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ORIGINAL ARTICLE

Addition of chromosomal microarray and next generation sequencing to FISH and classical cytogenetics enhances genomic profiling of myeloid malignancies

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Comprehensive genetic profiling is increasingly important for the clinical workup of hematologic tumors, as specific alterations are now linked to diagnostic characterization, prognostic stratification and therapy selection. To characterize relevant genetic and genomic alterations in myeloid malignancies maximally, we utilized a comprehensive strategy spanning fluorescence in situ hybridization (FISH), classical karyotyping, Chromosomal Microarray (CMA) for detection of copy number variants (CNVs) and Next generation Sequencing (NGS) analysis. In our cohort of 569 patients spanning the myeloid spectrum, NGS and CMA testing frequently identified mutations and copy number changes in the majority of genes with important clinical associations, such as TP53, TET2, RUNX1, SRSF2, APC and ATM. Most importantly, NGS and CMA uncovered medically actionable aberrations in 75.6% of cases normal by FISH/cytogenetics testing. NGS identified mutations in 65.5% of samples normal by CMA, cytogenetics and FISH, whereas CNVs were detected in 10.1% cases that were normal by all other methodologies. Finally, FISH or cytogenetics, or both, were abnormal in 14.1% of cases where NGS or CMA failed to detect any changes. Multiple mutations and CNVs were found to coexist, with potential implications for patient stratification. Thus, high throughput genomic tumor profiling through targeted DNA sequencing and CNV analysis complements conventional methods and leads to more frequent detection of actionable alterations.

Keywords Chromosomal microarray, myeloid malignancy, next generation sequencing, genomic profiling, actionable

Introduction

Routine testing of hematologic tumors for disease-specific molecular abnormalities consists of amplification methods such as Amplification Refractory Mutation System polymerase chain reaction (ARMS PCR), Sanger sequencing and conventional cytogenetic testing, including metaphase cytogenetics and fluorescence *in situ* hybridization (FISH). Metaphase cytogenetics is technically challenging and its sensitivity and resolution depends on good chromosome preparation that in turn is predicated on proliferating cells in culture and presence of clonal cells in the sample. FISH is less influenced by sample variation and able to detect gene rearrangements, but false

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negative results can occur when malignant cells represent a fraction of the population. Neither technology, however, can provide a genome-wide assessment of chromosomes at the gene level. Further, only about half of MDS and AML patients have abnormal metaphase cytogenetic results (1). These limitations, coupled with the clinical diversity in this patient population, points to the need for new techniques such as Next Generation Sequencing (NGS) and Chromosomal Microarrays (CMA) to detect additional molecular alterations to support diagnosis, prognosis and treatment.

NGS is a short-read, massively parallel deep sequencing technique that has revolutionized sequencing capabilities and has significantly increased the detection of clinically significant and therapeutically targetable mutations in various cancers. Clinically, molecular profiling by NGS primarily involves the detection of mutations, or somatic, oncogenic single nucleotide changes as well as small insertions and deletions in specific driver genes, and chromosomal rearrangements. Likewise, CMA has experienced increased adoption in genomic profiling. It is an effective tool for the detection of copy number variations (CNVs), which in our context refers to single copy gains or losses of at least 200 kb in genes associated with pathologic disease. Sometimes, however, genomic changes spanning smaller regions are also characterized as CNVs if they involve an amplification or gain, or a homozygous deletion in a known cancer gene. Used routinely for patients with developmental delay/intellectual disability, autism spectrum disorders, and multiple congenital anomalies (2), CMA is a cost-effective, high-resolution method for detecting global DNA copy number alterations, including chromosomal deletions (tumor suppressor genes) and amplifications (oncogenes), in oncology (3).

Myeloid malignancies are clonal disorders of hematopoietic stem and progenitor cells. Common myeloid malignancies include myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), neoplasms with an overlap of myelodysplastic and myeloproliferative components (MDS/MPN) and acute myeloid leukemia (AML). These disorders may present with a complex genetic profile, and can occur with multiple mutations (4) and CNVs (5). Coupled with clinical, morphologic, and immunophenotypic abnormalities, establishing a diagnosis of a myeloid malignancy in these instances is rather straightforward. However, in many cases, the morphologic features are subtle and/or routine testing such as single gene PCR analysis, classical cytogenetics and FISH are within normal limits, making a diagnosis difficult.

The role of mutations and CNVs in the diagnosis, prognosis and therapy of myeloid disorders has been apparent for some time now. Detection of somatic mutations are outside of the scope of discovery of conventional cytogenetics but can be detected by NGS. This has been particularly useful in the diagnosis of MPN. For example, identification of *CALR* mutations in patients with MPN who do not have *JAK2* or *MPL* mutations (6,7) can aid in the differentiation between reactive thrombocytosis and/or leukocytosis versus an MPN. Detection of CNVs is important for risk stratification of myeloid malignancies. For example, whole or partial deletion of chromosomes 5 and 7 are identified in 10-20% of myeloid malignancies including MDS and AML (8–10). Isolated 5q deletion is associated with good prognosis in patients with primary MDS (11). Generally, the presence of 5q deletion predicts response to Lenalidomide therapy. However, recent data indicate that the presence of a concurrent *TP53* mutation is necessary to refine prognostic risk stratification and likelihood of successful therapeutic outcomes in this context (12). In addition, copy neutral loss of heterozygosity (cnLOH), which can also be detected using SNP-based CMA, has been reported to be useful in determining prognosis in cytogenetically normal AML (13). Thus, we felt that there could be significant value in supplementing conventional techniques with emerging high-throughput technologies such as NGS and CMA in the work-up of myeloid malignancies.

In order to assess the extent to which incorporation of a multi-modal approach, including each of these technologies, contributes to improved characterization of myeloid neoplasms, we analyzed results from histomorphologic evaluation and comprehensive genomic profiling in patients with suspected myeloid disorders. These data are intended to refine diagnosis, establish prognostic risk stratification, and identify potential therapeutic targets.

Methods

Patients and tumor samples

All patients were referred to Pathgroup Labs, LLC for SmartGenomics Heme Profile testing, following morphologic evaluation and other routine diagnostic procedures including flow cytometry, FISH, conventional cytogenetic karyotyping, and PCR-based mutational analysis. Blood and bone marrow samples were classified according to standard hematopathology practice, as delineated by the World Health Organization (14). Classification was based on clinical, morphologic, immunophenotypic and molecular genetic features. Patients referred for expanded genomic profiling via next generation sequencing and cytogenomic microarray included those with morphologic evidence of acute leukemia, myelodysplastic syndrome, myeloproliferative neoplasm, or overlap myelodysplastic/myeloproliferative neoplasms. In addition, patients with a high clinical index of suspicion of a myeloid stem cell disorder with previously negative results by morphology, flow cytometry, and standard cytogenetic and molecular genetic testing were also profiled with our genomic panels. Examples of such patients with a high clinical index of suspicion included those with persistent CBC abnormalities where other etiologies had been clinically excluded.

Comprehensive genomic testing consisted of analysis of genetic targets by FISH and/or cytogenetics, as recommended by NCCN and other available guidelines and literature, concurrent with testing by CMA (Illumina CytoSNP-850K BeadChip, see Appendix A for a list of the genes related to available targeted therapies) and an 85 gene next generation sequencing panel consisting of somatic mutations (see Appendix B for a list of the genes and the corresponding codons used for our analysis) to find additional diagnostic, prognostic and therapy related targets. The study was performed on deidentified patient samples and deemed “Exempt” following review by the Institutional Review Board (IRB). In our particular context, “actionable” targets are characterized as mutations and CNVs whose presence would result in specific diagnostic, prognostic and therapeutic recommendations, both off-label and on-label. Our determination of actionability is supported by peer reviewed publications and guidelines, including the recently published recommendations by the Association of Molecular Pathology (15).

