Strategies for identifying *NUP98* rearrangements in adult myeloid neoplasms

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Abstract

Nucleoporin 98 rearrangements (*NUP98*r) are recurrent in myeloid neoplasms and are subtype-defining for acute myeloid leukemia (AML) in the World Health Organization Classification 5th edition (WHO5) and the International Consensus Classification (ICC). Identification of *NUP98*r is essential given the frequency of treatment resistance and possibility of sensitivity to targeted therapies. However, *NUP98*r is often cryptic on karyotype and has over 40 described partners. Therefore, it is underdiagnosed in the absence of dedicated testing that is not always routine practice, e.g., RNA-based next generation sequencing (NGS), *NUP98* break-apart fluorescence *in situ* hybridization, or real-time-quantitative polymerase chain reaction for specific *NUP98* fusions. Historically, AML with *NUP98*r has received the most attention in pediatric AML, where its incidence is highest, but has been increasingly characterized in adult AML. By contrast, the incidence and behavior of *NUP98* fusions in myelodysplastic syndromes (MDS) is less understood and based predominantly on case reports. In this study, we describe our adult institutional experience with a clinically validated anchored multiplex PCR RNA-based targeted NGS assay, explore strategies for rational use of specific testing for *NUP98*r including a proof-of-principle based on *WT1* and *FLT3*-ITD mutational status, and integrate our results with a review of the literature. In total, we identified 3 MDS and 15 AML patients with *NUP98r* as the genetic driver, including two novel fusion partners (*FGF14* and *LAMC3*), thus highlighting the utility of NGS testing to detect *NUP98* fusions. Recognition of *NUP98*r in myeloid neoplasms is crucial for accurate diagnosis and prognosis, with significant implications for therapy or enrollment in clinical trials.

Introduction

Acute myeloid leukemia (AML) with nucleoporin 98 rearrangement (*NUP98*r) comprises one of several genetically defined AML subtypes that have been newly incorporated into both the 2022 International Consensus Classification (ICC) and the 5th edition of the World Health Organization Classification (WHO5), where a diagnosis of AML can be made with a blast count under 20%.[1,2] AML with *NUP98*r has historically been associated with adverse clinical outcomes and chemotherapy resistance although recent preclinical models have raised the possibility of rational targeted therapy with menin inhibitors.[3] Thus, routine identification of *NUP98* rearrangement is important for clinical care of patients with AML and future improvement of risk-adapted therapy. However, the entity is underdiagnosed by many clinical practices since the rearrangements are frequently cryptic on conventional karyotype due to the location of *NUP98* at 11p15.4 near the terminal end of the short arm of chromosome 11. The *NUP98* gene, which encodes a component of the nuclear pore complex, rearranges with over 40 unique fusion partners, all involving the N-terminal end of *NUP98* and notable for partner-specific enrichments for monocytic, myelomonocytic, megakaryoblastic, or erythroid differentiation.[4] Accordingly, reliable detection across the entire spectrum of fusion partners requires complex testing modalities such as whole transcriptome RNA-seq, targeted RNA-based next generation sequencing (NGS) fusion assays with coverage of *NUP98* rearrangements, optical genome mapping (OGM), whole genome sequencing (WGS), or fluorescence *in situ* hybridization (FISH) break-apart probes for *NUP98*. Of these, *NUP98* FISH is cheapest and fastest

but does not identify the specific *NUP98* partner and would not detect other cryptic rearrangements that are potential drivers in the absence of *NUP98*r. By contrast, WGS is the most comprehensive and has started to become adopted clinically, with accurate risk categorization for AML and myelodysplastic syndromes (MDS) including reliable detection of *NUP98*r.[5,6]

In recent years, RNA-based NGS fusion assays have enabled estimates of the prevalence of *NUP98*r in AML, ranging from 7.2-8.0% of pediatric AML and 2.5-5.0% of adult AML.[6-12] NGS has also revealed distinct co-mutational patterns, including enrichment of *FLT3*-ITD and *WT1* variants, particularly in AML with *NUP98::NSD1*. *NUP98*r has been less studied in myeloid neoplasms outside of AML. Although presumed to be exceedingly rare, their frequency may be underappreciated.[13-15] Mouse models of *NUP98::NSD1* have generated conflicting data, with one study showing almost universal transformation to AML, compared to other studies indicating a weak leukemogenic potential alone, but increased when combined with *FLT3*-ITD.[16-18] The presence of a chronic or pre-leukemic phase of *NUP98*r AML may be clinically relevant for the possibility of earlier detection and intervention.

Here, we report morphological, clinical, and molecular findings at our institution of adults with *NUP98*r AML or MDS, and explore features in our data and in public datasets which could prompt specific testing for *NUP98*r, potentially providing the basis for cost-effective strategies in clinical practices that do not employ screening for *NUP98*r. In particular, a myelomonocytic morphology and immunophenotype, *WT1* mutations in MDS, and concurrent *FLT3*-ITD and *WT1* mutations in AML, in the absence of another subtype defining genetic aberration (e.g., *NPM1*), highly enrich for myeloid neoplasms with *NUP98::NSD1* or occasionally other *NUP98* rearrangement partners.

Methods

Nucleic acid extracted from blood, bone marrow (BM), or extramedullary disease sites was tested by one or more of several NGS assays: 1) a clinically validated targeted RNA assay (Heme Fusion Assay [HFA]; Integrated DNA Technologies) designed principally to detect fusions through anchored multiplex polymerase chain reaction (PCR) (AMP)[19] and performed on clinical samples as part of patient care from 2017-2024 (N=381; HFA clinical cohort) and on research samples for this study (N=7) at the Center for Integrated Diagnostics at Massachusetts General Hospital; 2) a clinically validated targeted DNA panel (Rapid Heme Panel [RHP*] version 3)[20] based on NEBNextDirect (New England BioLabs) to detect single nucleotide variants, small indels, and copy number alterations, and performed on clinical samples as part of patient care from 2019-2024 (N=21209; RHP cohort) at the Center for Advanced Molecular Diagnostics at Brigham and Women's Hospital; and 3) total RNA sequencing performed on research samples for this study (N=2) at the Dana-Farber Cancer Institute molecular biology core facility. The research RNA-based NGS testing was performed on nucleic acid extracted from archived cytogenetic pellets. Data were processed by default clinical pipelines (HFA, RHP) or by a custom pipeline (total RNA-seq) using adapter trimming by BBDuk, alignment to hg19 by bwa-mem, and manual analysis of bam files. *NUP98* break-apart FISH (Empire Genomics; 11p15.4) was performed on 100 interphase nuclei. Overall survival (determined from the date of first diagnosis to death from any cause) was assessed using the Kaplan-Meier method. Public RNA sequencing FASTQ files were downloaded from the Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) for 2 MDS datasets (SRP149374, SRP418365) and aligned to hg19 by bwa-mem.[21,22] Alignments were analyzed by: i) searching for select fusions via grep (restricted to alignments to partner gene regions) for exon-exon junctional sequences (30 nucleotides consisting of 15 from each exon) and their reverse complements across all possible exon combinations producing the fusion or its reciprocal as previously described,[23] followed by manual confirmation of hits; ii) outlier isoform analysis for aberrant expression of isoforms in select genes (*KMT2A, UBTF*) as previously described,[24]; and iii) custom variant detection based on pileup data across padded coding sequence of the *WT1* gene. Annotations (mutations, fusions, cytogenetics, diagnoses) for the IPSS-M MDS cohort and the Leucegene AML cohort were retrieved from previously published data.[30] The study was conducted in accordance with the Declaration of Helsinki and with the approval of the institutional review boards at the Dana-Farber Cancer Institute and Massachusetts General Brigham.

Results

NUP98 rearrangements are effectively detected by anchored multiplex polymerase chain reaction-based targeted RNA sequencing, revealing novel partners and a potential enrichment in high-risk myelodysplastic syndromes

*NUP98*r was identified through the targeted RNA sequencing HFA assay in 18 patients overall (Table 1 and *Online Supplementary Figure S1*), with diagnoses of AML (N=14), MDS (N=3), or B/myeloid mixed phenotype acute leukemia (MPAL) (N=1) at the time of initial *NUP98*r detection. Eight different partner genes were observed, comprising 6 established (*DDX10, HOXD13, KDM5A, NSD1, PRRX2, TNRC18*) and 2 novel (*FGF14, LAMC3*) partners (Online Supplementary Table S1). The novel partners both contained domains that form a coiled coil structure, similar to many other non-*HOX NUP98* partners.[30] *NSD1* was the most frequent partner (11/18 patients; 61.1%), with single occurrences of

Table 1. Summary of patient characteristics.

