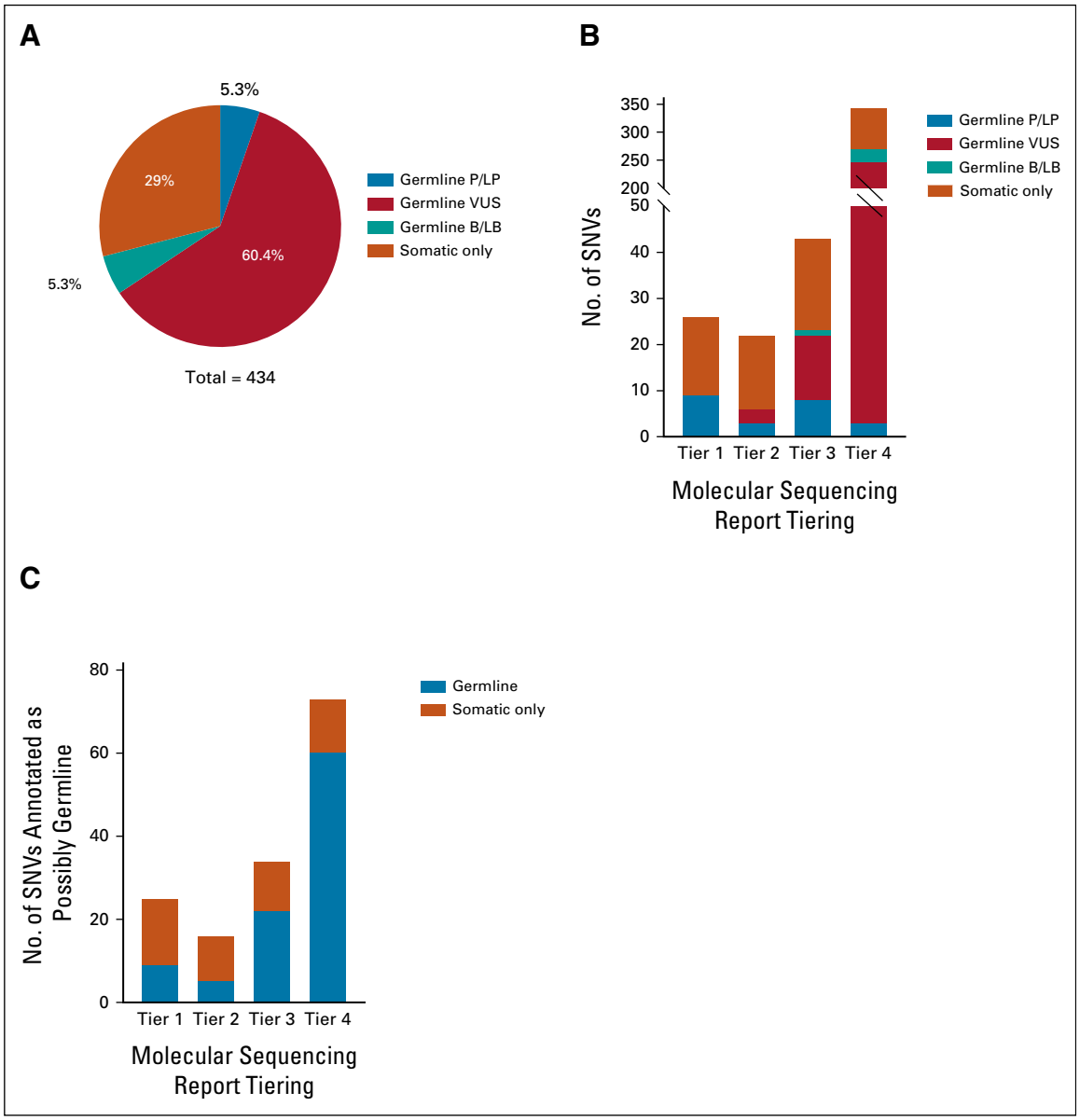

FIG 3. Comparison of germline variants within tumor-only sequencing reports. (A) Tumor-only sequencing SNVs. Pie chart demonstrating that only a minority (29%) of variants identified by tumor-only sequencing are somatic events with the rest being verified as germline variants by review of germline sequencing data. (B) Tumor-only sequencing SNVs by clinical tier. Germline P/LP variants were identified in all four clinical actionability tiers within the tumor-only sequencing report, including tiers 1 and 2. Although there were germline VUS present within tier 2, most of the VUS were within tiers 3 and 4. (C) SNVs annotated as potentially germline in tumor-only sequencing report. SNVs were flagged as possibly germline in all four clinical actionability tiers within the tumor-only sequencing report. More than half of the flagged variants within tiers 1 and 2 were somatic events, whereas the majority of flagged variants in tiers 3 and 4 were verified as germline. B/LB, benign or likely benign; P/LP, pathogenic or likely pathogenic; SNV, single-nucleotide variant; VUS, variant of uncertain significance.

risk pediatric cohort, 22% of participants had at least one P/LP germline autosomal dominant or recessive variant, similar to rates seen in other cohorts.