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Analysis of *NPM1* and *FLT3* Mutations in Patients with Acute Myeloid Leukemia in Jeddah, Saudi Arabia: A Pilot Study

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Abstract

Background: The outcome of acute myeloid leukemia (AML) is influenced by ethnicity, geographic variations, and the patient's molecular profile. We aimed to explore the mutation frequencies of the nucleophosmin 1 (*NPM1)* and the FMS-like tyrosine kinase 3 (*FLT3)* internal tandem duplication (ITD) or tyrosine kinase domain (TKD) with correlation to the cytogenetic profiles in patients with AML.

Methods and Results: Bone marrow/whole blood samples from 33 patients with AML were screened for NPM1/FLT3-ITD mutations by fragments analysis using a GeneScan analyzer. Depending on the fragment size, the *NPM1* and *FLT3* wild type (Wt) (170 bp and 330 bp) vs. mutated (170/174 bp and 330/351 bp) alleles, respectively, can be distinguished. The allelic ratio of *FLT3-ITD*$^+$ was calculated. FLT3-TKD$^+$ mutation was detected by Sanger sequencing. Samples were tested for chromosomal aberrations. According to the French-American-British (FAB) classification, the predominant type in the present cohort was AML-M5, accounting for 30.3%. *NPM1*$^+$, *FLT3-ITD*$^+$, and double mutations were found in 12.1%, 3.1%, and 6.1% of cases, respectively. The combined *NPM1*$^+$*/FLT3-ITD*$^+$*/FLT3-TKD*$^+$ profile was presented in one patient (3.1%). The dual positivity group (*NPM1*$^+$*/FLT3*$^+$) significantly had a higher WBC count with a median of 81.3×10^3/μL. A total of 63.6% of patients had abnormal cytogenetics. The *NPM1*$^+$*/FLT3-ITD*$^+$ patients had normal karyotypes. Patients with *NPM1*$^-$*/FLT3*$^-$ showed complex karyotype (24%) and t(8;21) (8%). The *FLT3-ITD*$^+$ patient had trisomy 8.

Conclusion: The frequency of *NPM1/FLT3* mutations in the study cohort showed less rate than in other studies with a distinct pattern. Due to the preliminary nature of the present work, more extensive screening is warranted to evaluate their usefulness as prognostic indicators in this region.(**International Journal of Biomedicine. 2023;13(1):73-83.**)

Keywords: acute myeloid leukemia • FLT3 • NPM1 • sequencing

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Abbreviations

AML, acute myeloid leukemia; **FLT3,** FMS-like tyrosine kinase 3; **ITD**, internal tandem duplication; **NPM1**, Nucleophosmin 1; **TKD**, tyrosine kinase domain.

Introduction

AML is one of the hematopoietic tumors caused by the atypical production and differentiation of myeloblasts in the bone marrow.[1] In 2017, AML accounted for 23.1% of the total leukemia cases with a current global incidence rate equal to 0.9-2.8 cases per 10^5 men and 0.4-2.2 per 10^5 women, raising the possibility of becoming one of the primary, worldwide public health concerns.[2,3]

According to the Saudi Cancer Registry, 2016, the leukemia age-standardized rate was 3.6 per 10^5 men and 3.0 per 10^5 women[4] with a slightly variable incidence of AML per Saudi Arabia regions, as the Eastern Region showed the highest rate of cases.[5]

The etiopathology of AML is heterogeneous and caused by different environmental and genetic aberrations associated with diverse outcomes.[6] Accumulating evidence indicates that the genetic profile alterations of patients with AML are considered essential workup for stratifying cases into "favorable, intermediate, or adverse prognostic risk groups," according to the 2017 European LeukemiaNet (ELN)guidelines, and are the strongest predictors of patients' outcome and planning therapeutic strategies.[7-9]

Among the molecular studies that are essential for AML case-risk stratification are the nucleophosmin 1 (*NPM1)* and FMS-like tyrosine kinase 3 (*FLT3)* mutations.[10] The phosphoprotein NPM1 is implicated in regulating the function and stability of various nuclear proteins and several intracellular activities, such as stress response, DNA replication/recombination, transcription, repair, preribosomal particle transport, centromere duplications, and the stability of tumor suppressor genes (Figure 1A).[11-13]

NPM1 is predominantly localized in the nucleolus and is thought to function as a molecular chaperone of proteins, facilitating the transport of ribosomal proteins through the nuclear membrane.[14-15] The role of NPM1 in human cancer has received a new spotlight with the discovery of mutations of exon 12 of NPM1 in approximately 30% of adult de novo AML, and in 50–60% in those with a normal karyotype.[16,17] The latest updates of the World Health Organization (WHO) classification of myeloid neoplasm recognized AML with mutated NPM1 as a distinct diagnostic entity.[18]

Several different types of mutations exist in exon 12 of the *NPM1* gene. The most common type (called type A), representing about 70%–80% of all mutations, features a four-base pair nucleotide (TCTG) insertion at the position encoding the 288th amino acid residue, resulting in a frameshift of the downstream sequence.[19] As a consequence, the C-terminal amino acid sequence 286DLWQWWRKSL-COOH changes to 286DLCLAVEEVSLRK-COOH. The mutant *NPM1* in AML leads to the cytoplasmic dislocation of NPM1.[20,21] Interestingly, this type of mutation has been reported to have a favorable prognosis within the context of other specific genetic changes in AML cases.[20,22-24]