DNA Extractions and QC

Genomic DNA was prepared from whole blood or bone marrow aspirate-derived white blood cells on the Qiagen (Germantown, MD) QIAsymphony® automated nucleic acid extraction platform, in combination with the QIAsymphony® DSP DNA mini Kit Version 1, as per the manufacturer's protocol, and concentrated using the Zymo Research DNA Clean & Concentrator-5 (Zymo Research, Orange, CA). For NGS testing, amplifiable DNA quantity (haploid genome copy number) was determined using a quantitative Taqman assay directed to the single copy gene FTH1 (Life Technologies). For CMA testing, DNA was quantified using Qubit (Life Technologies, Carlsbad, CA) and A260/A280 purity estimates determined using Nanodrop (Thermo Scientific, Waltham, MA).

Sanger sequencing

PCR reactions were performed using M13-tagged primers and the HotStart-IT Taq DNA Master Mix (Affymetrix, Cleveland, OH), starting from 15–50 ng of genomic DNA. PCR was followed by treatment with ExoI and SAP to remove residual primers (ExoSap-IT, Affymetrix, Cleveland, OH). Sanger sequencing was performed using the BigDye Terminator kit (version 3.1; Life Technologies, Carlsbad, CA) and M13 forward and reverse primers. The sequencing products were purified using the BigDye Xterminator purification kit (Life Technologies, Carlsbad, CA), separated using an ABI 3500xL Genetic Analyzer (Life Technologies, Carlsbad, CA) and analyzed using CLC Genomics Workbench (CLCbio, Waltham, MA).

Conventional cytogenetic and fluorescence in situ hybridization

Conventional G-banded chromosome studies were performed using standard techniques. A minimum of 20 metaphase cells were analyzed from unstimulated bone marrow aspirate or peripheral blood cultures, when available. The abnormal karyotypes were described using the International System for Human Cytogenetic Nomenclature (ISCN 2013) (16).

FISH was performed on interphase nuclei using disease specific panels of probes according to the manufacturer's protocol. The FISH probes used were based upon flow cytometry and morphologic evaluation by the hematopathologists and chosen from the following locus specific, translocation and break-apart probes—D5S23/D5S721 (5p15.2), *EGR1* (5q31), *PDGFRB* (5q33-34), *CEP7* (centromere 7), D7S486 (7q31), *CEP8* (centromere 8), *CEP9* (centromere 9), D13S319 (13q14.3)/*LAMP1* (13q34), D20S108 (20q12); *KMT2A* (*MLL*) (11q23) and *CBFB* (16q22) dual color break apart probes; *RPN1/MECOM* [t(3;3)(q21.3;q26.2)/ inv (3)(q21.3q26.2)] and *RUNX1T1 (ETO)/RUNX1* (*AML1*) [t(8;21)(q22;q22)], *BCR/ABL1* [t(9;22)(q34;q11.2)] dual color, dual fusion translocation probes, *SCFD2/LNX/PDGFRA* tri-color, tri-fusion translocation DNA probes specific for 4q12 rearrangements (Abbott Molecular, Inc., Des Plaines, IL), *TP53* (17p13.1) and *CEP19* (centromere 19) (Agilent Technologies, Inc., Santa Clara, CA), and the *FGFR1* (8p11) dual color break-apart probe, and *SCFD2* (4q12)/*TET2* (4q24) (MetaSystems Group, Inc., Newton, MA). A total of 200 nuclei per probe were evaluated with fluorescence microscopy using the Ikoniscope automated analysis system and the analysis was performed using the Ikonisoft viewer 2.0 (Ikonisys Inc., New Haven, CT). The specimen was considered abnormal if the results exceeded the laboratory-established cut-off for each probe set. A signal pattern of 3-5 additional copies was considered as a "gain" and $> = 6$ copies was considered as "amplification".

Quantitative and end-point real-time multiplex PCR

Somatic mutations (insertions and/or deletions) in exon 9 of the *CALR* gene are evaluated by target specific PCR to amplify the regions of interest using primer pairs that contain one fluorescently tagged primer to enable resolution of the PCR product on a capillary electrophoresis instrument. For the *CALR*, PCR of a genomic DNA template (20 ng) was performed using AmpliTaq Gold Polymerase (ThermoFisher Scientific, MA), CALR forward/reverse primers (3 uM), and nuclease-free water in a total volume of 15 uL. Amplification was performed on a GeneAmp PCR System 9700 (ThermoFisher Scientific, MA) thermal cycler as follows: 95 °C × 10 minutes; 35 cycles of 95 °C × 15 seconds, 59 °C × 15 seconds, and 72 °C × 30 seconds; and 72 °C × 10 minutes, followed by capillary electrophoresis on the 3500 xL Genetic Analyzer (ThermoFisher Scientific, MA). We determined our limit of detection (LOD) to be at 1% mutant allele frequency for the *CALR* assay. For the *FLT3* exon 14 insertion mutations (internal tandem duplication or ITD) and tyrosine kinase domain (TKD) mutations, each target region is amplified with target specific PCR primers. 3 uM of the forward/reverse primers are used in a total volume of 15 uL containing 20 ng of template, and amplified under identical conditions as *CALR*. The TKD PCR product is then digested with restriction enzyme EcoRV before both the ITD and TKD reactions are analyzed by fragment analysis via capillary electrophoresis. The LOD for this assay was also estimated at 1% mutant allele frequency. The quantitative detection of major and minor BCR/ABL1 fusions is performed using the Ipsogen BCR/ABL Mbcr IS-MMR Kit (Qiagen, Germany) following the manufacturer's instructions. Quantitative JAK2 V617F mutation detection was performed with the Ipsogen MutaQuant Kit (Qiagen, Germany) following the manufacturer's instructions.

Illumina infinium CNV processing and analysis by chromosomal microarray (CMA)

DNA samples (200 ng) were enzymatically fragmented, hybridized to an Illumina CytoSNP-850K BeadChip, stained using the Xstain HD BeadChip process and washed (17). BeadChips were scanned using the iScan system and raw data normalization, SNP clustering, CNV identification and SNP calling was performed using the GenomeStudio v3.3 Genotyping Module to generate genotype calls, B-allele frequency (BAF) and logR ratio (Illumina, San Diego CA). Systematic correction of probe distribution was performed using the quadratic correction algorithm of the Nexus Copy Number 7.5 software and copy number variation analysis was performed using SNPRank Segmentation algorithm. Each sample is compared to a cluster file generated from pooled normal control

samples provided by Illumina. The log ratio thresholds were set as follows—gain: 0.09, loss: –0.135, amplification: 0.3, homozygous loss: –0.45. The remaining parameters were set as follows—homozygous frequency threshold: 0.9, homozygous value threshold: 0.8, heterozygous imbalance threshold: 0.45. For quality control, only samples with quality scores (which represent robust probe to probe variance) less than 0.1 were considered for downstream cytogenetic data analysis. The segmented data were analyzed using Nexus Copy Number software. Of note, as a result of our own clinical validation, only gains and losses of 200 kb or more in size which include 16 or more consecutive SNP probes deflected beyond threshold, and containing RefSeq genes were considered true calls. Those true calls known to be normal copy number variants were considered benign and not included in the data analyzed in this project. Finally, terminal regions greater than 8 MB and interstitial regions greater than 10 MB which demonstrated cnLOH were included in the analysis. The terminology CNV refers to gain, loss, amplification or homozygous loss detected by CMA.

