

The Progress of Next Generation Sequencing in Assessment of Myeloid Malignancies

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With increasing characterization of disease associated molecular markers, it is becoming challenging to perform multiple single-gene molecular assays. Specific molecular markers are helpful for diagnostic, prognostic evaluation and management of hematologic malignancies. Introduction and rapid progress on next generation sequencing has led to extensive modifications and offers a novel way to integrate concurrent assessment of multiple target genes in routine laboratory analysis especially in view of increasing clinical demands for testing of multiple genetic aberrations. Despite challenges in next generation sequencing particularly involving bioinformatics support and clinical annotation of novel variants, assessment of myeloid neoplasms with targeted next generation sequencing panels showing

evidence to improve diagnosis, assist therapeutic decisions and provide better information about prognosis, as well as detection of minimal residual disease since it facilitates significant advantages over single-gene assays. Herein, we provide information for application and utilization of next generation sequencing studies with a focus on the most important mutations in clinical assessment of hematologic neoplasms such as acute myeloid leukemia, myelodysplastic syndrome, myeloproliferative neoplasms and other myelodysplastic/myeloproliferative neoplasms in order to integrate into daily clinical practice.

Keywords: Myeloid neoplasia, next generation sequencing, somatic mutation

Next-generation sequencing (NGS) technology has shown great progress during the past ten years, has been finding greater utility in clinical assessment of various neoplasms. Based on the marked improvements in sequencing platforms, the reliability of pipeline analyses, data interpretation and costs, NGS is rapidly integrated by the diagnostic laboratories and accepted by current clinical practice. However, the technology and language particularly bioinformatics and genetic code makes it difficult to understand especially for practitioners who had been far from the field of molecular biology. We will review the recent developments in NGS and technical progress and focus on approach of diseases affecting myeloid lineage, including acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), chronic myeloproliferative diseases (CMPDs) such as Polycythemia Vera (PV), Essential Thrombocythemia (ET), Primary Myelofibrosis (PMF), atypical CMPD, chronic myelomonocytic leukemia (CMML) and chronic eosinophilic leukemia (CEL) and mast cell disease.

Due to increasing number of mutations that are necessary to arrive a diagnosis, it is becoming very impractical to continue

to single gene testing analysis of these disorders. Furthermore, besides diagnostic aid of molecular tests, multigene assessment is providing guidance for determining prognosis in number of disease entities. Since many of the disease encountered in clinical practice show more than one molecular abnormality, it is becoming inevitable that we need to adapt NGS based assays in clinical assessment of these disorders. Simultaneous detection of multiple somatic mutations in tens or hundreds of target genes associated with specific diseases could enable diagnosis and further provide actionable information. During the past few years, NGS assays are becoming cheaper, faster, and rapidly adapted by many laboratories. Due to increasing awareness of sophisticated patients by the social networks, these tests are demanded and since there is an increasing number of actionable targets or specific treatment modalities that are recognized and many practicing hematologists requesting these assays. Based on this reality, both laboratory physicians and hematologists are now faced with utilizing these panels on a day-to-day basis. However, NGS technology requires highly complex bioinformatics and

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Received: 18 July 2018 Accepted: 24 September 2018 • DOI: 10.4274/balkanmedj.2018.1195

Available at www.balkanmedicaljournal.org

Cite this article as:

Öz Puyan F, Alkan S. The Progress of Next Generation Sequencing in Assessment of Myeloid Malinancies. *Balkan Med J*

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laboratory technologists to develop diagnostic pipeline and sustain knowledge in this evolving field. Currently, there are two commonly used NGS platforms that include instruments from Illumina and Thermo Fisher previously manufactured by Ion-Torrent. Though sequencing differs in these technologies, they use similar bioinformatics principles for determining abnormal sequences that are not matching reference sequences. There are also three different enrichment methods used for NGS sequencing: Hybrid capture, Amplicons and Anchored Multiplex PCR (1). Furthermore, there is no single database available for clinical annotations to determine significance of variants. Despite significant decrease in costs of the assays, these tests are still very expensive, and reimbursement remains to be poor by government agencies and insurance companies.

The introduction of NGS into clinical laboratories has significantly changed the usage of molecular techniques, because testing for multiple genes in a panel using NGS platforms is more practical and economical (2, 3). Furthermore, the cost of adding a new gene to the panel is relatively inexpensive, in comparison to performing of multiple single gene tests. The presence of specific genetic mutations could aid clinicians in 4 ways: 1) diagnostic assessment, 2) determination of prognosis, 3) use of targeted therapy and 4) assessment of residual disease and monitoring treatment response. Despite increased utilization, NGS assays remain challenging particularly for identifications of variants without determination of clinical significance. Therefore, new terms in NGS reports has been adapted: “actionable”, “potentially actionable”, “Variant of Undetermined Significance (VUS)”, “likely benign” or “benign”. Since the databases in various populations are not matured yet, some of the sequences observed by NGS may become difficult to assign (3, 4).

Mutation Profiles in Acute Myeloid Leukemia

The Cancer Genome Atlas Network performed many studies to report recurrent mutations and demonstrated a complex network of genetic mutations in the pathogenesis (5). Despite these exhaustive studies, there is no gene specific diagnostic entity or mutually exclusive with a specific diagnosis. However, there are some mutations leading to aberrant activation of a variety of proteins that have crucial effect on hematopoietic progenitor cell proliferation and differentiation. The driver mutations in AML involve epigenetic modifiers (TET2, IDH1/IDH2, DNMT3A, ASXL1, KMT2A, EZH2), activated signaling pathway (FLT3, KRAS, NRAS, KIT), tumor suppressor genes (TP53, WT1), RNA splicing (SF3B1), nucleophosmin mutation (NPM1) and genes coding for transcription-differentiation (CEBPA, RUNX1) (6-8) (Figure 1). Recurrent mutated genes in AML and MDS are listed on Table 1.

