Genomic profiling of pediatric hematologic malignancies and diagnosis of cancer predisposition syndromes: tumor-only *versus* paired tumor-normal sequencing

Next-generation sequencing (NGS) technology has dramatically enhanced genomic characterization of hematologic malignancies, aiding in diagnosis, risk stratification, and treatment.[1-3] Simultaneously, enhanced sequencing and more accessible germline testing has revealed that cancer predisposition syndrome (CPS) accounts for a greater proportion of pediatric cancer diagnoses than previously appreciated.[4,5] The diagnosis of a CPS in children is critical for informed treatment decision-making, future cancer surveillance, testing for family members, and family planning.[6-8] Although somatic DNA-based panel results may suggest the presence of a germline variant associated with cancer predisposition based on variant allele fraction (VAF) and/or other features of the variant and gene,[9] tumor-only sequencing cannot definitively distinguish somatic and germline alterations. Thus, tumor-only sequencing requires follow-up testing for assessment of potential germline mutations. Alternatively, up-front paired tumor and germline (referred to herein as tumor-normal [T/N]) testing at the time of diagnosis utilizes DNA isolated from both cancer cells and non-malignant cells (usually from skin biopsy), sequenced at the same time and on the same platform, such that data from tumor and normal DNA of the same individual can be analyzed together. This approach not only enhances the precision of identifying somatic alterations by comparing the patient's cancer genome to their own constitutional genome instead of a generic human reference genome, but can also simultaneously identify germline cancer predisposition within genes being sequenced.[10] We herein describe the implementation of DNA-based paired T/N testing as part of a large pediatric cancer program, compare diagnostic yield of tumor-only testing followed by germline confirmation *versus* paired T/N testing, assess clinical implications for patients diagnosed with a CPS, and examine the benefits and limitations of these two sequencing approaches.

A total of 1,190 pediatric and adolescent and young adult (AYA) patients (age 0-35 years) were retrospectively included in this cohort, 1,034 of whom underwent tumor-only testing between June 2016 and October 2022, and 156 of whom underwent paired T/N testing between January 2021 and August 2023 (Figure 1). This study was approved by the Children's Hospital of Philadelphia Institutional Review Board. Our targeted hematologic cancer panel (HEMEP)[2] and comprehensive hematologic panel (COHEM) interrogate 117 known cancer genes associated with hematologic malignancies for SNV, indel, and CNV. The COHEM panel also includes RNA-based fusion analysis for over 700 exons of 117 cancer genes for known and novel fusions.[2,11] The identified variants were categorized according to the guidelines.[12,13] Demographics and clinical characteristics of this patient cohort are described in *Online Supplementary Table S1*. Mean age was 9 years. The most common diagnosis was B-cell acute lymphoblastic leukemia (B-ALL; 58%), followed by acute myeloid leukemia (AML) / myeloid sarcoma (18%), and T-cell acute lymphoblastic leukemia/lymphoblastic lymphoma (T-ALL/T-LL; 9%). Most cases (1,068/1,190; 90%) underwent COHEM panel testing, while a smaller proportion had HEMEP panel sequencing (122/1,190; 10%).

Among 1,034 patients who initially underwent tumor-only molecular sequencing, 31 (3%) patients were found to have genomic alterations consistent with a pre-existing/known CPS. An additional 111 (11%) patients without a known CPS met criteria for follow-up germline testing as recommended on their diagnostic tumor NGS reports (Figure 1). Within the cohort of 111 patients recommended for germline follow-up testing, 47 cases were submitted from other institutions and had no follow-up clinical data available. Notably, none of these cases had germline specimens submitted to the CHOP diagnostic genomics laboratory. In the cohort of patients treated at our institution (N=64) recommended for follow-up testing, 29 (45%) had subsequent confirmatory germline testing facilitated by the cancer predisposition team or the patient's primary oncologist, whereas 35 patients (55%) had no documented confirmatory testing. Of these 35 patients, 16 died within six months of somatic testing, which may have precluded the possibility or intention to perform recommended follow-up testing. Notably, this high mortality rate may in part reflect the over-representation of children and AYA at our institution with relapsed / refractory disease, referred for early-phase clinical trial participation or other salvage therapies, who undergo molecular testing. One patient has germline testing pending insurance authorization, and another patient was recently referred to the cancer predisposition clinic. For the remaining 17 patients, germline predisposition was mentioned only in genomics reports, suggesting the information may have potentially been missed by clinicians and families. Among the 29 patients who initially underwent somatic-only tumor testing and subsequent recommended germline testing, 10 (34%) were confirmed to have a CPS, including genetic mutations associated with Noonan syndrome-like disorder (*CBL*), DNMT3A overgrowth syndrome and predisposition to hematologic