The FLT3 is a member of the class III receptor tyrosine kinase family that is generally expressed on early myeloid cell precursors and plays a central role in controlling the survival, proliferation and differentiation of hematopoietic cells (Figure 1B).[25] The in-frame internal tandem duplication (ITDs) and missense point mutation (D835) in the tyrosine kinase domain (TKD) are the most frequent activating mutations (i.e., lead to constitutive function of the TK receptor) explored in patients with AML.[25,26] The latter mutations have been associated with AML's poor prognosis for increased relapse risk and/or reduced overall survival.[27] Also, it is worth noting that the abnormal FLT3 kinase activation is one of the putative therapeutic targets in AML.[28]

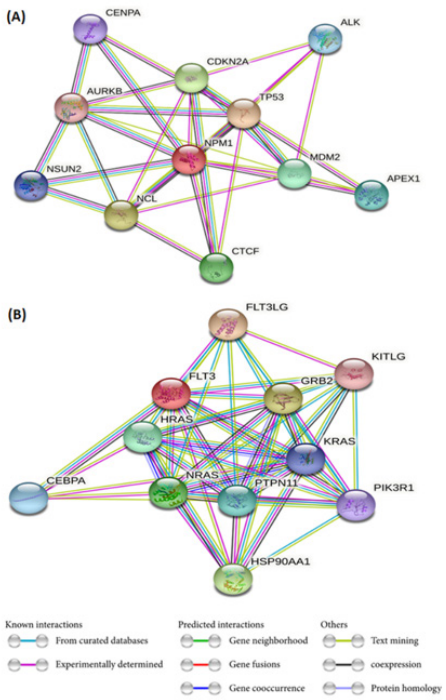

Figure 1. *The STRING analysis of NPM1 and FLT3 with the predicted functional partners. Each node represents a protein produced by a single gene locus. Edges represent protein-protein associations. (***A***) The NPM1 interacts with the tumor suppressor protein p53 (TP53), the major nucleolar protein nucleolin (NCL), cyclin-dependent kinase inhibitor 2A (CDKN2A), the transcriptional repressor (CTCF), the E3 ubiquitin-protein ligase Mdm2 (MDM2), the DNA-(apurinic or apyrimidinic site) lyase (APEX1), the ALK tyrosine kinase receptor (ALK), the NOP2/Sun RNA methyltransferase family member 2 (NSUN2), the Histone H3-like centromeric protein A (CENPA), and Aurora kinase B (AURKB) to mediate several cellular processes, including cell apoptosis, chromatin decondensation, transcriptional regulation, cellular response to oxidative stress, proper spindle assembly, and chromosome segregation, among others. (***B***) The FLT3 interacts with the Fms-related tyrosine kinase 3 ligand (FLT3LG), the growth factor receptor-bound protein 2 (GRB2), the heat shock protein HSP 90-alpha (HSP90AA1), the proto-oncogene (NRAS), the GTPases (HRAS and KRAS), the Tyrosine-protein phosphatase non-receptor type 11 (PTPN11), the CCAAT/enhancer-binding protein alpha (CEBPA), the Phosphoinositide-3-kinase regulatory subunit alpha/beta/delta (PIK3R1), and the Kit legend (KITLG), to stimulate the proliferation of early hematopoietic cells, cell maturation, structural maintenance, associate cell surface growth factor receptors with the Ras signaling pathway, coordinate proliferation arrest and the differentiation of myeloid progenitors, among others. [data source: https://string-db.org, version 11.5] (last accessed 20 Sep 2022).*

Cytogenetic abnormalities are detected in 50%–61% of AML patients at diagnosis.[29] For patients with normal karyotype, either the reverse transcriptase-polymerase chain reaction (RT-PCR) technique or the fluorescent in situ hybridization (FISH) is essential to detect any cryptic rearrangement of the relevant locus.[30] Based on the cytogenetic findings for AML patients, they are classified into three groups for their prognostic risk: (i) favorable group with balanced translocations, including t(15;17), t(8;21), inv(16) or t(16;16); (ii) intermediate risk group with normal karyotype, t(9;11) with no additional abnormalities, or isolated +8; (iii) adverse risk group with t(6;9), inv(3), or complex karyotype (more than three chromosomal abnormalities).[29-31]

As molecular genotyping and cytogenetic studies have an increasingly measurable impact on clinical decision-making for patients with AML, and there are limited related studies in our region, we were inspired to evaluate the molecular profiles of *NPM1* and *FLT3* mutations and correlate the results with the clinical and cytogenetic findings in a cohort of AML cases referred for molecular investigation in our hospital.

Materials and Methods

A total of 33 patients with AML referred to King Abdulaziz University Hospital from 2017 to 2020 were enrolled in the study. They have confirmed cases of AML based on the revised version of the myeloid neoplasms and acute leukemia definition of the WHO 2016 classification. Patients who started the treatment regimen and/or have low-quality extracted DNA were excluded.