Recent studies have demonstrated that genes mutated in AML with a normal karyotype (NK-AML) share significant overlap with genes mutated in MDS (9, 10). Updated WHO 2016 classification recognizes AML with mutated NPM1 and AML with biallelic mutation of CEBPA as specific AML classification categories and AML with mutated RUNX1 as provisional entity (8, 11). The most frequently mutated genes in NK-AML occur in the following genes: nucleophosmin (NPM1), Fms-related tyrosine kinase 3 (FLT3) and DNA methyltransferase 3A (DNMT3A), Ten-eleven translocation-2 (TET2), isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2), CCAAT/enhancer binding protein alpha (CEBPA), NRAS, Additional sex comb-like 1 (ASXL1), WT1 and runt-related transcription factor-1 (RUNX1). Mutations of FLT3, DNMT3A, and NPM1 are often present concurrently, while other mutations of NPM1, RUNX1, CEBPA, and TP53 are almost always mutually exclusive both at diagnosis and at the time of disease transformation (11-19). WHO classification of AML includes many specific chromosomal abnormalities, and these are typically determined by karyotyping (11). Although NGS is promising assay to provide karyotypic information with improving bioinformatics, currently this technology does not appear to reach that maturity to replace standard karyotyping and NGS is being primarily used for detection of copy number variations, single nucleotide aberrations and common translocations. A number of gene mutations are recognized as specific entity by the 2016 WHO classification of AML including NPM1, CEBPA, and RUNX1 mutations. However, since the publication of this classification, it became very important to clinically recognize other pathogenic variants such as those involving FLT3 (ITD and TKD) and IDH1/2. These drastically change the clinical

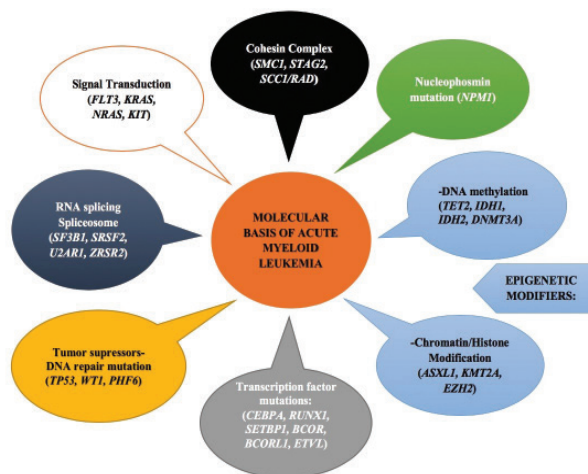


FIG. 1. The most frequent gene mutations and cellular pathways in acute myeloid leukemia.

management of AML patients since US Food and Drug Administration (FDA) have approved specific inhibitors for FLT3 and IDH2 positive AML, while IDH1 inhibitor already showing good outcome in early clinical investigations (20, 21). Hence, currently all of the AML patients particularly those with normal karyotype requires assessment of these genes. The

gene NPM1 is the most frequently mutated gene in adult type AML, occur approximately 50-60% of cytogenetically normal AMLs and 2-5% of MDS cases. It is often characterized by CD34 antigen negativity with bright expression of CD33 and monocytic differentiation. NPM1 mutations include small insertions (4-11bp in size), resulting in an aberrant cytoplasmic

TABLE 1. Common gene mutations in AML, MDS and their clinical significance

Gene	Locus	Functional classification	Approx. frequency in NK-AML %	Approx. frequency in MDS %	Effect on prognosis	Clinical significance
NPM1	5q35.1	Nucleophosmin mutation	50-60	2-5	Good	CD34 ⁺ blasts with cuplike nuclear invaginations, immunohistochemically detectable, favorable prognostic marker, cooccurrence with FLT3-ITD, DNMT3A, IDH, TET2 mutations
FLT3-ITD	13q12.2	Signal transduction	20-30	2-6	Poor	Unfavorable prognostic marker, NPM1 ⁺ /FLT3 ⁺ mutations occur early in MDS (risk of rapid progression to AML), usage of FLT3 inhibitors
CEBPA	19q13.11	Transcription factor mutation	10-18	<2	Good	Biallelic mutation has favorable prognosis, cooccurrence with FLT3-ITD adverse effect
RUNX1	21q22.12	Transcription factor mutation	4-16	9	Poor	Tumor suppressor properties, provisional entity in 2016 WHO classification, germline mutation associated with familial platelet disorder, unfavorable prognostic marker
RAS	12p12.1 (KRAS) 1p13.2 (NRAS)	Signal transduction	rare	5-10	None	Acquisition of RAS mutation on MDS patient is associated with leukemic transformation
KIT	4q12	Signal transduction	6	<2	Poor	Mostly found in CBF-AML and AML with t(8;21), presence of KIT mutation in CBF-AML worsens prognosis, usage of KIT inhibitors
DNMT3A	2p23	DNA methylation	20	2-8	Poor	Cooccurrence with FLT3, NPM1, IDH1 mutations. Associated with CHIP, CCUS. Early event in leukemogenesis. Frequency increases with age.
TET2	4q24	DNA methylation	10-15	10-25	Unclear-Poor	Cooccurrence with NPM1, IDH1 mutations. Associated with CHIP, CCUS. Early event in leukemogenesis. More frequent in CMML. Frequency increases with age.
IDH	2q34 (IDH1) 15q26.1 (IDH2)	DNA methylation	15-25	2-12	Unclear	Comutated with TET2 and WT1 mutations. Usage of IDH2 inhibitor and IDH1 inhibitor in clinical development
ASXL1	20q11	Chromatin-Histone modification	6-10	14-22	Poor	Early genetic event. More frequent in CMML and PMF. Associated with CHIP
EZH2	7q35-7q36	Chromatin-Histone modification	2	6-12	Poor	Loss of function mutations more common in secondary AML
TP53	17p13.1	Tumor suppressor-DNA repair	5-18	7-12	Poor	Associated with complex karyotype, resistance to chemotherapy
SF3B1	2q33.1	RNA splicing	<3	20-30	Good	More frequent in MDS with RS. RARS-T with SF3B1 mutation is regarded as MDS/MPN-RS-T in the 2016 WHO classification, associated with CHIP
SRSF2	17q25.1	RNA splicing	<8	10-15	Unclear-Poor	More frequent in CMML.