[2,20-22] Four major conclusions from our study emerged: (1) Germline variants are likely to be included in tumor-only sequencing reports, (2) some therapeutic recommendations may be made in reference to germline variants, (3) germline P/LP variants of clinical significance may be missed by tumor-only sequencing because of filtering on the basis of population frequency or masked by copy-number alterations; and (4) patients may be unnecessarily notified of a potential hereditary risk related to a finding that ultimately turns out not to be germline.

Within this pediatric cohort, more than half of the SNVs reported from tumor-only sequencing were actually germline, including 31% of tier 1 and 2 variants. Consistent with prior reports, we found that the distribution of VAFs of somatic and germline variants overlap and VAFs alone therefore cannot be

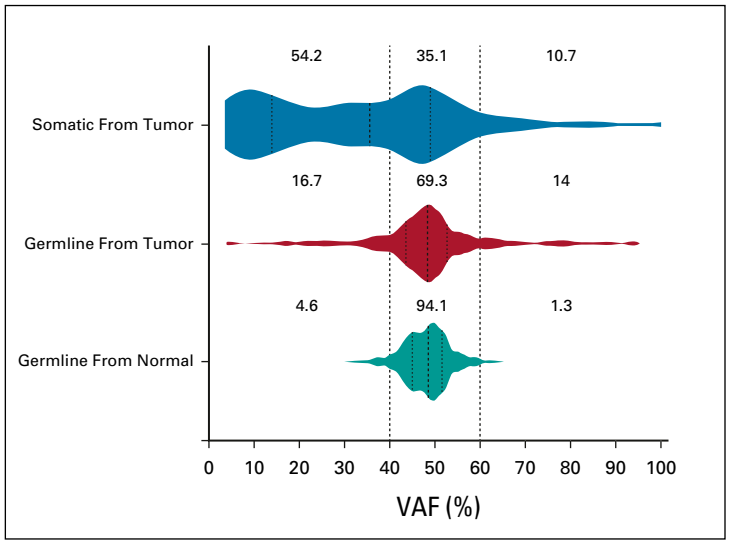

FIG 4. Comparison of somatic and germline VAFs in tumor and normal samples. Violin plot demonstrating the distribution of AF of variants identified from tumor-only sequencing (from tumor) and from germline sequencing (from normal). The height of the violin plot indicates the abundance of variants having AFs indicated along the *x*-axis. Vertical dashed lines embedded within the violin plots indicate the median AFs in the indicated cohort, and vertical dotted lines indicate the upper and lower quartiles of AFs within the data. Vertical dashes that run across the entire graph indicate AFs of 40% and 60%. Variants detected in tumor sequencing with AFs between 40%-60% are often interpreted to be likely germline when matching germline sequencing data are unavailable. AF, allelic fraction VAF, variant allele fraction.