Patient	WHO revised 4th ed. diagnosis (FAB)	WHO 5th ed. diagnosis	International Consensus Classification	BM blast %	PB blast %	Age at Dx, years	Sex	Fusion partner	Cryptic on karyotype	Karyotype	Mutations*	Initial treatment	N of SCT	N of relapse	Outcome
AML_1	AML-NOS (M0)	AML with *NUP98* rearrangement	AML with *NUP98::KDM5A*	65	59	68	F	*KDM5A*	Yes	46,XY[20]	*CDKN2A* *TP53*	7+3	1	0	Dead (GvHD) 6.1 months
AML_2	AML-NOS (M5b)	AML with *NUP98* rearrangement	AML with *NUP98::NSD1*	75	41	60	M	*NSD1*	Yes	46,XY,del(3)(p21)[cp3]/46,XY[cp20]	*FLT3*-ITD *WT1* x4 *TET2* *MYC*	7+3	1	0	Dead (intracranial hemorrhage) 4.5 months
AML_3	ET in blast phase/AML-NOS (M5a)	AML with *NUP98* rearrangement	AML with *NUP98::NSD1,* progressing from ET	30	24	77	F	*NSD1*	Yes	46,XX[cp20]	*FLT3*-ITD *TET2* x3 *JAK2 V617F*	Decitabine + venetoclax	1	0	Alive, in remission after SCT 21.3 months
AML_4	AML-NOS (M5a)	AML with *NUP98* rearrangement	AML with *NUP98::NSD1*	87	76	50	M	*NSD1*	Yes	46,XY[20]	*FLT3*-ITD (x2) *WT1*	7+3 and gilteritinib	1	1	Dead of relapsed/ refractory AML 18.3 months
AML_5	AML with mutated *RUNX1* (M5a)	AML with *NUP98* rearrangement	AML with *NUP98::TNRC18*	44	77	18	M	*TNRC18*	Yes	46,XY[20]	*RUNX1* *NRAS*	7+3	2	2	Dead of relapsed/ refractory AML 54.4 months
AML_6	AML-NOS (M4)	AML with *NUP98* rearrangement	AML with *NUP98::NSD1*	84	83	42	F	*NSD1*	Yes	46,XX[20]	*FLT3*-ITD	7+3 and midostarin	0	1	Dead of relapsed/ refractory AML 24.4 months
AML_7	AML-NOS (M4)	AML with *NUP98* rearrangement	AML with *NUP98::DDX10*	20	90	65	F	*DDX10*	No	46,XX,del(9)(q21q34),inv(11)(p15q22)[20]	*PTPN11*	Decitabine	0	1	Dead of relapsed/ refractory AML 10.9 months
AML_8	AML with mutated *RUNX1* (M0)	AML with *NUP98* rearrangement	AML with *NUP98::NSD1*	90	82	51	M	*NSD1*	Yes	46,XY,t(7;19)(q11.2;q13.2),del(11)(q21q24)[10]/46,XY[10]	*FLT3*-ITD *RUNX1*	7+3	1	2	Dead of relapsed/ refractory AML 14.3 months
AML_9	Therapy-related AML (M0)	AML with *NUP98* rearrangement, post cytotoxic therapy	AML with *NUP98::HOXD13,* therapy-related	58	1	39	F	*HOXD13*	No	46,XX,t(2;11)(q31;p15)[18]/46,XX[2]	None	7+3	2	1	Alive, in remission after second SCT 98.1 months

Continued on following page.

Patient	WHO revised 4th ed. diagnosis (FAB)	WHO 5th ed. diagnosis	International Consensus Classification	BM blast %	PB blast %	Age at Dx, years	Sex	Fusion partner	Cryptic on karyotype	Karyotype	Mutations*	Initial treatment	N of SCT	N of relapse	Outcome
AML_10	AML-NOS (M0)	AML with *NUP98* rearrangement	AML with *NUP98::LAMC3*	89	97	22	M	*LAMC3***	Yes	46,XY,t(4;17)(q21;q21), add(11)(p15)[9]/48, idem,+der(4)t(4;17), +21[cp11]	*WT1* *KRAS*	7+3	1	1	Dead of relapsed/ refractory AML 11.7 months
AML_11	AML with mutated *RUNX1* (M0)	AML with *NUP98* rearrangement	AML with *NUP98::NSD1*	79	87	24	M	*NSD1*	Yes	46,XY[20]	*WT1* (x2) *FLT3*-ITD (x2) *RUNX1* *NFE2* *MYC*	7+3 and midostaurin	1	0	Alive, in remission after SCT 9.4 months
MDS_1	MDS-EB2	MDS-IB2	MDS-EB	4	0	42	F	*FGF14*	Yes	46,XX[20]	*DNMT3A*	Decitabine + venetoclax	1	0	Alive, in remission after SCT 19.6 months
MDS_2	MDS-EB2	AML with *NUP98* rearrangement	AML with *NUP98::NSD1*	10	4.5	77	F	*NSD1*	Yes	47,XX,+8[3]/46,XX[17]	*WT1* (x2)	Decitabine	1	0	Alive 9.0 months day +42 after SCT
MPAL_1	Mixed-phenotype (B/ myeloid) acute leukemia (M5a)	Mixed-phenotype (B/ myeloid) acute leukemia	MPAL, B/myeloid, with *NUP98::NSD1*	98	19.5	30	F	*NSD1*	Yes	46,X,der(X)(X;5) (p22.1;q35),der(5)t(X;5) t(5;11) (q35;p15.4),der(11) t(5;11)(q35;p15.4) t(X;5)[20].ish der(X) (Xptel-,DXZ1+), der(5) (D5S723/D5S721+, EGR1+,Xptel+), der(11)(5'NUP98+)[5]	*WT1* (x3) *CEBPA* *KRAS* *PTPN11*	AYA induction	1	0	Alive 4.0 months day +26 after SCT
AML_12	AML-NOS (M5a)	AML with *NUP98* rearrangement	AML with *NUP98::NSD1*	83	37	48	F	*NSD1*	Yes	46,XX[cp20]	*WT1* (x2) *FLT3-ITD* *KRAS* *TET2* (x2)	7+3	1	1	Dead of relapsed/ refractory AML 31.8 months
AML_13	AML-NOS (M4)	AML with *NUP98* rearrangement	AML with *NUP98::NSD1*	87	5	82	F	*NSD1*	Yes	46,XX[20]	*WT1* (x2) *FLT3*-ITD	Decitabine + venetoclax	1	1	Dead of relapsed/ refractory AML 28.6 months
AML_14	AML with mutated RUNX1 (M5a)	AML with *NUP98* rearrangement	AML with *NUP98::PRRX2*	81	77	61	M	*PRRX2*	N/A	N/A	*WT1* *FLT3*-ITD (x2) *RUNX1* *DNMT3A*	7+3	0	0	Dead of AML 2 days

*Mutations include those that appeared in subsequent bone marrow (BM) biopsies. 7+3: induction with cytarabine days 1-7 and idarubicin days 1-3. AYA induction: vincristine, doxorubicin, methotrexate, cytarabine. **This same patient with *NUP98::LAMC3* was previously included in a workshop report.[49] AML: acute myeloid leukemia; Dx: diagnosis; ed.: edition; ET: essential thrombocythemia; F: female; FAB: French-American-British classification of AML; GvHD: graft-*versus*-host disease; M: male; MDS-EB2: myelodysplastic syndrome with excess blasts-2; N: number; N/A: not available; NOS: not otherwise specified; PB: peripheral blood; SCT: stem cell transplant; WHO: World Health Organization classification.

other partners. Fusions involving 6/8 (75%) partners (*FGF14, KDM5A, LAMC3, NSD1, PRRX2, TNRC18*) from 15/17 (88.2%) evaluated patients were cytogenetically cryptic on conventional karyotyping (Table 1), highlighting the utility of RNA-based NGS for comprehensive detection of *NUP98*r. Only one case (MPAL_1) had *NUP98* FISH performed during initial clinical workup. Nine *NUP98*r cases (50%) had a normal karyotype. Ten of the 15 (66.7%) AML/MPAL cases showed myelomonocytic or monocytic differentiation by morphology and flow cytometry (Table 1, *Online Supplementary Figure S2*), including 7/9 (77.8%) with *NUP98::NSD1* and 3/6 (50%) with non-NSD1 partners (*TNRC18, DDX10, PRRX2*). No other subtype-defining alteration was identified by any testing modality in any of the *NUP98*r cases, including those with novel fusion partners. *KMT2A*-PTD was also absent from *NUP98*r cases, akin to mutual exclusivity reported in pediatric AML; this alteration in AML has been associated with aberrant *HOXB* expression, similar to AML with *NUP98::NSD1*, mutated *NPM1*, *DEK::NUP214*, and *UBTF*-TD, although it is not subtype-defining.[6,31,32]

The majority of *NUP98* fusions were detected through clinical testing during the course of patient care, with clinically reported *NUP98*r in samples from 14 patients across the HFA clinical cohort, including 11/257 (4.3%) of all newly diagnosed AML patients treated at one institution (Massachusetts General Hospital) between 2017-2024 (*Online Supplementary Figure S1*). The other 3 clinically identified *NUP98*r patients accounted for 2/46 (4.3%) MDS and 1/3 (33.3%) MPAL from the HFA cohort; however, these test populations were subject to selection bias, particularly given the lack of clear guidelines on which MDS cases should be tested by an RNA fusion assay. Most MDS cases that were tested had high-risk features and clinical concern for AML, particularly elevated blast counts (28/46, 60.9%) and/or high to very high International Prognostic Scoring System (IPSS)-Molecular scores (29/46, 63.0%) (*Online Supplementary Table S2*). No cases tested clinically by HFA from patients diagnosed with MDS/myeloproliferative neoplasm (MPN) overlap (0/24), MPN (0/49), or another myeloid neoplasm (0/5) demonstrated a *NUP98*r.

