Evaluation of FLT3-ITD Mutation in Fifty Newly Diagnosed Iraqi Patients with Acute Myeloid Leukemia

Luay Attallah Fahad* , Zahraa Marwan Al-Omar* , Khalid Naf`i AL-Kheroo**
***Department of Pathology, College of Medicine, University of Mosul , ** Department of Internal Medicine, College of Medicine, University of Mosul, Mosul, Iraq**
Correspondence: loaiattallah@gmail.com

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ABSTRACT

Background: FMS-like tyrosine kinase-3 internal tandem duplication mutation (FLT3-ITD) ranked as the most ubiquitous sub-group of the FLT3 genetic aberration and was identified in about 20 to 30 percent of the entire acute myeloid leukemia (AML) cases. The patients harboring FLT3-ITD gene mutation carry dismal prognostic parameters.

Objective: This study is intended to investigate the distribution of clinical and laboratory characteristics in patients with AML, determine the incidence of FLT3-ITD mutations in adult newly diagnosed patients with AML, and compare the baseline characteristics between the FLT3-ITD mutated and FLT3 non-mutated cases.

Methods: In this study, fifty adult patients with newly diagnosed AML were prospectively studied. Every participant was investigated for peripheral blood film, bone marrow aspirate film, flow cytometry study, and complete blood count. To discover FLT3-ITD mutations, next-generation sequencing technique was used.

Results: Out of 50 AML patients, FLT3-ITD mutation was detected in 8 (16%) of the patients. The mean age of the FLT3-ITD mutation patients was lower than that of the non-mutant individuals. FLT3-ITD mutation is more likely to occur among females. Most FLT3-ITD mutations were found in the FAB classification M5 subtype (37.5%), followed by the M1 subtype (25%).

Conclusion: The frequency of the FLT3-ITD mutation in patients with AML was 16%. Fever was the most presenting symptom, and splenomegaly was the most presenting sign in patients with AML. FAB M5 was the most frequent subtype in FLT3-ITD mutation. There was non-significant rise in the white blood cell count and peripheral blood blast percentage in FLT3-ITD mutant patients compared with those without mutation.

Keywords: AML, Gene mutation, FLT3-ITD, Next-generation sequencing.

تقييم طفرة FLT3-ITD لدى خمسين مريضاً عراقياً تم تشخيص إصابتهم حديثاً بإبيضاض الدم النقوي الحاد

لؤي عطا الله فهد*، زهراء مروان العمر*، خالد نافع الخيرو**
* فرع علم الأمراض، كلية الطب، جامعة الموصل ، ** فرع الطب الباطني، كلية الطب، جامعة الموصل، الموصل، العراق

الخلاصة

خلفية البحث: تم تصنيف طفرة التضاعف الترادفي الداخلي الشبيهة ببروتين FMS التيروزين كيناز-٣ (FLT3-ITD) على أنها المجموعة الأكثر انتشارًا في الانحراف الوراثي FLT3 وتم تحديدها في حوالي ٢٠ إلى ٣٠ بالمائة من حالات ابيضاض الدم النقوي الحاد (AML). المرضى الذين لديهم طفرة FLT3-ITD يحملون مؤشرات تنبؤية مزرية.

الاهداف: تهدف هذه الدراسة إلى دراسة توزيع الخصائص السريرية والمخبرية لدى المرضى الذين يعانون من ابيضاض الدم النقوي الحاد، وتحديد مدى انتشار طفرات FLT3-ITD لدى المرضى البالغين الذين تم تشخيص إصابتهم حديثًا بمرض ابيضاض الدم النقوي الحاد، ومقارنة الخصائص الأساسية بين المرضى حاملي طفرة FLT3-ITD و غير حاملي طفرة FLT3.

طُرائق البحث: في هذه الدراسة، تمت دراسة ذات أثر استباقي لخمسين مريضًا بالغًا مصابين بإبيضاض الدم النقوي الحاد الذين تم تشخيصهم حديثًا. تم فحص كل مشارك لفيلم الدم المحيطي، وفيلم نخاع الدم العظمي، ودراسة قياس التدفق الخلوي، وتعداد الدم الكامل. لاكتشاف طفرات FLT3-ITD، تم استخدام تقنية الجيل التالي لتحديد التسلسل الجيني.