Next-generation sequencing (NGS) & analysis

All specimens were processed and analyzed in duplicate. A minimum of 2400 copies of genomic DNA from each specimen were subjected to multiplex pre-amplification using the Roche High Fidelity PCR System (Indianapolis, IN). The PCR product was cleaned using Affymetrix ExoSAP-it (Santa Clara, CA) following the manufacturer's recommended procedure and concentrated using Zymo Research DNA Clean & Concentrator-5 (Irvine, CA) following the manufacturer's recommended procedure. The cleaned products were used as template in the Fluidigm AccessArray system (South San Francisco, CA) and amplified using Roche High Fidelity PCR System. PCR products harvested from the AccessArray system were subjected to DNA indexing using Fluidigm barcodes following the manufacturer's recommended procedure. Indexed libraries were pooled and quantified using KAPA Biosystems Library Quantification Kit (Wilmington, MA). Diluted library pool was denatured and sequenced on an Illumina MiSeq or NextSeq using MiSeq Reagent Kit v2 (San Diego, CA) or NextSeq Reagent Kit v2. Sequencing was single index, 2 X 150 cycle paired end. Single-nucleotide variants and insertion/deletion events were reported. Bioinformatics analysis (trimming, alignment, variant calling, and variant annotation) was conducted using the MiSeq Reporter Custom Amplicon workflow (version 2.3) and the Genome Analysis Toolkit (GATK, version 2.3-9). The analytic sensitivity of the assay is 5%. However, sensitivity is contingent upon the specific mutation, the sequence context of the mutation, and the coverage of the amplicon. The average coverage for the panel was greater than 1000X. Coverage was amplicon dependent and ranged from 200X to greater than 10,000X within a specimen. Mutations were called if the observed variant met the following criteria: observed in at least 5% of the reads at that locus, present in both replicates, predicted to modify the sequence of the encoded protein, and not present in a database of common non-pathogenic inherited variants (dbSNP version 138). Insertion and deletion mutations were subjected to an additional filter, removing any indel larger than 25 nucleotides in length from review.

Statistical analysis for significant correlations

All specimens containing two or more variants detected by CMA and/or NGS were combined into a large dataset and examined for significant gene pair correlations. Specimens were removed if only a single gene variant was present or if more than one variant was located within the same gene region; as this analysis was focused on identifying correlations between two different genes and associations with chromosomal aberrations. The remaining dataset ($n = 289$) was examined for significant co-occurrences between gene pairs using frequency of observed variants per gene per specimen. A correlation matrix was created to examine Pearson correlation coefficients across all paired gene interactions using the stats package in R (https://www.r-project.org/; version 3.3.2. 10/2016). Samples that passed additional filtering ($r > 0.1$ and $n = 10$) were subjected to additional pairwise correlation to establish levels of significance (p-value) of those interactions. Several other significant gene pair correlations were detected however many of these correlations were found either within the same individual or within a small population ($n < 5$). To avoid small sample population overestimates, we limited our results to significant associations with populations greater than 10 samples.

Results

Distribution of specimen in the analysis

The sample cohort included 194 MDS patients, 34 patients with MDS/MPN, 156 non-CML MPN, 17 CML and 168 AML patients ($n = 569$ total cases). All 569 cases were analyzed by NGS, and subsets of these had concurrent CMA ($n = 477$), cytogenetics ($n = 493$) and FISH ($n = 426$) results (see Appendix C for the overlap of cases tested on various platforms).

Mutation spectrum in myeloid malignancies

To understand the benefit of performing NGS and CMA analysis, the number of cases with abnormal results was examined for each myeloid disorder. The test with the highest yield of abnormal results was NGS (65.4–94.1%), followed by CMA (34.4–62.6%) and conventional cytogenetics (10.9–59.7%) (Figure 1). The high degree of abnormal results in NGS and CMA is likely due to the higher resolution of these tests. Across all the myeloid diseases in this study, it is evident that genomic testing by NGS and CMA, for the most part, revealed a higher percentage of abnormal cases than conventional Cytogenetics and FISH, with the exception of CML. The primary driver genetic aberration in CML is the BCR-ABL1 translocation that is not detectable by array CGH and hence the lower diagnostic yield by CMA.

To understand if the increase in diagnostic yield translates into improved patient care, the identified abnormal results were restricted to actionable, medically relevant associations. These genes are targets of FDA approved drugs, both on and off-label, and those involved in pathways that are the subjects of clinical trials, as well as abnormalities with useful diagnostic and prognostic associations. When targeted for intervention, these mutation(s) are expected to influence

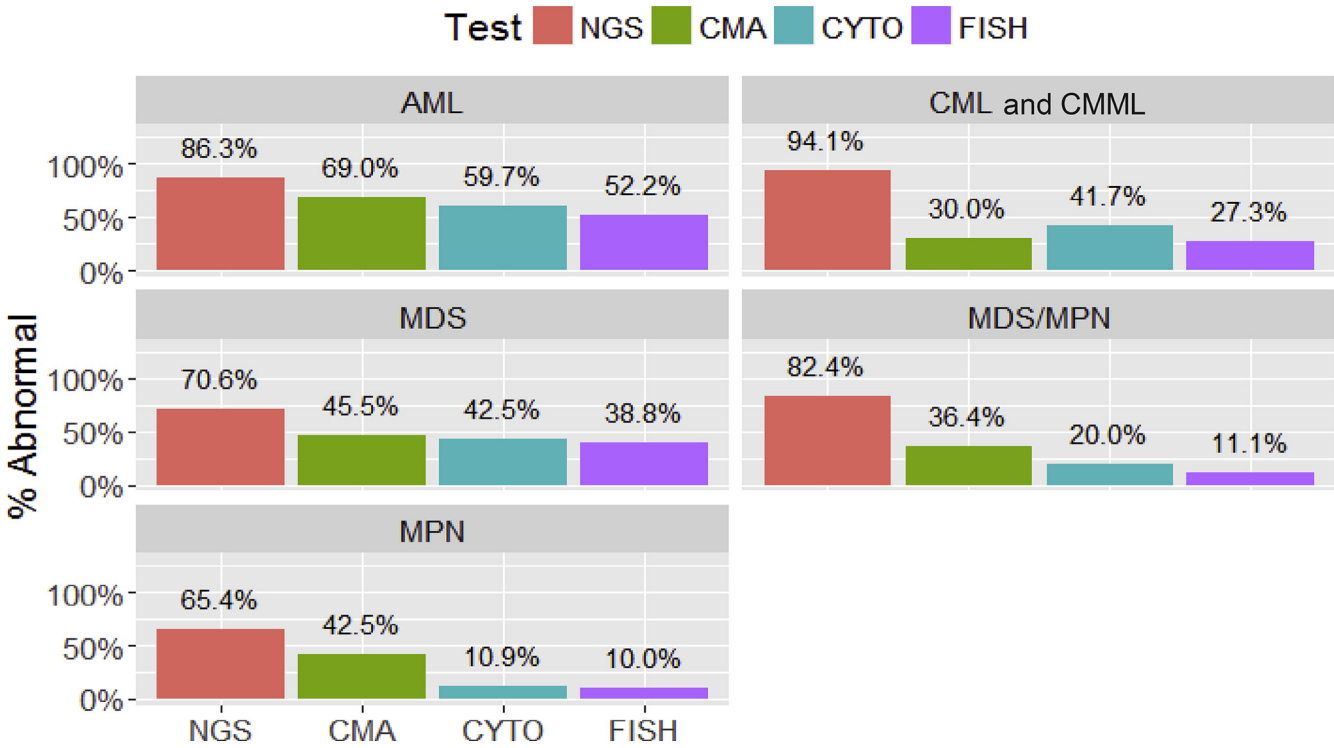

Figure 1 Distribution of abnormal cases by various testing platforms assessing diagnostic, prognostic and therapy related aberrations across the 5 myeloid cancer groups depicted.

outcomes. All of the genes on the NGS panel were chosen for actionability and mutations were detected in 428 of 569 cases (75.2%). Of 477 cases tested with CMA, 229 (48%) were abnormal, out of which 105 (45.9%) had copy number changes potentially targetable, both on-label and off-label, by FDA-approved treatments (Appendix A). The other cases had alterations with diagnostic and prognostic implications but did not contain genes listed in the targetable gene list (Appendix A). In 428 patients with mutations identified by NGS, mutations were observed in 49 genes (Table 1). Two patterns emerged from the data. First, certain mutations were predominantly observed in one disease type, such as *JAK2* in MPN patients or *SRSF2* in MDS/MPN and CML. Second, a few genes were frequently mutated in multiple myeloid disorders, such *TP53, TET2*, and *RUNX1*. CMA analysis also identified actionable CNVs in 49 genes, and some genes were recurrently affected across the entire myeloid spectrum, such as *APC*, *BRAF*, *ETV6* and *TP53* (Table 2). Of note, 26 (53%) of the actionable genes identified by NGS and CMA exhibit both mutations and CNVs (Figure 2).