WT1 mutations are recurrently observed with and without *FLT3*-ITD in acute myeloid leukemia with *NUP98::NSD1* and occur in myeloid neoplasms with other *NUP98* rearrangements

We investigated co-mutational profiles of the 14 *NUP98*r cases from the HFA cohort, as characterized by targeted DNA-based NGS testing (RHP). Consistent with prior studies, the most common co-occurring mutations at diagnosis were: i) *FLT3*-ITD in 6/14 cases (42.9%; all harboring *NUP98::NSD1*) with variant allele frequencies (VAF) ranging from <1% to 25%; and ii) *WT1* in 6/14 cases (42.9%; 5 with *NUP98::NSD1* and 1 with *NUP98::LAMC3*) with VAF ranging from 5.5% to 47.2%, where 4/14 (28.6%) had multiple (2-4) *WT1* mutations (Figure 1, *Online Supplementary Table S3*). All 6 cases with *WT1* mutations harbored one or more frameshift variants for a total of 12 frameshifts (*vs.* 1 nonsense) including frameshift insertions in all cases. Concurrent *FLT3*-ITD and *WT1* mutations were seen in 3/14 cases (21.4%). Mutations in *RUNX1, MYC, TET2, KRAS*, and *PTPN11* were also each seen more than once (Figure 1). Myelodysplasia-related (MR) gene mutations were not

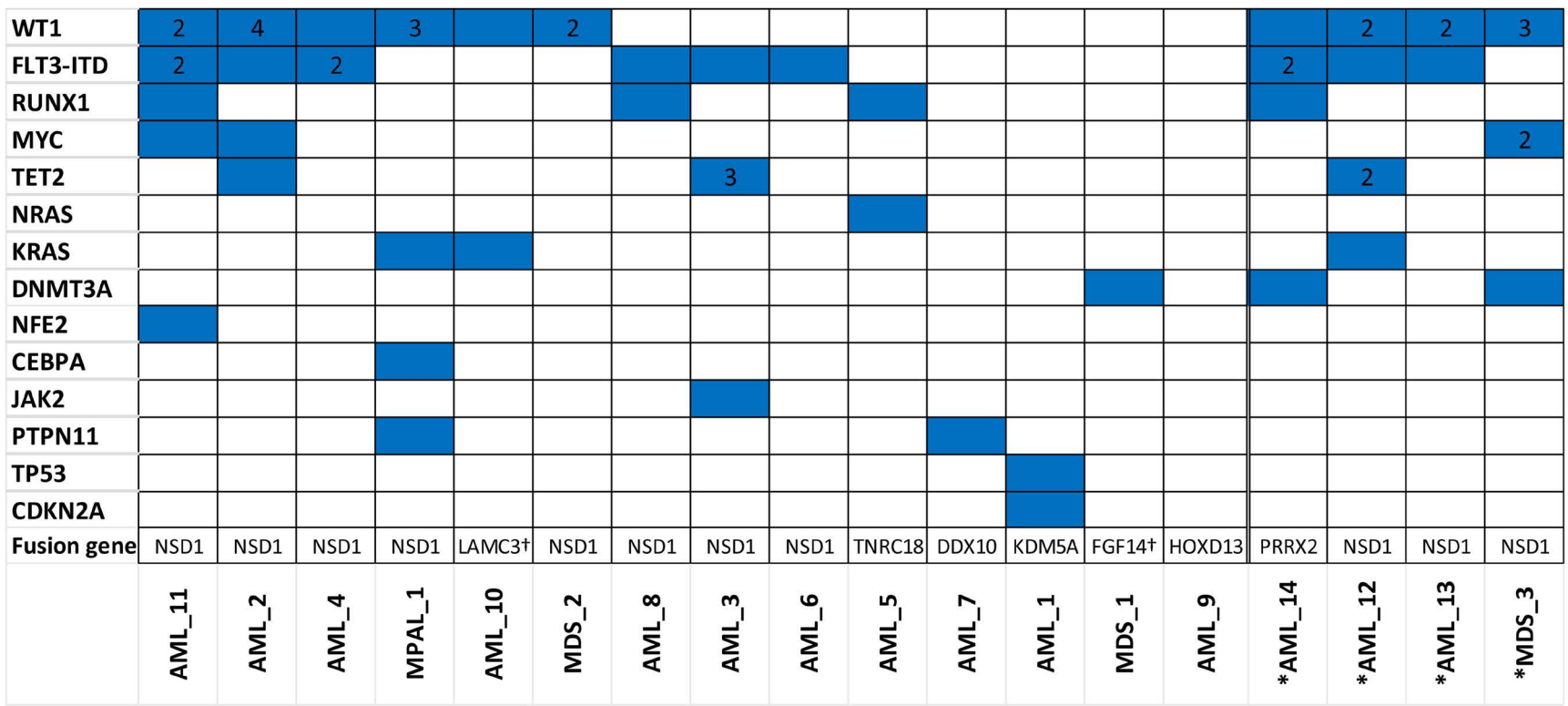

Figure 1. Co-mutation plot of the Heme Fusion Assay and Rapid Heme Panel (RHP) cohorts at initial diagnostic bone marrow biopsy. Pathogenic mutations identified in each *NUP98r* case are represented by blue boxes. If multiple mutations occur in the same gene, the number of concurrent mutations is indicated within the blue box. RHP: Rapid Heme panel. *RHP cohort. †Novel fusion partner.

identified, except in *RUNX1*, which is considered an MR gene by the ICC but not by the WHO5. *FLT3*-ITDs were detected exclusively in AML cases.[16,33] By contrast, 1/2 (50%) MDS cases demonstrated multiple *WT1* mutations up to 40% VAF. *WT1* mutations also developed after the initial diagnosis in 3 cases (AML_5, AML_6, AML_7) with fusions to *TNRC18, NSD1,* and *DDX10*, again with one or more frameshifts in every case. Thus overall, 9/14 (64.3%) cases presented with or developed a *WT1* mutation at various stages of their clonal hierarchies.

Mutational status of *WT1* identifies candidate cases for selective testing for *NUP98*r in myelodysplastic syndromes

We explored whether *WT1* mutations detected by up-front DNA-based NGS testing of MDS, in the absence of potential subtype-defining molecular or cytogenetic features determined from routine workups, might provide a rational strategy for initiation of HFA testing with the goal of detecting rare *NUP98*r cases. As a proof of principle, we interrogated DNA-based NGS testing across all MDS cases of the HFA cohort (N=45 with DNA NGS), revealing *WT1* mutations in 4/45 (8.9%) cases: an *NPM1*-mutated case by RHP, a *MECOM*-rearranged (*MECOMr*) case by cytogenetic studies, a case with *KMT2A*-PTD by RHP, and the *NUP98*r case described earlier that was cryptic on karyotype and characterized subsequently by HFA. Under a hypothetical tiered approach to MDS evaluation, the latter 2 cases would be candidates for dedicated *NUP98*r testing, whereas the first 2 cases were already characterized by their initial workups. However, *NUP98*r testing of *KMT2A*-PTD cases may have limited yield, given the mutual exclusivity of *KMT2A*-PTD with *NUP98*r (and most other molecular subtypes) reported in pediatric AML and the similar absence of *KMT2A*-PTD from the *NUP98*r HFA cohort. Therefore, the hypothetical yield of the proposed tiered strategy would be 1/1 (100%) if *KMT2A*-PTD cases were deliberately excluded from further testing or 1/2 (50%) otherwise or if a clinical practice did not include detection of *KMT2A*-PTD as part of their initial workup. Of note, one *NUP98*r MDS case from the HFA cohort would have been missed by this strategy, since that case harbored only a *DNMT3A* mutation, which is widely mutated across myeloid neoplasms and thus not amenable to a molecular strategy for rationed testing.

The above *WT1* mutational rate (8.9%; 4/45) and hypothetical HFA testing rate (2.2%; 1/45 after also excluding *KMT2A*-PTD) reflected a high-risk MDS cohort. As a broader estimate of testing rate using the public IPSS-M dataset, 37/2591 (1.4%) of its MDS (WHO4) cases harbored pathogenic *WT1* mutations with an enrichment in higher risk subtypes (MDS-EB2: 17/438 [3.9%]; MDS-EB1: 10/464 [2.2%]) and subsequent contribution to criteria for a proposed AML-like group of MDS.[25,34] Of these 37 *WT1*-mutated MDS cases, 14 showed key alterations considered mutually exclusive with *NUP98*r: mutated *NPM1* (N=6), *KMT2A*-PTD (N=4), biallelic *TP53* mutations (N=1), biallelic *DDX41* mutations (N=1), t(6;9)(p23;q34)/*DEK::NUP214* (N=1), and t(3;21)(q26;q22)/*RUNX1::MECOM* (N=1). After their exclusion, hypothetical HFA testing might then apply to 23/2591 (0.9%) MDS cases. These 23 cases demonstrated either single missense variants (N=8 cases), single nonsense variants (N=3), single splice variants (N=2), or purely frameshifts (8 insertions only, 1 deletion only, 1 both), thus an alternative minimalistic strategy might test only the 9/2591 (0.3%) harboring an insertion frameshift. The IPSS-M cohort, however, did not have *NUP98*r status (or RNA sequencing data) to measure testing yield.