used to reliably distinguish the germline or somatic origin of individual variants.[23] We also found that 24% of the iCat recommendations were made on the basis of variants later confirmed to be germline. Although the utility of targeting some germline variants remains unclear, there are, on the other hand, P/LP germline variants that have established therapeutic relevance: *BRCA1* and *BRCA2* variants and response to poly(ADP-ribose) polymerase (PARP) inhibitors, *KIT* and *PDGFRB* variants and tyrosine kinase inhibitors, and mismatch repair gene variants and immune checkpoint inhibitors.[24-26] Therefore, accurately identifying variants as germline first and then determining whether these have therapeutic relevance remains essential to consider.

Identifying hereditary P/LP variants in cancer predisposition genes may additionally affect future cancer screening or family planning for the patient and their family members. We found that tumor-only sequencing was not sufficient to identify all the clinically significant germline variants because of filtering of variants present above predefined thresholds in population databases, as well as copy-number gains and losses within the tumor. Although strategies to recover known P/LP variants in key genes can be part of the analytic pipeline, it can be difficult to make such recovery strategies comprehensive. Ideally, a paired tumor-normal analysis will allow for the optimal identification and recognition of significant germline alterations,

TABLE 2. Treatment Recommendations on the Basis of Tumor-Only Analysis Include Germline Variants

Variant	Germline Classification	Comments Within Report	Tier
CHEK2 c.1100delC (p.T367Mfs*15)	P	Caveat in report regarding gene association. Flagged as possible germline.	5
FAM175A c.1106dupG (p.S370Ifs*2)	P	Flagged as possible germline	4
SDHA c.91C>T (p.R31*)	P	None	3
SMARCA4 c.948delT (p.A317Pfs*9)	P	Flagged as possible germline	2
TP53 c.742C>T (p.R248W)	P	Flagged as possible germline	2
TP53 c.743G>A (p.R248Q)	P	Flagged as possible germline	2
TP53 c.392A>T (p.N131I)	LP	Flagged as possible germline	2
AKT1 c.1112C>A (p.T371K)	VUS	Caveat in report regarding VUS	2
NBN c.456G>A (p.M152I)	VUS	Caveat in report regarding VUS	4
TP53 c.949C>A (p.Q317K)	VUS	Caveat in report regarding VUS. Flagged as possible germline.	5
TSC2 c.2656G>C (p.V886L)	VUS	Caveat in report regarding VUS	2

NOTE. Eleven treatment recommendations were based on single-nucleotide variants that were later confirmed to be germline. Most (64%) were flagged as possibly germline during clinical review. The therapeutic recommendations were classified within tiers that indicate the level of evidence supporting recommendations (Tier 2: clinical evidence demonstrating a benefit for targeted therapy in patients with a different tumor and a variant in the same gene. Tier 3: preclinical evidence demonstrating a benefit for targeted therapy in models of the same tumor type. Tier 4: preclinical evidence demonstrating a benefit for targeted therapy in models of a different tumor type. Tier 5: expert panel feels there is insufficient information to qualify for treatment recommendation).

Abbreviations: LP, likely pathogenic; P, pathogenic; VUS, variant of uncertain significance.

because of the risk of masking important germline variants using a subtraction method.[26]

Our data support that paired tumor-normal sequencing would be best performed as an integrated analysis within the NGS pipeline. This approach would drastically decrease the workload for the molecular pathologist, shorten the time to clinical report, and greatly simplify the interpretation of results for the receiving provider or patient, which are time-intensive and potentially prohibitive.[27,28] It is important to acknowledge that many barriers exist to performing paired tumor-normal analysis including challenges in collecting tumor and blood samples simultaneously, limited access to molecular pathologists experienced in curating both somatic and germline cancer gene variants, and a potentially longer, more complicated consent process.

