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Original contribution

Multiplex high-throughput gene mutation analysis in acute myeloid leukemia

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Summary Classification of acute myeloid leukemia increasingly depends on genetic analysis. However, the number of known mutations in acute myeloid leukemia is expanding rapidly. Therefore, we tested a high-throughput screening method for acute myeloid leukemia mutation analysis using a multiplex mass spectrometry–based approach. To our knowledge, this is the first reported application of this approach to genotype leukemias in a clinical setting. One hundred seven acute myeloid leukemia cases were screened for mutations using a panel that covers 344 point mutations across 31 genes known to be associated with leukemia. The analysis was performed by multiplex polymerase chain reaction for mutations in genes of interest followed by primer extension reactions. Products were analyzed on a Sequenom MassARRAY system (San Diego, CA). The multiplex panel yielded mutations in 58% of acute myeloid leukemia cases with normal cytogenetics and 21% of cases with abnormal cytogenetics. Cytogenetics and routine polymerase chain reaction–based screening of *NPM1*, *CEBPA*, *FLT3-ITD*, and *KIT* was also performed on a subset of cases. When combined with the results of these standard polymerase chain reaction–based tests, the mutation frequency reached 78% in cases with normal cytogenetics. Of these, 42% harbored multiple mutations primarily involving *NPM1* with *NRAS*, *KRAS*, *CEBPA*, *PTPN11*, *IDH1*, or *FLT3*. In contrast, cases with abnormal cytogenetics rarely harbored more than 1 mutation (1.5%), suggesting different underlying biology. This study demonstrates the feasibility and utility of broad-based mutation profiling of acute myeloid leukemia in a clinical setting. This approach will be helpful in defining prognostic subgroups of acute myeloid leukemia and contribute to the selection of patients for enrollment into trials with novel inhibitors.

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1. Introduction

The classification, therapy, and prognosis of acute myeloid leukemia (AML) increasingly depend on molecular and cytogenetic analysis. Cytogenetic analysis stratifies patients into favorable, intermediate, and adverse prognostic groups. Approximately 40% to 50% of patients with AML have normal cytogenetics (CN-AML) and fall within the intermediate prognostic group in this classification system. However, there remains significant clinical heterogeneity in each cytogenetic group, particularly among patients with CN-AML.

In addition to cytogenetic abnormalities, a number of genetic mutations relevant to the pathogenesis of AML have been detected by traditional sequencing and polymerase chain reaction (PCR) methods and have become an integral part of routine clinical testing in AML. Mutation testing in conjunction with cytogenetics has provided insight into AML disease biology, defined prognostic subgroups, directed therapeutic decisions, and identified potential therapeutic targets. Somatic mutations in AML are broadly divided into 2 groups: class I and class II mutations. Class I mutations (*FLT3*, *KIT*, *FMS*, Ras-Braf pathway genes, etc) are activating mutations, which often target kinase pathways. Class II mutations (*NPM1*, *CEBPA*, chromosomal translocations, etc) are loss of function mutations involving genes important in transcription. An accepted model of AML pathogenesis proposes a multistep acquisition and collaboration of mutations where class II mutations occur early and block differentiation and class I mutations occur later in disease and promote survival and proliferation [1]. Chromosomal translocations affecting genes involved in transcription are now a part of the 2008 World Health Organization classification of AML and have prognostic significance. Similarly, mutations in several genes including *NPM1*, *FLT3*, and *CEBPA* have important prognostic as well as therapeutic implications in AML [2]. Isolated *NPM1* and *CEBPA* mutations confer a favorable prognosis, whereas *FLT3*-ITD mutations have been associated with a worse overall survival [3]. Moreover, AML with *NPM1* or *CEBPA* mutations have been incorporated in the 2008 World Health Organization classification as provisional diagnostic entities. Treatment is also guided by mutation status; patients with an *NPM1* mutation who are *FLT3*-ITD negative have been shown not to benefit from transplant [4], and clinical trials investigating *FLT3* inhibitors in patients with a *FLT3*-ITD mutation are currently underway. In addition, mutations in *RAS*; *TET2*; *IDH1/2*; and, most recently, *DNMT3A* have also been identified in AML [5-8]. The current recommendation is that molecular testing for *NPM1*, *FLT3*, and *CEBPA* mutations be routinely performed [9].

Cancer genomics in AML is a rapidly growing field aided by powerful and evolving genomic technologies. This raises the question of how best to perform large-scale mutation screening in the clinical setting with a limited amount of clinical sample, reasonable turnaround time, and affordable cost. In this study, we report our experience in AML genotyping using a high-throughput multiplex mass spectrometry–based approach.

2. Materials and methods

2.1. Patients and tissues

All samples were collected from the archives of the pathology and hematology-oncology departments, Oregon Health and Science University from 2008 to 2011. The study was approved by the Oregon Health and Science University Institutional Review Board. Peripheral blood or bone marrow aspirate was analyzed using a multiplex mass spectrometry–based approach (MassARRAY system; Sequenom, San Diego, CA) in 107 patients with AML. A single case of paraffin-embedded tonsillar myeloid sarcoma without peripheral blood or bone marrow involvement was included. Age, white blood cell count at diagnosis, AML subclassification, and results of additional molecular and cytogenetic studies were recorded (Table 1).