The study was conducted in accordance with the Declaration of Helsinki and approved by the Research Bioethics Committee of the Center of Excellence in Genomic Medicine Research (CEGMR). Written informed consent was obtained from patients (or the participant's parent/guardian) before collecting the data.

NPM1 and FLT3-ITD fragment analysis and mutation detection

A total of 5mL of blood/bone marrow were collected on EDTA and sodium heparin tubes for molecular and cytogenetic testing, respectively. Genomic DNA was isolated using the QIAamp DNA Kit (Cat# 51304, Qiagen, Hilden, Germany) following the manufacturer-supplied protocol. Nucleic acid concentration at 260nm and purity ratio at 260/280nm were determined by a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). The extracted DNA samples were stored at -70°C until use. PCR analysis for the extracted DNA was done using GoTaq® Green Master Mix (Cat# M7122, Promega, Madison, USA), which contains two types of identification dyes that allow monitoring of PCR product progress during the gel electrophoresis (https://worldwide.promega.com/resources/protocols/product-information-sheets/g/gotaq-green-master-mix-m712-protocol/). The PCR reaction was carried out in a 25-µL reaction volume containing 10µL GoTaq Green Master Mix and 1.0µL (10 pmol) of each primer, with 100ng genomic DNA completed to the final volume by the DNAsae- free distilled water. Appropriate negative (no template) and positive controls for both genes in each run were applied. The primers applied in the PCR reaction (Table 1) were designed by OligoPerfect™ Designer (ThermoFisher Scientific), available at www.lifetechnologies.com, in CEGMR laboratory.. The forward *FLT3* and the reverse *NPM1* primers were labeled with FAM and HEX dyes, respectively. These dyes are excited by the dual-mode argon laser of the 3500 Series Genetic Analyzer (Applied Biosystems), and the detector identifies the intensity of the emitted fluorescence during the capillary electrophoresis.

Table 1.

The applied primer sequences of the studied NPM1 and FLT3 genes

Gene name	Primer sequences (5′ to 3′)
NPM1-F	GTT TCT TTT TTT TTT TTT CCA GGC TAT TCA AG
NPM1-R	HEX-CAC GGT AGG GAA AGT TCT CAC TCT GC
FLT3-F	FAM-AGC AAT TTA GGT ATG AAA GCC AGC TA
FLT3-R	CTT TCA GCA TTT TGA CGG CAA CC
FLT3-D835H- F	CCG CCA GGA ACG TGC TTG
FLT3-D835H-R	GCA GCC TCA CAT TGC CCC

F: forward; R: reverse; D835H: the first nucleotide G of DNA sequence coding for D835H is substituted with C, resulting in an Aspartate to Histidine amino-acid change.

Capillary electrophoresis and data analysis

After the samples were amplified, each PCR product was loaded on the 3500 Series Genetic Analyzer with an internal lane size standard GeneScan™ 500 LIZ™ Size Standard (Life Technologies, USA) to normalize differences in electrophoretic mobility between injections or gel lanes and to allow for automatic sizing of the products.[32,33] The analyzer performs fragment sizing during data collection, and the data were managed by GeneScan® Analysis Software. The sample electropherograms showed fluorescence intensity as a function of fragment size. Depending on the fragment size, the *NPM1* and *FLT3* wild type (Wt) (170 bp and 330 bp) vs. mutated (170/174 bp and 330/351 bp) alleles, respectively, can be distinguished.

FLT3-ITD$^+$ allelic ratio calculation

The allelic ratio of *FLT3-ITD*$^+$ was calculated by the following formula: [mutated allele peak area/(mutated allele peak area + Wt allele peak area)].[34] The allelic ratio of *FLT3-ITD*+ can be used to specify the prognosis of *FLT3-ITD*$^+$ carrier patients following the ELN recommendations. Patients with a high allelic ratio (≥0.5) have a poor prognosis, while patients with a low allelic ratio (<0.5) have a favorable prognosis, particularly if combined with *NPM1* mutation.[7]

FLT3-TKD$^+$ mutation detection by DNA sequencing

The purified PCR products were cycle sequenced in an ABI 3500/3500XL Genetic analyzer using BigDye® Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on ABI 3730XL Gene Analyzer (Applied Biosystems, USA) following the manufacturer's protocol. Briefly, the master mix for cycle sequencing included 2µl sequencing buffer (5x), 1µl Big Dye Terminator ready

reaction premix, 1µl for either primer (3.2 pmol) (Table 1), and 5µl nuclease-free water to be added to 1µl of purified PCR (10 µl) product. The ready-made pGEM®-3Zf(+) DNA and M13 forward primer (Applied Biosystems) were applied as control during the sequencing analysis. The cycle sequencing conditions were the first stage of initial denaturation at 95°C for 1 min, then 35 cycles each of denaturation at 96°C for 10 sec, annealing at 59°C for 5 sec, and extension at 60°C for 4 min, followed by cooling stage for 10 min at 4 °C. The sequencing results were analyzed by FinchTV Software and compared to the *FLT3* sequence on the ensemble (https://useast.ensembl.org/).