We attempted to further validate the *WT1*-based strategy by applying it to the RHP cohort (Figure 2A). Screening yielded 17 adult patients with MDS harboring *WT1* mutations, of which 7 were found to have an alteration considered mutually exclusive with *NUP98*r by either RHP or kayotype: *NPM1* mutation (N=5), *MECOM*-r (N=1), or *TP53* multi-hit (N=1) (Figure 2A). Cases from 6 additional patients harbored *KMT2A*-PTD, which we decided not to test further for the reasons discussed earlier. Of the remaining 4 patients, 2 had already undergone testing within the HFA clinical cohort, with one positive for *NUP98*r and one negative for any fusions by HFA. The final 2 patients underwent retrospective HFA testing for this research study, with one positive for *NUP98*r and one failing sequencing quality control metrics. Thus, the overall HFA yield for *NUP98*r within *WT1*-mutated MDS without a key driver (including *KMT2A*-PTD) was 2/3 (66.7%) in this limited dataset.

We also reanalyzed public RNA sequencing data from two adult MDS cohorts, resulting in detection of *NUP98*r in 4 more MDS cases involving 2 different partner genes (3 cases with *NUP98::NSD1*, 1 with *NUP98::HOXA9*), for cohort frequencies of 2/215 (0.9% in SRP418365) and 2/109 (1.8% in SRP149374) (*Online Supplementary Table S4*). No evidence was found for *NUP98* fusions involving 22 other partner genes that have been reported previously in MDS, CMML, or AML across predominantly adult studies (Tables 2, 3) (*DDX10, EMX1, FGF14, HHEX, HMGB3, HOXA11, HOXA13, HOXC13, HOXD12, HOXD13, KAT7, KDM5A, LNP1, NSD3, PHF23, PRRX1, PRRX2, PSIP1, RAP1GDS1, TLX1, TNRC18, TOP1*). We lacked knowledge of *WT1* mutational status on the DNA level to fully evaluate the *WT1*-based strategy. As a proxy, we instead screened the RNA-seq data for expressed WT1 loss-of-function (LOF) mutations (frameshift, nonsense, or splice site) while adopting an approach prioritizing sensitivity in order to partially offset inherent limitations posed by RNA, including variably low *WT1* expression, nonsense mediated decay of mutant RNA transcripts, splicing mutations that may not appear within mature RNA transcripts (e.g., by conferring exon skipping rather than intron retention), and shallow sequencing coverage. Our analysis detected expressed *WT1* LOF mutations in 3/4

(75%) *NUP98*r cases (3/3 *NUP98::NSD1*; 0/1 *NUP98::HOXA9*) and 15/324 (4.6%) MDS cases overall, with individual cohort frequencies of 5/215 (2.3% in SRP418365) and 10/109 (9.2% in SRP149374) (*Online Supplementary Table S5*). The higher frequency of the latter cohort was hypothesized to be a consequence of CD34$^+$ enrichment and was associated with higher expressed VAF; however, the possibility of a component of false positives also existed. Outlier isoform analysis demonstrated 2 *KMT2A*-PTD and one *UBTF*-PTD within non-*NUP98*r cases expressing *WT1* LOF mutations. After excluding *KMT2A*-PTD cases, the hypothetical yield of the *WT1*-based strategy was 1/3 (33.3% in SRP418365) and 2/10 (20% in SRP149374) but potentially could be greater, given the lack of annotations (e.g., cytogenetics) and the possibility of additional findings upon standard workups (e.g., *MECOM*r). Of note, all 3 *NUP98::NSD1* cases exhibited *WT1* frameshift insertions, similar to cases in our local cohort. Thus, the alternative strategy of using *WT1* frameshift insertions might largely maintain sensitivity while increasing specificity, with a hypothetical yield across both public cohorts of 3/4 (75%) for *NUP98*r, where the non-*NUP98*r case harbored *UBTF*-TD and would also benefit from subtyping.

Mutational status of *WT1/FLT3*-ITD identifies candidate cases for selective testing for *NUP98*r in acute myeloid leukemia

In AML, RNA-based NGS testing was a part of routine workups in some but not all our local institutional practices during the study period. As access to testing expands, it is likely that clinical practices will increasingly adopt either universal upfront RNA-based NGS or a tiered approach with reflex testing of all AML cases that do not have a characterized subtype after routine workup. However, in a setting of limited resources with goals of maximizing positive predictive value, we explored the utility of *WT1/FLT3*-ITD dual mutations in the absence of subtype-defining genetic features (by RHP or cytogenetics) as another potential rational strategy in AML for initiation of HFA testing. Screening of the RHP cohort identified 41 adult patients with AML harboring both a *WT1* mutation and *FLT3*-ITD, of which 31 were found to have a genetic abnormality considered mutually exclusive with *NUP98*r, including *NPM1* (N=15), *KMT2A*-PTD (N=7), *PML::RARA* (N=6), *DEK::NUP214* (N=1), *MECOM*r (N=1), and *CEBPA* bZIP domain mutation (N=1) (Figure 2B), of which all but *KMT2A*-PTD further enabled AML classification by the ICC or the WHO5. Of the remaining patients, 3 had al-

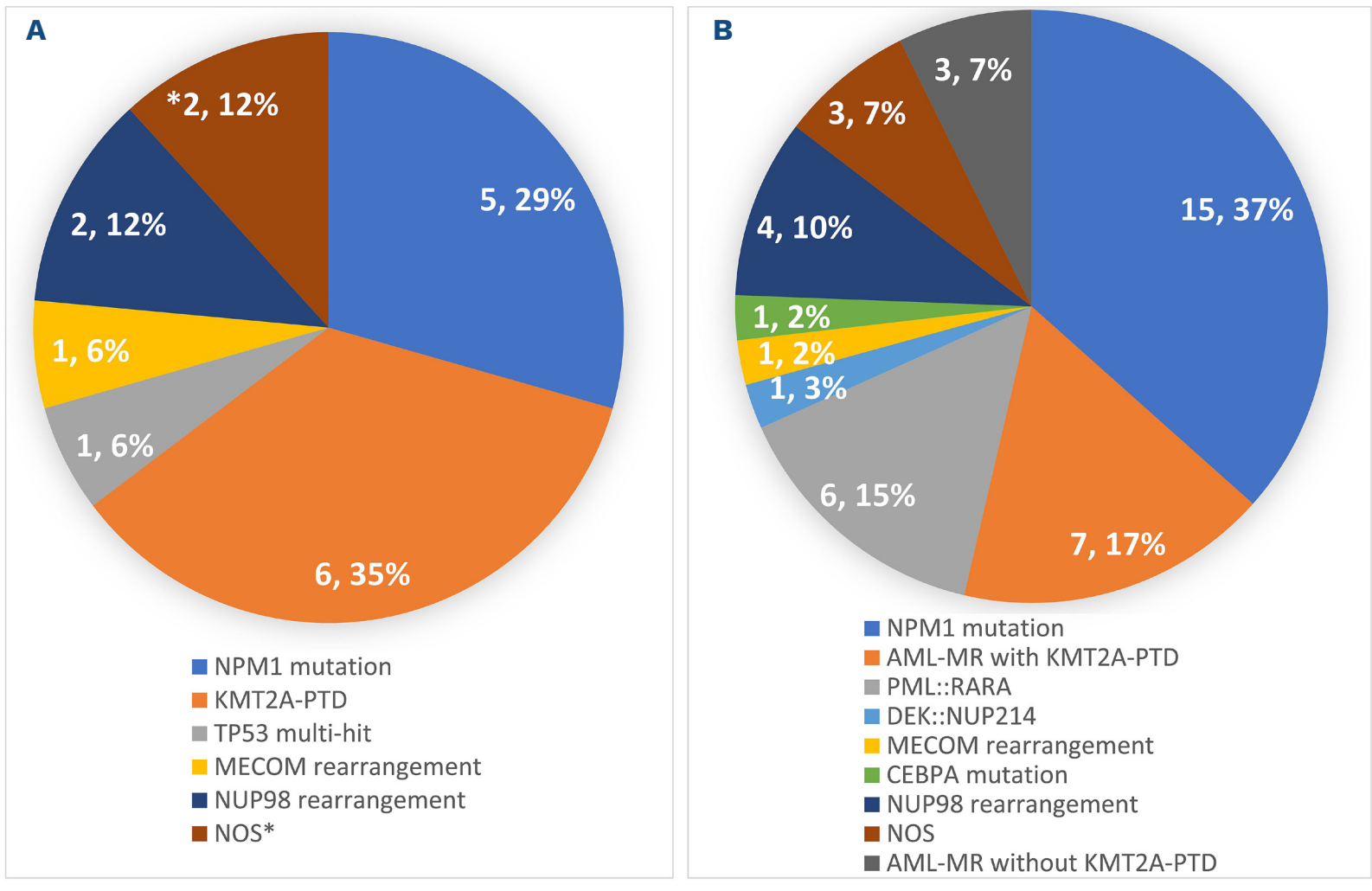

Figure 2. Mutually exclusive genetic alterations in myelodysplastic syndromes with *WT1* mutations and acute myeloid leukemia with *WT1* and *FLT3*-ITD mutations. (A) Adult myelodysplastic syndrome (MDS) cases with *WT1* mutation in the Rapid Heme Panel database (N=17), labeled with detected genetic driver. NOS: no genetic driver detected. *1 case sample with no known genetic driver failed QC metrics on Heme Fusion Assay. (B) Adult acute myeloid leukemia (AML) cases with *WT1* mutation and *FLT3*-ITD in the Rapid Heme Panel database (N=41), labeled with detected genetic driver. AML-MR: myelodysplasia-related, by the International Consensus Classification (ICC); NOS: no genetic driver detected. Of note, 2 of the 3 AML-NOS cases subsequently underwent total RNA-sequencing, and both were found to harbor *UBTF*-TD.