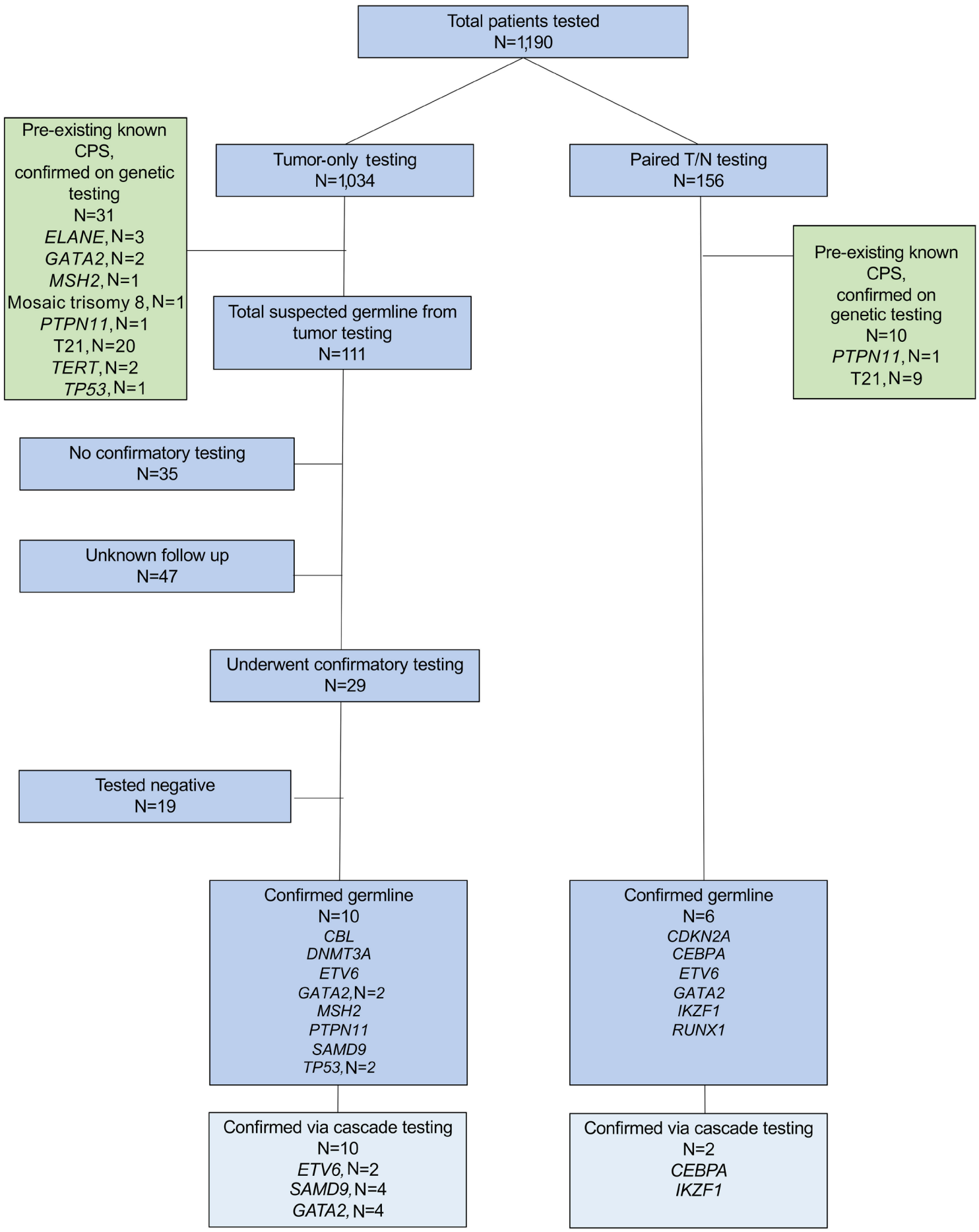

Figure 1. Consort diagram of patients undergoing genomic testing with tumor-only or paired tumor-normal testing and cancer predisposition syndrome diagnoses.

malignancy/ Tatton-Brown-Rahman syndrome (*DNMT3A*), ETV6 thrombocytopenia and leukemia predisposition syndrome (*ETV6*), GATA2 deficiency syndrome (GATA2), Lynch syndrome (*MSH2*), Noonan syndrome (*PTPN11*), Mirage syndrome (*SAMD9*), and LFS (*TP53*).

