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Characterization of the FLT3 Mutation in Newly Diagnosed Patients with Acute Myeloid Leukemia

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ABSTRACT

Background: The FMS-like tyrosine kinase 3 mutation is one of the most prevalent recurrent genetic aberrations in acute myeloid leukemia (AML). Two modules of mutation are subsumed under the term FLT3 mutation, including internal tandem duplication (ITD) within the juxtamembrane domain and point mutations in the tyrosine kinase domain (TKD).
Objectives: To determine the frequency of FLT3 mutations in adults with newly diagnosed AML and to find any correlation between the existence of FLT3 mutations and the clinical or laboratory findings of the disease.
Materials and methods: Fifty newly diagnosed AML patients were investigated prospectively in this study. A complete blood count, peripheral blood film, bone marrow aspirate, and flow cytometry study were conducted for each patient. The detection of FLT3 mutations was performed using next-generation sequencing technology.
Results: Out of 50 AML patients, FLT3 mutations were detected in 12 (24%). 8 (16%) of them expressed FLT3-ITD, whereas FLT3-TKD was expressed in 4 (8%). There was a non-significant rise in the medians of the total leucocyte count and the peripheral blood blast percentage in both FLT3-ITD and FLT3-TKD mutated patients compared to those without mutation (P-value = 0.15, 0.28, 0.74, and 0.66, respectively). Most cases of both FLT3-ITD and FLT3-TKD mutations were female (65.8% and 75%, respectively). The majority of FLT3-ITD mutations were found in the FAB classification M5 subtype (37.5%), followed by the M1 subtype (25%). FLT3-TKD mutations were mostly detected in the M3 subtype (50%), followed by M4 and M5 (25%).
Conclusion: The FLT3 mutations were frequently seen in patients with AML disease. They were associated with a higher white blood cell count and peripheral blood blast percentage; therefore, they are considered a predictive and prognostic marker in AML disease.
Keywords: Acute Myeloid Leukemia; Gene mutation; FLT3-ITD; FLT3-TKD; Next-generation sequencing .

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INTRODUCTION

Acute myeloid leukemia (AML) is an abnormal clonal rapid proliferative disease of the bone marrow myelogenous hematopoietic stem cells (HSCs). Genetically, this cancerous disease is a diverse group of disorders marked by the rapid, unchecked growth of hematopoietic precursor cells that don't work properly, along with the stopping of differentiation of these precursor cells into mature cells [1–3].

The FLT3 trans-membrane receptor tyrosine kinase is made from the FLT3 gene, which is found on chromosome 13 band q12. Only myeloid and lymphoid progenitor cells express FLT3 in a lineage-restricted manner, which is triggered by the FLT3 ligand (FL) [4].

After binding FL with the FLT3 receptor, this complex leads to active conformational changes and dimerization of the receptor. As a result, kinetic phosphorylation of its tyrosine residues. FLT3 receptor phosphorylation occurs within 5–15 minutes, then internalization and degradation occur af-

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ter 20 minutes from the stimulation. The phosphorylation of tyrosine residues initiates the activation of three main kinds of signaling cascade pathways: RAS/MAPK/ERK, PI3K/AKT/mTOR, and JAK/STAT5. These signaling pathways play a fundamental role in the enhancement of the growth, development, and survival of hematopoietic progenitor cells [5, 6].

Internal tandem duplications (ITDs) within the structure of the juxtamembrane domain and point mutations in the tyrosine kinase domain (TKD) are the two principal types of FLT3 mutations. These mutations cause FLT3 signaling to be constantly active, which causes leukemic cells to proliferate abnormally, differentiate poorly, and survive longer. A higher white blood cell (WBC) count, a greater blast proportion, and a higher chance of recurrence only represent some other adverse clinical characteristics linked to FLT3 mutations. Additionally, they are a significant independent prognostic factor for patients with AML who have poor overall survival and lower remission rates [7].

About 25–30% of AML patients have mutations in the FLT3 gene. The effect of this mutation on treatment and prognosis has been thoroughly investigated and disputed ever since it was first identified in AML more than 25 years ago. The incidence rises with age; however, the mutation appears to be less common in the oldest patients (> 60 years) [8]. Accordingly, FLT3 mutations have a significant effect on the prognosis and treatment of AML patients, and due to the lack of relevant local research in our city (Nineveh province), we conducted this study. The objective of this study was to determine the distribution of the FLT3 mutations among adult patients with AML disease and their impacts on the clinical and laboratory characteristics of the disease.