Chromosomal analysis

Karyotyping was carried out for all cases according to the International System for Human Cytogenetic Nomenclature[35] to detect structural and numerical abnormalities using CytoVision Software v3.6 (Leica Biosystems, USA). For each case, 20 metaphases were analyzed, and in the case of mosaicism, the number was raised to 50 metaphases. Based on the cytogenetic findings, the patients were classified into three groups for their prognostic risk.[31]

Statistical analysis was performed using GraphPad Prism 7.0 and *s*tatistical software package SPSS version 20.0 (Armonk, NY: IBM Corp.). The normality of distribution of continuous variables was tested by the Shapiro-Wilk test. Baseline characteristics were summarized as frequencies and percentages for categorical variables. Continuous variables with normal distribution were presented as mean (standard deviation [SD]); non-normal variables were reported as median. Means of 2 continuous normally distributed variables were compared by independent samples Student's t test. Group comparisons with respect to categorical variables are performed using chi-square test. A probability value of $P<0.05$ was considered statistically significant.

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Research Bioethics Committee of the Center of Excellence in Genomic Medicine Research (CEGMR) (approval no. 06-CEGMR-Bioeth2019).

Results

The mean age of AML patients was 36.7±20.6 years, and 63.6% of patients were men (Table 2). According to the French-American-British (FAB) classification, the predominant type in the present cohort was AML-M5, accounting for 30.3%, followed by the AML-M4 at 9.1% and the AML-M3 at 6.1%. AML-M1, M2, M6, and therapy-related AML patients each accounted for 3%, while the remaining 42.4% were not classified.

NPM1 and FLT3 mutational profile

Following a screening of the study participants using fragment analysis and sequencing, the majority of patients (25/75.8%) had wild-type *NPM1/FLT3*, one patient (3.1%) had *FLT3-ITD*$^+$, four (12.1%) patients had *NPM1*$^+$, two (6.1%) had combined *NPM1*$^+$ with *FLT3-ITD*$^+$ and one patient (3.1%) had combined *NPM1*$^+$*/FLT3-ITD*$^+$*/FLT3-TKD*$^+$ profile. Examples of an electropherogram of FLT3 and NPM1 results are shown in Figure 2. The Sequencing result of one FLT3-TKD case is shown in Figure 3. In the combined *NPM1*$^+$*/FLT3-ITD*$^+$ group, the allelic ratio of *FLT3-ITD* was calculated as explained previously, to conclude the prognostic impact of *FLT3-ITD*$^+$. The calculated ratios for the three patients were 0.48, 0.23, and 0.37, respectively.

Cytogenetic study findings

Out of 33 AML patients investigated, 12(36.3%) patients had normal karyotypes (Table 2). The t(8;21) was presented in 9.1% of cases, inv(16) in 6.1%, del(7) in 6.1%, and the other types of cytogenetic abnormalities represented 3.1% of cases for each. The complex karyotype (more than three chromosomal abnormalities) was found in 18.2% of cases (Table 2).

Table 2.
Demographic and clinical data of the study patients with AML

Parameters		n=33
Age	Age (Mean ± SD), years	36.7±20.6
Sex	Male : Female	21:12
CBC	WBC (Mean ± SD), (cell//µL)	32.5±31.7
	Blast (Mean ± SD), count (%)	41.1±32.8
AML Subtypes	M1	1(3.1%)
	M2	1(3.1%)
	M3	2(6.1%)
	M4	3(9.1%)
	M5	10(30.3%)
	M6	1(3.1%)
	Therapy-related AML	1(3.1%)
	Not Classified	14(42.4%)
Cytogenetics	Normal	12(36.3%)
	Complex	6(18.2%)
	t(8;21)	3(9.1%)
	inv(16)	2(6.1%)
	del(7)	2(6.1%)
	t(8;21), -Y	1(3.1%)
	t(8;21), del (9)	1(3.1%)
	t(15;17)	1(3.1%)
	+8	1(3.1%)
	del(13)	1(3.1%)
	t(1;11)	1(3.1%)
	t(18,22), trisomy 22	1(3.1%)
	+9, ins(11)	1(3.1%)

CBC: complete blood count; WBC: white blood cells; complex: more than 3 chromosomes have abnormality; t: translocation; inv: inversion; del: deletion; ins: insertion.