2.2. Multiplex mutation screening

DNA was extracted and purified from peripheral blood, bone marrow, or formalin-fixed, paraffin-embedded tissue. Multiplex mutation screening was performed using the Sequenom MassARRAY system, as previously described [10]. Assay Designer software (Sequenom, San Diego CA, USA) was used to design multiplex PCR's targeting point mutations in genes known to be associated with leukemia (Table 2). Initial PCR reactions used 10 ng DNA per multiplex in a total volume of 5 μL, with 100 nmol/L primers, 2 mmol/L $MgCl_2$, 500 μmol/L dNTPs, and 0.1 U Taq polymerase. Amplification included 1 cycle at 94°C for 4 minutes, followed by 45 cycles at 94°C for 20 seconds, 56°C for 30 seconds, and 72°C for 1 minute, and 1 final cycle at 72°C for 3 minutes. Unincorporated nucleotides were inactivated by addition of 0.3 U shrimp alkaline phosphatase and incubation at 37°C for 40 minutes, followed by heat inactivation of shrimp alkaline phosphatase at 85°C for 5 minutes. Single base primer extension reactions were carried out with 0.625 to 1.25 μmol/L extension primer and 1.35 U TypePLEX thermosequenase DNA polymerase (Sequenom). Extension cycling included 1 cycle at 94°C for 30 seconds and 40 cycles at 94°C for 5 seconds, with 5 cycles at 52°C for 5 seconds and 80°C for 5 seconds, followed by 1 cycle at 72°C for 3 minutes. Extension products were purified with an ion exchange resin, and approximately 10 nL of product was spotted onto SpectroChip II matrices (Sequenom). A Bruker matrix-assisted laser desorption/ionization time of flight mass spectrometer (MassARRAY Compact; Sequenom) was used to resolve extension products. MassARRAY Typer

Analyzer software (Sequenom) was used for automated data analysis, accompanied by visual inspection of extension products. Mutations detected by mass spectrometry were confirmed by conventional bidirectional DNA sequencing.

2.3. *KIT* method

KIT gene exons 8 and 17 were screened by a combination of real-time PCR and high-resolution melting curve analysis on a Roche Lightcycler LC480 (Indianapolis, IN, USA). The primers used were as follows: exon 8 forward GACATATGGCCATTTCTGTTT; exon 8 reverse GAATCCTGCTGCCACACATT; exon 17 forward TCGGATCACAAAGATTTGTG; exon 17 reverse GCAGGACTGTCAAGCAGAGA. Amplifications were performed with 100 ng DNA in 20 μL reactions using the Roche LightCycler 480 Probes Master Mixcontaining LC+ green dye. Cycling was as follows: 94°C for 8 minutes, followed by 40 cycles at 94°C for 20 seconds, 58°C for 2 seconds, and 72°C for 10 seconds. All suspected mutations were confirmed by direct DNA sequencing.

2.4. *CEBPA* method

For the assay performed in our laboratory, DNA is extracted from blood or bone marrow. PCR amplification of the entire *CEBPA* coding sequence (1 large exon; divided into 2 separate PCR products) is followed by direct DNA sequencing (with 8 different sequencing primers) to detect the presence or absence of mutations [11]. The low-level sensitivity limit of sequencing is approximately 20%, such that a mutant allele population below this detection limit is not reliably detected.

2.5. *FLT3-ITD* and *NPM1* methods

The detection of the internal tandem duplication mutation in the *FLT3* gene was assessed by PCR amplification of the juxtamembrane domain (with fluorescent primers) and determining the size of the resulting *FLT3* amplicons (by capillary electrophoresis). The *NPM1* C-terminal insertion mutation causing cytoplasmic localization was assessed by PCR amplification and direct DNA sequencing of *NPM1* exon 12.

2.6. Cytogenetics

Standard cytogenetic karyotype analysis was performed by the OHSU Cytogenetics Laboratory on peripheral blood or bone marrow aspirate/biopsy material. The specimen was cultured for 24 to 48 hours in complete RPMI 1640 medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA). Cells were harvested, and slides were prepared according to standard laboratory protocol. Slides were treated with 10% trypsin (Invitrogen) for 40 to 55 seconds followed by Wright stain (Sigma, St Louis, MO) for 2 minutes and 30 seconds. These Trypsin-Wright (GTW)-banded preparations were analyzed on a Nikon Eclipse E800 microscope (Nikon Instruments, Melville, NY) with Applied Imaging CytoVysion software (Genetix, San Jose, CA). When possible, at least 20 metaphase cells were examined for each case.

2.7. Statistics

Statistical analysis was performed using the χ^2 test.