Our study had several limitations. First, our study was restricted to patients with pediatric solid tumors, well known to have lower somatic mutation rates than other cancers. Data also suggest that germline alterations may contribute more to the transformation process of early-onset cancers.[29] In addition, our study was limited to the analysis of variants within 147 of 447 genes included in the tumor sequencing. It is possible that the proportion of germline variants would be different if every gene were examined within the larger gene list. We also observed that there were very few cases within our cohort of tumor samples with elevated tumor mutational burden and, therefore, were unable to assess the impact that tumor-only sequencing may have had on these estimations, but we acknowledge that this remains another important consideration.[30] Finally, it is important to note that sequencing assays and associated analytic pipelines may be distinct, such that the results shown here may not overlap perfectly with other precision medicine programs that rely on different analytic tools for their tumor-only sequencing. Despite these limitations, the benefits of integrated germline sequencing, when feasible, are expected to be broadly applicable.

In summary, we demonstrate several potential gaps in the interpretation of tumor-only sequencing data in pediatric high-risk tumors and suggest that paired tumor-normal sequencing and analysis may offer substantial benefits for cancer precision medicine programs.

AFFILIATIONS

[1]Department of Pediatric Oncology, Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Boston, MA
[2]Department of Pathology, Boston Children's Hospital, Boston, MA
[3]Department of Pathology, Brigham and Women's Hospital, Boston, MA
[4]Department of Pediatrics, University of Chicago, Chicago, IL
[5]Department of Pediatrics, Children's Hospital of Philadelphia/University of Pennsylvania, Philadelphia, PA
[6]Department of Pediatrics, Memorial Sloan Kettering Cancer Center, New York, NY
[7]Center for Research Informatics, University of Chicago, Chicago, IL
[8]Center for Cancer and Blood Disorders, Children's National Hospital, Washington, DC
[9]Children's Hospital Colorado and University of Colorado Anschutz Medical Campus, Aurora, CO
[10]Division of Pediatrics (Pediatric Hematology and Oncology University of Utah), Huntsman Cancer Institute, University of Utah, Salt Lake City, UT
[11]Division of Pediatric Hematology/Oncology, University of Washington, Seattle, WA
[12]Department of Pediatrics, University of California, San Francisco, CA, San Francisco, CA
[13]Division of Pediatric Hematology, Oncology, and BMT, Nationwide Children's Hospital, Columbus, OH
[14]Division of Pediatric Hematology, Oncology, and Cellular Therapy, Children's Hospital at Montefiore, Bronx, NY
[15]Broad Institute of Harvard and MIT, Cambridge, MA

CORRESPONDING AUTHOR

Junne Kamihara, MD, PhD, 450 Brookline Ave, Boston, MA 02215; e-mail: junne_kamihara@dfci.harvard.edu.

EQUAL CONTRIBUTION

J.S. and A.J.C. contributed equally to this work. B.D.C. and J.K. jointly supervised this work.

PRIOR PRESENTATION

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AUTHOR CONTRIBUTIONS

Conception and design: Jaclyn Schienda, Alanna J. Church, Catherine M. Clinton, Theodore W. Laetsch, Samuel L. Volchenboum, Neal I. Lindeman, Katherine A. Janeway, Brian D. Crompton, Junne Kamihara
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Collection and assembly of data: Jaclyn Schienda, Alanna J. Church, Laura B. Corson, Brennan Decker, Catherine M. Clinton, Deirdre Reidy, Gianna R. Strand, Rochelle Bagatell, Steven G. DuBois, Julia L. Glade-Bender, Wenjun Kang, AeRang Kim, Theodore W. Laetsch, Margaret E. Macy, Amit J. Sabnis, Susan I. Colace, Neal I. Lindeman, Katherine A. Janeway, Brian D. Crompton, Junne Kamihara
Data analysis and interpretation: Jaclyn Schienda, Alanna J. Church, Laura B. Corson, Brennan Decker, Danielle K. Manning, Alma Imamovic-Tuco, Mark A. Applebaum, Rochelle Bagatell, Julia L. Glade-Bender, AeRang Kim, Theodore W. Laetsch, Margaret E. Macy, Luke Maese, Navin Pinto, Amit J. Sabnis, Joshua D. Schiffman, Susan I. Colace, Daniel A. Weiser, Jonathan A. Nowak, Neal I. Lindeman, Katherine A. Janeway, Brian D. Crompton, Junne Kamihara
Manuscript writing: All authors
Final approval of manuscript: All authors
Accountable for all aspects of the work: All authors