Among 156 patients who underwent paired T/N testing, 10 patients (6%) were found to have genomic alterations consistent with a pre-existing / known CPS. Six patients (4%) were diagnosed with a new CPS (Figure 1), including CDNK2A-associated predisposition (*CDKN2A*), CEBPA-associated predisposition to AML (*CEBPA*), ETV6 thrombocytopenia and leukemia predisposition syndrome (*ETV6*), GATA2 deficiency syndrome (*GATA2*), IKZF1-associated leukemia predisposition (*IKZF1*), and RUNX1 familial platelet disorder with associated myeloid malignancies (*RUNX1*).

If tumor-only testing, instead of T/N, were performed on

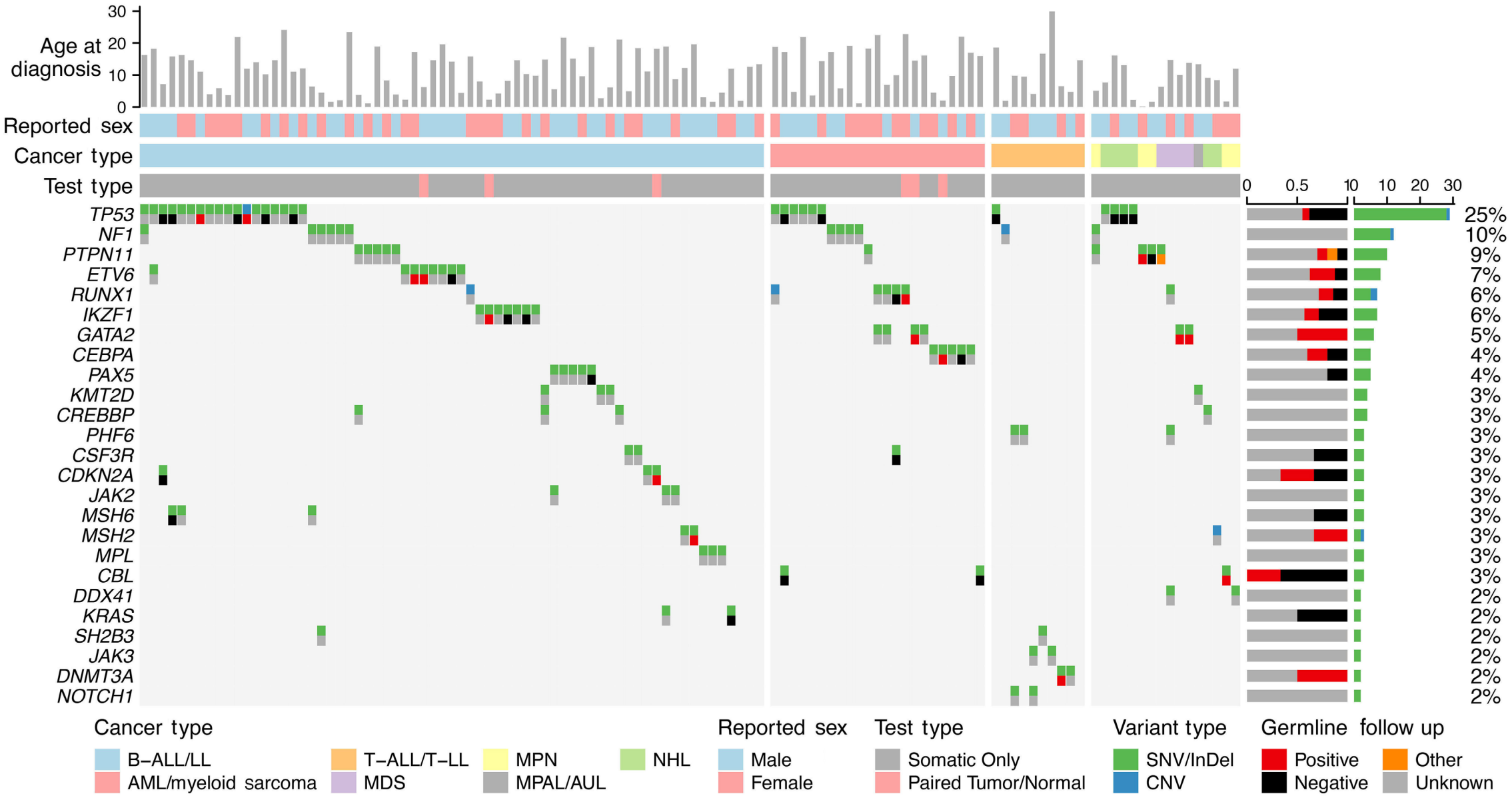

Figure 2. Oncoprint of variants recommended for germline follow up, identified through somatic testing, or confirmed germline variants, identified through paired tumor/normal testing. Top icon demonstrates variant type (SNV / indel, CNV). Bottom icon demonstrates germline follow-up result. Annotations for age at diagnosis, sex, cancer type, and test type (somatic *vs*. tumor/normal [T/N]) are displayed on the top of the oncoprint. Barcharts to the right show the proportion of patients with a germline variant identified that underwent follow up by gene (left) and the frequency of each gene within the whole cohort (right).

Table 1. Clinical characteristics of patients diagnosed with cancer predisposition syndrome.

Case	Syndrome	Gene	Diagnosis	Age at onset in years	Clinical profile	Clinical implications of knowing CPS
Cases diagnosed from somatic testing with follow-up germline testing						
1	Noonan syndrome-like disorder	*CBL*	JMML	1	- Patient presented with splenomegaly and thrombocytopenia as a toddler. Genetic testing notable for *CBL* mutation in tumor and subsequently heterozygous germline specimen. - Treated with cytarabine and fludarabine for 2 cycles and required no additional treatment; remains in remission.	- Management of JMML impacted by knowing germline predisposition. - Connected to other multidisciplinary subspecialists.
2	DNMT3A overgrowth syndrome and predisposition to hematologic malignancy (Tatton-Brown-Rahman)	*DNMT3A*	T-ALL	2	- Patient had a history of paraspinal neuroblastoma and subsequently presented with cervical adenopathy and a mediastinal mass consistent with T-cell lymphoblastic lymphoma. Progressed during therapy and subsequently died from disease progression.	- Connected to genetics team for follow up and familial testing.