3. Results

3.1. Patient demographics

Bone marrow aspirate, peripheral blood, or soft tissue was analyzed in 107 patients. The cohort consisted of 43 females and 64 males with a median age of 57 years. The white blood cell count ranged from 0.7 to 359.4 with a median value of 16.25. De novo and relapsed AML accounted for 64% (68/107) and 20% (21/107) of the cases, respectively. Sixteen percent (17/107) of cases were transformed AML arising from previously diagnosed myelodysplastic syndromes (MDSs), chronic myelomonocytic leukemia (CMML), or chronic myelogenous leukemia. A single case of tonsillar myeloid sarcoma without documented peripheral blood or bone marrow involvement was included. Patients were stratified into favorable, intermediate, and adverse cytogenetic risk groups according to published guidelines [9]. Favorable cytogenetic abnormalities accounted for 12% (13/107) of cases including inversion 16, t(8;21) and t(15;17). The intermediate-risk group comprised 62% (65/107) of total cases. Of these, 37% (40/107) had normal cytogenetics. Most of the normal cytogenetic cases had routine karyotypes with at least 20 metaphases analyzed as well as concomitant fluorescence in situ hybridization analysis. In 4 cases, less than 20 metaphases were analyzed: of these, 1 case had 16, 2 had 8, and 1 had only 1 metaphase. All of these cases, however, were normal by interphase fluorescence in situ hybridization panel analysis [includes probes for chromosome 5, chromosome 7, t(15;17), t(8;21), t(9;22), t(16;16)/inv16, and *MLL*(11q23)]. Finally, 27% (29/107) of cases harbored high-risk cytogenetic abnormalities including complex karyotype, monosomy 7, and *MLL* rearrangements.

3.2. Results of multiplex mutation screening

We previously developed a multiplexed mass spectrometry–based panel for screening oncogene mutations in solid tumors [10]. Mass spectrometry supports a rapid, quantitative readout with a lower limit of sensitivity of approximately 10% mutant allele. For this study, we developed a similar panel consisting of 270 assays covering 344 mutations across 31 genes known to play a role in hematologic malignancies (Table 2). Among the 344 mutations represented on the panel, 128 were validated by

Table 1 Characteristics of cases with mutations detected by multiplex analysis

Case	Age	Sex	WBC	Diagnosis	Mutated gene(s)	Codon	Chromosome	Start (hg18)	End (hg18)	Ref	Var	Additional mutations detected by standard PCR-based testing	Cytogenetics
1	76	M	1	AML-M2	*PTPN11*	T73I	12	111372585	111372585	C	T	ND	Normal
					FLT3	D835A	13	27490641	27490641	T	G		
					NPM1	W288fs*12	5	170770153	170770153	G	TCTGG		
2	41	M	0.8	AML	*NPM*	W288fs*12	5	170770153	170770153	G	TCTGG	ND	Normal
					IDH1	R132H	2	208821357	208821357	C	T		
3	46	F	70	AML-M2	*NPM1*	W288fs*12	5	170770153	170770153	G	TCTGG	*FLT3*-ITD	Normal
					KRAS	T58I	12	25271552	25271552	G	A		
4	37	M	109	AML-M5	*NRAS*	Q61H	1	115058051	115058051	T	A	ND	Normal
					IDH1	R132H	2	208821357	208821357	C	T		
					NPM1	W288fs*12	5	170770153	170770153	G	CCTGG		
5	78	M	214	AML-M5	*FLT3*	S451F	13	27508138	27508138	G	A	ND	Normal
					NPM1	W288fs*12	5	170770153	170770153	G	TCTGG		
6	75	F	277	AML-M1	*NPM1*	W288fs*12	5	170770153	170770153	G	TCTGG	ND	Normal
7	79	M	96	AML-M5	*NPM1*	W288fs*12	5	170770153	170770153	G	TCTGG	ND	Normal
8	52	M	60.5	AML-M2	*NPM1*	W288fs*12	5	170770153	170770153	G	TCTGG	*FLT3*-ITD	Normal
9	69	F	64.8	AML-M1	*NPM1*	W288fs*12	5	170770153	170770153	G	TCTGG	ND	Normal
10	68	F	9.3	AML-M5	*NPM1*	W288fs*12	5	170770153	170770153	G	TCTGG	ND	Normal
11	73	M	94	AML-M1	*NPM1*	W288FS*12	5	170770153	170770153	G	TCTGG	*FLT3*-ITD	Normal
12	61	M	26	AML-M1	*NPM1*	W288fs*12	5	170770153	170770153	G	TCTGG	*FLT3*-D835	Normal
13	27	F	66.6	AML-M1	*NPM1*	W288fs*12	5	170770153	170770153	G	CATGG	*CEBPA* and *FLT3*-ITD	Normal
14	70	F	105	AML	*NPM1*	W288fs*12	5	170770153	170770153	G	TCTGG	*FLT3*-ITD	Normal
15	17	M	90	AML-M1	*NPM1*	W288fs*12	5	170770153	170770153	G	TCTGG	*FLT3*-ITD	Normal
16	58	M	26.8	AML	*NPM1*	W288fs*12	5	170770153	170770153	G	TCTGG	*FLT3*-ITD	Normal
17	36	F	Normal	myeloid sarcoma	*NPM1*	W288fs*12	5	170770153	170770153	G	TCTGG	ND	Normal