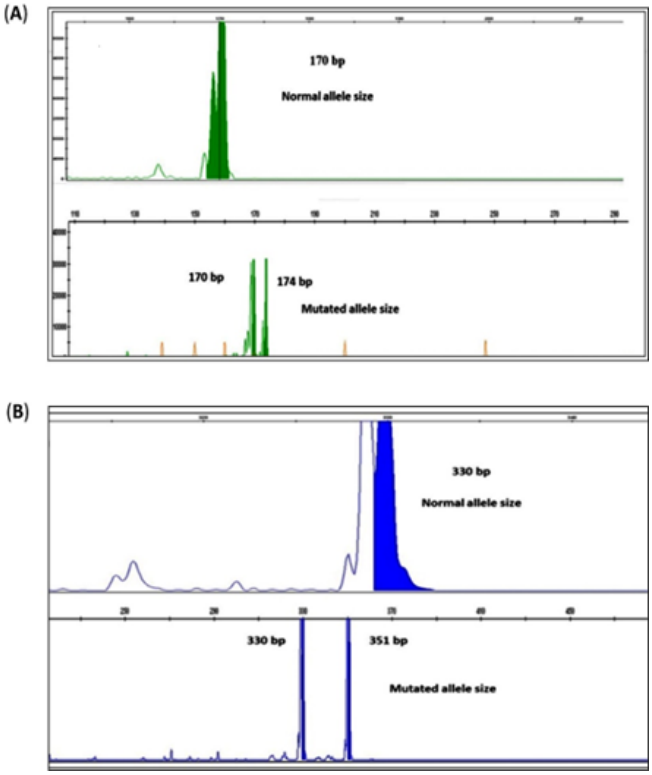

Figure 2. *An example of an electropherogram for NPM1 and FLT3 alleles. (***A***) For NPM1, the electropherogram shows one peak of 170 bp in Wt NPM1 (upper panel) and two peaks of 170 bp and 174 bp in the case of heterozygous NPM1 mutation (lower panel). (***B***) For FLT3-ITD, the electropherogram shows one peak of 330 bp in wild-type (Wt) FLT3 (upper panel) and two peaks of 330 bp and 351 bp in the case of heterozygous FLT3-ITD$^+$ (lower panel).*

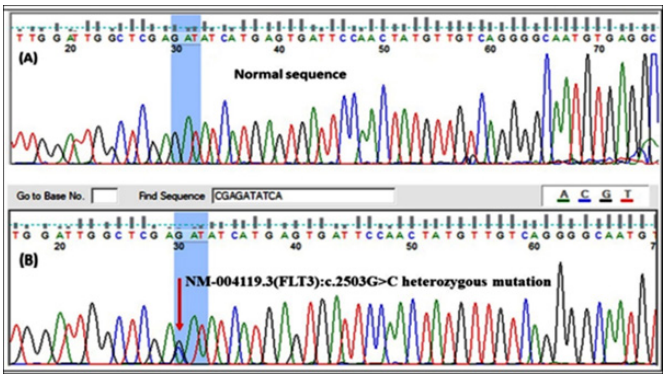

Figure 3. *An example of FLT3-TKD Sequencing results. (A) Normal sequence. (B) Heterozygous FLT3-TKD mutation, in which the first nucleotide G of D835 is substituted with C, resulting in an Aspartate to Histidine amino-acid change (D835H).*

Association of NPM1 and FLT3 mutational profiles with the clinical characteristics

Based on the *NPM1* and *FLT3* mutational profiles of the study participants, patients were classified into four different groups: *NPM1*$^-$/*FLT3*$^-$ (Wt), *NPM1*$^+$, *FLT3*$^+$, and dual positivity group *NPM1*$^+$/*FLT3*$^+$. Carriers of both gene mutant alleles had the highest median age of 42 (a range of 37-63). Males predominated in all subgroups. Carriers of either dual wild or mutant alleles of both study genes exhibited 60% (15/25) and 66.6% (2/3) mortality rates, respectively (Table 3).

Table 3.

The clinical features among different mutational profiles in the study cohort

	NPM1$^-$/ FL3$^-$ n = 25	NPM1$^+$ n = 4	FLT3-ITD$^+$ n = 1	NPM1$^+$/ FLT3-ITD$^+$ n = 3
Median age	35	22.5	14	42
Age range	5-76	9-38	14	37-63
Male: Female	15:10	3:1	1:0	2:1
M1	1(4%)	-	-	-
M2	1(4%)	-	-	-
M3	1(4%)	-	-	1(33.3%)
M4	3(12%)	-	-	-
M5	8(32%)	1(25%)	-	1(33.3%)
M6	1(4%)	-	-	-
Therapy related	-	-	-	-
NOS*	-	1(25%)	-	-
Unknown classification	10(40%)	2(50%)	1(100%)	1(33.3%)
Favorable risk karyotype	7(28%)	1(25%)	-	-
Intermediate risk karyotype	8(32%)	2(50%)	1(100%)	3(100%)
Un-favorable risk karyotype	8(32%)	1(25%)	-	-
Death	15(60%)	2(50%)	-	2(66.6%)
In remission	7(28%)	1(25%)	1	1(33.3%)
Without remission	3(12%)	1(25%)	-	-

**Not otherwise specified, according to the WHO classification of AML.*

The subgroups were compared according to the CBC results (Figure 4). The dual positivity group (*NPM1*$^+$/*FLT3*$^+$) significantly had a higher WBC count with a median of 81.3×10^3/μL compared to other groups: Wt (20.9×10^3/μL), P=0.008; *NPM1*$^+$ (16.26×10^3/μL), P=0.036. Also, the FLT3$^+$ subgroup showed a significantly higher WBC median than the *NPM1*$^+$ subgroup (P=0.029) (Figure 4A).

Furthermore, the combined mutations subgroup (*NPM1*$^+$/*FLT3*$^+$) significantly had a higher blast count (88.5%) in the peripheral blood compared to other subgroups (Wt subgroup (34%), P=0.024; *FLT3*$^+$ (39%), P=0.009; NPM1$^+$ (35%), P=0.049) (Figure 4B). The *NPM1*$^+$ subgroup had the highest Hb level among the other subgroups, and there was a significant difference between the Wt subgroup and *NPM1*$^+$ subgroup (P=0.002) (Figure 4E). Regarding the count of RBCs and platelets, there were no significant differences between the different subgroups (Figure 4C&D).