Abbreviations: NK-AML, acute myeloid leukemia with a normal karyotype; MDS, myelodysplastic syndrome; CBF-AML, Core binding factor-AML; CHIP, clonal hematopoiesis of indeterminate potential; CCUS, clonal cytopenia of undetermined significance; CMML, chronic myelomonocytic leukemia.

localization of the mutant protein nucleophosmin which can be detected immunohistochemically (21, 22). NPM1 mutations increase disease free and overall survival in patients with AML. Secondary mutations (FLT3-ITD, DNMT3A (21), IDH, TET2 mutations) are frequently accompanying these patients. FLT3-ITD mutations are observed in 40% of NPM1 mutated AML patients. In general, FLT3 ITD positive patients without other molecular abnormalities show poor clinical outcome while NPM1 positive patients have better clinical response. Coexistence of FLT3-ITD mutation in NPM1 positive patients is associated with a poorer prognosis in young AML patients compared with patients with NPM1^{mut}/FLT3^{wild} type (4, 11, 17, 23).

Among transcription factor mutations in myeloid neoplasia, CEBPA and RUX1 mutations are recognized as important by WHO due to their prognostic implications. CCAAT Enhancer Binding Protein-alpha (CEBPA) is a transcription factor responsible for promoting granulocytic maturation in regulatory cellular function. CEBPA mutations are observed in approximately 10% of de novo AML patients and are commonly biallelic. The biallelic mutation is a favorable prognostic marker. Germline mutations of CEBPA predispose to the development of AML in young age groups. Concurrence of FLT3-ITD mutation is an adverse prognostic finding in CEBPA mutated-AML patients (23). RUNX1 encodes the DNA binding alpha subunit of core binding factor (CBFalpha), which is involved in the normal differentiation of hematopoietic cells. RUNX1 has also tumor suppressor properties and loss of function of the gene contributes to tumorigenesis in myeloid cancers. De novo AML with mutated RUNX1 in the absence of MDS related cytogenetic abnormalities appears to represent a distinct entity with adverse outcome. Thus, this type of mutation is included as a provisional entity in the new WHO 2016 classification of myeloid neoplasms. RUNX1 mutations are detected in approximately 4% to 16% of AML and 9% of MDS (11). They are commonly seen in older patients with NK-AML, in therapy-related and MDS-associated AML. They have been associated with worse overall survival in multivariate analysis (23-25).

FLT3, is a class III receptor tyrosine kinase coding for signal transduction gene, is important for proliferation and differentiation of hematopoietic stem cells. Its expression is downregulated during differentiation. These FLT3 mutation events occur in 30% - 40% of AML patients with normal cytogenetics and 2% to 6% of MDS cases. Two types of mutations are observed in FLT3 gene: FLT3-ITD (internal tandem duplications) mutations (with a frequency of 75% - 80%) and FLT3-TKD (tyrosine kinase domain) (with a frequency of 20% - 25%). FLT3 mutated patients show higher white blood cell count, loss of HLA-DR and CD34 antigen

negativity, they are associated with impaired survival in AML (26). AML patients with FLT3-ITD mutation appear to benefit from allogenic stem cell transplantation. However the prognostic effect of FLT3-TKD remains controversial (27). The FDA approved treatment with midostaurin in 2017 for newly diagnosed patients with FLT3-mutated AML due to observation of significant beneficial outcome (28) and there are many other Tyrosine kinase inhibitors (TKI) in clinical investigation (29). In MDS, FLT3 mutations are observed in high risk subgroups and are associated with complex karyotype (30, 31). NPM1 and FLT3 mutations are found primarily in AML; however, the presence of these mutations in a MDS patient should alert the clinician higher chance of rapid progression to AML (22). Alterations on FLT3 signaling pathway seems to be the most important prognostic factor for overall survival in AML patients younger than 60 years. Overactivation of FLT3 is associated with a poor prognosis (32-34).

The other signal transduction gene namely KIT mutations, frequent in gastrointestinal stromal tumors, germ cell tumors, melanomas and systemic mast cell diseases, can be detected in approximately 6% of patients with newly diagnosed AML and 20% of AML patients with t(8;21) RUNX1-RUNX1T1 and 30% of AML patients with inv(16) or t(16;16) CBFβ-MYH11 (the core binding factor AMLs - CBF-AML). KIT mutation in AML increases risk of relapse rate and worsens the good prognosis of CBF-AML. In addition, the presence of KIT mutations may also have therapeutic relevance (4, 23, 31, 35, 36).