18	60	M	65.2	AML	*NPM1*	W288fs*12	5	170770153	170770153	G	TCTGG	*FLT3*-ITD	Normal
19	72	M	132	AML, CMML	*CBL*	R420P	11	118654461	118654461	G	C	ND	Normal
20	61	M	2.2	AML, MPN	*CBL*	C404Y	11	118654201	118654201	G	A	ND	Normal
21	66	F	4	AML-M4	*NRAS*	G12D	1	115060270	115060270	C	T	ND	Normal
22	59	M	5	AML-M0	*NRAS*	G13D	1	115060267	115060267	C	T	ND	Normal
23	22	M	47.3	AML-M1	*IDH1*	R132H	2	208821357	208821357	C	T	*FLT3*-ITD	Normal
24	80	F	106	AML-M4, MDS	*FLT3*	D835Y	13	27490642	27490642	C	A	ND	t(3;12)
25	70	F	61.5	AML	*NPM1*	W288fs*12	5	170770153	170770153	G	TCTGG	*FLT3*-ITD	Extra isochrome 1q
26	54	F	Unknown	AML	*NRAS*	G12D	1	115060270	115060270	C	T	ND	11q23
27	46	F	13.4	AML-M4eo	*NRAS*	G12S	1	115060271	115060271	C	T	ND	Inv16, trisomy 22
28	23	M	280	AML-M4eo	*NRAS*	G13D	1	115060267	115060267	C	T	ND	Inv16
29	61	F	40.9	AML-M4	*NRAS*	Q61K	1	115058053	115058053	G	T	ND	Inv16
30	76	M	105	AML-M4	*KRAS*	G12D	12	25289551	25289551	C	T	ND	Additional 11q attached to 16
31	72	M	174.4	AML-M5	*KRAS*	G12V	12	25289548	25289548	C	A	ND	Monosomy 7
32	53	M	1.9	AML	*KRAS*	G12V	12	25289548	25289548	C	A	ND	Monosomy 7,8q
33	40	F	12.5	AML-M3	*KRAS*	Q61P	1	25271543	25271543	T	G	ND	t(15;17)
34	45	M	22	AML, CMML2	*IDH1*	R132H	2	208821357	208821357	C	T	ND	11;19 (involves MLL)
35	61	M	0.7	AML-M0	*IDH1*	R132H	2	208821357	208821357	C	T	ND	t(9p;11p)
36	24	M	3.3	AML with myeloid sarcoma	*NRAS*	G12D	1	115060270	115060270	C	T	ND	Inv16, trisomy 8, -Y
					KIT	D816Y	4	55294077	55294077	G	C		
37	37	M	2.2	AML-M0	*PTPN11*	E69K	12	111372572	111372572	G	A	ND	Monosomy 7

Patient demographics and diagnoses. Abbreviations: M, male; F, female; ND, not detected.

Table 2 Genes tested using a multiplex mass spectrometry–based approach

R-tyrosine kinase	*FLT3*	*KIT*	*FMS*	*PDGFRB*	*FGFR4*	*NTRK1*	*MET*
C-tyrosine kinase	*JAK1*	*JAK2*	*JAK3*	*FES*	*ABL1*		
Signaling molecule	*CBL*	*CBLB*	*NRAS*	*KRAS*	*HRAS*	*SOS1*	
Serine/threonine kinase	*AKT1*	*AKT2*	*AKT3*	*BRAF*			
Cytokine receptor	*MPL*						
Receptor	*NOTCH1*						
Phosphatase	*PTPN11*						
Metabolic pathway	*IDH1*	*IDH2*					
Tumor suppressor	*FBXW7*						
Transcriptional factor	*PAX5*	*NPM1*	*GATA1*				

follow-up sequencing during initial pilot work and through the course of this study. The remaining mutations were not available in our archive; however, every assay includes a positive control (wild-type allele) to show that the PCR and primer extension reactions worked.

NPM1 mutations were the most common among the cases analyzed. Most (78%) of the cases underwent routine screening of this gene by standard DNA sequencing, allowing us to compare the performance of the multiplex panel with the routine clinical assay. All cases positive for an *NPM1* mutation on the multiplex panel were also positive by standard DNA sequencing (15/15). In 4 additional cases, standard sequencing picked up an *NPM1* mutation when the *NPM1* assay in the multiplex mutation failed. PCR failure is detected when no extension products are generated and only the peak of the unextended primer is identified by mass spectrometry. Thus, when using a multiplex approach, it is necessary to have a backup test for any assays that fail and could result in a clinically important mutation being missed.

The multiplex panel demonstrated that 35% of all cases harbored at least 1 detectable mutation (Fig. 1). Recurring mutations were identified in *NPM1* (17%, 18/107), *NRAS* (7%, 8/107), *KRAS* (5%, 5/107), *IDH1* (5%, 5/107), *PTPN11* (2%, 2/107), *CBL* (2%, 2/107), and *FLT3* (point mutations) (3%, 3/107). Nearly 6% of cases harbored multiple mutations by mass spectrometry analysis alone; *NPM1* was most commonly involved in cases with multiple mutations. The multiplex panel yielded a mutation frequency of 58% in cases with normal cytogenetics and 22% in cases with abnormal cytogenetics (Fig. 2). Differences in mutation frequency and distribution are discussed in more detail below.