Table 2. Acute myeloid leukemia literature review, adult only.

Institution	Year	N of patients		Screening method	Fusion partner	Median age, years (range)	Sex	FAB subtype	Cytogenetics	Mutations	Outcome	Reference
		Total	Fusion									
National Taiwan University Hospital, Taipei, Taiwan	2009	493 AML	11	t(7;11) (p15;p15) only	*HOXA9*	31 (23-59)	18.2% male	91% M2, 9% M4	t(7;11) (p15;p15) in all	36.3% with *WT1*, 27.3% with *FLT3*-ITD, 18.2% with *KRAS*, 18.2% with *NRAS*	13.5 mo OS, 6 mo RFS	PMID: 19225539
Erasmus Medical Center - Sophia Children's Hospital, Rotterdam, the Netherlands	2011	808 AML	10	*NSD1* only	10 *NSD1*	range 15-77 of entire cohort	40% male	10% M2, 30% M4, 60% M5	90% normal, 10% with inv(5)	90% with *FLT3*-ITD, 30% with *WT1*	4-year pOS 11%, 4-year pEFS 0%	PMID: 21813447
			1	11p15 aberration on karyotype	1 *DDX10*		Male	M6	+8,inv(11) (p15;q22)	*NRAS*	EFS 13.9 mo, OS 22.3 mo	
Cancer Genomic Atlas Research Network	2013	200 AML	3	Comprehensive	3 *NSD1*	Enitre cohort: 55.0±16.1	Entire cohort: 54% male	N/A	N/A	N/A	EFS 0.54, OS 0.81	PMID: 23634996
Hannover Medical School, Hannover, Germany	2013	504 AML, 193 MDS	7 AML, 0 MDS	*NSD1* only	*NSD1*	34 (23-48)	43% male	14.3% M1, 28.6% M2, 57% M4	71.4% normal, 14.3% with del(9q), 14.3% with inv(3) (q21q26) and monosomy 7	71.4% with *FLT3*-ITD, 28.6% with *WT1*, 14.3% with *NRAS*	43% complete response rate. OS and RFS no difference compared to *NUP98::NSD1*-neg patients	PMID: 22929522
Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin, China	2013	17 AML with t(7;11) (p15;p15)	16	t(7;11) (p15;p15) only	*HOXA9*	34 (18-67)	23.5% male	52.9% M2, 23.5% M4, 17.6% M5b, 6% M6	t(7;11) (p15;p15) in all, 6% with trisomy 8	6.7% with *FLT3*-ITD	OS 8 months, DFS 4 months	PMID: 23800796
MLL Munich Leukemia Laboratory, Munich, Germany	2013	378 AML	8	*NSD1* only	*NSD1*	42.3 (20.9-71.4)	50% male	62.5% M1, 25% M2, 12.5% M4	12.5% with ins(5;11) (q35;p13p15)	75% with *FLT3*-ITD, 50% with *WT1*	1.8 mo EFS	PMID: 22945772
Shanghai Institute of Hematology, Shanghai, China	2022	655 AML	18	Comprehensive	6 *HOXA9*, 6 *NSD1*, 2 *PRRX2*, 1 *HMGB3*, 1 *HOXA11*, 1 *HOXD12*, 1 *TNRC18*	Entire cohort: 48 (IQR 34-60)	Entire cohort: 51.8% male	N/A	N/A	N/A	N/A	PMID: 36442087

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Institution	Year	N of patients		Screening method	Fusion partner	Median age, years (range)	Sex	FAB subtype	Cytogenetics	Mutations	Outcome	Reference
		Total	Fusion									
Oregon Health & Science University, Portland, OR, USA	2022	316 AML	8 + 1 from another institution	Comprehensive	N/A (FISH breakapart probe)	49 (24-77)	44.4% male	11.1 M0, 44.4 M1, 44.4 M4, 11.1 M5	33.3% normal, 44.4% with 11p15 rearrangement, 22.2% with del(5q), 33.3% with del(7q)	62.5% with *WT1*, 50% with KRAS, 37.5% with *CEBPA*, 37.5% with *FLT3*-ITD, 25% with *IDH2*, 25% with *PTPN11*	13 mo OS	PMID: 35258401
Ningbo First Hospital, Ningbo, China	2023	11 AML	11	*NSD1* only	*NSD1*	30 (14-59)	45% male	N/A	81.8% normal, 9.1% with trisomy 8, 9.1% with del(9q13)	63.6% with *FLT3*-ITD, 27.3% with *WT1*, 27.3% with *CEBPA*	1yr OS 54.5-68.6%	PMID: 36751862
Hokkaido University Hospital, Sapporo, Japan	2023	97 *FLT3*-ITD-positive AML	6	*NSD1* only	*NSD1*	55 (21-61)	67% male	33.3% M1, 66.7% M4	83.3% normal, 16.6% with trisomy 8	*FLT3*-ITD in all (by screening criteria), 50% with *WT1*	2yr OS 53.5% , 2-year RFS 33.3%; 83% induction failure rate	PMID: 37465857
Erasmus Medical Center - Sophia Children's Hospital, Rotterdam, the Netherlands	2023	825 adult AML, 2235 pediatric AML	13 adult	Comprehensive	Adult: 11 *NSD1*, 1 *TOP1*, 1 *RAP1GDS1*	N/A	N/A	N/A	Entire cohort: 18.8% of *NUP98::NSD1* have trisomy 8	Entire cohort: 74% have *FLT3*-ITD and 42% have *WT1* in *NUP98::NSD1*; 25% have *WT1* in *NUP98::X*	Entire cohort: *NUP98::NSD1* 17% EFS and 36% OS; *NUP98::X* 35% EFS and 35% OS; overall *NUP98r* 35% OS	PMID: 36815378
Josep Carreras Leukaemia Research Institute, Barcelona, Spain	2024	291 AML	8	Comprehensive	5 *NSD1*, 1 *EMX1*, 1 *KMT2A*, 1 *KDM5A*	45 (31-67)	87.5% male	12.5% M0, 12.5% M1, 25% M4; 50% MRC	62.5% normal, 37.5% with chr 11 aberration	62.5% with *FLT3*-ITD, 62.5% with *WT1*, 25% with *CEBPA*	5/8 died	PMID: 38536941
Shanghai Jiao Tong University School of Medicine	2024	1099 adult and pediatric AML	48 adult	Comprehensive	22 *NSD1*, 10 *HOXA9*, 4 *PRRX2*, 2 *HMGB3*, 2 *TOP1*, 2 *KDM5A*, 1 *TNRC18*, 1 *HOXA11*, 1 *HHEX*, 1 *DDX10*, 1 *PSIP1*, 1 *KMT2A*	43 (19-79)	46.6% male	16.7% M2, 37.5% M4, 14.6% M5, 6.3% t-AML; 6.3% secondary-AML	47.8% normal, 19.6% with t(7;11)(p15;p15), 13.0% with trisomy 8, 10.9% with complex karyotype	54.2% with *FLT3*-ITD, 31.3% with *WT1*	*NUP98::NSD1* 8.0% CR rate and 65.2% 4yr OS; overall 37.3% CR rate; non-*NUP98::NSD1* 66.7% 4yr OS	PMID: 38744828

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Institution	Year	N of patients		Screening method	Fusion partner	Median age, years (range)	Sex	FAB subtype	Cytogenetics	Mutations	Outcome	Reference
		Total	Fusion									
Yonsei University College of Medicine, Severance Hospital, Seoul, Republic of Korea	2024	260 AML	13 (10 with data)	Comprehensive	4 *NSD1*, 1 *HOXA9*, 1 *DDX10*, 1 *TOP1*, 1 *PHF23*, 1 *HMGB3*, 1 *HOXC13*	35.5 (20-74)	50% male	N/A	2/10 normal, 1 with trisomy 8, 1 with t(7;11)(p15;p15), 1 with complex karyotype	6/10 with *FLT3*-ITD, 1 with *WT1*, 3 with *NRAS*, 1 with *DNMT3A*, 1 with *RAD21*, 1 with *RUNX1*	6/10 in CR, 3 died, 1 lost to follow-up	PMID: 39158088
Oregon Health & Science University, Portland, OR, USA	2024	746 AML	15	Comprehensive	6 *NSD1*, 1 *KDM5A*, 1 *DDX10*, 1 *HMGB3*, 1 PSIP1, 1 *HOXC13*, 1 *TLX1* (*HOX11*), 1 *HHEX*, 2 N/A	52 (18-77)	67% male	13.3% M0, 46.7% M1, 33.3% M4, 6.7 M5; 20% t-AML	11/15 cryptic; 7 normal, 3 with complex karyotype	10/15 with *FLT3*-ITD, 6 with *WT1*, 3 with *KRAS*, 2 with mono-allelic *CEBPA*, 2 with *TET2*, 2 with *IDH2*, 2 with *PTPN11*	6/15 died, 3 alive with relapsed/ persistent disease, 3 alive in CR	PMID: 39701595

AML: acute myeloid leukemia; CR: complete remission; EFS: event-free survival; FAB: French-American-British classification of AML; IQR: interquartile range; M: male; MDS: myelodysplastic syndrome; mo: months; N/A: not available; OS: overall survival; PB: peripheral blood; pEFS: probability of EFS; pOS: probability of OS; RFS: relapse-free survival; yr: years.

