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Original Article

The Importance of Targeted Next-Generation Sequencing Usage in Cytogenetically Normal Myeloid Malignancies

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Abstract. Advanced diagnostic methods give an advantage for the identification of abnormalities in myeloid malignancies. Various researchers have shown the potential importance of genetic tests before the disease's onset and in remission. Large testing panels prevent false-negative results in myeloid malignancies. However, the critical question is how the results of conventional cytogenetic and molecular cytogenetic techniques can be merged with NGS technologies. In this paper, we drew an algorithm for the evaluation of myeloid malignancies. To evaluate genetic abnormalities, we performed cytogenetics, molecular cytogenetics, and NGS testing in myeloid malignancies. In this study, we analyzed 100 patients admitted to the Medical Genetics Laboratory with different myeloid malignancies. We highlighted the possible diagnostic algorithm for cytogenetically normal cases. We applied NGS 141 gene panel for cytogenetically normal patients, and we detected two or more pathogenic variations in 61 out of 100 patients (61%). NGS's pathogenic variation detection rate varies in disease groups: they were present in 85% of A.M.L. and 23% of M.D.S. Here, we identified 24 novel variations out of total pathogenic variations in myeloid malignancies. A total of 18 novel variations were identified in A.M.L., and 6 novel variations were identified in M.D.S. Despite long turnaround times, conventional techniques are still a golden standard for myeloid malignancies but sometimes cryptic gene fusions or complex abnormalities cannot be easily identified by conventional techniques. In these conditions, advanced technologies like NGS are highly recommended.

Keywords: Hematologic malignancies; NGS; Karyotype; FISH.

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Introduction. Myeloid malignancies originate from hematopoietic progenitor cells and are characterized by defective differentiation of myeloid progenitor cells.[1] Advanced molecular detection techniques have changed the diagnostic algorithm of cancer. Increased next-generation sequencing (NGS) usage can help change the scope, timing, and suitability of genetic testing in hematologic malignancies.[2] Despite the advances in NGS technology and the rising number of study findings that support the diagnostic and prognostic usage of mutational profiling in myeloproliferative neoplasms (M.P.N.), the clinical decision-making role is still not

fully utilized.[3]

Diagnostics algorithms of acute myeloid leukemia (A.M.L.), myelodysplastic syndromes (M.D.S.), and myeloproliferative neoplasms (M.P.N.) have evolved in recent years.[4,5] Due to NGS technology advances, various myeloid NGS panels are commercially available and generally analyze 25–50 genes classified into several functional categories including the splicing machinery, epigenetic modifiers, and transcription factors signaling molecules and chromatin modifiers.[6,7,8,9] The increased knowledge of genetic abnormalities has led to a reclassification of Acute Myeloid Leukaemia (A.M.L.).[10] The World Health Organization[11] and European LeukemiaNet[12] added new subgroups of diseases, and molecular genetic abnormalities have also been added in diagnostic criteria. An increased number of mutational, epigenetic, and expression studies will help identify the novel markers in myeloid malignancies.

National Comprehensive Cancer Network (NCCN) has added mutations in *FLT3*, *NPM1*, *CEBPA,* and *K.I.T.* genes to evaluate risk;[13] moreover, the ELN guidelines suggest to add *TP53*, *RUNX1,* and *ASXL1* mutations in the evaluation of risk. According to some studies, *SF3B1, IDH1,* and *IDH2* should also be included.[3,6,7,14,15] NGS based myeloid gene panels will help for the identification of multiple recurrent somatic mutations in many A.M.L. patients, and additional molecular genetic mutations can be detected in most cases, even within defined A.M.L. entities.[2] In myelodysplastic syndrome (M.D.S.), NGS allows detecting molecular mutations in approximately 90% of patients.[6,16,17] As a result, NGS data should be interpreted in the context of other laboratory findings, including cytomorphology, histopathology, immune-phenotyping, conventional molecular genetics, cytogenetics, and clinical diagnostic parameters. In this study, we analyzed 100 patients referred to Medical Genetics Laboratory with different hematologic malignancies. We performed conventional cytogenetics, molecular cytogenetics, and NGS analysis in these cases. According to our results, we highlighted a possible algorithm for cytogenetically standard cases.

Materials and Methods.