3.3. Combining multiplex mutation screening and standard PCR-based molecular testing

Most of the cases in our series underwent routine PCR-based screening for mutations in *FLT3*, *CEBPA*, and *KIT*.

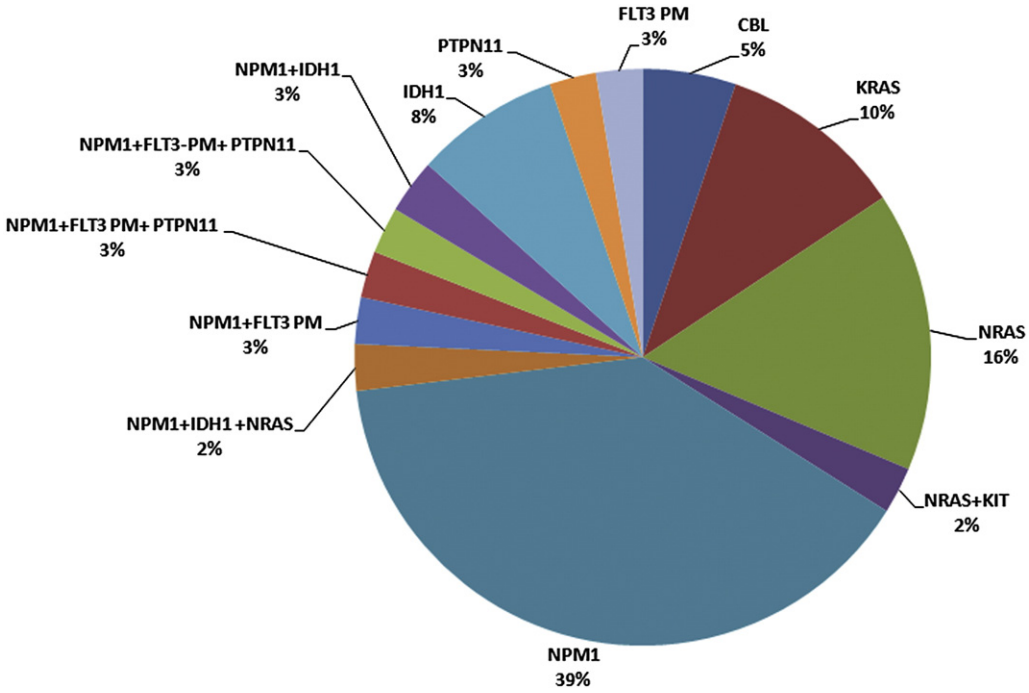

Fig. 1 Mutation distribution by multiplex analysis across all cases.

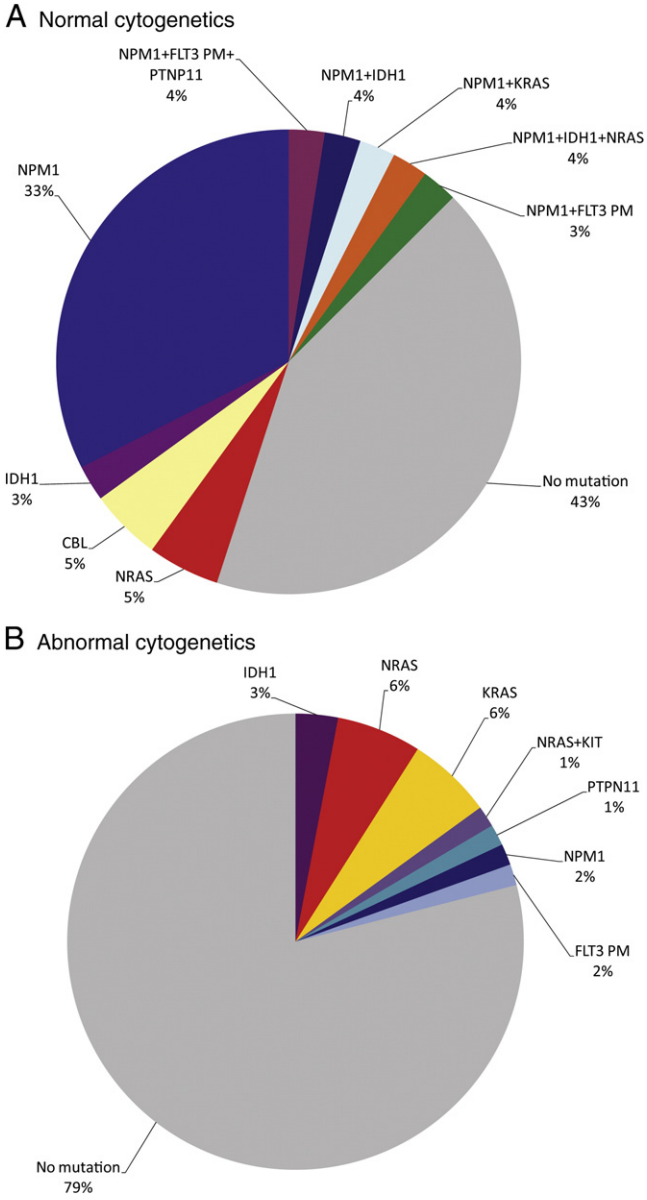

Fig. 2 Mutation frequency by multiplex analysis. A, Normal cytogenetics. B, Abnormal cytogenetics.