The clinical association between the mutations and the cytogenetic findings

A total of 63.6% of patients had abnormal cytogenetics. The most common abnormality was complex karyotype, found in the Wt-*NPM1*$^-$/*FLT3*$^-$ group only (Table 4). The *FLT3-ITD*$^+$ patient had trisomy 8. The *NPM1*$^+$ group had two patients with normal karyotype, one patient with t(8;21), and one patient with del(13). The *NPM1*$^+$/*FLT3-ITD*$^+$ group had normal karyotypes in all patients (Table 4). The cytogenetic abnormalities of prognostic significant and molecular mutations results are summarized in Table 3.

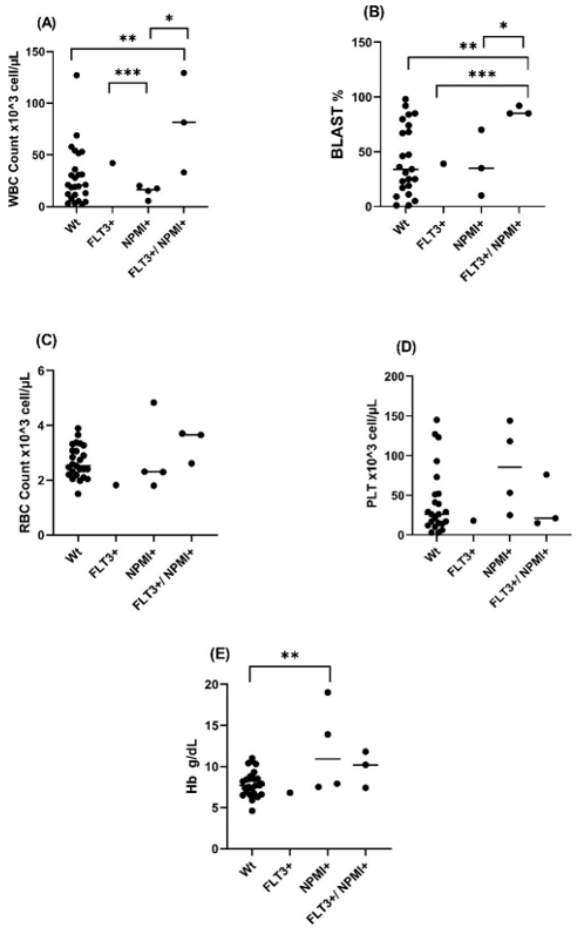

Figure 4. *Comparison of the hematological parameters among the different AML subgroups.* **(A)** *White blood cell (WBC) count, *P=0.036, **P=0.008. ***P=0.029,* **(B)** *Blast percentage, *P=0.049, **P=0.024, ***P=0.009,* **(C)** *Red blood cell (RBC) count,* **(D)** *Platelet (PLT) count, and* **(E)** *Hemoglobin (Hb) concentration, **P=0.002. An unpaired t-test was applied.*

Table 4.

Cytogenetic findings frequencies among different mutation profiles in the study cohort

	NPM1^{-}/ FLT3^{-} n = 25	NPM1^{+} n = 4	FLT3-ITD^{+} n = 1	NPM1^{+}/ FLT3-ITD^{+} n = 3
Normal karyotype	7(28%)	2(50%)	0	3(100%)
Complex karyotype	6(24%)	0	0	0
t(8;21)	2(8%)	1(25%)	0	0
inv(16)	2(8%)	0	0	0
del(7)	2(8%)	0	0	0
t(8;21),-Y	1(4%)	0	0	0
t(8;21), del(9)	1(4%)	0	0	0
t(15;17)	1(4%)	0	0	0
Trisomy 8	0	0	1(100%)	0
del(13)	0	1(25%)	0	0
Trisomy 9, ins(11)	1(4%)	0	0	0
t(1;11)	1(4%)	0	0	0
t(18,22), trisomy 22	1(4%)	0	0	0

Complex: more than 3 chromosomes have abnormality;
t: translocation; inv: inversion; -: loss; del: deletion; ins: insertion.

Discussion

Recent evidence supports the diagnostic/prognostic utility of molecular aberrations for AML cases.[29] The *NPM1* and *FLT3* mutations are the most common genetic mutations in AML, associated with a favorable prognosis in the case of the *NPM1*mutation and a poor prognosis in the *FLT3*.[21,36] Unravelling the prevalence of these mutations in patients with AML is greatly valuable in predicting patients' prognosis, planning for the appropriate therapeutic regimen, and monitoring patients' response to the therapy.[37,38] In this sense, we aimed to explore *NPM1* and *FLT3* mutation frequencies and features in our tertiary health center.