Patient samples. The present study included 100 patients (52 were male, and 38 were female) from December 2017 to August 2020. Written informed consent was obtained from all cases. If patients are under 18 (5 children (≤15 years)), a consent form was signed by a parent and/or legal guardian. The study was approved by the Ethics Committee of our university and conducted following the ethical principles established in the Declaration of Helsinki. The median age of cases was 54 years, ranging from 1 to 90 years, and there were five children (≤15years) in 100 adults. The distribution of patients was shown in **Table 2**. Our cohort consists of 100 patients diagnosed with A.M.L. (61) and M.D.S. (39). D.N.A. was isolated from bone marrow (QIAamp D.N.A. Blood Mini Kit (bone marrow = 100) (Qiagen, Germany) and peripheral blood (MagNA Pure system Roche Diagnostics). D.N.A. was quantified using a Qubit fluorometer (Thermo Fisher Scientific). The patients who have normal karyotype and fluorescence in situ hybridization (FISH) report were enrolled in this study. Patients enrolled in this study were newly diagnosed. Therefore the treatment protocols were not determined yet.

Next-generation sequencing. For evaluating myeloid neoplasm specific 141 genes, the Human Myeloid Neoplasms QIAseq Targeted D.N.A. Panel (Qiagen, Germany) was used. This panel covers exon/intron boundaries shown in **Figure 1** and covered genes as listed in **Table 1**.[18] MiSeq sequencing-by synthesis

Table 1. The list of covered genes and related diseases in NGS panel.

Disease	Genes covered
Acute myeloid leukaemia (AML)	ANKRD26, ASXL1, ATM, BCOR, BCORL1, BIRC3, BRAF, C17orf97, CALR, CARD11, CBLC, CDKN2A, CEBPA, CHEK2, CREBBP, CSF1R, CSF3R, CTCF, DAXX, DDX41, DNM2, DNMT1, ELANE, EP300, FLRT2, FLT3, GATA1, GATA2, HNRNPK, IDH1, IDH2, IKZF1, IL7R, JAK1, JAK3, KDM6A, KDR, KIT(CD117), KMT2A, KMT2C, KRAS, LRRC4, MAP2K1, MPL, MSH6, MYC, NBN, NOTCH1, NPM1, NRAS, NSD1, NTRK3, OR13H1, OR8B12, P2RY2, PCDHB1, PDGFRA, PHF6, PRAMEF2, PRPF8, PTEN, PTPN11, RAD21, RUNX1 (AML1), SF1, SF3A1, SMARCB1, SMC1A (SMC1L1), SMC3, SRP72, SRSF2, STAG2, STXBP2, U2AF1, U2AF2, WT1
Myelodysplastic syndromes (MDS)	ATRX, CALR, CDKN2A, CEBPA, CSF1R, CSF3R, EP300, ETNK1, GNAS, HRAS, KDM6A, KMT2A, KMT2C, RAD21, RB1, SETBP1, SF1, SF3A1, SMC3, SRSF2, STAG2, U2AF1, U2AF2, XPO1, ZRSR2
Myeloid malignancies	CBL, CBLB, DNMT3A, EED, ETV6, EZH2, PRPF40B, SUZ12, TET2, TP53
Myeloproliferative neoplasm (MPN)	ABL1, ASXL1, CALR, CSF1R, JAK2, JAK3, KAT6A (MYST3), KRAS, MPL, NF1, NRAS, RB1, SETBP1, SF3B1, SH2B3, SRSF2, STAG2.
Myelofibrosis (MF)	CALR, CHEK2, IDH1, IDH2, CSF1R, SRSF2
Other myeloid neoplasms	BRAF, CDKN2A, CEBPA, FBXW7, HRAS, IKZF3, KLHDC8B, KMT2C, MSH6, NTRK3, PTEN, SRP72, TPMT
Other myeloid neoplasm genes	BRCA1, BRCA2, BRINP3, CUX1, FAM47A, FAS, KCNK13, MYD88, PML, PRF1, SAXO2, STAT3, TERC, TNFRSF13B

Table 2. WHO classification of our cohort and results of genetic analysis.

WHO classification	n	Detected pathogenic variant	Detected likely pathogenic variant	Detected VOUS	FISH	Karyotype
Acute myeloid leukemia (AML)	61	52	18	25	N	N
Myelodysplastic syndrome (MDS)	39	9	6	14	N	N
Total	100	61	24	39	N	N