These assays complemented the multiplex panel, which is not suitable for detecting insertions and deletions of variable length (eg, *FLT3* and *KIT*) or loss-of-function mutations that cannot be readily predicted (eg, *CEBPA*). Overall, 26% (22/85) of the cases demonstrated *FLT3*-ITD mutations, with 18 cases in the intermediate cytogenetic risk group and only 4 in the high-risk cytogenetic group. *KIT* mutations in exons 8 and 17 were screened in 57 cases; 10% (6/57) were positive for either deletion/insertion mutations in exon 8 or point mutations in exon 17. Interestingly, 83% (5/6) of the *KIT* mutation–positive cases were within the inversion 16 cytogenetic group. Finally, *CEBPA* mutations were detected in 16% (5/31) of the tumors; all but 1 case were in the normal cytogenetic group.

With the addition of routine testing for *FLT3*-ITD, *CEBPA*, and *KIT*, the mutation frequency in the normal cytogenetic group increased to 78%, predominantly due to additional *FLT3*-ITD and *CEBPA* mutations (Fig. 3). In the abnormal cytogenetic risk group, the mutation frequency reached 42%, mostly due to *FLT3*-ITD mutations in the high-risk group and *KIT* mutations in the low-risk group.

3.4. Comparison of mutation frequency and distribution among cytogenetic risk groups

There was a distinctly different mutation frequency and distribution between the normal and abnormal cytogenetic risk groups. Multiplex mutation analysis demonstrated mutations in 58% (23/40) of cases with normal cytogenetics, including *NPM1* (40%, 16/40), *NRAS* (8%, 3/40), *IDH1* (8%, 3/40), and *CBL* (5%, 2/40). When combined with results of standard PCR-based tests, 78% of cases with normal cytogenetics had a detectable mutation. Of these, 41% harbored multiple mutations primarily involving *NPM1* together with *NRAS*, *KRAS*, *CEBPA*, *PTPN11*, *IDH1*, or *FLT3*. As depicted in Fig. 4, the normal cytogenetic group demonstrated a significantly higher total mutation frequency ($P < .001$) as well as more *NPM1* mutations ($P < .001$), *FLT3*-ITD/*NPM1* concurrent mutations ($P < .001$), and multiple mutations ($P < .001$). *IDH1* mutations were also more common in cases with normal cytogenetics, although this did not reach statistical significance.

In contrast, multiplex mutation analysis demonstrated mutations in only 21% (14/67) of AML with abnormal cytogenetics, including *IDH1* (3%, 2/67), *NRAS* (7%, 5/67), and *KRAS* (6%, 4/67). The mutation frequency reached 42% when combined with standard PCR based tests. Only 2 *NPM1* mutations were identified in this group, and 1 *CEBPA*-positive case was seen. On the other hand, *KIT* ($P < .05$), *RAS* and isolated *FLT3*-ITD mutations were more frequent in cases with abnormal cytogenetics. In contrast to cases with normal cytogenetics, only 1 case (1.5%) harboring multiple mutations was detected, which had concomitant *KIT* and *RAS* mutations, an uncommon example of coexisting class I mutations.

The mutation distribution in all 107 cases among the different cytogenetic risk groups is summarized in Fig. 5. In the low-risk group, only *KIT* and *RAS* mutations were seen, with most of them in association with inversion 16 (core binding factor leukemia). One *KRAS* mutation was found in acute promyelocytic leukemia. The intermediate-risk group was the largest group in our study, including normal cytogenetics, trisomy 8, and other non–high-risk cytogenetic changes. *NPM1* was the most frequent mutation in the normal cytogenetic group occurring as a sole mutation (30%), in combination with *FLT3*-ITD (60%), or in combination with a *FLT3* point mutation (10%). Overlapping *NPM1-IDH1* mutations were identified in 1 case. Interestingly, the mutation frequency was much lower in the high-risk cytogenetic groups, and most mutations were class I mutations involving *KIT*, *RAS*, *PTPN11*, and *FLT3*-ITD.