There are a number of other genes playing role in the pathogenesis of AML and appear to be important for overall survival despite these are currently are not recognized as specific entities. DNA methylation mutations and chromatin/histone modifications are belonging to epigenetic modifiers. DNMT3A encodes an epigenetic regulator and mediates de novo methylation of CpG dinucleotides and are seen approximately 20% of AML with normal karyotype. The prognostic effect of this mutation tends to shorten overall survival (14, 37). TET2 is also involved in the epigenetic regulation of DNA methylation. TET2 mutations occur in approximately 10% to 15% of AMLs. As TET2 is a key regulator in hematopoietic stem cell renewal and differentiation, their mutations result in increased stem cell renewal and myeloid hyperplasia with impaired differentiation. These tend to have poorer prognosis in NK-AML. TET2 mutations predict response to hypomethylating agents (4, 38). IDH1/IDH2 mutations prevent conversion of isocitrate to alpha-ketoglutarate in the Krebs cycle, create an oncometabolite which ultimately inhibits the function of TET2. IDH1/IDH2 mutations frequently concurrently mutates with TET2 and WT1 mutations (39). 15% to 25% of AML and approximately 2% to 12% of MDS cases display IDH mutations (40). The

prognostic significance of IDH mutations in AML is currently unclear. IDH2 inhibitor had been successful in management of IDH2 positive AML patients while IDH1 inhibitor appears to be showing promising results in ongoing clinical trials (4, 20, 34, 40).

Chromatin/Histone Modification mutations such as Additional Sex Coms Like Transcriptional Regulator 1 (ASXL1) mutations and Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2) mutations are a part of the epigenetic modifiers. ASXL1 gene is involved in the epigenetic regulation of gene expression. They are uncommon in de novo AML (6.5%), but more frequent up to 30% of AML arising from a prior myeloid neoplasm. (41-43). These mutations are associated with shorter overall survival and resistance to chemotherapy. KMT2A: lysine(K)-specific methyltransferase 2A (KMT2A/MLL) gene plays a role in hematopoiesis and cell differentiation. Close to all KMT2A mutations co-occur with an additional gene mutation (IDH2/DNMT3A/U2AF1/TET2). This mutation is frequently associated with trisomy 11 (90%) and poorer prognosis (17, 44, 45).

TP53 protein is a transcription factor and tumor suppressor gene which determines whether the cell undergoes repair, senescence or apoptosis. Although somatic TP53 mutations are frequently seen in 50% of solid tumors, they are encountered uncommonly in de novo AML (5% to 18%) and they are more commonly associated with secondary or therapy-related AMLs. TP53 mutations often coexist with complex karyotypes, chemotherapy resistance and reduced overall survival compared

with TP53^{wild} AML patients. TP53^{mut} AML patients have also high relapse rates after stem cell transplantation (SCT) (46-48). Even so, a recent study indicated that AML patients with cytogenetic abnormalities associated with unfavorable risk, TP53 mutations, or both had favorable clinical responses to decitabine (49, 50). Therefore, the presence of TP53 mutation alters prognosis and management of the disease.

According to the guidelines for management of AML in adults by the European LeukemiaNet released last year, AML risk stratification by genetics have been determined. Figure 2 represents these risk categories associated with distinct molecular mutations that should be screened by NGS in AML patients.

For monitoring of minimal residual disease (MRD) in AML; NGS based technologies can be used besides multiparameter flow cytometry (MFC). Although MFC is a reliable method for monitoring remission than conventional morphology-based assessment in AML patients, there are also cases that MFC lacks sensitivity for detecting residual disease. Therefore, combined results of sequencing and flow cytometry detects residual disease more specifically. A recent study about molecular MRD in AML showed the detection of molecular MRD is associated with significantly higher relapse rate than without detection (51). During the complete remission persistent mutations associated with clonal hematopoiesis (DNMT3A, TET2, and ASXL1 referred as DTA mutations) and non DTA mutations can be detected by NGS. While DTA mutations do not have prognostic value for MRD, non DTA mutations (such as