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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Alanna J. Church
Honoraria: The Jackson Laboratory
Consulting or Advisory Role: AlphaSights, The Jackson Laboratory, Bayer
Travel, Accommodations, Expenses: Bayer

Laura B. Corson
Employment: Sema4
Stock and Other Ownership Interests: Alnylam, Inovio Pharmaceuticals, Agios
Consulting or Advisory Role: Biomatics
Patents, Royalties, Other Intellectual Property: Patent from work done at H3 Biomedicine before 2016

Brennan Decker
Employment: Foundation Medicine
Stock and Other Ownership Interests: Avidea Technologies, Roche
Consulting or Advisory Role: Foundation Medicine, Avidea Technologies

Catherine M. Clinton
Employment: Helix OpCo LLC

Mark A. Applebaum
Consulting or Advisory Role: Fennec Pharma

Rochelle Bagatell
Uncompensated Relationships: Ymabs Therapeutics Inc

Steven G. DuBois
Consulting or Advisory Role: Bayer, Amgen
Research Funding: Merck (Inst), Roche/Genentech (Inst), Lilly (Inst), Curis (Inst), Loxo (Inst), BMS (Inst), Eisai (Inst), Pfizer (Inst), Turning Point Therapeutics (Inst), Bayer (Inst), Salarius Pharmaceuticals (Inst)
Travel, Accommodations, Expenses: Roche/Genentech, Salarius Pharmaceuticals
Uncompensated Relationships: Ymabs Therapeutics Inc

Julia L. Glade-Bender
Research Funding: Eisai (Inst), Lilly (Inst), Loxo (Inst), Roche/Genentech (Inst), Bayer (Inst)
Patents, Royalties, Other Intellectual Property: Patent on a T lympboblastic lymphoma cell line, CUTLL1
Travel, Accommodations, Expenses: Amgen (Inst)
Uncompensated Relationships: SpringWorks Therapeutics, Bristol Myers Squibb, Merck, Eisai
Open Payments Link: https://openpaymentsdata.cms.gov/physician/708514

AeRang Kim
Research Funding: Esai (Inst); AstraZeneca (Inst); Oncternal (Inst); Ascentage (Inst); BioAlta (Inst).

Theodore W. Laetsch
Consulting or Advisory Role: Novartis, Bayer, Cellectis, Aptitude Health, Clinical Education Alliance, Deciphera, Jumo Health, Massive Bio, Med Learning Group, Medscape, Physicans' Education Resource, Y-mAbs Therapeutics
Research Funding: Pfizer (Inst), Novartis (Inst), Bayer (Inst), AbbVie (Inst), Amgen (Inst), Atara Biotherapeutics (Inst), Bristol Myers Squibb (Inst), Lilly (Inst), Epizyme (Inst), GlaxoSmithKline (Inst), Janssen (Inst), Jubilant Pharmaceuticals (Inst), Novella Clinical (Inst), Servier (Inst), Foundation Medicine (Inst), Merck Sharp & Dohme (Inst)