In this study, the most frequent FAB leukemia subtype was M5, which agrees with another study in Jeddah, which indicated that this subtype is common in the Saudi [39] population. Meanwhile, other studies showed M3 as the most frequent AML subtype.[40] In Egypt, Jordan, and Pakistan, M2 was reported to be the most frequent subtype.[40-43]

The *NPM1* mutation was implicated in apoptosis and cell cycle regulation in AML. The related mutation shifts the NPM1 protein, which is essential for the stability of FBW7γ (important in the degradation of the oncoprotein MYC) and ARF (important in the stability of P53), to the cytoplasm with subsequent malignant transformation. This occurs because the mutated NPM will bind and enhance degradation of the above-mentioned proteins, contributing to attenuation of the oncosuppressor pathway and augmentation of oncogenic mechanisms augmentation in the cell.[44]

For the *NPM1* mutation, the frequency in the present study was 21% (including the combined cases with FLT3; 9%). This rate is higher than reported by Alrajeh et al.,[40] with a 12% recorded frequency rate. Other studies reported a range between 6%-48.6% for *NPM1* mutation frequency (Table 5).[45-51] This variation could be attributed to the differences in the ethnicity and epidemiology of AML in different countries.

Table 5.

Prevalence of NPM1 mutations in AML in other studies

Authors	Country	Number of patients	% NPM1 mutation
Alsobhi et al. [39]	Saudi Arabia (Jeddah)	87	11.5%
Alrajeh et al.[40]	Saudi Arabia (Riyadh)	100	12%
Wang et al.[45]	China	76	26.3%
Haferlach et al.[46]	Germany	805	48.6%
Dalal et al.[47]	Canada	83	46%
Daver et al. [48]	USA	145	16%
Chauhan et al.[49]	India	161	21%
Marshall et al.[50]	South African	160	7.5%
Koczkodaj et al.[51]	Poland	50	6%
Rubio et al.[65]	Argentina	216	4.2%

For the *NPM1*$^+$ group, which was reported to have a favorable outcome, the patients showed a poor prognosis, which seems to differ from other studies. However, this finding confirms that AML prognosis is variable and depends on the clinical features of the patients, such as "patient age, performance status, and comorbidities," as well as a constellation of "leukemia-specific genetic features."[52,53]

The second gene studied in the current work is coding for the FLT3 receptor protein. On legend binding, this receptor synergizes with other interleukins and growth factors in the bone marrow to stimulate the progenitor cells to survive, proliferate and differentiate.[54] The pro-survival function of FLT3 may be mediated by phosphorylation of the proapoptotic BAD protein, by induction of the anti-apoptotic BCL-2, or by preventing the induction of the proapoptotic BAX. FLT3 receptor with ITD initiates the activation of the Ras and PI3K pathways in a similar way to the wild-type receptor, although STAT5 may play a more critical role in *FLT3-ITD*$^+$ signaling.[55] *FLT3-ITD*$^+$ has also been shown to inhibit the Forkhead transcription factor (FOXO3)function, thereby inhibiting induction of the proapoptotic BIM protein.[56]

The *FLT3-ITD* mutation found in exons 14 and 15 of the gene results in the addition of many amino acids in the juxta membrane domain that impact the auto-inhibitory function of the FLT3 receptor. The length of the duplicated region varies from 3bp to 400bp, but always, the resultant transcripts are in-frame. Approximately 20%–30% of AML patients carry the *FLT3-ITD* mutation in the *FLT3* gene, which leads to uncontrolled cellular proliferation, survival, and differentiation through constitutive activation of *FLT3*.[36,57,58]

The total frequency of *FLT3-ITD* in the current study was 12% (including the combined cases with *NPM1*). This number is consistent with other studies that stated *FLT3* more commonly occurs in patients with mutated *NPM1* than Wt *NPM1*.[59,60] Alrajeh et al.[40] and Gari et al.[61] reported frequencies of 9% (9/100) and 11.6% (15/129), respectively. In contrast, Alsobhi et al.[39] reported a frequency of 4.6% (4/87) (Table 6). However, the frequency was lower (2.3%) in Jastaniah et al.[62] The reason for this discrepancy could be due to the difference in sample size and the age of the study participants as the later study, for example, enrolled children only. The study frequency for *FLT3-ITD*$^+$ was lower than in other countries. The reported range in the literature was between 8% and 47%. This difference could be due to the variable epidemiology of AML in different countries. (Table 6).[41,45-51,63-68]

The patient with the *FLT3-ITD*$^+$ mutation alone had trisomy and was in remission. Different factors can impact the prognosis of patients with the *FLT3-ITD*$^+$ mutation, including patient age, the number of *FLT3* mutations the same patient has, co-excising mutations, and the type of chemotherapy used.[58] The patient with the *FLT3-ITD*$^+$ mutation in the current study was 14 years old. In elderly patients with AML carrying the *FLT3-ITD*$^+$ mutation, the survival rate is low due to many factors, including the increased risk of having high-risk cytogenetic abnormalities (i.e., monosomy 5, monosomy 7, or complex karyotypes) or resistant disease and poor performance status.[48] However, here, it is difficult to determine the mutation impact based on only one patient, and it can be considered a limitation in the current study.

Table 6.