Table 3. MDS and MDS/MPN literature review, adult and pediatric.

Institution	Year	N of patients		Detection method	Fusion partner	Age,* years	Sex	Blasts	Cytogenetics	Mutations	Outcome	Reference
		Total	Fusion									
National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan	1997	52 *MDS*,	1 MDS, 1 t-MDS	Karyotype and Southern blot	*DDX10*	7, 4	1 male, 1 female	N/A	inv(11)(p15q22)	N/A	N/A	PMID: 9166830
Saitama Cancer Center Hospital, Saitama, Japan	1999	11 *t-MDS*	2 t-MDS	Karyotype, Southern blot, and RT-PCR	1 possible *HOXB* 1 unknown	8, 15	1 female, 1 male	N/A	1 with t(11;17)(p15;q21) 1 with add(11)(p15)	N/A	Died 34 mo, died 24 mo	PMID: 10502319
Akita University School of Medicine, Akita, Japan	1999	98 *t-AML* or t-MDS	1 CMML, 1 MDS-EB2	Southern blot and RT-PCR	*HOXA9*	45, 69	1 male, 1 female	8.4%, 4.5% with Auer rods	t(7;11)(p15;p15)	N/A	Both progressed to AML within 1 year	PMID: 10583265
Roswell Park Cancer Institute, Buffalo, NY, USA	1999	1 *CMML*, 1 *MDS-EB2*	2	Southern blot and RT-PCR	*TOP1*	13, 15	1 female, 1 male	>2%	2 with t(11;20)(p15;q11.2)	N/A	N/A	PMID: 10556215
University Hospital, Lund, Sweden	2002	2 *t-MDS*	1	RT-PCR for *NUP98::TOP1*	*TOP1*	60	Female	12% with Auer rods	t(10;20;11)(q24;q11;p15)	N/A	Alive in remission after induction and consolidation	PMID: 11979559
The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan	2002	1 *t-MDS*	1	Southern blot and RT-PCR	*HOXA13*	45	Male	2%	t(7;11)(p15;p15)	N/A	Relapsed 6 mo after induction, died 14 mo after diagnosis	PMID: 11830496
University of Perugia, Perugia, Italy	2004	1 *CMML*	1	RT-PCR for *NUP98::NSD1*	*NSD1*	65	Male	3% in peripheral blood	add(11)(p15)	N/A	Died 4 mo	PMID: 15382262
Centre Hospitalier Universitaire Necker-Enfants Malades, Paris, France	2006	71 patients with 11p15 abnormality	1 t-MDS 1 t-MDS 1 CMML 1 CMML 1 MDS 1 MDS 1 t-MDS 1 t-MDS	FISH and RT-PCR	*DDX10* *DDX10* *HOXA9* *HOXA9* *LNP1* (*NP3*) *NSD1* *NSD3* *TOP1*	55 50 45 31 3 65 70 4	Female Male Male Male Female Male Female Female	N/A N/A N/A N/A N/A N/A N/A N/A	t(11;11)(p15;q22) inv(11)(p15q22) t(7;11)(p15;p15) t(7;11)(p15;p15) t(3;11)(q12.2;p15) add(11)(p15) t(8;11)(p11;p15),add(16)(q22) t(11;20)(p15;q11)	N/A N/A N/A N/A N/A N/A N/A N/A	N/A N/A N/A N/A N/A N/A N/A N/A	PMID: 16467868
Cedars Sinai Medical Center, Los Angeles, CA, USA	2007	1 *t-MDS*	1	FISH	*PRRX1*	74	Male	3.5%	t(1;11)(q23;p15)	N/A	AML progression 8 mo, died 10 mo	PMID: 17889707

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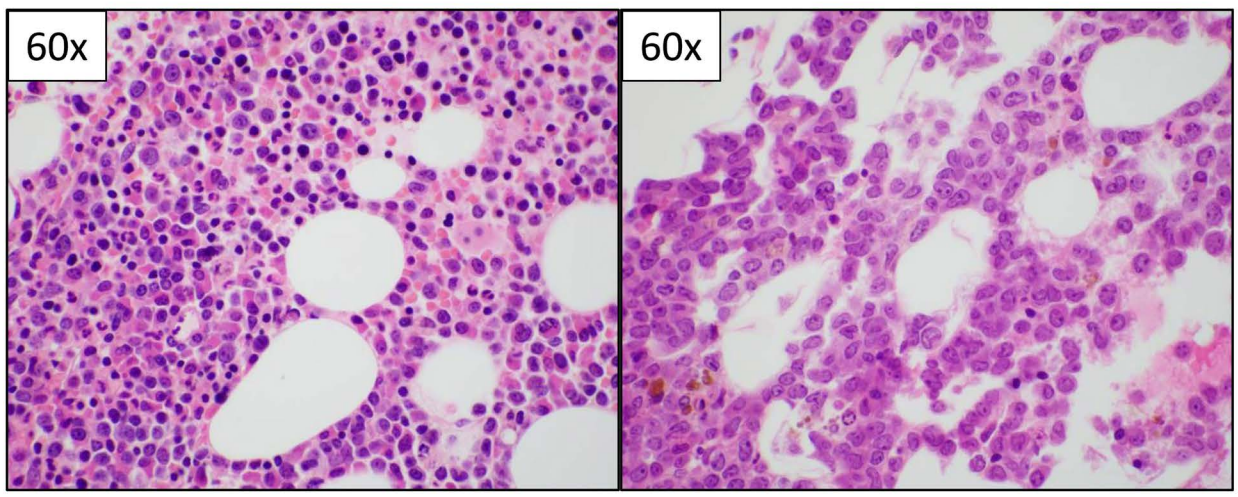
Institution	Year	N of patients		Detection method	Fusion partner	Age,* years	Sex	Blasts	Cytogenetics	Mutations	Outcome	Reference
		Total	Fusion									
Shimane University Hospital, Izumo, Shimane, Japan	2009	Died 10 mo	PMID: 17889707	Southern blot and RT-PCR	*NSD3*	60	Male	0%	t(8;11)(p11;p15),del(1)(p22p32)	N/A	AML progression 12 mo, died 23 mo	PMID: 19380029
Kobe University Graduate School of Medicine, Kobe, Japan	2012	1 *MDS-EB2*	1	FISH and RT-PCR	*PSIP1*	64	Female	9.2%	t(9;11)(p22;p15)	N/A	AML progression and died 7 mo	PMID: 22103895
Hannover Medical School, Hannover, Germany	2013	193 *MDS*	0	RT-PCR for *NUP98::NSD1*	--	--	--	--	--	--	--	PMID: 22929522
Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan	2019	1 *CMML*	1	RT-PCR	*KAT7* (*HBO1*)	69	Female	15%	t(11;17)(p11.5;q21)	N/A	AML progression and died 3 mo	PMID: 30944097
Benioff Children's Hospital, University of California San Francisco, San Francisco, CA, USA	2022	1 *JMML*	1	RNA seq	*NSD1*	2	Female	5%	44,X,-X,del(9)(q13q22),-16[1],46,XX[20]	*NRAS* p.Gly12Val (8%)	Alive, *NUP98::NSD1* not detected, 5 months after SCT	PMID: 32815876
The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA	2022	101 *MDS*	2	Optical genome mapping	1 *PRRX2*, 1 *NSD1*	Entire cohort: 72 (25-92)	Entire cohort: 71% male	N/A	Cryptic	N/A	N/A	PMID: 35915143
Sunkyunkwan University School of Medicine, Seoul, Republic of Korea	2024	15 patients with 11p15 translocation	1	FISH and/or RT-PCR	*DDX10*	50	Female	-	inv(11)(p15q22)	N/A	Died 5 mo	PMID: 39344146
			1		*HOXA9*	37	Female	-	t(7;11)(p15;p15)	N/A	AML progression 28 mo, died 32 mo	
			2		*PSIP1*	55	Female	-	t(9;11)(p22;p15)	N/A	AML progression 4 mo, died 12 mo	

AML: acute myeloid leukemia; CMML: chronic myelomonocytic leukemia; FISH: fluorescence *in situ* hybridization; JMML: Juvenile CMML; MDS: myelodysplastic syndrome; MDS-EB2: MDS wit excess blasts-2; MDS/MPN: MDS/myeloproliferative neoplasm; mo: months; N: number; N/A: not available; RT-PCR: reverse transcription polymerase chain reaction; SCT: stem cell transplant; t-AML: treatment-related AML; t-MDS: treatment-related MDS. *Age of fusion patient(s) in years; JMML: Juvenile CMML.