Drug-targetable CNVs identified by CMA were observed mostly in AML and MDS patients. The most frequently observed CNVs affected the *TP53*, *APC*, *RB1*, *NF1*, *BRCA2* and *ATM* genes. The CNVs observed were overwhelmingly losses/homozygous losses affecting tumor suppressor genes, owing to large deletions in MDS and AML. Only 8 of 105 specimens analyzed (7.6%) showed gains/amplifications, the majority of which were oncogenes, and mostly observed in AML patients. Although we identified fewer affected genes in CML and MDS/MPN, overall the percentage of cases with a mutation or CNV was very high in these groups, 100% and 85.3%, respectively. Detection of fewer numbers of affected genes in these two sub-groups is a likely reflection of both the smaller number of patients tested in our cohort, especially for CNVs, as well as the relatively limited genomic diversity in these populations (18,19).

Diagnostic yield of conventional testing by cytogenetics and FISH

FISH and conventional cytogenetics are the standard of care for myeloid disorders. FISH results were available for 426 patients, of which 152 showed gene rearrangements or copy number variations. A majority, 62.5% (95/152), showed deletions of 5q, 7q, and 20q, reflected in the high percentage of patients with loss of *APC* and *BRAF*. In addition, FISH identified rearrangements and amplifications involving *KMT2A*, *PML/RARA*, *CBFB*, *BCR/ABL1*, *RPN1/MECOM*. For samples tested with conventional cytogenetics, 183 of 493 (37%) showed abnormal results. Cytogenetic testing revealed several heterogeneous chromosomal abnormalities, a significant fraction of which involved monosomies or deletions of part or all of chromosomes 5 or 7 (51.8% in AML and 63.5% in MDS) that also include complex karyotypes involving other chromosomes such as 12p, 17p, 20q and trisomy 8.

Diagnostic yield of NGS and CMA testing in samples normal by cytogenetics and FISH

To determine the usefulness of NGS and CMA testing in patients that were normal by conventional cytogenetics and FISH testing, we examined the distribution of mutations and all CNVs in these patients (Table 3). Of the 227 cases that were normal

Table 1 Percentage of mutated cases by NGS for each gene, stratified by myeloid malignancy

	AML (n = 145)	CML (n = 16)	MDS (n = 137)	MDS/MPN (n = 28)	MPN (n = 102)		AML (n = 145)	CML (n = 16)	MDS (n = 137)	MDS/MPN (n = 28)	MPN (n = 102)
JAK2	2.8%	18.8%	2.2%	17.9%	57.8%	*MPL*	1.4%				3.9%
TP53	20.0%	6.3%	24.1%	10.7%	3.9%	*TNFAIP3*	2.1%		1.5%		1.0%
TET2	12.4%	25.0%	15.3%	21.4%	10.8%	*CCND3*	0.7%		0.7%		2.9%
RUNX1	14.5%	25.0%	11.7%	17.9%	8.8%	*GATA2*	2.8%		0.7%		
ATM	8.3%	31.3%	10.2%	7.1%	19.6%	*NOTCH1*	1.4%		2.2%		
SRSF2	11.0%	25.0%	12.4%	25.0%	6.9%	*ABL1*		25.0%			
SF3B1	4.1%	18.8%	19.0%	25.0%	2.0%	*KIT*	2.8%				
IDH2	13.8%		8.0%	3.6%	1.0%	*U2AF2*			0.7%	7.1%	1.0%
DNMT3A	13.1%		5.8%	10.7%	2.0%	*STK11*	1.4%		0.7%		
NPM1	18.6%		1.5%	3.6%	1.0%	*TRAF3*			0.7%		2.0%
U2AF1	6.9%	6.3%	10.2%	7.1%	2.9%	*EGFR*			1.5%		
NRAS	11.0%	12.5%	4.4%	14.3%	1.0%	*EZH2*	0.7%				1.0%
ASXL1	1.4%		5.1%	3.6%	8.8%	*FGFR3*					2.0%
CREBBP	2.1%		5.8%	10.7%	2.9%	*FGFR4*			0.7%	3.6%	
KRAS	6.2%	12.5%	0.7%	14.3%	1.0%	*PLCG2*		6.3%			1.0%
PTPN11	8.3%		2.2%	3.6%		*SF3A1*	1.4%				
IDH1	8.3%		1.5%			*STAT3*			1.5%		
PHF6	6.9%		2.2%		1.0%	*B2M*				3.6%	
CEBPA	5.5%		2.9%	3.6%		*BCL2*					1.0%
WT1	6.2%		2.2%			*BCOR*			0.7%		
SETBP1	2.1%		1.5%	14.3%	1.0%	*BTK*	0.7%				
CALR	1.4%		0.7%		4.9%	*CTNNB1*					1.0%
CSF3R	2.8%		0.7%	10.7%		*FBXW7*		6.3%			
CBL	3.4%		1.5%			*MYD88*			0.7%		
FLT3	2.8%		0.7%		1.0%						

Table 2 Percentage of abnormal cases by CMA for each gene, stratified by myeloid malignancy

	AML (n = 48)	CML (n = 3)	MDS (n = 41)	MDS/MPN (n = 5)	MPN (n = 8)		AML (n = 48)	CML (n = 3)	MDS (n = 41)	MDS/MPN (n = 5)	MPN (n = 8)
Gene losses						*BCOR*			4.9%		12.5%
APC	47.9%		58.5%	40.0%	12.5%	*STAG2*			4.9%		12.5%
BRAF	50.0%	33.3%	46.3%			*ZRSR2*			4.9%		12.5%
ETV6	20.8%	33.3%	29.3%		12.5%	*GATA1*			4.9%		12.5%
TP53	29.2%		19.5%	20.0%	12.5%	*CDKN2A*	2.1%	33.3%			
NPM1	18.8%		26.8%			*SMARCB1*	2.1%	33.3%			
NSD1	14.6%		24.4%			*IDH2*	2.1%		2.4%		
RB1	12.5%		14.6%	20.0%	12.5%	*PTCH1*			4.9%		
NF1	14.6%		4.9%	40.0%	12.5%	*IDH1*			4.9%		
TET2	12.5%		12.2%		12.5%	*TSC1*	2.1%				
BRCA2	8.3%		9.8%			*CALR*	2.1%				
SETBP1	10.4%		2.4%	20.0%		*SF3B1*			2.4%		
RUNX1	8.3%		2.4%	20.0%	12.5%						
ATM	4.2%		7.3%		25.0%	***Gene amplifications***					
WT1	8.3%		4.9%			*FLT3*	4.2%				
STK11	6.3%		7.3%			*ROS1*	2.1%				
CBL			7.3%	20.0%	25.0%	*JAK3*	2.1%				
GATA2	8.3%		2.4%			*CCND3*	2.1%				
FBXW7	6.3%		4.9%			*KRAS*	2.1%				
BAP1	8.3%					*CCND2*	2.1%				
CEBPA	6.3%		2.4%			*KMT2A*	2.1%				
PTPN11	4.2%		4.9%			*JAK2*	2.1%				
DNMT3A	4.2%		2.4%		12.5%	*BCL2*	2.1%				
PHF6			7.3%		12.5%	*ERG*	2.1%		2.4%		12.5%
BRCA1	2.1%		2.4%		12.5%	*BRD4*	2.1%				
SRSF2			2.4%	20.0%	12.5%	*TMPRSS2*					12.5%