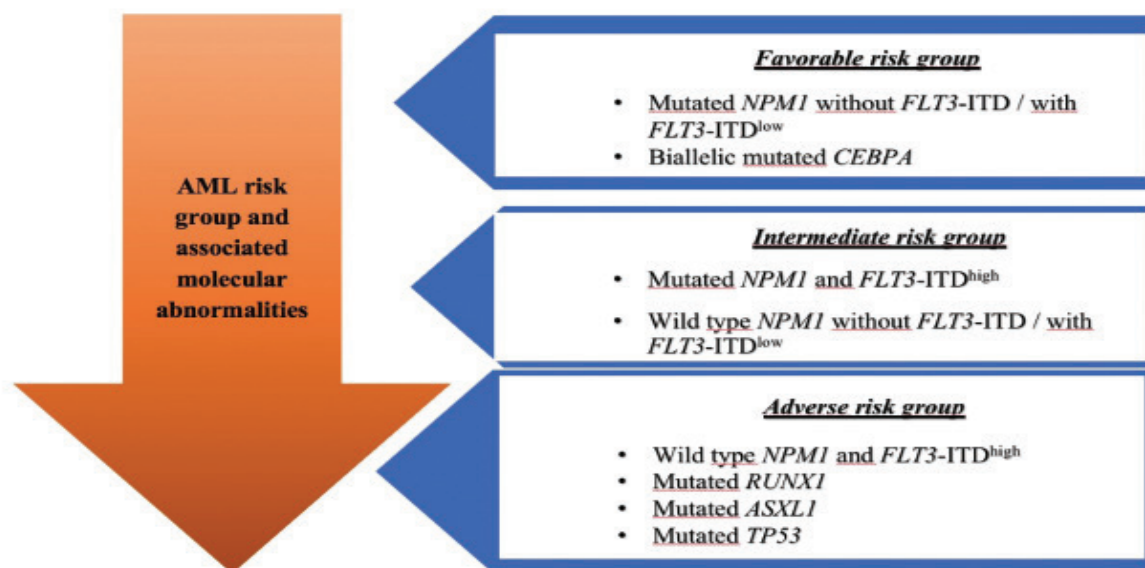


FIG. 2. AML risk categories and associated molecular abnormalities.
(*FLT3-ITD*^{low}: *FLT3-ITD*/wild type allelic ratio <0.5 ; *FLT3-ITD*^{high}: *FLT3-ITD*/wild type allelic ratio > 0.5)

mutations in TP53, IDH1, IDH2 and genes related to the RAS pathway) with a higher allele frequency predicts higher relapse rates (51, 52).

Among AML with myelodysplasia-related changes (AML-MRC) and therapy-related AML (t-AML), mutations in splicing factors genes such as SRSF2, SF3B1, U2AF1, ZRSR2, mutations about chromatin/histone modifications like ASXL1, EZH2, BCOR, STAG2, and TP53 mutations are relatively frequent (53). NPM1 mutations in the absence of FLT3-ITD and double CEBPA mutations are fairly uncommon in AML-MRC and are associated with favorable overall survival. Patients in this category with TP53 mutations had poor prognosis regardless of age (46, 54-56). Most of the t-AML patients show an abnormal karyotype. After treating patients with alkylating agents/ionizing radiation, patients often have increased blasts with associated multilineage dysplasia thus causing similar mutation profiles like in AML-MRC. Mutations of TP53 can be seen in as many as 50% of t-AML cases with worse survival (11, 53, 57, 58). Following a topoisomerase II inhibitor therapy, 20-30% of patients develop overt acute leukemia without a preceding myelodysplastic phase. These patients often show balanced chromosomal translocations involving 11q23 (KMT2A/MLL) or 21q22 (RUNX1) and are associated with monoblastic or myelomonocytic morphology (11, 53, 59, 60). Mutations in TP53, TET2 and PTPN11 (protein tyrosine phosphatase, non-receptor type 11), IDH1/2, NRAS are frequent in t-AML, but FLT3 and NPM1 mutations are less frequent than de novo AML (58, 61).

Mutation Profiles in Myelodysplastic Syndrome

Approximately half of myelodysplastic syndrome patients are associated with recurrent cytogenetic abnormalities such as -5/del(5q), -7/del(7q), +8del(20q) or complex karyotypes (62). Although observation of cytogenetic abnormalities by karyotyping or FISH are supportive of MDS, this finding is limited (63). Targeted panels and whole-genome NGS assay detect somatic mutations in up to 90% of MDS patients (64, 65). Although some of the mutations overlap with those observed in AML, none of these mutations are specific for MDS diagnosis. However, having multiple genetic abnormalities with high variant allele frequency, is beginning to be recognized for supportive evidence of evolving MDS (66).

The most frequently mutated genes in MDS are SF3B1 (~25%), TET2 (~25%-20%), ASXL1 (~15%), SRSF2 (~15%), RUNX1 (~15% - 10%), DNMT3A (~15% - 10%) (23). Mutations in ASXL1, TP53, EZH2, ETV6 and RUNX1 are also found to be recurrent in MDS patients and predict poor overall survival (41). DNMT3A mutations occur early in the course of MDS that suggests an early genetic event in leukemogenesis. Patients

with DNMT3A mutations have worse overall survival and more rapid progression to AML. They are associated with mutations in FLT3 (ITD or TKD), NPM1, IDH1 genes (67). TP53 mutations in MDS is associated with very aggressive disease. Even in low risk MDS with del(5q), presence of TP53 mutation at low frequency is correlated with a higher rate of leukemic transformation and poor response to lenalidomide (11, 68).

Mutations in splicing factors are commonly detected in MDS patients with a frequency of 50%. The most common of these mutations are SF3B1, SRSF2, U2AF1 and ZSR2 mutations. These mutations can occur with mutations in epigenetic modifiers. Comutations of TET2 and SRSF2 genes are associated with monocytic differentiation (69). SF3B1 mutations are the most common among splicing mutations in MDS. These mutations are highly correlated with the presence of ring sideroblast (RS). They are observed in 20% to 28% of MDS, more frequent in MDS with RSs. On the other site the frequency of SF3B1 mutation in AML is < 5% (11, 23, 70, 71). Mutations in Cohesin Complex genes (SMC1, SMC3, SCC1/RAD, STAG2) can be germline resulting in congenital malformation syndrome. These mutations can be detected in 10% to 25% of MDS and 10% to 15% of de novo AML (17, 69).