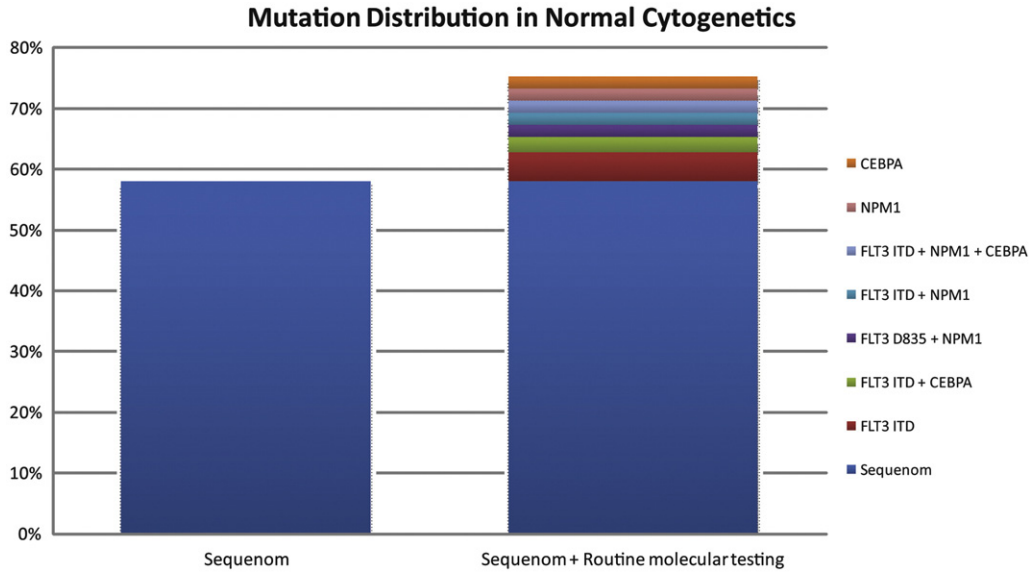

Fig. 3 Additional mutations detected by routine molecular testing in cases with normal cytogenetics.

These results suggest that different molecular mechanisms are involved in different cytogenetic groups.

4. Discussion

Our understanding of molecular genetics in AML is advancing, and as the number of known mutations in AML with prognostic and predictive utility continues to grow, so does the need for efficient methods for routine genotyping in the clinical setting. Practically speaking, it is time consuming and expensive to use multiple different assays in the diagnostic workup of a patient with AML. Moreover, the number of individual assays that can be performed is often limited by sample size. In this study, we report our experience in AML genotyping using a multiplex mass spectrometry–based approach. Previous panels built on this technology have been used primarily to study solid tumors [10,12,13]. To our knowledge, this is the first reported application of this approach focused on the study of leukemia. The platform is ideal for clinical specimens, as it requires a relatively small amount of DNA and can be used with DNA extracted from blood, bone marrow, and fresh/frozen tissue and formalin-fixed, paraffin-embedded tissue. The sensitivity is approximately 10% mutant allele [10]. Using this approach, we were able to quickly and efficiently screen for 344 mutations across 31 genes as a means to genotype AML.

Our genotyping studies identified recurring mutations in *NPM1*, *RAS*, *IDH1*, *PTPN11*, and *CBL* (Fig. 1). There was a distinctly different mutation frequency and distribution between the normal and abnormal cytogenetic risk groups, strongly suggesting differences in underlying biology (Figs. 4 and 5). In combination with standard PCR-based tests (*CEBPA*, *FLT3*-ITD, *NPM1*, and *KIT*), the normal cytogenetic risk group had a mutation frequency of 78%, nearly

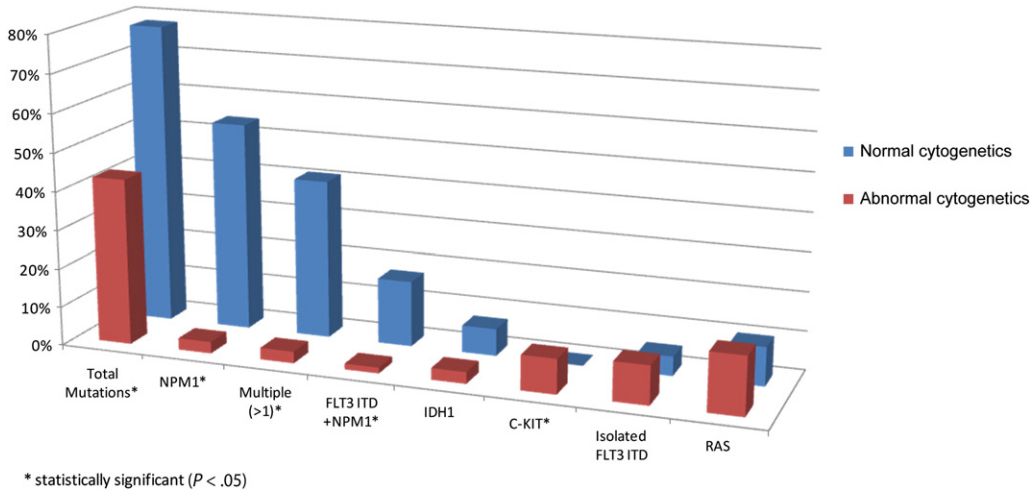

Fig. 4 Comparison of mutation distribution between normal and abnormal cytogenetic risk groups.