A

Time	day 0	day 41	day 231	day 451
BM diagnosis	MDS-MLD	MDS-IB2	AML	AML
BM blast %	2%	8%	83%	60%
DNMT3A p.R749C	43.4%	56%	Unknown	43.8%
WT1 p.R380Vfs*	9.5%	13.2%	Unknown	
WT1 p.K141*	7.3%	21.9%	Unknown	37.7%
WT1 p.R462W	7%	5.7%	Unknown	
WT1 p.S381Gfs*			Unknown	48.6%
MYC p.P74L	5.5%	2.9%	Unknown	
MYC p.P72S	3.6%	3.6%	Unknown	
MYC p.P74Q	0.9%	16.7%	Unknown	
FLT3-ITD (18 bp)	0.1%	0.5%	Unknown	
FLT3-ITD (84 bp)		1.5%	Unknown	23.3%
NRAS p.G12D				42.4%
NUP98r detection	HFA	FISH		HFA

B



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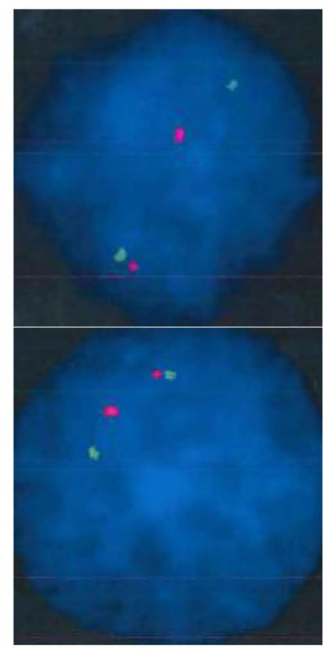

Figure 3. MDS_3 progression to acute myeloid leukemia. (A) Summary of aspirate blast count and mutations present in bone marrow (BM) biopsies for this patient at the time of myelodysplastic syndrome with multilineage dysplasia (MDS-MLD), MDS with excess blasts/increased blasts 2 (MDS-IB2), and acute myeloid leukemia (AML) diagnoses. (B) Representative histology of BM cores from MDS-IB2 and final AML biopsies (HE, 60x magnification). (C) Fluorescence *in situ* hybridization (FISH) testing on the MDS-IB2 biopsy using *NUP98* (11p15.4) break-apart probe; two representative interphase nuclei each showing one intact *NUP98* signal and one split 3' green signal (t) and 5' red signal (c), indicating a rearrangement. HFA: Heme Fusion Assay.

ready undergone testing within the HFA clinical cohort, with one positive for *NUP98::NSD1* and 2 negative for fusions. The final 7 patients included 5 with available material for retrospective HFA testing for this research study, yielding detection of *NUP98*r in 4/5 (80%) cases (*Online Supplementary Figure S1*). One of the 4 patients with successful confirmation of *NUP98*r at the AML stage also had *NUP98*r detected at an earlier MDS stage through the *WT1* screen. This case (MDS_3) progressed from MDS-MLD to MDS-IB2 together with rising peripheral blasts and relapsed quickly after transplant as AML, with emergence and outgrowth of *FLT3*-ITD across the serial samples (Figure 3A, B). FISH analysis performed for this study supported the early clonal nature of the *NUP98*r at the initial MDS timepoint (Figure 3C). Thus, the overall HFA yield for *NUP98*r within FLT3-*ITD*$^{+}$/*WT1*$^{+}$ AML without a mutually exclusive molecular alteration by RHP or karyotype was 5/8 (62.5%). To further characterize the 3 cases which remained unresolved after HFA, 2 had available material for total RNA-sequencing, revealing *UBTF*-TD in both.

Examination of the well-characterized Leucegene AML cohort (n=452) demonstrated similar findings (*Online Supplementary Table S6*). Out of 17 AML cases positive for both *FLT3*-ITD and *WT1* mutations, 15 harbored a genetic alteration considered mutually exclusive with *NUP98*r, again with NPM1 mutations (N=8) and *KMT2A*-PTD (N=5) as the most common, along with *PML::RARA* (N=1) and classic biallelic *CEBPA* mutations (N=1). Of the 2 remaining cases, one harbored *NUP98::NSD1* while the other could be considered AML-MR. Thus, the hypothetical yield of a *WT1/FLT3*-ITD strategy would be 1/2 (50%) if *KMT2A*-PTD status is determined up front. The Leucegene cohort also contained 16 additional AML cases with *WT1* mutations but lacking *FLT3*-ITD, of which 11 harbored a genetic alteration considered mutually exclusive with *NUP98*r, including *PML::RARA* (N=6), NPM1 mutations (N=2), classic biallelic CEPBA mutations (N=1), *RUNX1::RUNX1T1* (N=1), and *KMT2A::AFDN* (N=1). After their exclusion, 5 cases remained, with 2 harboring *NUP98::NSD1, 2* potential AML-MR, and one AML-NOS. Thus, the hypothetical yield of a *WT1*-based strategy regardless of *FLT3*-ITD status would be 3/7 (42.9%) if *KMT2A*-PTD cases are excluded.

*NUP98*r myeloid neoplasms have an aggressive clinical course with poor outcomes even after stem cell transplantation

In our two cohorts, 11 of the 14 AML patients plus the one patient with MPAL were initially treated with induction

chemotherapy (daunorubicin plus cytarabine [7+3] or vincristine, doxorubicin, methotrexate, plus cytarabine). Three of 14 patients with AML and all 3 MDS patients received hypomethylating agent (HMA)-based therapy with decitabine and venetoclax or with decitabine alone. One patient with AML died two days after starting 7+3 induction chemotherapy, and 2 patients failed to achieve remission, while the other 15 proceeded to hematopoietic stem cell transplant (SCT) in first complete remission (CR1). Post transplant relapse was seen in 60.0% (9/15) of patients transplanted in CR1, including MDS_3 who relapsed with AML (Figure 3). Of the remaining patients, 5 are in remission at 26 days, 42 days, 9.4 months, 19.6 months, and 21.3 months after SCT, and one has achieved sustained remission (98 months) after a second SCT (Table 1). In the HFA cohort, 8 patients died with a mean overall survival (OS) of 14 months. The median OS of the RHP cohort was 12 months (Figure 4).

Discussion

*NUP98*r is a rare genetic finding that is AML-defining in new classification systems but prone to under-detection without dedicated or complex testing. It portends a poor prognosis and likely requires dedicated therapeutic approaches. Here, utilizing a clinically validated targeted RNA sequencing approach, we studied the frequency of *NUP98*r in myeloid neoplasms in adult patients at two large academic centers and found 18 cases overall, including 11/257 (4.3%) of all newly diagnosed AML patients treated at one institution. In doing so, we also detected *NUP98*r in patients with MDS, uncovered novel *NUP98* fusion partners, and identified frequent co-mutations which could be leveraged to prompt dedicated testing for *NUP98*r.

In our review of the literature, less than 200 adult *NUP98*r with AML (Table 2) and far fewer adult *NUP98*r cases with other myeloid diagnoses (14 MDS, 4 CMML) (Table 3) have been described to date. The frequency of *NUP98*r in MDS is difficult to estimate precisely, given the lack of large comprehensive studies (Table 3). The most applicable study tested 101 consecutive adult MDS patients at a single institution by OGM, resulting in detection of *NUP98*r in 2/101 (2.0%) cases (1 *NUP98::NSD1*, 1 *NUP98::PRRX2*). Similarly, our reanalysis of public RNA-sequencing data from 2 adult MDS cohorts revealed *NUP98*r in 2/215 (0.9%) and 2/109 (1.8%) patients (3 *NUP98::NSD1*, 1 *NUP98::HOXA9*). Our study of the HFA clinical cohort revealed *NUP98*r at a slightly greater incidence in 2/46 (4.3%) adult MDS patients (1 *NUP98::NSD1*, 1 *NUP98::FGF14*). However, this cohort was subject to non-universal testing patterns and enriched for high-risk MDS. We also identified another high-risk MDS case with *NUP98::NSD1* through our dedicated strategies. Although relatively small, these studies suggest that this genetic aberration may be more common in MDS than previously thought, particularly in high-risk patients. Of note, an older study testing only for *NUP98::NSD1* by RT-PCR detected no cases out of 193 MDS patients.[35] Finally, since *NUP98*r is AML-defining in both the ICC (if >10% blasts) and the WHO5 (if >5% BM / >2% blood blasts) when with increased blasts, the 2/3 of the *NUP98*r MDS reported in the literature with at least 5% blasts would now be diagnosed as AML. Therefore, screening of MDS cases will also be important to identify cases that are actually AML, if they meet the blast criteria and have *NUP98*r.