3	ETV6 thrombocytopenia and leukemia predisposition syndrome	*ETV6*	B-ALL	16	- Teenager presented with flu-like symptoms, diagnosed with high-risk ALL. Mother and sibling also had a history of B-ALL; both underwent testing and found to have the same pathogenic mutation. Patient is currently in remission.	- Follows with CPP. - Guidance provided on bleeding phenotype in some patients with this syndrome. - Cascade testing performed.
4	GATA2 deficiency syndrome	*GATA2*	MDS	10	- Patient initially presented with lymphedema, neutropenia and monocytopenia. BM biopsy revealed monosomy 7 MDS and *GATA2* mutation, which was then confirmed as germline. Recommended for HSCT.	- Identification of predisposition influenced recommendation for HSCT. Also guided familial cascade testing to identify the optimal donor.
5	GATA2 deficiency syndrome	*GATA2*	MDS	10	- Patient presented with fevers and pancytopenia, found to have monosomy 7 MDS with *GATA2* mutation which was then confirmed as germline. Underwent BM transplant, in remission and doing well.	- Identification of predisposition influenced recommendation for HSCT. Also guided familial cascade testing to identify the optimal donor.
6	Lynch syndrome	*MSH2*	B-ALL	19	- Presented with abdominal pain, found to have high-risk B-ALL. Somatic testing showed a *MSH2* variant which was confirmed as germline. In remission and doing well.	- Follows with CPP. - Family members underwent genetic testing. - Connected with gastroenterology for colonoscopies and dermatology for regular skin exams.
7	Noonan syndrome	*PTPN11*	B-ALL	8 weeks	- Presented with JMML in infancy and on presentation noted to have heart murmur and dysmorphic features. Cancer panel demonstrated *PTPN11* variant, confirmed as germline. JMML spontaneously resolved. - Patient in remission and doing well.	- Management of JMML impacted by knowing germline predisposition. - Connected to .subspecialists (genetics, cardiology, urology, endocrine, ophthalmology).
8	Mirage syndrome	*SAMD9*	MDS	6	- Presented with lymphedema, neutropenia and monocytopenia. BM biopsy revealed hypocellular marrow with a chromosomal abnormality in small fraction of cells (46, XX der(1;7)) resulting in gain of 1q and loss of 7q. NGS testing revealed a pathogenic *GATA2* mutation consistent with GATA2 haploinsufficiency and monosomy 7 myelodysplasia. Given monosomy 7 MDS, patient was referred for BM transplant. In remission post transplant and doing well.	- Identification of predisposition influenced recommendation for HSCT. Also guided familial cascade testing to identify the optimal donor. - Family cascade testing performed.