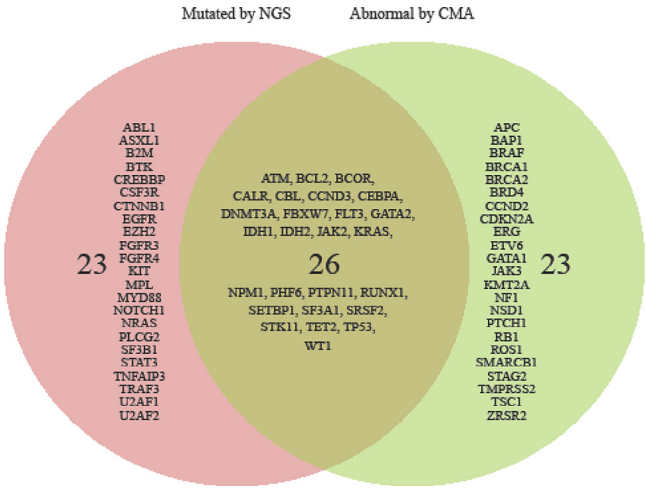

Figure 2 Genes affected by mutations and copy number variations as detected by NGS and CMA, respectively.

by conventional testing, NGS testing uncovered mutations in 158/227 (69.6%), and CMA in 51 of the 204 cases (25%) for which results were available. Overall, 164/217 (75.6%) of the Cytogenetics/FISH normal cases were abnormal by either CMA and/or NGS testing. Analysis of each myeloid disorder showed that the percentage of cases with abnormal results was very high (Figure 3). It should be noted that this analysis excludes 10 cases from the set of 227. NGS did not detect pathogenic or actionable mutations in these patients, and since results from CMA testing were not available, it was not possible to determine definitively whether they contained any genomic aberrations or not.

Table 3 Genetic aberrations detected by NGS and CMA in normal cytogenetics & FISH specimens

Abnormal NGS and CMA results in Normal Cytogenetics & FISH specimens		
NGS	Mutations detected	158/227 (69.6%)
	Mutations not detected	69/227
CMA	CNV Detected	51/204 (25%)
	CNV Not Detected	153/204

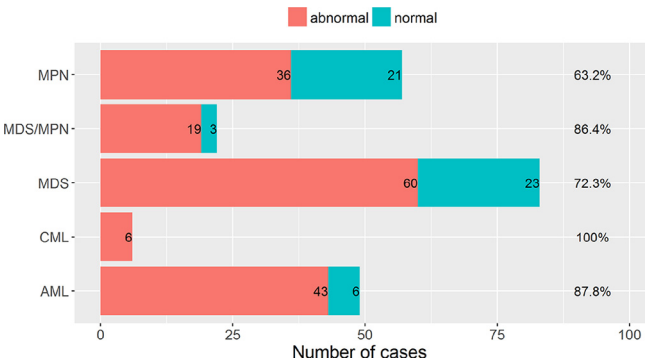

Figure 3 Distribution of cases with mutations detected by NGS and CMA in Normal Cytogenetics & FISH cases.

Table 4 Comparison of CMA and NGS in myeloid neoplasms with normal cytogenetics and FISH

		Copy Number Variant (CMA)	
		Not Detected	Detected
Mutations (NGS)	Not Detected	53 (26%)	6 (2.9%)
	Detected	100 (49%)	45 (22.1%)

The cytogenetics/FISH normal specimens were further examined for mutations and CNVs to understand the individual contributions of NGS and CMA. Table 4 represents the cases with or without CNVs and mutations, all of which were normal by cytogenetics and FISH. Thus, 100 of 204 patients in this cohort (49%) were abnormal by NGS testing only, whereas CMA detected aberrations in 6 (2.9%). These data indicate that addition of NGS and CMA to the routine workup of myeloid disorders provides additional, potentially actionable, information in otherwise normal specimens.

In a previous study that examined NGS and CMA in solid tumors (lung and colorectal), 6.6% of specimens otherwise normal by CMA, had mutations identified by NGS (20). This is in contrast to the results in this study, which shows 65.5% (100/153) in the CMA normal population (Table 4), pointing to the greater diagnostic yield from mutations detectable by NGS in myeloid diseases compared to lung and colorectal tumors.

We also routinely perform PCR-based single analyte molecular tests such as *BCR-ABL1* analysis by quantitative PCR, Calreticulin (*CALR*) mutation and *FLT3* ITD analysis, to supplement the other genomic testing in myeloid neoplasms, currently not included in the NGS testing protocols. These provide additional information for the diagnosis and monitoring response to therapy and disease recurrence. In our cohort, there were 48 such cases with mutations detected by NGS and PCR-based single analyte molecular tests. Four of these had *BCR-ABL1* fusions (3 with concurrent *ABL1* mutations), and 7 had *FLT3* ITD mutations. Most importantly, mutations were detected in 10 of 241 (4.1%) cases that were normal by all 4 testing methodologies (6 with *JAK2*, 3 with *CALR* and one with *FLT3* ITD) (data not shown). This further underscores our contention that none of these techniques is completely replaceable and that as our repertoire of testing methods increases, we would need to implement comprehensive testing for effective patient management.

CMA provides additional clinical information

NGS, Cytogenetics and FISH positive specimens were analyzed for the percentage of cases to which CMA testing provided additional diagnostic, prognostic and therapeutic information. CMA results were available in 94 cases positive for NGS, FISH and Cytogenetics. Of those, 59 (63%) had medically actionable gene losses or gains/amplifications, thus providing another level of clinically relevant information. More importantly, CMA testing was able to detect additional alterations in 6 of 59 (10.1%) cases which were normal by NGS, FISH and cytogenetics. Overall, it is evident that CMA studies provide additional diagnostic, prognostic, or therapeutic information beyond information rendered by NGS, conventional

Table 5 Distribution of non-targetable alterations detected by CMA by disease type

CMA Abnormality	AML (n = 39)	CML (n = 3)	MDS (n = 32)	MDS/MPN (n = 6)	MPN (n = 44)	Total
Trisomy	8		5		5	18
Trisomy + cnLOH	2	1	3			6
cnLOH	17		9	3	24	53
Loss	8	2	10	3	5	28
Gain	3		4		5	12
Complex (Loss/Gain +/– cnLOH)	1		1		5	7

karyotyping, and FISH. The additional abnormalities detected by CMA included submicroscopic losses and gains that were cytogenetically undetectable; and those targets not assessed by FISH at the time of testing (for example, loss of *TET2, CDKN2A/2B, NF1*, amplification of *JAK2/3, CCND2/3, BRD4, KRAS, ROS1*).

Non-targetable aberrations characterized by CMA analysis

Of the 229 cases abnormal by CMA, 124 (54%) had large copy loss/gain and copy neutral loss of heterozygosity (cnLOH) that are not directly gene-targetable (and hence not included in the CMA targetable subset of cases) but have important implications for diagnosis and prognosis of myeloid disease. The distribution of events included 18 cases with trisomy (most frequently seen were +8, +9, +11, +21), 53 cases with cnLOH (most frequently involved chromosome regions were 1p, 2p, 4q, 7q, 11q and 9p), 6 cases with trisomy and cnLOH, 28 cases with losses (most frequent losses were -Y with/without other alterations, 20q-, 5q- and 7q-), 12 cases with gains (including gain on 1q, 9p (*JAK2*), 8p, 11q, 21q), and 7 cases with a complex CMA result including gains/losses/cnLOH (Table 5). Of note, out of 59/124 (48%) cases with cnLOH (53 with sole cnLOH and 6 with trisomy and cnLOH) 35/59 (59%) cases had an associated point mutation in the gene within the cnLOH region, indicative of a double hit or homozygous mutation. The majority of the cnLOH (21 cases) included 9p cnLOH with *JAK2* mutation in the MPN group. Others included 2p cnLOH with *DNMT3A* mutation (3 cases), 1p cnLOH with associated *MPL* mutation (2 cases); 4q cnLOH with *TET2* mutation (2 cases); and one case each with 11q cnLOH and *ATM* mutation, and 17p cnLOH with *TP53* mutation. Of note, cases with cnLOH and associated mutations were not included in the CMA targetable subset, as these have been included as a targetable event under the NGS testing methodology.