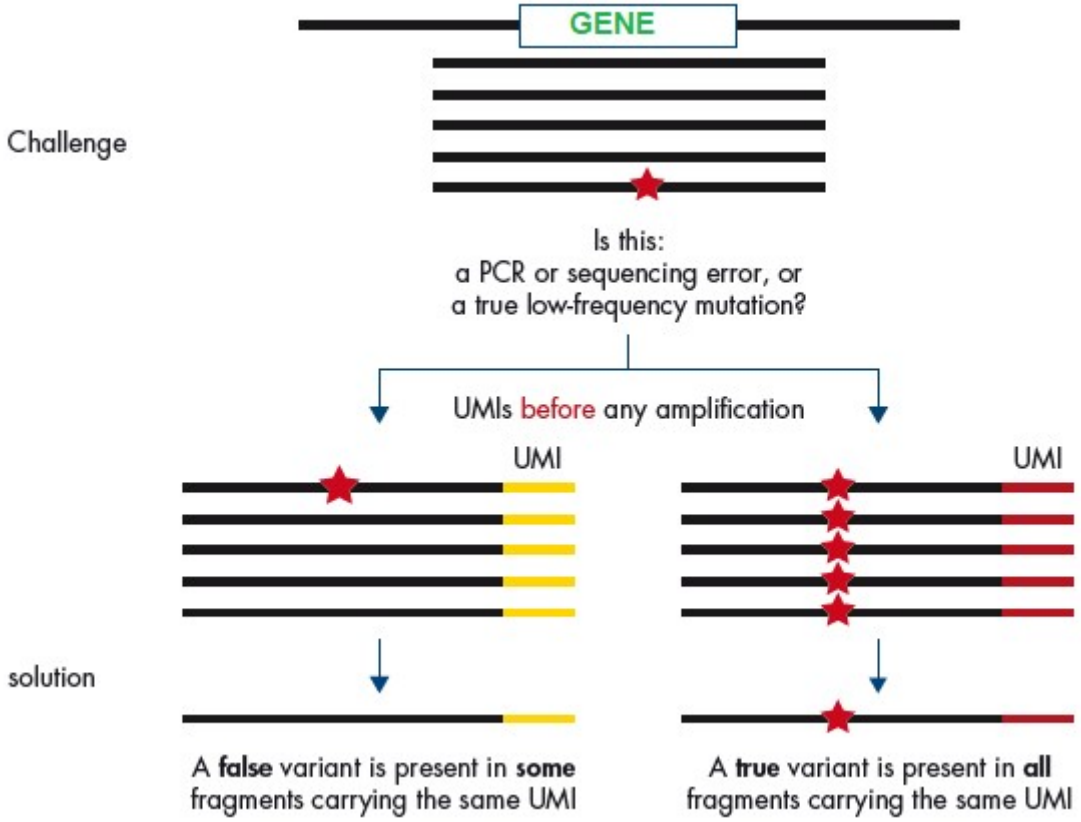

Figure 1. Mechanism of unique molecular indices (U.M.I.s).

benchtop sequencer was used for sequencing of amplified targets according to the manufacturer's protocol for paired-end sequencing (Illumina, San Diego, CA, U.S.A.). Data analysis and quality assessment for calling of single-nucleotide variants and analysis of short insertions and deletions were evaluated using Ingenuity Variant Analysis (I.V.A.) program. Amplicons were noted as a dropout and excluded from analysis if the coverage at any analyzed position in any of the two paired-end sequences (minimal coverage) was 100x, with allele frequency >5% were included for subsequent investigation. Libraries covering the target genes were prepared according to the QIAseq Targeted D.N.A. Panel protocol (Qiagen, Hilden, Germany). Following the target enrichment process, libraries were sequenced on the MiSeq System and NextSeq 550 System (Illumina, San Diego, CA, U.S.A.). O.C.I. analysis (Qiagen, Hilden, Germany) was used for Quality control and Variant Call Format file generation. Variant analysis has been performed in Ingenuity software (Qiagen, Hilden, Germany). Variants were interpreted according to the American College of Medical Genetics, and Genomics 2015 (ACMG-2015) recommended standards. The candidate variants were annotated by ANNOVAR with SIFT, PolyPhen-2, MutationTaster, and the Exome Aggregation Consortium (ExAC) and other databases. Known hotspot or clinically actionable variants detected below these thresholds were verified using orthogonal methods such as Sanger sequencing.

Cytogenetic Assessment.

Karyotyping: Marrow Max and Chang media were used for cultures of bone marrow and peripheral blood specimens in a CO_2 incubator. After 24, 48, or 72 h of incubation, cultures were harvested. Colcemid was used to arrest metaphase cells, and chromosome slides were stained using G banding protocol. International System for Human Cytogenetic Nomenclature (ISCN 2016)[19] was used for reporting, and 25 metaphases were analyzed in each culture. The best metaphases were chosen for karyotype analysis, and the total chromosome count was usually determined in 25 cells.