As nearly all cases of MDS show at least one mutation, demonstration of such a specific mutation with a targeted NGS panel is highly useful for the diagnosis of MDS. However, determination of mutational burden/variant allele frequency (VAF) (usually >25% for MDS) is critical for the diagnostic assessment to distinguish MDS from clonal hematopoiesis of indeterminate potential (CHIP) or clonal cytopenia of undetermined significance (CCUS). CHIP is the proposed term for healthy individuals, lacking a known hematological malignancy or clonal disorder, but carrying a detectable hematological somatic mutation. More than 10% of healthy individuals over 70 years old show clonal mutations in leukemia driver genes. Typically, single gene mutations (mostly DNMT3A, TET2 or ASXL1 mutation) and increased risk of developing hematological malignancy, markedly increased rate of cardiovascular mortality regardless of their cancer risk are the key properties of CHIP. The single mutation burden in CHIP has a VAF more than 2% but typically not exceeding 20% - 30%, which allows the distinguishing from underlying dysplastic process (3). Patients who are not meeting the WHO defined criteria for a hematologic neoplasm, but show a clonal mutation with unexplained cytopenia, are referred as CCUS. The mutation spectrum on CCUS patients are similar to mutations seen in MDS. Mutations in spliceosome genes (SF3B1, SRSF2, U2AF1, ZRSR2) have highest predictive value for CCUS (72). Comutations of DNMT3A, TET2 and ASXL1 genes remain also common in CCUS. There are no clear recommendations

to follow up CCUS patients. However, increase in degree of cytopenia, mutation burden, number and type of the mutations or the detection of additional mutations in the follow up of a patient with suspected MDS appear to have a course similar to MDS (72-76).

Mutation Profiles in Myeloproliferative and Myelodysplastic/Myeloproliferative Neoplasms

Disorders altering the myeloid elements include following disorders: Polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), chronic myelogenous leukemia (CML), chronic neutrophilic leukemia (CNL),

hypereosinophilic leukemia/chronic eosinophilic leukemia (CEL), chronic myelomonocytic leukemia (CMML), and mast cell disease (MCD). Among CMPD (PV, ET, PMF and CML), molecular markers had been essential for diagnosis of these disorders. Clinically, recurrently somatic mutations in BCR-ABL2 negative MPNs and MDS/MPNs are listed on Table 2.

The most frequent mutation in BCR-ABL2 negative MPN, namely JAK2 exon 14 (V617F) mutation is observed 95% of PV patients (77). The majority of remaining PV patients may harbor mutation in JAK2 exon 12 (missense mutation, deletion, insertion). JAK2 (V617F) mutation occurs in approximately 50 - 60 % of ET or PMF patients. Homozygous mutations of JAK2 (V617F) (with a VAF value > 50%) are more common

TABLE 2. Common gene mutations in MPN, MDS/MPN and their clinical significance

Gene	Locus	Functional classification	Approx. frequency in MPN%			Approx. frequency in MDS/MPN %	Clinical significance
			PV	ET	PMF		
JAK2	9p24.1	Signal transduction	95	50-60	50-60	<1	Intermediate prognosis and higher risk of thrombosis compared to patients with CALR mutation.
CALR	13q12.2	Signal transduction	<1	20-25	20-30	2-6	Associated with better overall survival than JAK2 V617F or MPL mutation (especially for type 1/type 1-like mutation). Lower risk of thrombosis compared to JAK2 mutation
MPL	19q13.11	Transcription factor mutation	<1	5-10	5-10	<2	Intermediate prognosis increased risk for thrombosis compared JAK2 and CALR mutation
SF3B1	2q33.1	RNA splicing	1	1	5-10	20-30 (70-90 in MD/MPN RS-T)	The presence of at least 1 of these “adverse variants/mutations” is associated with inferior overall survival, independent of age and karyotype
TP53	17p13.1	Tumor suppressor DNA repair	<1	<1	2-4	7-12	Associated with inferior leukemia-free survival in multivariate analysis
DNMT3A	2p23	DNA methylation	5-10	1-5	5-12	<5	Might be associated with adverse outcome and greater risk for AML transformation
CBL	4q24	DNA methylation	<1	0-2	5-10	40-60	Might be involved in leukemic transformation
IDH	2q34 (IDH1) 15q26.1 (IDH2)	DNA methylation	<2	<1	3-5	<10	The presence of at least 1 of these “adverse variants/mutations” is associated with inferior overall survival
ASXL1	20q11	Chromatin-Histone modification	2-7	5-10	15-35	40-50	The presence of at least 1 of these “adverse variants/mutations” is associated with inferior overall survival
EZH2	7q35-7q36	Chromatin-Histone modification	2	2	5-10	6-20	The presence of at least 1 of these “adverse variants/mutations” is associated with inferior overall survival
SRSF2	17q25.1	RNA splicing				35-50	The presence of at least 1 of these “adverse variants/mutations” is associated with inferior overall survival

Abbreviations: AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; CBF-AML, Core binding factor-AML; CHIP, clonal hematopoiesis of indeterminate potential; CCUS, clonal cytopenia of undetermined significance; CMML, chronic myelomonocytic leukemia; MD/MPN RS-T, myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis

in PV than in ET (78, 79). JAK2 (V617F) mutation may not be present in half of the MPN patients. This type of mutation has rarely been reported in patients with MDS/MPN. As they are also found in patients with MDS, CMML, atypical CML, de novo AML (approx. <5%) and MDS/MPN-RS-T (approx. 50%), the presence of this mutation should not be used in the context of distinguishing MPN from other myeloid neoplasia's. In PV and ET; JAK2 (V617F) allele burden is also associated with a more aggressive behavior. Thrombotic events and fibrotic transformation are more common in patients with a high JAK2 (V617F) allele burden (higher JAK2 VAF). These findings suggest that monitoring JAK2 (V617F) allele burden could be useful to identify patients at higher risk of myelofibrotic transformation. JAK2 mutation in PMF is associated with intermediate prognosis, when compared with CALR mutation (3, 11, 17, 23, 80).