Only 2 *NUP98*r cases in our cohort were recognized by karyotype (11.2% of 17 evaluable) (Table 1), highlighting the need for testing beyond conventional cytogenetics. Indeed, the most common *NUP98*r gene partners in adult and pediatric AML (*NSD1* and *KDM5A*) are well-known to produce karyotypically cryptic fusions. Moreover, a substantial proportion of uncommon *NUP98*r gene partners may similarly generate cryptic fusions according to recent comprehensive studies of adult AML enabled by RNA-based NGS. The largest such study reported 4 uncommon partners that were always cryptic by karyotype (*HMGB3, KMT2A, PSIP1,* and *TNRC18*), 4 that were never karyotypically cryptic (*HOXA9, TOP1, DDX10*, and *HHEX*), and one that was variably cryptic (*PRRX2*).[10] In our study, uncommon fusions involving both

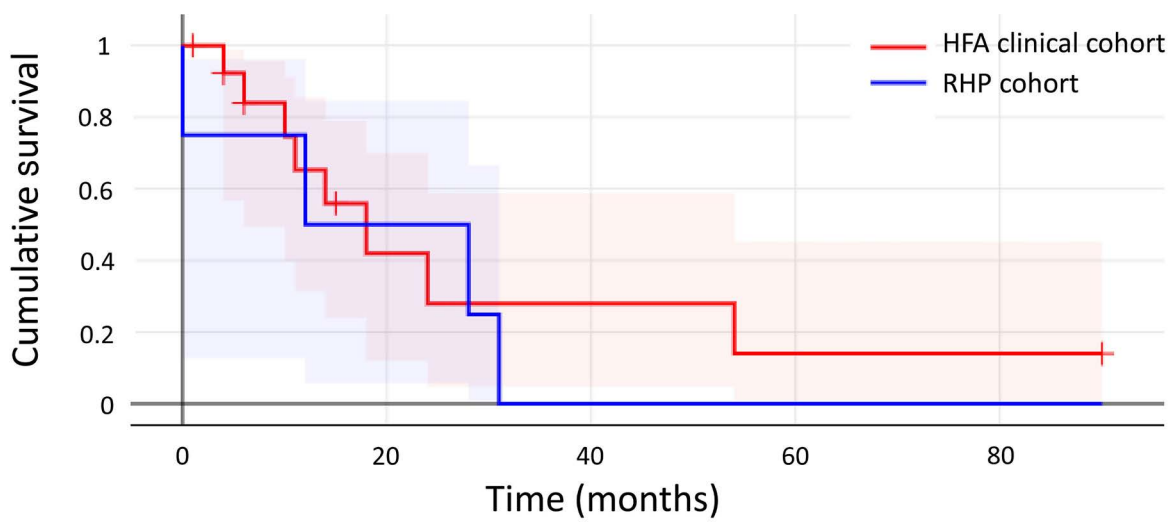

Figure 4. Overall survival curve of the Heme Fusion Assay clinical cohort and the Rapid Heme Panel cohort. Mean overall survival was 14 months and 12 months, respectively.

novel partners (*FGF14* and *LAMC3*) as well as *TNRC18* were karyotypically undetectable, *versus* 2 (*DDX10* and *HOXD13*) that were characterizable. By contrast, in a study of pediatric AML with *NUP98*r, uncommon partners were mostly detectable by conventional karyotype G-banding.[7]

Until universal screening for *NUP98*r becomes widely adopted as part of routine workups, strategies for rational test utilization are critical to ensure accurate detection of this entity. These strategies may be particularly beneficial for MDS, where guidelines for RNA-based NGS testing are lacking. We propose a tiered approach in the absence of universal screening and show here that it is possible to identify a subset of cases with high likelihood of *NUP98*r based on results from standard molecular testing. Specifically, MDS with *WT1* mutations and AML with *FLT3*-ITD and *WT1* co-mutations are enriched for *NUP98*r and thus represent candidates for follow-up dedicated testing in the absence of AML subtype defining alterations and *KMT2A*-PTD. These cases alternatively could harbor *UBTF*-TD, another high-risk alteration that is more common in pediatric MDS/AML but also occurs rarely in adults, including the 2 AML-NOS cases in our *FLT3*-ITD$^+$/*WT1*$^+$ AML cohort and a *WT1*$^+$ MDS case from the public RNA sequencing data; of note, most *UBTF*-TD should eventually be detectable during up-front testing by adding *UBTF* exon 13 to DNA-based panels. Further development of strategies to detect *NUP98*r may be warranted to leverage other known features, such as its association with FAB subtypes M4 and M5 (e.g., 10/15 cases in our *NUP98*r cohort) or the high frequency of a normal karyotype (9/17 evaluable cases in our cohort).

Although identification of *NUP98*r cases is critical for appropriate diagnosis and prognosis of AML, the optimal approach to *NUP98*r testing must balance cost and turnaround time with sensitivity. The most economical and fastest testing option is *NUP98* FISH, with a proposed reimbursement in the United States of $145.28 per test (CPT code 88368) and a turnaround time as short as 1-2 days but longer if run in batches/infrequently. However, since the incidence of *NUP98*r cases is less than 5% of cases of adult AML, the overall cost of universal testing for all AML patients would be quite high relative to the very low pre-test probability – the cost to the healthcare system is effectively greater than 20 times the individual FISH cost, or more than $2,905.60 per each *NUP98*r case detected. In MDS, where *NUP98*r is rarer (potentially 2% of cases), universal testing would be even more costly. Selective testing, such as through *WT1* or *FLT3*-ITD/*WT1* strategies, is, therefore, a much better fit for *NUP98* FISH. Larger studies will be needed to better characterize yield and to further develop and optimize strategies.

On the surface, RNA sequencing appears to be more costly, with a proposed reimbursement in the United States of $2,919.60 per test for a targeted RNA sequencing panel (CPT code 81455, 2025 Clinical Diagnostics Laboratory fee schedule) and a longer turnaround time (at least 4-7 days) than FISH. However, universal RNA sequencing allows for essentially 100% detection of *NUP98*r fusions, identification of gene partners, and appropriate disease subclassification. In addition, RNA sequencing approaches capture not only *NUP98*r cases but a wide spectrum of clinically important alterations that are critical for diagnosis, prognosis, and treatment of AML, some of which may also be cryptic by metaphase karyotype. Similar results may be obtained by WGS-based or whole transcriptome-based methods. On the other hand, a tiered approach with only karyotype and a DNA panel upfront would have an initial turnaround time of 3-5 days. Based on the results, reflex testing for RNA sequencing or FISH could be added. This strategy increases pre-test probability and decreases costs compared to universal testing, but results in longer turnaround times and lower sensitivity of *NUP98*r detection. Importantly, all testing algorithms are institution-specific, influenced by the availability of individual tests, testing schedules, and local logistics. Thus, testing decisions are ideally managed/supervised by pathology, as algorithmic testing in hematopathology has previously been shown to improve cost-effectiveness.[36]

*NUP98*r has consistently been associated with worse outcomes in studies of both pediatric and adult AML.[7,11,37-40] In our study, we observed high relapse rates even after SCT in CR1 (60% of patients). Therefore, there is a need to identify *NUP98*r at diagnosis and to develop more effective treatment strategies. In pre-clinical models, *NUP98*r AML has demonstrated sensitivity to Menin inhibition, with eviction of both *NUP98* fusion proteins and *KMT2A* (*MLL1*) from chromatin at a critical set of pro-leukemic genes.3 Given the recent approval of Menin inhibitors for AML with *KMT2A* rearrangement and their active development for *NPM1*-mutated AML, there are several phase I clinical trials (e.g., clinicaltrials.gov NCT05326516 and NCT05453903) that also recruit patients with *NUP98*r AML.[41-44] Recent PDX mouse models of *NUP98*r have also indicated that the combination of a Menin inhibitor with a CDK4/6 inhibitor (palbociclib) or a FLT3 inhibitor (gilteritinib) has a synergistic anti-leukemic effect.[45] In addition, several alternative treatments may be promising for *NUP98*r AML. One example is venetoclax, a BCL-2 inhibitor, which may be effective against AML with HOXA/B gene overexpression.[46,47] Another example is dasatinib, an inhibitor of ABL and SRC family kinases, which has synergistic effects on cells with *NUP98::NSD1* and *FLT3*-ITD.[48]

In conclusion, our results indicate that AML with *NUP98*r cases are usually cytogenetically cryptic and can be missed with conventional molecular testing, such as karyotype testing, FISH for common translocations, and myeloid-directed NGS panels looking at DNA mutations. Targeted RNA sequencing with anchored multiplex PCR or hybrid capture enrichment, whole transcriptome sequencing or other genome wide technologies, such as optical genome mapping, should be considered to detect *NUP98*r alterations.

Our high-yield tiered approach could be used to perform dedicated testing in the subset of AML and MDS that are enriched for *NUP98*r, which we, like others, demonstrated to be associated with poor prognosis. In fact, *NUP98*r should be specifically investigated in MDS as well, since it could lead to a change in diagnosis to AML and since the ability to detect *NUP98*r prior to leukemic transformation may allow for earlier intervention.

Disclosures

The authors have no conflicts of interest related to this research. RCL reports consulting for Qiagen, Bluebird Bio, Vertex Pharmaceuticals, Verve Therapeutics, Geron Corporation, Takeda Pharmaceuticals and Jazz Pharmaceuticals. ATF reports consulting for Servier, Brystol Myers Squibb, Astellas, Amgen, Kura, Syndax, AstraZeneca, Daiichi Sankyo, Prelude, AbbVie, Schrödinger, Takeda, Rigel, Gilead, Genentech, Autolus, Genmab, and has received clinical trial support from AbbVie, Servier, Kura, and Brystol Myers Squibb. MRL has received research funding to the institution from Novartis, AbbVie, and is part of the advisory board and provides consulting for Pfizer, Novartis, Kite and Jazz. VN reports consulting for Predicta Biosciences. All other authors have no disclosures.

Contributions

LDY, HKT, and VN designed the study, collected the data, performed analysis and interpreted the data, and wrote, reviewed, and revised the manuscript. All authors critically reviewed and edited the manuscript for publication.

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Data-sharing statement

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

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