MATERIALS AND METHODS

Patients

This prospective analytic case-series study was approved by the Ethics Committee of the Nineveh Health Directorate Training and Development Center, Ministry of Health in Iraq, (protocol number 2023039 issued on March 1, 2023). The study was conducted on 50 recently diagnosed adult AML patients who attended Ibn-Sina Teaching Hospital in Mosul between the 1st of October 2022 and the 26th of September 2023. We included only the recently diagnosed adult ($\geq$ 18 years old) patients who gave their consent to participate. We excluded the child age (< 18 years old) group, previously diagnosed patients, FLT3 inhibitors-treated patients, relapsed AML patients, and those who declined in participate to the current study. Before the study was established, all participants were informed, and their signed agreement was obtained.

We calculated the sample size according to this formula: n = Z^2 pq/e^2. Where the n is the sample size, Z is the standard deviation corresponding to the confidence level, which is 1.96 at 95%, p is the expected frequency (20% according to a previous study [9]), q is the complement of the expected frequency (1-20%), and e is the precision; we took it at 10%, so the equation will be as follow: n = $1.96^2\times$ 0.2$\times$ 0.8/0.1^2. Therefore, n = 61. However, our sample size was 50 because we couldn't collect the desired sample within the specified duration of the study.

Detailed information was obtained from each patient concerning the gender, age, and symptoms of the present illness. For each enrolled patient, 3 mL of peripheral blood was aspirated and preserved in an EDTA tube for analysis of hematological indices, which is performed at the laboratory department of the hospital. Another 3 mL of bone marrow aspirate or peripheral blood if the sampling from the bone marrow was difficult were obtained from each patient at the time of diagnosis, which were preserved in EDTA tubes and stored in a deep freeze (-20°C) until the day of FLT3 gene analysis. The flow cytometry result was obtained from the case files that were performed in the Hematology Centre of the Medical City complex in Baghdad City, Iraq.

FLT3 gene mutation analysis

The isolation of genomic DNA (gDNA) from the samples was done using a DNA extraction kit (Thermofisher^{TM}, USA). Digestion and cell lysis were done by adding 20 μL of proteinase K solution and 200 μL of genomic lysis buffer solution. After a sequence of washing procedures, gDNA samples were obtained by elution using spin columns, which were supplied with the kit. Evaluation of the gDNA quality was carried out by a spectrophotometric nanodrop device (Biometrica^{TM}, Taiwan). Highly purified with a suitable concentration of gDNA, the exons 13–15 and 20 of the FLT3 gene were amplified by polymerase chain reaction (PCR) performed by a thermal cycler (SimpliAmp^{TM}, Singapore).

The PCR reaction of 20 μL in total volume is composed of 10 μL of PCR Master Mix (2X), 1 μL of forward primer and 1 μL of reverse primer, and 8 μL of purified extracted gDNA sample with a desired dilution of 25 ng. The primer sequences designed by (Oligomer Biotechnology©, Turkey) are as follows: (FLT3-ITD forward primer: 5'-GT CGA GCA GTA CTC TAA ACA-3', FLT3-ITD reverse primer: 5'-TC CTA GTA CCT TCC CAA ACT-3', FLT3-TKD D835Y forward primer: 5'-CCG CCA GGA ACG TGC TT-3', and FLT3-TKD D835Y reverse primer: 5'-GCA GAC GGG CAT TGC CC-3'). The thermal cycler program was set according to the manufacturer's instructions illustrated in Table 1.

PCR products belonging to each sample were mixed to obtain PCR pools. PCR pools were qualified by electrophoresis on a 2% agarose gel. Adequate samples were purified using the NucleoFast 96 PCR kit (MACHEREY-NAGEL GmbH).

Library preparation was performed using the NexteraXT DNA Library Preparation Kit (Illumina Inc., USA). A specific sequence of adaptors and indices oligonucleotides were ligated at both ends of amplicons. Next-generation sequencing of the samples was carried out using the Miseq system (Illumina Inc., USA) with paired-end reads per fragment of 150 base pairs.

Raw reading data was gathered in FASTq format. The

Table 1. Genomic DNA amplification thermal cycler program.