Margaret E. Macy
Stock and Other Ownership Interests: Johnson & Johnson, GE Healthcare, Varian Medical Systems
Consulting or Advisory Role: Ymabs Therapeutics Inc
Research Funding: Bayer (Inst), Ignyta (Inst), Roche (Inst), Lilly (Inst), Merck (Inst), Oncternal Therapeutics Inc (Inst), AbbVie (Inst), Jubilant DraxImage (Inst), Actuate Therapeutics (Inst)
Patents, Royalties, Other Intellectual Property: Patent for non-invasive methods of leukemia cell detection with MRI/MRS—Patent No. 8894975

Luke Maese
Honoraria: Jazz Pharmaceuticals
Consulting or Advisory Role: Jazz Pharmaceuticals

Amit J. Sabnis
Consulting or Advisory Role: Cardinal Health

Joshua D. Schiffman
Employment: PEEL Therapeutics
Leadership: PEEL Therapeutics
Stock and Other Ownership Interests: ItRunsInMyFamily.com, PEEL Therapeutics
Honoraria: Affymetrix
Consulting or Advisory Role: N-of-One, Fabric Genomics

Samuel L. Volchenboum
Stock and Other Ownership Interests: Litmus Health
Consulting or Advisory Role: Accordant
Travel, Accommodations, Expenses: Sanford Health

Daniel A. Weiser
Consulting or Advisory Role: Illumina Radiopharmaceuticals

Jonathan A. Nowak
Research Funding: NanoString Technologies, Illumina

Katherine A. Janeway
Honoraria: Foundation Medicine, Takeda
Consulting or Advisory Role: Bayer, Ipsen
Travel, Accommodations, Expenses: Bayer

Brian D. Crompton
Employment: Acceleron Pharma (I)
Stock and Other Ownership Interests: Acceleron Pharma (I)
Research Funding: Gradalis

Junne Kamihara
Stock and Other Ownership Interests: PanTher Therapeutics (I), ROME Therapeutics (I), TellBio (I)
Honoraria: Pfizer (I), NanoString Technologies (I), Foundation Medicine (I), Ikena Oncology (I)
Consulting or Advisory Role: ROME Therapeutics (I), Third Rock Ventures (I), Tekla Capital Management (I)
Research Funding: PureTech (I), Ribon Therapeutics (I), ACD Biotechne (I)
Patents, Royalties, Other Intellectual Property: Patent on drug delivery device licensed to PanTher Therapeutics (I), Patents on Repeat RNA biomarkers and therapeutics licensed to Rome Therapeutics (I), Patents on Circulating Tumor Cell Biomarkers Licensed to TellBio Inc (I)