Patients with ET have mutations in CALR gene that encodes the endoplasmic reticulum-associated chaperone calreticulin with a frequency of 20% to 25%. CALR mutations are frequently seen in ET and PMF patients with JAK2^{wild} and MPL^{wild} type. They are rarely detected in PV, CMML, MDS/MPN patients. CALR mutations are not seen in AML, mastocytosis, lymphoid neoplasia and solid tumors (81, 82). CALR mutated MPNs demonstrate distinct features; such as younger age group, lower hemoglobin levels, higher platelet counts, reduced risk of thrombosis and improved overall survival compared to patients with JAK2 or MPL mutations (83, 84). In ET and PMF patients, CALR show 2 different mutations: type-I that typically has 52-bp deletion (p.L367fs*46), and type-II characterized by 5-bp TTGTC insertion (p.K385fs*47). Type-I CALR mutation is detected more frequently in PMF than ET. Type-II CALR mutations tend to show leukemic transformation (namely increased leucocyte count and higher circulating blasts) than type-I CALR mutation (81, 85, 86). Platelet count is significantly higher in type-II vs. type-I CALRmut ET patients, but no difference was noted in thrombosis-free survival in a study with a large cohort of ET patients. However, there is a significant survival difference in the setting of PMF, as CALR

type-I positive PMF patients do much better than CALR type-II or JAK2 (V617F) positive PMF patients (84).

The MPL gene, located on chromosome 1p34, encodes the thrombopoietin receptor. The activating mutation on codon W515 of MPL gene is seen in 5-10% of ET and PMF patients who are negative for JAK2 (V617F) (87). Patients that are JAK2^{wild} and CALR^{wild}, but harboring ET or PMF clinical features, should be screened for MPL mutation. MPL mutated patients have increased risk for thrombotic episodes and transfusion requirement compared with JAK2^{mut} and CALR^{mut} patients (80, 88, 89). These data show the significance of JAK2, CALR and MPL mutations in BCR-ABL negative MPNs, in order to confirm the clonal nature of the disease.

Patients with PMF have mutations for JAK2 in 60%, CALR in 20-30% and MPL in 5-10% of the cases (Figure 3). The remaining 10% to 15% of patients with ET or PMF patients have none of the above mutated genes and these are referred to as "triple negative" (85, 90, 91). This is a heterogeneous category and some of the ET patients may have rare noncanonical mutations in JAK2, MPL and SH2B3; however, the large majority remain without a specific mutation. For "triple negative" PMF patients, a detailed search for the mutation profiles regarded as a marker of clonality (such as ASXL1, EZH2, TET2, IDH1/IDH2, SRSF2, SF3B1) are currently recommended (77). "Triple negative" patients tend to show inferior outcome compared to JAK2^{mut}/CALR^{mut} and MPL^{mut} CMPD patients. The presence of at least one of the 3 variants/mutations (ASXL1, SRSF2, and IDH2) is associated with inferior overall and myelofibrosis-free survival in patients with PV. SH2B3, IDH2, U2AF1, SF3B1, EZH2, and TP53 mutations are identified as significant risk factors for inferior overall, myelofibrosis-free survival, and leukemia-free survival in patients with ET (85).

Recent studies about leukemic transformation in MPNs show ASXL1, SRSF2, IDH1, IDH2, RUNX1 mutations has been associated with leukemic transformation in PMF, whereas SRSF2, IDH2 mutations in PV and TP53, EZH2 mutations in ET predicts more leukemic transformation (92). These findings show that mutation spectrum in primary NK-AML is different

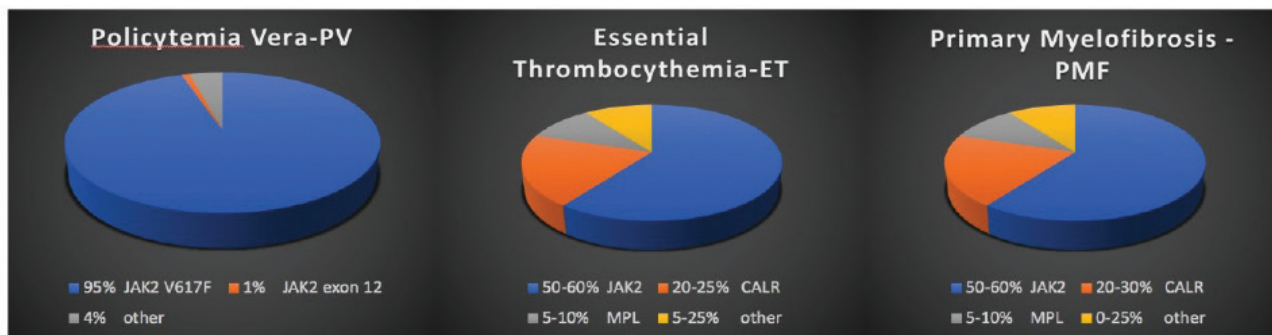


FIG. 3. Distribution of mutation profiles in myeloproliferative neoplasm.