Fluorescence in situ hybridization (FISH): FISH was applied according to the manufacturer's

recommendations. A total of 200 interphase cells were analyzed for each sample, and images were captured/stored by using the Applied Imaging/Cytovision system. Final results were reported by using the cutoff established in the laboratory for each of the tested probes.[20] Specific gene panels for FISH was applied for each malignancy. FISH panels for each of the malignancies are listed below.

FISH Panel for AML: 5q-, -5 (5p15, 5q31, 5q33), 7q-, -7 (Cen 7, 7q22, 7q31), Trisomy 8 (Cen 8), MLL (11q23), 20q- (20q12,20qter), RUNX1/RUNX1T1 (ETO/AML1) t(8;21), PML/RARA t(15;17), CBFB inv(16), t(16;16)

FISH Panel for MDS: 5q-, -5 (5p15, 5q31, 5q33), 7q-, -7(Cen 7, 7q22, 7q31), Trisomy 8 (Cen 8), MLL(11q23), 20q- (20q12, 20qter)

Results. Characteristics of the patients are summarized in **Table 2**. Among these patients, 52 were male, and 38 were female. The median age was 54 years, ranging from 1 to 90 years, and there were 100 adults and five children (≤15 years).

Results of cytogenetic and molecular cytogenetic analysis. Cytogenetic and molecular cytogenetic analyses were performed on all of the patients. Cytogenetics and molecular cytogenetic evaluations were reported as standard in all of the cases.

Results of next-generation sequencing. Next-generation sequencing of hotspot regions of 141 genes has been performed in 100 bone marrow samples referred from the Department of Hematology. Variables with a depth of coverage > 100x and an allele frequency of > 5% were included in this study. Known hot spots or variants identified below the threshold that may require clinical intervention were confirmed using the Sanger sequencing. Variables of unknown significance were excluded from the clinical benefit analysis. Variants were classified as pathogenic and possible pathogenic according to the gene and clinical effects. Two or more pathogenic variations were identified in 61 out of 100 patients (61%). A list of the variants is presented in **Table 3**. A total of 24 novel pathogenic or likely pathogenic variations have been described. In A.M.L., novel pathogenic and likely pathogenic variations were identified in EP300, STAG2, CUX1, U2AF1, RUNX1, GNAS, CHEK2, CREBBP, and PHF6 genes. In M.D.S., novel pathogenic and likely pathogenic variations were identified in SRSF2, ASXL1, A.T.M., RUNX1, and TET2 genes (**Table 3**).

The distribution of frequent mutations in AML includes, TET2, TP53, FLT3 and IDH2 genes. A total 7 different variants of TET2 (TET2; c.2746C>T, c.2656C>T, c.945del, c.3543_3544delCT, c.4478delA, c.1184delC, c.1184delC) were detected in 6 different AML patients and 6 different variants of TP53 (TP53; c.537T>A, c.596G>A, c.503A>G, c.460G>A, c.467G>C, c.844C>G) were detected in 5 different AML patients. Additionally, pathogenic FLT3 variants identified in 3 AML patients, including: c.1770_1793dupCTACGTTGATTTCAGAGAATATGA, c.2503G>T, c.1837+1G>A, c.2678C>T. The other common pathogenic variants identified in IDH2 gene in AML, including; c.419G>A, c.419G>A and c.419G>A.

The common pathogenic variants in MDS was SRSF2 and identified in two different cases, includes: c.284C>T and c.284_307del. More than one pathogenic variants identified in 2 different cases (Case1: BCOR c.2428C>T, BRCA2 c.4446_4451dupAACAGA, U2AF1 c.101C>T and case 2: SRSF2:c.284_307del and IDH1:c.395G>A).

These results show us that clonality could be observed in the lowest percentages. The literature recommends that to determine clonally, up to 5% allelic fraction should be evaluated.[21] NGS's pathogenic variation detection rate varies in disease groups: in A.M.L. was 85% (52 out of 61) and M.D.S. was 23% (9 out of 39). Likely pathogenic variation detection rate and VOUS detection rate of NGS have been listed in **Table 2**, and the mutation list of disease groups has been shown in **Table 3**.

Table 3. The list of identified mutations and their distributions of diseases. Novel mutations have been shown in table as a red labeled. Green color demonstrates the VOUS, pink color demonstrates the likely pathogenic mutations and red color demonstrates the pathogenic mutations.