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APPENDIX

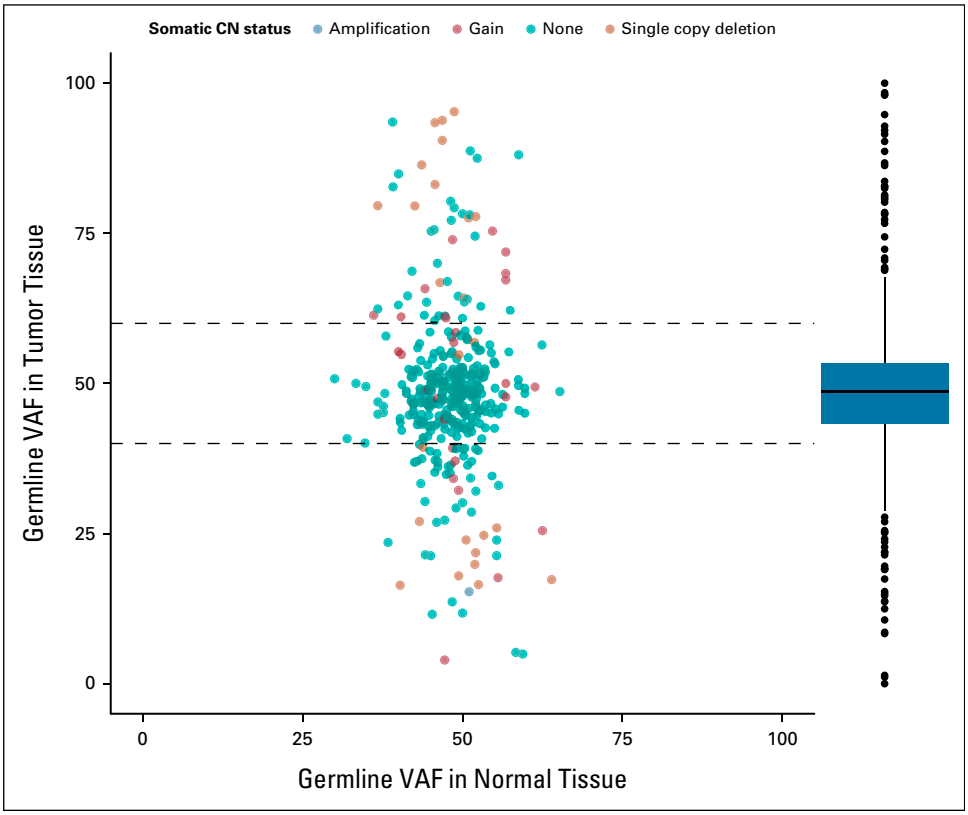

FIG A1. Pairwise comparison of germline VAFs in tumor versus normal tissue. Pairwise comparison of the AF of germline variants identified from tumor-only sequencing (tumor tissue) to the AF from germline sequencing (normal tissue). Each variant is color-coded to indicate the copy-number status (amplification, gain, single copy deletion, or neutral) of the corresponding gene. Copy-number gains and loss could not explain many germline VAFs outside of 40%-60% in tumor tissue. AF, allelic fraction; CN, copy number; VAF, variant allele fraction.

TABLE A1. Pathogenic or Likely Pathogenic Germline Variants

Gene (Ensembl Reference Transcript), Variant	Cancer Risk Classification	Tumor Diagnosis	Present on Tumor-Only Sequencing Report	Second Mutation in Tumor	Gene Previously Associated With Cancer Type
APC (ENST00000457016), c.3920T>A (p.I1307K)	AD	Osteosarcoma	No	No	No
APC (ENST00000457016), c.3920T>A (p.I1307K)	AD	Wilms tumor	Yes	No	No
BLM (ENST00000355112), c.1933C>T (p.Q645*)	AR	Ewing sarcoma	Yes	No	No
BRCA1 (ENST00000357654), CNV intron9/exon 10 partial deletion	AD	Neuroblastoma	No	No	No
CHEK2 (ENST00000404276), c.1100delC (p.T367Mfs*15)	AD	Wilms tumor	Yes	No	No
DICER1 (ENST00000393063), c.3591C>A (p.C1197*)	AD	CNS sarcoma	Yes	*DICER1* c.5125G>A (p.D1709N)	Yes
DICER1 (ENST00000393063), c.1525C>T (p.R509*)	AD	Pleuropulmonary blastoma	Yes	*DICER1* c.5113G>A (p.E1705K)	Yes
DICER1 (ENST00000393063), c.2781C>A (p.Y927*)	AD	Uterine embryonal rhabdomyosarcoma, botryoid type	Yes	*DICER1* c.5125G>A (p.D1709N)	Yes
DICER1 (ENST00000393063), c.904-1G>C	AD	Sertoli-Leydig cell tumor of the ovary	Yes	*DICER1* c.5439G>T (p.E1813D)	Yes
DOCK8 (ENST00000453981), c.700delA (p.R234Gfs*40)	AR	Alveolar rhabdomyosarcoma	Yes	No	No
ELANE (ENST00000263621), whole gene deletion	AD	*CIC* rearranged sarcoma	Yes	No	No
ERCC3 (ENST00000285398), c.325C>T (p.R109*)	AR	Mesenchymal chondrosarcoma	No	No	No
FAM175A (ENST00000321945), c.1106dupG (p.S370Ifs*2)	AD	Osteosarcoma	Yes	Deletion of *FAM175A*	No
FANCA (ENST00000389301), CNV exons 11-29 deletion	AR	Hepatoblastoma	Yes	No	No
GBA (ENST00000368373), c.1226A>G (p.N409S)	AR	Mesenchymal chondrosarcoma	No	No	No
GBA (ENST00000368373), c.1226A>G (p.N409S)	AR	*CIC* rearranged sarcoma	No	No	No
GBA (ENST00000368373), c.222_224delTAC (p.T75del)	AR	Osteosarcoma	Yes	No	No
MITF (ENST00000352241), c.1255G>A (p.E419K)	AD	Ewing sarcoma	No	No	No
MUTYH (ENST00000372098), c.1178G>A (p.G393D)	AR	Spindle cell neoplasm (infantile myofibroma)	Yes	No	No
MUTYH (ENST00000372098), c.1178G>A (p.G393D)	AR	Alveolar rhabdomyosarcoma	Yes	No	No