Summary of the prevalence of FLT3 mutations in patients with AML in other studies

Authors	Country	Number of patients	% FLT3
Alsobhi et al.[39]	Saudi Arabia (Jeddah)	87	4.6% ITD
Alrajeh et al.[40]	Saudi Arabia (Riyadh)	100	9 % ITD
Ishfaq et al.[41]	Pakistan	55	13.3% ITD
Wang et al.[45]	China	76	19.7% ITD
Haferlach et al.[46]	Germany	805	27.2% ITD
Dalal et al.[47]	Canada	83	47%
Daver et al.[48]	USA	390	12% ITD
Chauhan et al.[49]	India	161	25% ITD
Marshall et al.[50]	South Africa	160	12% ITD
Koczkodaj et al.[51]	Poland	50	8% ITD
Gari et al.[61]	Saudi Arabia (Jeddah)	129	11.62% ITD 8.5% TKD
Zaker et al.[63]	Iran	212	18% ITD
Stirewalt et al.[64]	USA	140	34 % ITD
Rubio et al.[65]	Argentina	216	10.2% ITD 7.9% TKD
Kumsaen et al.[66]	Thailand	52	19.2 % ITD
Perry et al.[67]	France	126	20.6% ITD 12.7% TKD
Kandeel et al.[68]	Egypt	257	21.7 % ITD
Fröhling et al.[72]	Germany	523	32% ITD 14% TKD
Bacher et al.[73]	Germany	3082	4.8% TKD

The prognosis of *NPM1*$^+$*/FLT3-ITD*$^+$ patients was determined according to the calculated *FLT3-ITD*$^+$ mutated allelic ratio, which showed low allelic ratios for all patients. In contrast to Lyu et al.,[34] who considered a low ratio a good prognosis, two patients with a low allelic ratio died in our study. This event agrees with others, where a low mutated allelic ratio was not associated with a favorable prognosis.[7,34,69] Further studies for patients with *NPM1*$^+$*/FLT3-ITD*$^+$ mutations are recommended to investigate the impact of allelic ratio on patients' prognosis.

The second most common type of *FLT3* mutation is found on exon 20, and it is a missense point mutation that occurs within the activation loop of the TKD of the receptor. This mutation is found in 5%-10% of the patients with AML and currently has no clinically significant impact.[36]

In the present study, only one patient had the *FLT3-TKD* mutation, and interestingly, it was combined with mutated *NPM1*$^+$*/FLT3-ITD*$^+$. There was no adverse effect of the three mutations on the prognosis, as the patient was in remission status. A study by Boddu et al. found that the effect of combining the triple mutations has equivalent survival. Further studies are also recommended to investigate the effect of this type of three combined mutations.[70]

For the *FLT3-TKD* mutation, the frequency was 3%, combined with *NPM1*$^+$ and *FLT3-ITD*$^+$. In Saudi Arabia, three studies for *FLT3-TKD* were found, and one of these studies was for AML in childhood, in which the frequency was 4.2%.[62] The second study included only five AML patients, and the rate was

40%.[71] Gari's group found *FLT3-TKD* in 8%.[61] Internationally, the rate was as follows: Argentina -7.9%, France - 12.7%, and Germany - 4.8% and 14% in two different studies (Table 6).[72,73]

By comparing the available laboratory parameters among patient subgroups with different genetic profiles, we found that the WBC count and blast percentage were high in the *NPM1*$^+$/*FLT3-ITD*$^+$ and *FLT3-ITD*$^+$ subgroups. Similarly, Marshal and colleagues had the same findings and traced this increment to the presence of the *FLT3* mutation, which results in continuous proliferation and a decrease in the apoptosis of cells.[50,59]

The chromosomal analysis showed abnormal karyotypes in 63.6% of the cases. The most frequent karyotype result was the complex karyotype (18%). In Alrajeh's study,[40] the abnormal karyotype percentage was 64%, which is very close to our percentage; however, the difference was that the most frequent cytogenetic abnormalities were trisomy 8 and trisomy 21, representing 8% for each. Another study,[39] reported t(15;17) and complex karyotype as the most frequent abnormalities, with 17.2% and 13.8%, respectively.

Although the small sample size could limit the present study, the findings reflected our patients' genetic profiles regarding two common mutations that are helpful to be incorporated into the routine workup of the patients to pave the way for personalized medicine in AML in this region.

It is recommended that patients of intermediate risk group, based on karyotyping analysis with Wt-*NPM1/FLT3* genes, be further subjected to screening of other molecular mutations with prognostic significance, such as *IDH1*, *IDH2*, *WT1*, and *c-KIT*.[31,36,74]

Conclusion

The prevalence of *FLT3* was found to be similar to that of other studies conducted in Saudi Arabia and lower than found in international studies. However, *NPM1* frequency was higher than in other Saudi studies. The *FLT3* mutation was primarily found combined with the *NPM1* mutation. Furthermore, the *FLT3* mutation was associated with increased WBC and blast percentages. Although AML with non-mutated *FLT3* or *NPM1* was the most prominent genetic subgroup of the present cohort, the *NPM1*$^+$/*FLT3-ITD*$^+$ subgroup was associated with normal karyotype, which agrees with other studies. The complex karyotype is the most frequent cytogenetic abnormality in the study cohort. Further molecular work for other gene mutations with prognostic significance in AML is strongly recommended to complete the big picture.

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Competing Interests

The authors declare that they have no competing interests.

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