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9	Li Fraumeni syndrome	*TP53*	B-ALL	12	- Presented with fevers, diagnosed with low hypodiploid B-ALL. Now off therapy and in remission; doing well.	- Follows with CPP and undergoes routine LFS surveillance. - Family members underwent cascade testing.
10	Li Fraumeni syndrome	*TP53*	B-ALL	10	- Presented with weight loss, fevers and diagnosed with hypodiploid B-ALL. Somatic testing showed TP53 variant in tumor, confirmed in germline and thought to be mosaic given low VAF.	- Follows with CPP. - Will undergo routine LFS surveillance.
Cases diagnosed from paired T/N testing						
1	CDKN2A-associated predisposition	*CDKN2A*	B-ALL	15	- Presented with fever, bleeding gums and diagnosed with B-ALL. Found to have germline *CDKN2A* variant.	- Recommended for follow up with CPP.
2	CEBPA-associated predisposition to AML	*CEBPA*	AML	2	- Presented with fever and increased breathlessness, diagnosed with AML. T/N testing showed a germline mutation in *CEBPA* and additional *CEBPA* mutation in the tumor only. - In remission and doing well.	- Follows with CPP. - Family underwent cascade testing.
3	ETV6 thrombocytopenia and leukemia predisposition syndrome	*ETV6*	B-ALL	6	- Presented with fatigue and pallor and diagnosed with B-ALL. T/N demonstrated pathogenic *ETV6* variant in germline and tumor, hyperdiploid ALL. Now in remission and doing well.	- Follows with CPP. - Guidance provided on bleeding phenotype in some patients with this syndrome. - Cascade testing performed.
4	GATA2 deficiency syndrome	*GATA2*	AML	14	- Presented with splenomegaly, petechiae and purpura, diagnosed with AML. T/N testing demonstrated somatic and germline *GATA2* (0.50 tumor/0.47 germline) and additional somatic *CEBPA* mutation. Given *GATA2* predisposition, patient was recommended for HSCT. Underwent transplant and is now in remission.	- Identification of predisposition influenced recommendation for HSCT. Also guided familial cascade testing to identify the optimal donor.
5	IKZF1-associated leukemia predisposition	*IKZF1*	B-ALL	2	- Presented with prolonged fevers, diagnosed with high-risk B-ALL. Somatic testing demonstrated *P2RY8::CRLF2* fusion; paired T/N with IKZF1 variant in the skin and tumor specimen. Currently in remission.	- Follows with CPP. - Family underwent cascade testing; parent referred to adult CPP program.
6	RUNX1 familial platelet disorder with associated myeloid malignancy	*RUNX1*	AML	2	- Initially followed by Hematology for congenital thrombocytopenia and subsequently diagnosed with AML. Genetic testing demonstrated germline *RUNX1* pathogenic variant. Received HSCT, in remission and doing well.	- Identification of predisposition influenced recommendation for HSCT. Also guided familial cascade testing to identify the optimal donor. - Follows with CPP.

ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; B-ALL: B-cell acute lymphoblastic leukemia; BM: bone marrow; CPP: cancer predisposition program; CPS: cancer predisposition syndrome; HSCT: hematopoietic stem cell transplantation; JMML: juvenile myelomonocytic leukemia; LFS: Li-Fraumeni syndrome; MDS: myelodysplastic syndromes; NGS: next-generation sequencing; T-ALL: T-cell acute lymphoblastic leukemia; T/N: tumor/normal; VAF: variant allele fraction.

those 6 patients with a new CPS, the results would trigger a germline testing.