Process description	Temperature (°C)	Time (minutes)	Cycles
Initial Denaturation	95	10:00	1
Denaturation	95	00:45	
Annealing	60	00:45	45
Extension	72	00:45	
Final Extension	72	10:00	1
Storage (Hold)	12	∞	1

data were analyzed on Integrated Genomics Viewer version 2.3 software (Broad Institute). Raw reads were aligned to Human Genome 19 using the BWA algorithm MEM (0.7.17) [10]. Variant calling was made using two separate algorithms: GATK Unified Genotyper and GATK Haplotype Caller, which were both used to complement each other [11].

Statistical analysis

Statistical analysis was performed using the Windows version of IBM-SPSS (Statistical Package for the Social Sciences) 26.0 (IMB Inc., Armonk, NY, USA). Shapiro-Wilk analysis verified the normality of these data. For the determination of statistical significance differences between continuous variables, we used the Mann-Whitney U test, and the results of these variables were expressed in the median (range). The Pearson Chi-square test was used for qualitative variables, which were expressed in numbers or frequencies. A Pearson correlation test was used to assess the relationship between FLT3 mutations and clinical or laboratory variables among each mutation sub-group. Statistically significant events had a P-value < 0.05.

RESULTS

For the 50 AML-enrolled patients, the median age was 48 (age range 18–83) years. FLT3 gene mutations were identified in 12 (24%) patients; 8 (16%) of patients harboured the FLT3-ITD mutation, whereas the FLT3-TKD mutation was found in 4 (8%) of them. The incidence of both FLT3 mutation sub-groups was insignificantly decreased with the advance of age for FLT3-ITD and FLT3-TKD (P-value = 0.30, P-value = 0.44, respectively). Female predominance was noticed in both the FLT3-ITD mutation (65.8%) and the FLT3-TKD mutation (75%). The lab results showed that the FLT3 mutant patients had a higher WBC count and a higher percentage of peripheral blood blasts (PBBs) compared to the FLT3-WT patients, but the difference was not statistically significant. The median WBC count in FLT3-TKD patients was slightly higher than in FLT3-WT patients, going from 12.62×10^9/L to 14.69×10^9/L (P-value = 0.74). In contrast, the median WBC count in FLT3-ITD patients was the highest, at 26.2×10^9/L, which was also not statistically significant (P-value = 0.15). The median PBB percentages of FLT3-ITD and FLT3-TKD were 65% and 63.5%, respectively. Both were somewhat higher than those of patients with FLT3-WT, where the median PBB percentage was 59%. However, the pairwise differences were not significant (P-value = 0.28, P-value = 0.66, respectively). The Hb levels of FLT3-ITD mutant patients went from 8.15 g/dL in FLT3 non-mutated patients to 8.9 g/dL, which was not statistically significant (P-value = 0.26). On the other hand, the median Hb level in FLT3-TKD mutant patients was 8.05 g/dL, which was also not statistically significant (P-value = 0.91). The median platelet count was higher in FLT3-TKD mutant patients (68×10^9/L) and slightly lower in FLT3-ITD mutant patients (44.5×10^9/L) compared to the median platelet count in FLT3-WT patients (47×10^9/L). However, neither of these differences were statistically significant (P-value = 0.11 for FLT3-TKD and 0.85 for FLT3-ITD). Concerning the distribution of FLT3 mutations among FAB subtypes, FLT3 mutations were not identified in the M7 subtype in this study. With an incidence of 37.5%, the study showed that M5 is predominant in FLT3-ITD mutant patients, with M1 coming in second (25%), and M0 coming in third. Both M3 and M4 had an incidence of 12.5% for each remnant subtype. The M3 was the most frequent subtype in FLT3-TKD mutant patients, with an incidence of 50% followed, by M5 and M6, with an incidence of 25%, respectively, for each subtype. However, none of them revealed a significant difference (P-value = 0.29), as shown in Table 2.

This study did not show any observable correlation between the existence of FLT3 mutations and age, and hematological parameters (P-value > 0.05), which are demonstrated in Table 3 and Table 4.