Mutation List	Disorders
TET2 NM_001127208.2(TET2):c.2746C>T	AML
TET2 NM_001127208.2(TET2):c.2656C>T	AML
TET2 NM_001127208.2(TET2):c.945delC	AML
TET2 NM_001127208.2(TET2):c.3543_3544delCT	AML
TET2 NM_001127208.2(TET2) :c.4478delA	AML **Novel**
TET2 NM_001127208.2(TET2):c.4182+1G>A	AML **Novel**
TET 2 NM_001127208.2 (TET2):c.1184delC	AML **Novel**
TP53 NM_000546.5(TP53):c.537T>A	AML
TP53 NM_000546.5(TP53):c.596G>A	AML

TP53 NM_000546.5(TP53):c.503A>G	AML
TP53 NM_000546.5(TP53):c.460G>A	AML
TP53 NM_000546.5(TP53):c.467G>C	
TP53 NM_000546.5(TP53):c.844C>G	AML
FLT3 NM_004119.2(FLT3):c.1770_1793dupCTACGTTGATTTCAGAGAATATGA	AML
FLT3 NM_004119.2(FLT3): c.1784_1804dupGAGAATATGAATATGATCTCA	AML
FLT3 NM_004119.2(FLT3):c.2678C>T	AML
FLT3 NM_004119.2(FLT3):c.1837+1G>A	AML **Novel**
FLT3 NM_004119.2(FLT3):c.2503G>T	AML
ASXL1 NM_015338.5(ASXL1):c.2122C>T	AML
ASXL1 NM_015338.5(ASXL1):c.2128delG	AML **Novel**
ASXL1 NM_015338.5(ASXL1):c.1926_1927insG	AML **Novel**
ASXL1 NM_015338.5(ASXL1):c.2122C>T	AML
IDH2 NM_002168.3(IDH2):c.515G>A	AML
IDH2 NM_002168.3(IDH2):c.419G>Ax3	AML
IDH1 NM_005896.3(IDH1):c.394C>T x3	AML
DNMT3A NM_022552.4(DNMT3A):c.2644C>T	AML
DNMT3A NM_022552.4(DNMT3A):c.2645G>A	AML
RUNX1 NM_001754.4(RUNX1):c.502G>T	AML
RUNX1 NM_001754.4(RUNX1):c.400G>C	AML
MPL NM_005373.2(MPL): c.1544G>T	AML
MPL NM_005373.2(MPL):c.1771T>G	AML **Novel**
WT1 NM_024426.5(WT1):c.1153_1157dupCGGTC	AML **Novel**
PHF6 NM_001015877.2(PHF6):c.110dupA	AML **Novel**
PTPN11 NM_002834.4(PTPN11): c.227A>G	AML
ATM NM_000051.3(ATM):c.7328G>A	AML
JAK2 NM_004972.3(JAK2):c.1849G>T	AML
BCORL1 NM_021946.4(BCORL1):c.2916T>A	AML
NF1 NM_000267.3(NF1):c.4537C>T	AML
NOTCH1 NM_017617.5(NOTCH1):c.4721T>C	AML
SRSF2 NM_003016.4(SRSF2):c.284C>T	AML
GATA2 NM_032638.4(GATA2):c.1076T>C	AML
EP300 NM_001429.3(EP300): c.6627_6638delCCAGTTCCAGCA	AML **Novel**
STAG2 NM_001282418.1(STAG2): c.733C>T	AML **Novel**
CBL NM_005188.3(CBL):c.1192C>T	AML
CUX1 NM_181552.4(CUX1):c.976C>T	AML **Novel**
U2AF1 NM_006758.2 (U2AF1) :c.470A>G	AML **Novel**
SETBP1 NM_015559.2(SETBP1):c.2602G>A	AML
SF3B1 NM_012433.3(SF3B1):c.2098A>G	AML
NPM1 NM_002520.6(NPM1):c.859_860insTCTG x2	AML
NPM1 NM_002520.6(NPM1):c.860_863dupTCTG	AML
RUNX1 NM_001754.4(RUNX1):c.423_424insAAGGAG	AML **Novel**
RUNX1 NM_001754.4(RUNX1):c.482T>C	AML