(Continued on following page)

TABLE A1. Pathogenic or Likely Pathogenic Germline Variants (Continued)

Gene (Ensembl Reference Transcript), Variant	Cancer Risk Classification	Tumor Diagnosis	Present on Tumor-Only Sequencing Report	Second Mutation in Tumor	Gene Previously Associated With Cancer Type
MUTYH (ENST00000372098), c.303C>A (p.Y101*)	AR	Hepatocellular carcinoma	Yes	No	No
RB1 (ENST00000267163), c.1216-3A>G	AD	Osteosarcoma	Yes	No	Yes
SBDS (ENST00000246868), c.258+2T>C	AR	Osteosarcoma	No	No	No
SDHA (ENST00000264932), c.91C>T (p.R31*)	AD	GIST	Yes	Deletion of *SDHA*	Yes
SERPINA1 (ENST00000440909), c.1096G>A (p.E366K)	AR	Osteosarcoma	No	No	No
SERPINA1 (ENST00000440909), c.1096G>A (p.E366K)	AR	Osteosarcoma	No	No	No
SERPINA1 (ENST00000440909), c.1096G>A (p.E366K)	AR	Osteosarcoma	No	No	No
SERPINA1 (ENST00000440909), c.1096G>A (p.E366K)	AR	Alveolar rhabdomyosarcoma	No	No	No
SERPINA1 (ENST00000440909), c.1096G>A (p.E366K)	AR	Osteosarcoma	No	No	No
SERPINA1 (ENST00000440909), c.187C>T (p.R63C)	AR	Ewing sarcoma	Yes	No	No
SERPINA1 (ENST00000440909), c.739C>T (p.R247C)	AR	Ganglioneuroblastoma	No	No	No
SMARCA4 (ENST00000344626), c.948delT (p.A317Pfs*9)	AD	Malignant neoplasm with rhabdoid-like morphology	Yes	CN-LOH	Yes
TP53 (ENST00000269305), c.392A>T (p.N131I)	AD	Chondrosarcoma	Yes	No	Yes
TP53 (ENST00000269305), c.742C>T (p.R248W)	AD	Osteosarcoma	Yes	Deletion of *TP53* within loss of 17p	Yes
TP53 (ENST00000269305), c.743G>A (p.R248Q)	AD	Osteosarcoma	Yes	CN-LOH	Yes
UROD (ENST00000246337), c.239C>G (p.A80G)	AD	Neuroblastoma	Yes	No	No
WRN (ENST00000298139), c.2194C>T (p.R732*)	AR	Sertoli-Leydig cell tumor of the ovary	Yes	No	No
XPA (ENST00000375128), c.555G>C (p.Q185H)	AR	Osteosarcoma	Yes	No	No

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; CN-LOH, copy neutral loss of heterozygosity; CNS, central nervous system; CNV, copy-number variant; GIST, gastrointestinal stromal tumor.