DISCUSSION

AML is a quite heterogeneous and particularly aggressive hematological malignant illness associated with higher risks of treatment failure and dismal survival outcomes [12]. Larger cohort studies revealed that around one-third of patients with AML disease expressed FLT3 gene mutations [13, 14]. 12 (24%) of the 50 AML patients who were identified at in this study had changes found in the FLT3 gene. Of these, 8 (16%) had the FLT3-ITD mutation and 4 (8%) had the FLT3-TKD mutation. FLT3 mutation incidence was analogous with many other studies that reported the incidence of FLT3 mutations, which were 24%, 24%, 25.13%, 25.4%, and 26% in the United States, Indian, German, Japanese, and Syrian patients, respectively [15–19]. However, the incidence was higher in Iran (31.8%) [20], Turkey (34.6%) [21], and Malaysia (34.2%) [22]. These slight discrepancies in FLT3 mutation incidence may be attributed to the ethnic and geographical diversities in each study.

The incidence of the FLT3 mutations in the present study was insignificantly decreased with age which, was comparable to the study conducted by Sarojam et al. [16]. The actual cause of these various FLT3 mutations with age is not fully understood, although it might be linked to the interaction between the FLT3 mutation and other genetic or environmental variables that impact the initial development and progression of AML, which may have different frequencies or effects in different age groups [23]. Female gender predominates in both ITD and TKD mutations; the results were in concurrence with other studies [17, 22, 24, 25].

According to the current study, FLT3-ITD mutations were more frequently distributed in the FAB M5 subtype. This result was consistent with previous studies by Hamed et al. [26] and Sarojam et al. [16]. The FLT3-TKD mutation was found in only four patient samples; two of them expressed the FAB M3 subtype, and the other two patients expressed FAB M4 and M5, respectively. FAB M5 and FAB M3 were the two most prevalent subtypes in FLT3-TKD mutant patients, according to a sizable cohort study by Bacher et al. [27]. This may be attributed to the ethnic and geographical differences among the patients in the different study samples.

FLT3 mutant receptors were found to be constitutively stimulated in a manner independent of the FLT3 ligands, which enhances the proliferation and survival of the aberrant leukemic blast cells [28]. Initial inspection of the WBC count in patients with AML is considered a prognostic marker [29].

This study found that the mutated FLT3-ITD patients had a non-insignificant rise in the median WBC count and the PBB percentage compared to those without mutation. These results agreed with other previous studies [30, 31], although the increment in the PBB percentage in these studies was significant (P-value = 0.013, P-value = 0.04, respectively). This may be attributed to the ethnic variation or may be due to the differences in the assay methods.

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Table 2. Clinical and laboratory characteristics of the 50 patients with acute myeloid leukemia*.

Variables	All (n = 50)	FLT3-WT (n = 38)	FLT3-ITD (n = 8)	P-value (ITD vs WT)	FLT-TKD (n = 4)	P-value (TKD vs WT)
Median age (range) years	48 (18-83)	49.5 (18-83)	35 (28-64)	0.25	49.5 (22-60)	0.68
Age > 50 No.(%)	21 (42)	21 (55.3)	2 (25)	0.30	1 (25)	0.44
Age ≤ 50 No. (%)	29 (58)	17 (44.7)	6 (75)		3 (75)	
Gender No. (%)						
Male	29 (58)	25 (37.5)	3 (34.2)	0.13	1 (25)	0.11
Female	21 (42)	13 (62.5)	5 (65.8)		3 (75)	
Median Hb (range) mg/dL	8.5 (4.3-13.3)	8.15 (4.3-13.3)	8.9 (6.5-11.7)	0.26	8.05 (7.6-9)	0.91
Median WBC count (range) $\times 10^9$/L	15.22 (1.27-204.24)	12.62 (1.27-204.24)	26.2 (12.43-120.4)	0.15	14.69 (7.4-20.34)	0.74
Median Platelet count (range) $\times 10^9$/L	51.5 (1-196)	47 (1-170)	44.5 (10-196)	0.85	68 (52-155)	0.11
Median PBB (range) %	59 (8-95)	55.5 (8-91)	65 (19-95)	0.28	63.5 (20-80)	0.66
FAB classification No. (%)						
M0	3 (6)	2 (5.3)	1 (12.5)	0.35	0	0.29
M1	4 (8)	2 (5.3)	2 (25)		0	
M2	5 (10)	5 (13.2)	0		0	
M3	13 (26)	12 (31.6)	1 (12.5)		2 (50)	
M4	11 (22)	8 (21.1)	1 (12.5)		1 (25)	
M5	12 (24)	8 (21.1)	3 (37.5)		1 (25)	
M6	2 (4)	1 (2.6)	0		0	
M7	-	-	-		-	

* AML: acute myeloid leukemia; FLT3-WT: Fms-like kinase3-wild gene type; FLT3-ITD: Fms-like kinase3-internal tandem duplication; FLT3-TKD: Fms-like kinase3-tyrosine kinase domain; Hb: hemoglobin; WBC: white blood cell; PBB: peripheral blood blast; FAB: French-American-British; No. = number.