STAG2 NM_001282418.1(STAG2):c.3243_3244insATTT STAG2 NM_001282418.1(STAG2):c.1414G>T	AML
ASXL1 NM_015338.5(ASXL1):c.2056A>T	AML
ETV6 NM_001987.4(ETV6):c.163+1G>T	AML
FBXW7 NM_033632.3(FBXW7):c.1393C>T	AML
GNAS NM_080425.2:c.1376C>G(GNAS): c.1376C>G	AML **Novel**
DNMT3A NM_022552.4 (DNMT3A):c.2645G>A	AML
CHEK2 NM_007194.4(CHEK2): c.480A>G	AML **Novel**
CREBBP NM_004380.2(CREBBP): c.5213_5216dupATGC	AML **Novel**
CEBPA NM_004364.4(CEBPA):c.779_783delACCCCinsG	AML
BCORL1 NM_021946.4(BCORL1):c.2916T>A	AML
PHF6 NM_001015877.2(PHF6):c.309C>A	AML **Novel**
IDH1 NM_005896.3(IDH1) :c.394C>T	AML
ATM NM_000051.3(ATM):c.5723C>T	AML
ATM NM_000051.3(ATM):c.7237A>G	AML
ATM NM_000051.3(ATM):c.5723C>T	AML
DNMT3A NM_022552.4(DNMT3A): c.1555-8_1555-1delCTGTCTAG	AML
DNMT3A NM_022552.4(DNMT3A):c.976delC	AML
DNMT3A NM_022552.4(DNMT3A):c.2114T>C	AML
NPM1 NM_002520.6(NPM1):c.733G>C	AML
NPM1 NM_002520.6(NPM1):c.863_864insCCTG	AML
EP300 NM_001429.3(EP300):c.7238T>A	AML
ETV6 NM_001987.4: c.1254-2A>G	AML
JAK1 NM_002227.3(JAK1):c.1951G>A	AML
AKAP13 NM_006738.5(AKAP13):c.7265G>A	AML
DNAH9 NM_001372.3(DNAH9):c.10555C>A	AML
SMC1A NM_006306.3(SMC1A):c.2152G>A	AML
OR8B12 NM_001005195.1(OR8B12):c.353C>T	AML
CALR NM_004343.3(CALR):c.682C>T	AML
KAT6A NM_006766.4(KAT6A):c.4108G>T	AML
ASXL2 NM_018263.6(ASXL2):c.833T>A	AML
NF1 NM_001042492.2(NF1):c.1921A>G	AML
PRPF40B NM_001031698.2(PRPF40B):c.1103C>T	AML
PMS2 NM_000535.6(PMS2):c.2321A>T	AML
ADA NM_000022.3(ADA):c.179A>G	AML
MPL NM_005373.2(MPL):c.121T>C	AML
SETD2 NM_014159.6(SETD2):c.6685G>A	AML
SMC1A NM_006306.3(SMC1A):c.2152G>A	AML
WT1 NM_024426.5(WT1): c.470A>G	AML
SRSF2 NM_003016.4(SRSF2):c.284_307del	MDS **Novel**
SRSF2 NM_003016.4(SRSF2):c.284C>T x2	MDS
SF3B1 NM_012433.3(SF3B1):c.1866G>T	MDS
SF3B1 NM_012433.3(SF3B1):c.2098A>G	MDS
TET2 NM_015559.2(SETBP1):c.2602G>A	MDS
ASXL1 NM_015338.5(ASXL1):c.2128delG	MDS **Novel**
ATM NM_000051.3(ATM):c.7466C>T	MDS **Novel**
BRCA2 NM_000059.3(BRCA2):c.4446_4451dupAACAGA	MDS
RUNX1 NM_001754.4(RUNX1):c.482T>C	MDS **Novel**

BCOR NM_001123385.1(BCOR):c.2428C>T	MDS
TET2 NM_001127208.2(TET2):c.1916dupA	MDS **Novel**
TET2 NM_001127208.2 (TET2):c.5067delT	MDS **Novel**
IDH1 NM_005896.3(IDH1):c.395G>A	MDS
U2AF1 NM_006758.2(U2AF1):c.101C>T	MDS
	MDS
ADA NM_000022.4(ADA):c.937C>T	MDS
ATM NM_000051.3(ATM):c.3605G>C	MDS
BLM NM_001287246.1(BLM):c.3416G>C	MDS
CNOT NM_014516.3(CNOT):c.1847C>T	MDS
CBLB NM_170662.5(CBLB): c.815G>A	MDS
KRAS NM_033360.4(KRAS) :c.503T>A	MDS
CALR NM_004343.3(CALR):c.682C>T	MDS
NTRK3 NM_002530.3(NTRK3):c.121A>G	MDS
DNMT1 NM_001379.3(DNMT1):c.4393G>A	MDS
STAG2 NM_001282418.1(STAG2): c.607C>T	MDS
IKZF1 NM_006060.6: c.949_951delAAC	MDS