Potential or confirmed germline variants were identified in 35 cancer predisposition genes from 117 patients (Figure 2, *Online Supplementary Table S2*). Potential germline variants were most frequently identified in *TP53* (N=36), *NF1* (N=15), and *PTPN11* (N=10). Among patients diagnosed with a CPS, there were several immediate, clinically relevant implications (Table 1). Two patients with germline *CBL*- or *PTPN11*-mutant juvenile myelomonocytic leukemia (JMML) were treated according to guidelines for patients with germline predisposition to JMML, which differ from guidelines for children with somatic Ras pathway mutation-driven JMML.[14] An additional 3 patients were diagnosed with germline GATA2 deficiency syndrome, one with AML and 2 with monosomy 7 myelodysplastic syndromes. All 3 were recommended for hematopoietic stem cell transplantation (HSCT) with genetic testing of family members for optimal *GATA2* wild-type transplant donor selection and predisposition screening. Two patients were diagnosed with Li-Fraumeni syndrome (LFS) portending lifelong increased risk of malignancy and prompting referral to the cancer

predisposition team for optimal tumor surveillance, genetic counseling, and cascade testing for at-risk family members. Of the 16 patients with hematologic malignancies and a CPS newly diagnosed from genomic testing (Figure 1), there was no known cancer predisposition or early onset cancer in any other immediate family members. To date, 33 of 48 (69%) family members recommended for germline testing based upon diagnoses of a CPS in their probands have undergone testing (*Online Supplementary Figure S1*). Among those tested, 12 family members tested positive for germline mutations and a CPS (including *CEBPA*, *ETV6*, *GATA2*, *IKZF1*, *SAMD9*) with indications for follow up in the pediatric or adult cancer predisposition clinic, and in some instances referral for HSCT.

Overall, 9% of patients in this cohort who underwent tumor-only testing with follow-up germline confirmation or up-front paired T/N testing (10 of 29 and 6 of 156, respectively) at our institution were diagnosed with a new CPS, consistent with reported frequency in pediatric cancer.[5] Among patients with suspected germline predisposition from somatic tumor-only testing, a substantial number (32%) did not undergo confirmatory germline testing.

The results of our study suggest that paired T/N testing for pediatric and AYA patients with hematologic malignancies has several advantages over somatic tumor-only testing. From a patient care perspective, this approach identifies somatic variants and hereditary predisposition simultaneously,[10] obviating barriers to germline testing, mitigating loss to follow-up, and reducing undue anxiety for patients with findings suggestive of germline alteration that are, in fact, somatic events and do not have familial implications. Even with improvement in data reporting and recognition of a possible CPS, challenges with insurance approval for testing and additional clinical visits create substantial barriers to obtaining follow-up germline testing for many patients. Furthermore, with declining sequencing costs, there is now minimal extra cost of sequencing a paired normal sample on a single, streamlined platform. Thus, at our institution, the cost of up-front paired T/N is less than the cumulative cost of tumor-only sequencing followed by confirmatory sequencing with specific primer design and lab implementation. From a genomic perspective, paired T/N sequencing allows for the subtraction of variants in matched normal tissue from tumor tissue to reveal acquired genetic alterations that are truly somatic in origin and aid in variant classification.

Our study suggests that up-front paired T/N testing should be pursued, when possible, with thorough pre-test counseling,[15] as the impact on clinical decision-making and long-term management is significant when a CPS is identified. Furthermore, this strategy can potentially alleviate undue emotional burden and mitigate economic barriers associated with follow-up testing in most patients with somatic mutation-driven cancers who do not require further germline testing. Future studies should explore patient and family experiences utilizing mixed methods approaches incorporating qualitative data, as well as assess implementation of broader predisposition genomic panels in the context of pediatric malignancies. At present, our comprehensive hematologic panel does not include all cancer predisposition-related genes. We are currently working to implement a more comprehensive panel, as the continuing reduction in sequencing costs enables expanded genomic profiling without additional expense.

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SPH owns common stock in Amgen and has received honoraria or consulting fees from Amgen, Jazz, Novartis, and Servier. SKT has received research funding from Kura Oncology and Incyte Corporation, has served on scientific advisory boards for Aleta Biotherapeutics, AstraZeneca, Jazz Pharmaceuticals, Kura Oncology, and Syndax Pharmaceuticals, and has received travel support from Amgen (all for unrelated studies). All the other authors have no conflicts of interest to disclose.

Contributions

HN, MEC, DW, YZ and ML conceived and designed the study, analyzed the data, and wrote the initial version of the manuscript. All authors interpreted the results, and reviewed and contributed to the final version of the manuscript.

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Data-sharing statement

The datasets generated and analyzed for this study are available from the corresponding author on reasonable request.

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