Table 3. Correlation of the FLT3-ITD mutation with age and hematological parameters*† .

ITD mutation	Correlation Coefficient value (r)	Approximate T-test value*	P-value†
Age /years	0.153	1.074	0.28
Hemoglobin level g/dL	– 0.176	– 1.238	0.22
White blood cell count $\times 10^6$/L	– 0.227	– 1.614	0.11
Platelet count $\times 10^6$/L	0.057	0.394	0.69
Peripheral blood blast cells %	– 0.148	– 1.034	0.30

* Using the asymptotic standard error assuming the null hypothesis.
† Based on the normal approximation.

Table 4. Correlation of the FLT3-TKD mutation with age and hematological parameters*† .

ITD mutation	Correlation Coefficient value (r)	Approximate T-test value*	P-value†
Age /years	0.041	0.284	0.77
Hemoglobin level g/dL	0.028	0.195	0.84
White blood cell count $\times 10^6$/L	0.018	0.124	0.90
Platelet count $\times 10^6$/L	– 0.230	– 1.637	0.10
Peripheral blood blast cells %	– 0.041	– 0.284	0.77

* Using the asymptotic standard error assuming the null hypothesis.
† Based on the normal approximation.

The current study also found that the median WBC count and blast cell percentage were higher in mutated FLT3-TKD patients compared to other patients with FLT3-WT. This is similar to what Sarojam et al. found, but the differences were not statistically significant (P-value < 0.001 for WBC count and P-value = 0.012 for blast cell percentage) [16]. This rise in the median WBC count and PBB percentage in FLT3 mutated patients may be because these mutations can cause FLT3 receptors to be constitutively active through ligand-independent dimerization, which allows leukemic cells

to proliferate autonomously, leading to leukocytosis [5].

The present study showed that both sub-groups of the FLT3 mutation had no obvious correlation between gender, age, or other hematological parameters and the presence of the FLT3 mutation. Previous studies observed that the clinical presentation of AML disease cannot be attributed to FLT3 aberrant activation [19, 32].

However, it is imperative to recognize some limitations that we confronted which impinge on the application of this study. Firstly, only a small number of the patients can meet the inclusion criteria of the study, which takes a lot of time and effort. Secondly, the bone marrow aspiration kit is not always available in the hospital, which compels us to exclude some patients who are already meeting the inclusion criteria. Lastly, the unavailability of the next-generation sequencing device in Nineveh province requires a lot of effort to preserve and transport the patients' samples. A larger study encompassing all age groups is recommended to provide more illuminating information about the distribution of FLT3 mutations and their clinical and laboratory characteristics in Iraq.

CONCLUSION

FLT3 mutations were found in a quarter of the patients with AML disease investigated in the study. FLT3 mutations occur more in the female gender. FLT3-ITD mutations were mostly distributed in the FAB M5 subtype, while FLT3-TKD mutations were mostly found in the FAB M3 subtype. It is applicable to routinely check for these mutations in newly diagnosed AML patients since they are associated with a higher WBC count and peripheral blood blast percentage.

ETHICAL DECLARATIONS

Acknowledgments

None.

Ethics Approval and Consent to Participate

The study was approved by the Ethics Committee of the Nineveh Health Directorate Training and Development Center, Ministry of Health in Iraq, (protocol number 2023039 issued on March 1, 2023). Informed consent was obtained from each participant.

Consent for Publication

No personal data included.

Availability of Data and Material

The datasets produced and/or analyzed during the present study can be obtained from the corresponding author upon reasonable request.

Competing Interests

The authors declare that there is no conflict of interest.

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Authors' Contributions

All the listed authors have made significant, direct, and intellectual contributions to the work and have approved it for publication.

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