from secondary AML (also known as blast phase of MPN). Recurrent somatic gene mutations are observed in up to 90% of CMML patients. TET2 (~60%), SRSF2 (~50%), ASXL1 (~50%), RUX1, NRAS and TP53 are the most frequent mutations. Co-mutation of TET2 and SRSF2 with a higher mutation burden as compared to MDS and accompanying monocytosis on a patient indicate CMML. However, these mutations can also be encountered in healthy aging people as CHIP with a single mutation of a VAF <20-30%. Therefore, a low VAF of these mutations should not be used as a definitive diagnosis for CMML (23). As ASXL1 mutations are associated with a higher white blood cell count (WBC), lower hemoglobin, extramedullary disease and an abnormal karyotype, ASXL1 mutations lead to aggressive clinical course on CMML patients (93, 94). JAK2 mutation also occurs in CMML patients sharing some mutual features with JAK2^{mut} MPNs. These cases predict more reticulin fibrosis, erythroid and megakaryocytic hyperplasia, occasional megakaryocytic clustering with atypia (95). NPM1 mutations are rarely seen in CMML. In case of presence, NPM1^{mut} AML with monocytic differentiation should be suspected. Variable results are also expected on JAK^{mut} CMML patients treated with JAK2 inhibitors (11, 96, 97). Juvenile myelomonocytic leukemia (JMML) patients harbor also clonal cytogenetic abnormalities. The commonly mutated genes are KRAS/NRAS, NF1, PTPN11, CBL, which encode proteins of RAS oncogene pathway. PTPN11 mutations are the most frequent alterations (~ %35). Somatic NRAS and KRAS mutations occur %20-25 of JMML cases. Germline mutations in CBL and NF1 genes are also frequent which are associated with Noonan Syndrome-like disorder and neurofibromatosis type 1 on JMML patients, respectively (98, 99).

SETBP1 and ethanolamine kinase 1 (ETNK1) mutations are relatively common in atypical chronic myeloid leukemia (aCML). JAK2, CALR and MPL mutations are typically not seen on these patients. Although CSF3R mutation is frequently associated with CNL, this type of mutation is uncommon (<10%) in aCML and rarely encountered in CMML and AML cases (100, 101). Correlation of CSF3R mutation with CNL has been included in the diagnostic criteria in the revised 2016 WHO classification (10, 21).

In myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T), SF3B1 mutations are present approximately in 70-90% of cases. They are frequently commutated with JAK2 (V617F) (50-65%) and less frequently with CALR / MPL (11, 23, 70, 71).

Among myeloid neoplasms with eosinophilia; chronic eosinophilic leukemia, not otherwise specified (CEL-NOS), idiopathic hypereosinophilic syndrome (IHES), myeloid/

lymphoid neoplasms with eosinophilia and gene rearrangement are considered in the revised 2016 WHO classification. However, hypereosinophilia can be encountered in systemic mastocytosis, CML, other types of MPNs, MDS/MPNs, AML, MDS and T cell lymphoproliferative disorders. Once, cytogenetically PDGFRA, PDGFRB, FGFR1 rearrangements and PCM1-JAK2 fusion and other causes of hypereosinophilia are excluded, NGS should be performed for the patients with eosinophilia. A recent study showed that a significant number of IHES patients harbor mutations in DNA methylation and chromatin modification (such as ASXL1, EZH2, TET2) and indicated that patients with clonal mutations should be regarded as CEL-NOS (11, 102).

Lastly, mast cell diseases show point mutations of the KIT gene (D816V) in 95% of cases. Although the presence of D816V mutations is important at diagnosis for systemic mastocytosis, a minority of patients harbor mutations elsewhere on exon 17 or other exons (103) Additionally; TET2, ASXL1, RUX1, SRSF2 and JAK2 mutations can be seen on the patients with mastocytosis (103, 104).

Despite the technology and platforms used in NGS is still evolving, the impact of NGS in understanding the biological processes of hematological diseases and clinical implications has already reached to stage to adapt these assays in current practice of hematology and oncology. We outlined some of the common genes that are important to assess in various hematologic neoplasms with emphasis on myeloid neoplasia. Despite the significant progress has been noted for lymphoid neoplasms, particularly for understanding in pathogenesis, NGS has not yet reached to a point to integrate in routine clinical testing. It is expected that this will be soon changed, and unique opportunities will be described for lymphoid neoplasms. Most of the NGS panels offered for assessment of myeloid neoplasms contain between 30-60 targeted genes. The market for NGS-based diagnostic procedures is rapidly growing, therefore it is impossible to list all the available tests. However, based on current literature, a panel with following genes likely be informative in clinical assessment for most of the myeloid neoplasms: FLT3, NPM1, CEBPA, TP53, IDH1/2, DNMT3A, TET2, CSF3R, SRSF2, KIT, NRAS, RUNX1, WT1, ASXL1, SF3B1 for AML; NRAS, KRAS, IDH1/2, TET2, EZH2, ASXL1, RUNX1, TP53, DNMT3A, SF3B1, U2AF1, ETV6 for MDS; JAK2, CALR, MPL, IDH1/2, ASXL1, TET2, EZH2, SRSF2, SF3B1, TP53 for MPN; TET2, SRSF2, ASXL1, RUNX1, NRAS, TP53 for CMML; SF3B1 for MD/MPN RS-T; CSF3R for CNL, SETBP1 for aCML, KIT (D816V) for mast cell disease.

In conclusion; the progress of NGS and widen utilization on hematologic malignancies is highly complex and it will facilitate more precise diagnosis and treatment for patients with

hematologic neoplasms. The discovery of genetic alterations on myeloid neoplasms alters the target mutation spectrum. AML, MDS, and MPNs are characterized by morphologic or phenotypic similarities but these disorders harbor different mutations types with different prognostic implications. Myeloid neoplasms that lack a cytogenetic alteration but show a somatic mutation can be diagnosed by the above-mentioned mutation profiles. These mutations facilitate also new novel therapeutic agents. In the coming years, it appears that major efforts with more comprehensive high through put NGS sequencing and inclusion of genomic data clinical management will enable diagnostician and clinician much more efficient delivery of precision medicine.

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