VOUS: **Likely Pathogenic:** **Pathogenic:**

Discussion. Genetic and epigenetic alterations play an important role in leukemogenesis.[22] Several techniques have been used to identify genetic alterations in hematologic malignancies, including; FISH, cytogenetics, NGS, RT-PCR (real time-PCR).[23] Advances in next-generation sequencing (NGS) technology help transform gene sequencing into a considerably faster and less expensive test, making it more practical in clinical practice. The validation of NGS panels is critical, and generally, a two-step approach is recommended for validation. The first one is related to the optimization and analysis of relevant errors during the testing, and the second step is related to the establishment of thresholds of the depth of coverage and V.A.F. (low variant allele frequency of variations) for each type of identified variant.[24]

In recent years, NGS has been used to identify T-cell clonality, recurrent cytogenetic translocations, and identification of the Philadelphia chromosome in Acute Lymphoblastic Leukaemia.[2] In addition to these conditions, in lymphoproliferative diseases, NGS has also been used to identify clonal I.G.H. and TCR rearrangements in M.R.D. (Minimal Residual Disease).[21] NGS technology can be used to identify mutant or clonal D.N.A. in several circulating tumor cells. It is also essential for clinical trials based substantially on next-generation sequencing (NGS) parallel it with the increasing number of molecular markers.[25]

An increased number of studies in this field will discover new mutations and update the WHO classification for myeloid malignancies. Moreover, those studies will help develop novel targeted therapeutic agents and novel therapeutic targets.[26] Discovering new mutations in myeloid neoplasms enables us to understand the variable prognosis and pathogenesis of these diseases. The use of cytogenetic-based techniques allows identifying "gross" chromosomal abnormalities such as translocations, amplifications, and deletions.[22] However, the technique's limitation is based on the abnormality size because genes can change in various ways (mutations, methylation, etc.) that may be critical for the onset and/or progression of malignant hemopathies. The major advancement in NGS is identifying the molecular basis of leukemia because now we can classify malignant hemopathies at a molecular level that is more informative than the cytological classification.[27]

Delic and colleagues analyzed a 28-gene testing panel in different hematologic malignancies (myeloproliferative neoplasms, essential thrombocythaemia, primary myelofibrosis, polycythemia vera). Different mutations were identified in splicing related genes (SF3B1, SRSF2, and U2AF1), chromatin modification genes (ASXL1 and EZH2), and methylation related genes (DNMT3A, IDH1, IDH2, and TET2).[28] Maes et al. analyzed 155 newly diagnosed myeloid neoplasm patients and identified mutation in 81% of the cases.[29] They highlighted the importance of targeted NGS testing in myeloid neoplasms' routine diagnostic approach and demonstrates that NGS helps improve diagnosis, subclassification, and prognosis of cases.[29]

Our study analyzed 100 myeloid malignancies and identified variations in 61% of cases, and the mutation frequency was similar to the literature. The critical patient inclusion criterium of the study was the cytogenetically normal report because we aim to show the importance of further testing in cytogenetically normal cases during the evaluation of prognosis of disease and treatment design. Another interesting point of our study was identifying 24 novel pathogenic and likely pathogenic variations in myeloid malignancies.

Northrup and colleagues applied a targeted NGS panel to a total of 178 patients diagnosed with myeloid neoplasms. They identified gene variants in 53% of patients, and they conclude that NGS was a more sensitive test than conventional cytogenetics, so they

suggested that NGS should become a part of the routine workup of patients.[30] Kawata and colleagues used cytogenetics and NGS for the evaluation of 134 MDS cases.[31] According to Kawata's work, abnormal NGS was identified in 44 cases (32.8%). They highlighted together with NGS; the cytogenetic evaluation also provided more frequent diagnostic information in M.D.S. cases.[31] Studies suggested that NGS can help identify over 80% of recurrent mutations in M.D.S. cases.[8,32] In our study, NGS's variation detection rate was 61% in myeloid malignancies, and the detection rate for NGS in M.D.S. was lower (23%) than what has been described in other studies[8,31,32] because of a limited number of cases and our inclusion criteria. The patients who have abnormal cytogenetic reports were excluded from our study. Because of this reason, our mutation frequency was lower than the previous studies. Abnormal cytogenetics were closely correlated with the accumulation of mutations in the transcription factors; cell cycle checkpoints related genes were associated with normal and abnormal karyotypes.[33] Therefore, the differences in variation rates reported in this study were related to our patient selection criteria, which we enrolled in cases with average karyotype results. Our present results suggest that NGS could be the right choice for cases without any cytogenetic alteration, but this approach would require validation in more extensive studies.

Yu and colleagues analyzed 43 genes in 93 de novo M.D.S. and 325 non-M3 A.M.L. patients by NGS and conventional cytogenetics. In 60.1% of cases carries a complex karyotype, and mutation frequency was detected as 85.8% in A.M.L. cases.[33] In our study, the detection rate for NGS in A.M.L. was 85%, which was similar to Yu's study, which confirms the importance of NGS testing as a diagnostic tool.