Conventional cytogenetics and FISH testing complements new technologies in samples with low tumor burden

While our data clearly highlights the importance of the role of NGS and CMA testing in the clinical workup of myeloid neoplasms, we also looked at the subset of patients where these methodologies failed to detect genomic changes. Of the 92 cases that were normal by both NGS and CMA, 85 had either cytogenetics or FISH results available (83 cytogenetics, 67 FISH cases). In all, 12 (14.1%) cases normal by NGS and CMA were abnormal either by FISH or cytogenetics (abnormalities include t(9;22), inv (16), t(16;16), t(11;19) and low-level clones of losses of 7q and 20q), thus highlighting the continued contribution of conventional testing in the diagnostic and prognostic delineation of myeloid malignancies. Additionally, this may attribute to enrichment of the low number of circulating tumor cells in culture, which results in increased diagnostic yield in a small proportion of samples.

Based on cases for which results from all testing platforms were available, 43.3% of MDS and 39.6% of MPN specimens had normal results for NGS, CMA, FISH and conventional cytogenetics, whereas AML had only 11.3%. This may indicate the involvement of additional genes affected in the development of MDS or MPN that are not yet identified or routinely assessed with current technologies.

Pairwise correlations between gene variants detected by NGS and CMA in myeloid cohort

Pairwise correlations across all gene variants detected by both CMA and/or NGS in our entire myeloid cohort found significant gene interactions (p-value < 0.01) spanning six genes (Table 6). In order to estimate statistical association of the genomic alterations, we considered the targetable genes within the deleted and amplified regions as detected by CMA. Associations of *APC* (with or without *NPM1* and *NSD1*) demonstrate the strongest correlations within the dataset with four significant gene pairs (*BRAF*, *ETV6*, *RB1*, and *TP53*). Since *APC*, *NPM1* and *NSD1* (along with *EGR1*) all cluster together on chromosome 5q and most often are co-deleted due to loss of 5q, we considered all subjects containing *APC*, with or without *NPM1*, and *NSD1*, as a separate group and examined correlations across those subjects containing both CMA and NGS information. Our analysis found two gene variants showing significant correlations with this 5q loss cluster, *ETV6* loss (r = 0.25, p-value < 0.008) and *TP53* loss/mutations (r = 0.48, p-value < 0.001) (data not shown).

Table 6 Pairwise correlations between gene alterations detected by CMA and/or NGS

Gene 1	Gene 2	R	p-value	95% CI		n
APC	BRAF	0.424	<0.001	0.325	0.514	23
APC	ETV6	0.399	<0.001	0.297	0.491	16
APC	RB1	0.327	<0.001	0.220	0.426	10
APC	TP53	0.650	<0.001	0.578	0.712	40
BRAF	ETV6	0.417	<0.001	0.317	0.508	15
BRAF	TP53	0.389	<0.001	0.287	0.483	12
DNMT3A	NPM1	0.228	<0.001	0.116	0.335	14
ETV6	TP53	0.292	<0.001	0.182	0.394	16

When we examined the correlations of CNVs and/or mutations in genes on different chromosomes, four of these genes show relatively strong interactions with *APC* loss (r = 0.32-0.65), with *TP53* loss/mutations again showing the strongest interactions as a large number of patients had alterations in both genes. All of this data corroborates the well-established notion that karyotypic loss of 5/5q- and loss of 7/7q- and 12p abnormalities are associated with *TP53* alterations (loss or mutations) in myeloid disorders. Defects in *TP53* are independent poor prognostic factors for overall and disease-free survival in myeloid diseases. Several reports have demonstrated that loss of *TP53* in hematopoietic stem and progenitor cells haploinsufficient for both *EGR1* and *APC* lead to the development of AML in 17% of mice (21). Some recent studies suggest that haploinsufficiency of genes on chromosome 5 is strongly associated with loss of *TP53* activity in the development of therapy related myeloid neoplasms (21). We observed similar association of *TP53* point mutations with chromosome 5 and 7 abnormalities detected by conventional cytogenetics in MDS and AML cohorts (22/25, 88% in AML, and 29/29, 100% in MDS). Taken together, these observations are in tandem with published findings that indicate *TP53* alterations occurred more frequently in patients with unfavorable-risk cytogenetics and a higher degree of karyotypic complexity (22). Interestingly, we also observed significant correlation (r = 0.417, p < 0.001) between loss of the *BRAF* and *ETV6* genes as detected by CMA analysis.

Although correlation of the *SF3B1* point mutation with alterations in *TET2* did not meet our criteria of p-value < 0.01, we observed correlation of weaker significance (R = 0.122988; p- value 0.03665). This is most likely due to association of *TET2* with other mutations and CNVs across the entire myeloid spectrum, which would dilute its association with *SF3B1*. *SF3B1*, which encodes for splicing factors and is prognostically favorable, is commonly mutated in MDS patients with ring sideroblasts and has been shown to frequently coexist with mutations in the DNA methylation gene *TET2* in chronic myelomonocytic leukemia (CMML), long considered a MDS subtype (23).

Correlation of gene variants detected by CMA and NGS with disease states

We further focused our mutation and CNV correlation analyses to MDS, AML and MPN, since the majority of aberrations detected by either genomic platform predominate in these disease states. The recurrently mutated genes in any of the myeloid diseases belong to distinct functional groups and are mostly mutually exclusive. For example, in patients with MDS, mutations in genes associated with the spliceosome machinery (*SF3B1, U2AF1*, and *SRSF2*) were among those identified most frequently (19%, 10.2% and 12.4%, respectively). Defects in the *U2AF1* and *SRSF2* genes, also associated with unfavorable prognosis in MDS patients without ring sideroblasts (24), did not occur in the same specimen. Our NGS panel detects point mutations in the activation loop of the tyrosine kinase domain of the *FLT3* gene (*FLT3*/TKD mutations). *FLT3*/TKD mutations likely associate with cytogenetically favorable risk AML (25). We found *FLT3* point mutations in 4 out of 145 (2.8%) patients, consistent with data that suggest that these mutations occur at far lower frequency in AML patients (26,27). In addition, as expected, defects in the *JAK2* gene were observed primarily in MPN patients. Gene correlations with disease state of observed patients indicate the strongest associations occur with *JAK2* with MPN (r = 0.48; p-value < 0.001). Compared to patients with MDS or AML, relatively fewer other defects were identified in these patients. Patients with additional defects beyond *JAK2* commonly had mutations in *ASXL1*, a negative prognosticator, and *TET2*. Association with *ASXL1* with MPN, MDS and MDS/MPN suggested a lower but still significant correlation (r = 0.144; p-value = 0.002). *TET2* alterations are common in the various myeloid cancers (28). It frequently coexists with *JAK2* mutations and are temporally linked to outcomes (29). Twenty MPN specimens (19.6%) also contained mutations in the DNA repair gene *ATM*, which was reported only recently in patients with this neoplasm (30).

Examination of *NPM1* alteration frequency with disease state indicated a small but statistically significant correlation with AML (r = 0.26; p-value < 0.001). Mutations and CNVs in *NPM1*, associated with favorable prognosis, were significantly associated with *DNMT3A* alterations. This conforms to observations of *NPM1* mutated patients more likely to present with concomitant *TET2* and *DNMT3A* mutations in cytogenetically normal AML (31). Mutations in *ASXL1*, which is an independent poor prognostic factor for survival in MDS and MPN patients, occurred with other gene mutations at diagnosis, such as *RUNX1*, *JAK2* and *TET2* (17/19, 89%). This is consistent with other studies where *ASXL1* mutations frequently coexisted with acquisition of mutations of other genes during disease progression (32).