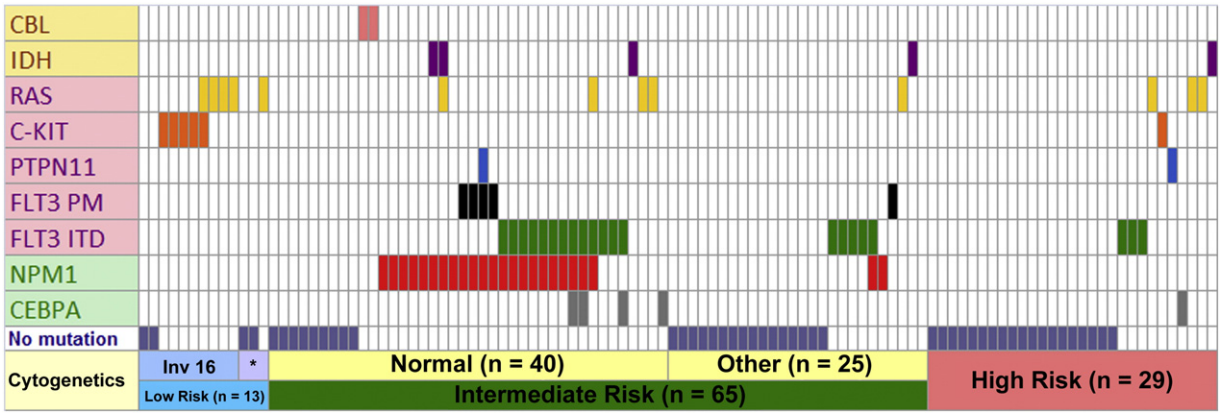

Fig. 5 Distribution of mutations across cytogenetic risk groups *t(15;17).

twice that seen in abnormal cytogenetics. Our mutation frequency in normal cytogenetics was slightly lower than what has been reported (85%), although prior studies also included *MLL*–partial tandem duplications and *WT-1* mutations, not analyzed in this study [9]. Key differences in mutation distribution include more frequent *IDH1*, *NPM1*, and *NPM1-FLT3* overlapping mutations in the normal cytogenetic group and increased *RAS*, *KIT*, and isolated *FLT3*-ITD mutations in the abnormal cytogenetic group.

Another striking difference arose when comparing concurrent mutations between cases with abnormal and normal cytogenetics. Concurrent mutations in 2 or more genes were seen almost exclusively in patients with normal cytogenetics. Of 40 cases with normal cytogenetics, 17 (42%) harbored more than 1 mutation, whereas this was true for only 1 case (1/67, 1.5%) with abnormal cytogenetics. Different patterns of overlapping mutations were also evident. Within the low-risk cytogenetic group, *KIT* and *NRAS* mutations were only seen in cases with inversion 16 and did not occur with other mutations. In cases with normal cytogenetics, *FLT3*, *NPM1*, *IDH1*, and *CEBPA* mutations were commonly seen in the context of multiple mutations and rarely occurred in isolation.

An understanding of what mutations occur together provides important prognostic information. The association between *FLT3*-ITD and *NPM1* mutations is a good example. Patients with *NPM1* have a good prognosis, which is abrogated in the setting of a concomitant *FLT3*-ITD mutation. Other similar relationships have been proposed. Mutations in *IDH1*, a metabolic enzyme, have been reported in approximately 7% to 16% of CN-AML [7,14,15]. *IDH1* mutations are often seen with other mutations especially *NPM1* and have been shown to be associated with a worse prognosis when they occur with unmutated *NPM1* [16], although an adverse prognosis in the setting of mutated *NPM1* has also been reported [17]. Several recent studies have integrated gene mutations and gene expression profiles to improve on the current risk stratification in AML [18-20].

Acquisition of *RAS* mutations has been shown to be associated with progression of MDS to AML [21], although multiple large studies have not demonstrated prognostic significance of *RAS* mutations in AML [4,22,23]. However, a recent study suggested that *RAS* mutations may predict the sensitivity of tumors to MEK inhibitors [24], and clinical trials investigating MEK inhibitors in myeloid malignancies with *NRAS* and/or *KRAS* mutations are currently underway. In our study, *RAS* mutations were identified in 40% (4/10) of cases with inversion 16; 2 mutations were identified at amino acid 12, 1 at 13, and 1 at 61. Similar results have been reported by others who also found that the cytogenetic groups inv16/t(16;16) and inv3/t(3;3) showed a higher frequency of *NRAS* mutations (37.6% and 26.8%, respectively) as compared with other cytogenetic groups [22]. Also consistent with previously published data, the *RAS* mutations in this study most frequently occurred at amino acid 12 (50%, 4/8).

In summary, using a multiplexed mass spectrometry–based approach, we were able to quickly screen for 344 mutations across 31 genes known to be associated with leukemia. This approach allows for genotyping of AML with a clinically useful turnaround time and limited sample. Moreover, in CN-AML, we demonstrate a mutation frequency nearly twice that seen in cases with abnormal cytogenetics with concurrent mutations occurring almost exclusively in CN-AML strongly suggesting differences in underlying pathogenic mechanisms between these 2 groups. Finally, we demonstrate molecular heterogeneity within CN-AML, which our current risk stratification scheme in AML does not account for; however, investigations are underway. Broad-based mutation profiling of AML will not only provide insight into disease biology and define prognostic subgroups but is being used now to guide therapy and select patients for enrollment into clinical trials with novel inhibitors.

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