Vantyghem and colleagues conducted a study to show the real-life setting of chronic myeloid malignancies by NGS testing in a total of 177 chronic myeloid malignancies patients.[34] They concluded that NGS's daily practice helps for the final diagnosis of 83% of the patients.[34] Reinig et al. applied a 42-gene panel in 109 cases of myelodysplastic syndrome (M.D.S., n: 38), chronic myelomonocytic leukemia (CMML, n: 14), myeloproliferative neoplasm (M.P.N., n: 24), and M.D.S. and/or M.P.N. transformed to acute myeloid leukemia (A.M.L., n: 33).[35] A pathogenic mutation was identified in 74% of cases of M.D.S., 100% of CMMLs, and 96% of M.P.N.s cases.[35] Levy and colleagues used a cohort of 380 patients and performed clinical validation of a gene panel within 50.5% of diagnostic yield. They concluded that targeted NGS testing should be an alternative to targeted molecular testing in patients with suspected hematologic malignancies.[36] Yun et al. used NGS analysis for evaluation of 157 patients (MDS [n = 95]; secondary-AML (sAML) [n = 52]; CMML [n = 10]) and they highlighted the clinical importance of NGS during treatment planning of cases.[37] In making the comparison with our cases, we must make some considerations. We focused on cases with normal cytogenetic and FISH results, which is a critical inclusion criterium of patients. All of the cytogenetically abnormal cases have been excluded from the study. We sequenced 141 genes in a cohort consisting of 100 patients diagnosed with A.M.L. (61 cases) and M.D.S. (39 cases). We identified two or more pathogenic variations in 61% of patients. Previous studies aimed to improve NGS usage in all cases without prior analysis. This study chose the patients who had normal cytogenetic and FISH results to show the possible false-negative results depending on the cytogenetic evaluation. Our results also confirm this hypothesis, showing that those who had normal cytogenetic evaluation should need further testing by using NGS. We suggested NGS in routine clinical testing for myeloid malignancies, which are cytogenetically reported as normal. Here, we identified variations in different genes related to epigenetic modifications, R.N.A. modifications, transcription factors, D.N.A. repair, and cohesin complex. We identified novel variations in *EP300, STAG2, CUX1, U2AF1, RUNX1, GNAS, CHEK2, CREBBP, PHF6, SRSF2, ASXL1, A.T.M., RUNX1,* and *TET2* genes which were not previously described in the literature.

This procedure will help prevent false-negative results and apply correct treatment strategies and give prognostic information. Our suggested algorithm was shown in **figure 2**, which shows that only cytogenetic analysis is not sufficient to evaluate diseases.

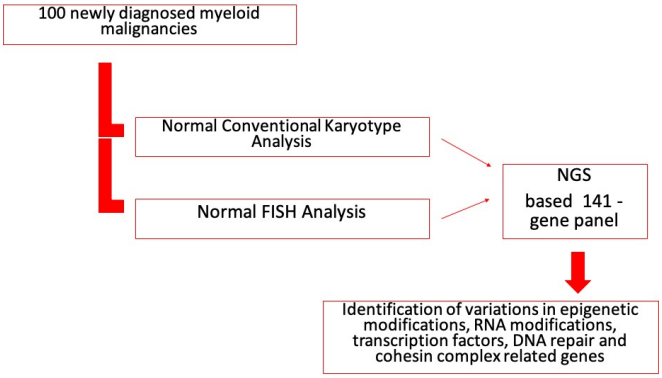

Figure 2. Suggested algorithm for cytogenetically normal cases.

NGS-based panel testing is widely accepted in clinical practice, and this can facilitate the construction of well-designed comprehensive NGS panels, especially during initial diagnosis. Albeit, the targeted NGS panels can evaluate the genome-wide numerical imbalances. NGS testing gives a chance to analyze the genomic copy number alteration of interest gene and which triggers different questions for conventional cytogenetics during the evaluation of myeloid neoplasms. However, NGS testing cannot identify structural abnormalities, lacking single-cell resolution, and low target density, so

simultaneous cytogenetic analysis needs to have a complete picture of the genomic profile. Therefore, after clinical and diagnostic evaluation, it may be advantageous to perform cytogenetic analysis for patients whose NGS results show significant clonal evolution. This procedure has financial consequences, the requirement of well-trained technical staff, problems during the bioinformatics analysis of NGS testing. However, despite all of these conditions, the collected clinical and molecular information should be led to develop targeted therapeutics in this field.

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