Discussion

In this study, we showed that our approach of combining mutation detection with our panel of 85 genes and CNV detection by CMA analysis with conventional cytogenetics and FISH testing is effective for the characterization of chromosomal and gene changes with important diagnostic, prognostic and therapeutic associations in myeloid malignancies. Coupled with histomorphologic and immunophenotypic information, this approach will enable clinical decisions to better align with evidence-based guidelines and best practices. In myeloid disorders where common testing technologies are within normal limits, such technologies will be critical to help establish a diagnosis and refine prognostic risk stratification. This strategy additionally will identify genomic alterations for which available targeted therapy (FDA approved, off label, or available through clinical trials) may be available. Finally, thoughtful and cost effective integration of these promising technologies may result in more cost-effective clinical management and improved outcomes of these patients, particularly when traditional technologies yield negative results. These disorders are driven by multiple variables and are not always easily diagnosed, necessitating the use of multiple markers and platforms for a complete picture to emerge.

Genomic biomarkers such as *NPM1*, *FLT3*, *JAK2*, *KIT*, and *TP53* associated with myeloid neoplasms have been incorporated into clinical guidelines for diagnosis, risk assessment and the establishment of treatment options. For example: 1) in the absence of concurrent *FLT3* ITD mutations, *NPM1* mutations confer a favorable prognosis (33); 2) a high *FLT3*-mutant allelic ratio characterizes an AML subtype potentially

sensitive to *FLT3*-inhibitor therapy (34), several of which are in development; 3) loss-of-function in *CEBPA* caused by a biallelic mutation of this gene, which occur in approximately 10% of patients with AML and normal karyotype, is associated with a favorable prognosis compared with wild-type (33). The presence of mutations in genes encoding for signaling molecules (*CBL*, *JAK2*), transcription factors (*RUNX1*), epigenetic regulators (*ASXL1*, *EZH2*, *IDH2*, *TET2*) or splicing factors (*SRSF2*, *SF3B1*, *U2AF1*) have been identified in myeloid neoplasms (35,36), and several major studies have demonstrated their negative effect on survival (37), with the increasing number of mutated genes significantly associated with more advanced phenotypes and worse prognosis. A more recent study underscored the importance of genomic profiling to classify AML into 1 of 11 molecular-based categories, each of which portended a favorable or aggressive clinical course (38). Targeted therapies directed against many of these genomic aberrations are in various stages of clinical development.

In our study, NGS testing identified medically relevant mutations in *JAK2*, *TP53*, *TET2*, *CSF3R*, *SRSF2*, *RUNX1* and *ATM*, among other genes. CMA analysis detected copy number changes mostly in *TP53*, *APC*, *RB1*, *NF1*, *BRCA2* and *ATM*. A study by the Cancer Genome Atlas Research Network (39) analyzed the genomes of 200 cases of de novo AML, and reported that AML genomes possess a relatively limited number of mutations. The most frequently mutated genes include *FLT3*, *NPM1*, *DNMT3A*, *IDH1*, *IDH2*, *TET2*, *RUNX1*, *TP53*, *NRAS*, *CEBPA*, and *WT1*, similar to the mutational frequency profile observed in our cohort. The majority of chromosomal changes detected by FISH correspond to the common -5/del (5) and -7/del (7) abnormalities observed in MDS and AML. Across the entire myeloid spectrum, we observed that only a few genes were more frequently affected than others. For example, SRSF2 was mutated more often in MDS/MPN and CML. Similarly, loss of APC was observed more frequently in MDS and AML and not in CML. Although beyond the scope of this study, we feel that as more data around these associations becomes available and as more genes and pathways are elucidated, it will provide a basis for molecular stratification and prognostication for myeloid and other hematologic malignancies. From the list of actionable genes assessed by NGS and CMA, 26 (53%) exhibited both mutations detected by NGS and CNVs detected by CMA. This suggests that both mechanisms may be responsible for the resulting haploinsufficiency or overexpression driving tumorigenesis. It also highlights the importance of both testing modalities, especially if these alterations occur independently of each other and associate with distinct diagnosis and/or prognosis. For example, some reports indicate that *NPM1* deletion and *NPM1* exon 12 mutations are mutually exclusive and are associated with two distinct cytogenetic subsets of MDS and AML (40). Another important observation in our study is the presence of medically relevant changes detected by the NGS and CMA platforms in 75.6% of the cases normal by conventional testing. In addition, NGS identified mutations in 65.5% of specimens that were negative by all other testing modalities, whereas CNVs were detected in 10.1% cases that were normal by NGS, cytogenetics and FISH. Such findings are consistent with multiple other studies, both at our institution (20) and at elsewhere (41) that show that mutation profiling by NGS increases diagnostic precision and adds prognostic and therapy related information. Finally, 14.1% of cases where NGS or CMA failed to detect any genomic aberrations had clinically relevant chromosomal changes identified by either by FISH or cytogenetics, and 4.1% by PCR techniques. This suggests the continued role of conventional tests, beginning from the morphologic examination of peripheral blood, bone marrow aspirates or trephine biopsies that raise the suspicion of a myeloid stem cell disorder. In our estimation, presence of mutations and copy number changes in the absence of well-defined morphologic dysplasia need to be interpreted carefully. For example, in cytopenias and/or minimal dysplasia being evaluated for MDS, genomic testing would result in a higher frequency of clonal hematopoiesis of indeterminate potential (CHIP) and clonal cytopenias of undetermined significance (CCUS) cases that are “clonal” but are not necessarily predictive for clinical outcome (42).

In line with many other studies, we detected the coexistence of multiple mutations and CNVs. Such analysis could contribute to the improved classification and risk stratification in the context of disease-based panels. For example, mutations in *TP53*, an independent poor prognostic factor for overall and disease-free survival, was strongly associated with complex chromosomal abnormalities including large copy losses on 5q. When we looked at individual genes, loss of *APC* showed strong correlations with losses in *BRAF*, *ETV6*, and *RB1.* While many of the strong correlations reported here might be a result of the interplay of complex chromosome abnormalities such as 5q and 7q deletions, consistent with their involvement in myeloid malignancies, characterizing them on a gene level may also be informative for correlating with genes located on different chromosomes.

Unlike our previous study published for lung and colorectal tumors (20), there seems to be a greater contribution of NGS testing compared to CMA testing in the genomic profiling of myeloid disorders. This is most likely due to the larger number of genes in the panel as well as the higher degree of genomic instability in solid tumors, reflected in greater numbers of gene losses and gains. Nevertheless, because CMA yielded actionable results in cases that were negative by other profiling technologies, one cannot discount its importance in the work-up of hematologic malignancies. Of the 477 cases in this study with CMA results, 229 (48%) were abnormal and 248 (52%) were normal. Of the 229 abnormal cases, 105 (46%) cases had events that were directly or indirectly correlated to drug targets. The remaining 124 cases included trisomy, cnLOH, large copy losses and gains and some with complex CMA patterns. Although not “druggable”, the diagnostic and prognostic importance of these alterations are well characterized.

Thus, in conclusion, high throughput genomic tumor profiling through targeted DNA sequencing and analysis of copy number alterations complements conventional methods of tumor interrogation and leads to more frequent detection of actionable alterations. In the context of morphologic and/or clinical suspicion of a myeloid stem cell disorder, our data indicates that integrating multiple strategies to identify informative biomarkers can enhance diagnosis, prognosis and/or therapy in myeloid neoplasms.

Appendix

Appendix A List of genes which are targets of FDA approved drugs, both on and off-label, and those involved in pathways that are the subjects of clinical trials, thus used in designating medically-relevant copy number variations (MR-CNVs) (from www.selleckchem.com)

Gene Gain/ Amplification	Targeted Therapy	*Gene Gain/ Amplification*	Targeted Therapy	*Gene Loss/ Homozygous Loss*	Targeted Therapy
ABL1-3	**Imatinib**	*JAK1-3*	**Pacritinib**	*APC*	**PKF115-584**
AKT1-3	**Ipatasertib**	*KIT*	**Dasatinib**	*ATR*	**Olaparib**
ALK	**Crizotinib**	*KMT2A (MLL)*	**EPZ-5676**	*ATM*	**Veliparib**
AR	**Andarine**	*KRAS*	**Trametinib**	*BAP1*	**Vorinostat**
ARAF	**Encorafenib**	*LYN*	**Saracatinib**	*BRCA1-2*	**Olaparib**
AURORA A-C	**Tozasertib**	*MAP2K1,2K2,3K1 (MEK)*	**Selemetinib**	*CEBPA*	**Panobinostat**
BCL2	**ABT-737**	*MAPK1 (ERK2)*	**Ulixertinib**	*CDKN2A-2B*	**Flavopiridol**
BRAF	**Vemurafenib**	*MCL1*	**Obatoclax Mesylate**	*CDKN1A-1B*	**Dinaciclib**
BRD2-4	**RVX-208**	*MDM2,4*	**MI-773**	*FBXW7*	**Everolimus**
BTK	**Ibrutinib**	*MET*	**Golvatinib**	*NF1-2*	**Alpelisib**
CCND1-3	**Sirolimus**	*MST1R*	**BMS-777607**	*PTCH1*	**Taladegib**
CCNE1	**Flavopiridol**	*mTOR*	**Sirolimus**	*PTEN*	**Ipatasertib**
CDK1,4,5,6,7,9	**Flavopiridol**	*NOTCH1,2*	**Semagacestat**	*RB1*	**PD-0332991**
CRKL	**Dasatinib**	*NRAS*	**Cetuximab**	*SMARCA4*	**Entinostat**
CSF1R	**PF-477736**	*NTRK1-3*	**LOXO-101**	*SMARCB1*	**Taladegib**
CTNNB1	**iCRT3**	*PDGFRA*	**Ponatinib**	*STK11*	**Dasatinib**
DDR2	**Dasatinib**	*PDGFRB*	**Axitinib**	*TP53*	**RG-7112; Nutlin-3**
DNMT3A	**Azacitidine**	*PIK3CA*	**Sirolimus**	*TSC1*	**Sirolimus**
EGFR	**Erlotinib**	*PIK3R1*	**Selemetinib**		
EPHA3-5	**Dovitinib**	*RET*	**Regorafenib**		
ERBB2 (HER2)	**Trastuzumab**	*ROS1*	**Crizotinib**		
ERBB3 (HER4)	**Sapitinib**	*SMAD4*	**Galunisertib**		
ERBB4	**Lapatinib**	*SRC*	**Dasatinib**		
ERG	**Degrasyn**	*SYK*	**Fostamatinib**		
ESR1	**Tamoxifen**	*TBK1*	**GSK2334470**		
EZH2	**Tazemetostat**	*TEK*	**Dovitinib**		
FGFR1-3	**Lucitanib**	*TGRB1-2*	**LY2157299**		
FLT3	**Dovitinib**	*TMPRSS2*	**Vorinostat**		
HDAC9-11	**Panobinostat**	*TNFSF11*	**Denosumab**		
HRAS	**Lonafarnib**	*TORC1-2(CRTC1-CRTC2)*	**Omipalisib**		
IDH1-2	**AG-120**	*VEGFR1-3*	**PF-477736**		
IGF1R	**Linsitinib**	*XPO1*	**Selinexor**		

Appendix B List of 85 genes interrogated by next generation sequencing

Gene	RefSeq	Codons Included in Analysis	Gene	RefSeq	Codons Included in Analysis
ABL1	NM_005157	244-255, 262-493	*JAK2*	NM_004972	505-592, 606-618, 682-683
AKT1	NM_005163	17	*JAK3*	NM_000215	501-511, 572-576, 657
ALK	NM_004304	542, 808-811, 1209	*KIT*	NM_000222	416-422, 541-546, 550-592, 642, 796-850
ASXL1	NM_015338	630-643, 1102-1107	*KRAS*	NM_004985	12-13, 58-61, 146
ATM	NM_000051	All protein coding sequences	*MAP2K1*	NM_002755	56, 67, 121-124
B2M	NM_004048	1-15	*MAP3K14*	NM_003954	639-640
BCL2	NM_000633	7-20, 57-60, 129-135	*MEF2B*	NM_005919	77-81
BCL6	NM_001706	587-615	*MLL*	NM_005933	2462, 3440
BCOR	NM_017745	1329-1351	*MPL*	NM_005373	490-522
BIRC3	NM_001165	123	*MYD88*	NM_002468	219-220, 265
BRAF	NM_004333	593-602	*NOTCH1*	NM_017617	1574-1578, 1585-1607, 1674-1680, 2438-2444, 2459-2467, 2492-2503, 2512-2523
BTK	NM_000061	1-47, 175-196, 281-298, 327-367, 426-450, 463-522	*NOTCH2*	NM_024408	2400
CALR	NM_004343	352-418	*NPM1*	NM_002520	287-292
CARD11	NM_032415	230-251	*NRAS*	NM_002524	11-13, 60-61, 101-149
CBL	NM_005188	371-384, 416-420	*PAX5*	NM_016734	75-80
CCND3	NM_001760	260-289	*PDGFRA*	NM_006206	560-566, 659, 824, 841-849
CD79A	NM_001783	167-214	*PHF6*	NM_032458	All protein coding sequences
CD79B	NM_000626	196	*PIK3CA*	NM_006218	542-545, 1045-1047
CDKN2A	NM_000077	78-80	*PIM1*	NM_002648	1-16, 28-56, 68-171, 210-251
CEBPA	NM_004364	All protein coding sequences	*PIM2*	NM_006875	199-224, 277-312
CREBBP	NM_004380	All protein coding sequences	*PLCG2*	NM_002661	161-188, 256-322, 330-355, 496-519, 526-569, 646-676, 686-724, 746-769, 864-908, 961-1005
CSF3R	NM_156039	601-621, 706-802	*PRDM1*	NM_001198	59-62
CTNNB1	NM_001904	23-66	*PRPF40B*	NM_001031698	696
DDX3X	NM_001356	330-341, 410-411	*PTEN*	NM_000314	233-247
DNMT3A	NM_022552	867-913	*PTPN11*	NM_002834	60-76, 502-503
EGFR	NM_005228	321, 709, 719, 768, 833, 858, Exon 19, Exon 20	*RUNX1*	NM_001754	56, 98, 106-114, 141, 162-166, 198-204, 320
EP300	NM_001429	1625-1639	*SETBP1*	NM_015559	852-892
ETV6	NM_001987	102-105	*SF3A1*	NM_005877	478
EZH2	NM_152998	646, 682-693	*SF3B1*	NM_012433	622-626, 662-666, 700-701, 742
FBXW7	NM_018315	465, 479, 505	*SRSF2*	NM_003016	95-107
FGFR1	NM_023110	441, 537	*STAT3*	NM_139276	640-661
FGFR2	NM_000141	221-249, 250-294, 362-398, 521-558, 628-662	*STK11*	NM_000455	36-38, 60-66, 170-171, 194-199, 281-282, 354
FGFR3	NM_000142	373-384, 650	*SYK*	NM_003177	67-116, 306-372, 395-407, 426-463, 534-574
FGFR4	NM_022963	535, 550, 576, 641, 681-689	*TET2*	NM_017628	All protein coding sequences
FLT3	NM_004119	597-612, 834-842	*TNFAIP3*	NM_006290	All protein coding sequences
GATA1	NM_002049	1-30	*TP53*	NM_000546	68-331
GATA2	NM_032638	317-321, 359-362	*TRAF3*	NM_145725	118
HRAS	NM_005343	12-13, 59-61	*U2AF1*	NM_006758	34-35, 156-160
ID3	NM_002167	56	*U2AF2*	NM_007279	143, 190
IDH1	NM_005896	132	*WT1*	NM_024426	301-314, 394-396
IDH2	NM_002168	140, 171-172	*XPO1*	NM_003400	571
IL7R	NM_002185	237-245	*ZRSR2*	NM_005089	147-153
JAK1	NM_002227	652-658			

Appendix C Distribution of cases with results from NGS, CMA, cytogenetics, FISH

NGS Exist	FISH Exist	CYTO Exist	CMA Exist	Count
X	X	X	X	348
X	X	X		56
X		X		23
X		X	X	66
X				10
X			X	44
X	X		X	19
X	X			3

Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.cancergen.2017.07.010.

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