النتائج: من بين ٥٠ مريض مصاب بإبيضاض الدم النقوي الحاد، تم اكتشاف طفرة FLT3-ITD في ٨ (١٦%) من المرضى. كان متوسط عمر مرضى طفرة FLT3-ITD أقل من متوسط عمر الأفراد غير الحاملين للطفرة. من المرجح أن تحدث طفرة FLT3-ITD اكثر احتمالاً بين الإناث. تم العثور على معظم طفرات FLT3-ITD في النوع الفرعي M5 من تصنيف FAB (٣٧.٥%)، يليه النوع الفرعي M1 (٢٥%).
الاستنتاج: كان تواتر طفرة FLT3-ITD في المرضى الذين يعانون من ابيضاض الدم النقوي الحاد ١٦٪. كانت الحمى هي أكثر الأعراض ظهوراً، وكان تضخم الطحال هو العلامة الأكثر ظهوراً لدى مرضى المصابين ابيضاض الدم النقوي الحاد. كان FAB M5 هو النوع الفرعي الأكثر شيوعًا في طفرة FLT3-ITD. كان هناك ارتفاع طفيف في عدد خلايا الدم البيضاء ونسبة الخلايا الجذعية في الدم المحيطي لدى المرضى الذين يعانون من طفرة FLT3-ITD مقارنة مع أولئك الذين ليس لديهم طفرة.

الكلمات المفتاحية : ابيضاض الدم النقوي الحاد، طفرة جينية، طفرة FLT3-ITD، تسلسل الجيل القادم.

INTRODUCTION

Hematopoiesis is an essential process that keeps the body's supply of immune and blood cells in a constant state throughout life. This is done by special stem cells within the bone marrow with unique self-renewal capacity called hematopoietic stem cells (HSCs), which are responsible for replenishing all kinds of these required cells. The hematopoiesis process is controlled by signals from various chemokines that are found in the microenvironment, which preserve the physiological balance between differentiation and renewal of the HSCs [1,2].

One of these signals is the FMS-like tyrosine kinase-3 ligand (FLT3L) and its receptor. Through their cooperative interactions with several other chemokines, they are essential to every step of hematopoiesis, and any aberrant regulatory activity can lead to the development of numerous cancer types [3].

Transcription of the FLT3 transmembrane receptor tyrosine kinase occurs in the FLT3 gene, which is located in chromosome 13 band q12. The expression of the FLT3 receptor is confined to myeloid and lymphoid progenitor cells and is triggered by the FLT3L [4].

Acute myeloid leukemia is an abnormal clonal rapid proliferative disease of the bone marrow myelogenous HSCs. This malignant disease is genetically defined as a heterogeneous group of disorders characterized by fast, progressive, uncontrolled proliferation of dysfunctional hematopoietic precursor cells with differentiation arrest of these precursor cells towards the mature cells [5-7].

FLT3-ITD gene mutations are considered the most significant genetic abnormality in AML disease and are found in around 20% to 30% of the entire cases of the disease [8].

The majority of kinase FLT3 receptor mutations (70%) are ITD mutations, and their molecular constructor has been extensively researched. In-frame amino acid sequence duplications and insertion of an unpredictable length of base pair fragment comprising three to a few hundred base pairs in the region that encodes for the juxtamembrane domain (JMD) are the primary impacts of ITDs on the FLT3 gene's exons 14 and 15 [9-11].

The exact reason behind FLT3-ITD's duplicated segment insertion events and in-frame sequence duplication is still undefined [12,13].

Conformational alterations in the JMD structure have the potential to disrupt the kinase activity's auto-inhibitory regulatory mechanism, which in turn stimulates kinase domain activation by constitutively activating the FLT3 receptor dimerization process in a ligand-independent manner [14,15].

Compared to FLT3-ITD non-mutated AML patients, patients with FLT3-ITD mutations had greater recurrence rates and a worse prognosis. They also experience shorter remission periods [16]. Considering this study, the purpose of this research was to examine the clinical characteristics of adult AML patients as well as the distribution of FLT3-ITD mutations and their effects on the disease's laboratory and clinical features.

Method

Patients

Fifty newly diagnosed adult AML patients who attended Ibn-Sina Teaching Hospital in Mosul City between October 2022 and August 2023 were the target population of this prospective case series analytic study which was subjected to the research committee of the Ninevah Health Directorate, protocol number (2023039). Patients with AML <18 years old, relapsed or FLT3 inhibitor-treated AML patients are not included in the current study. All participants were notified before the study's establishment, and their written consent was acquired. For each patient, a three-milliliter sample was taken from the peripheral blood and stored in an EDTA tube for hematological parameter analysis, which is carried out at the hospital's

laboratory department at the time of each patient's initial AML disease presentation. An additional 3 ml samples of bone marrow aspirate or peripheral blood were obtained if the bone marrow aspirate sample was insufficient (dry tap); these were preserved in EDTA tubes and kept in a deep freeze (-20°C) until the FLT3-ITD gene analysis day. The flow cytometry reports were documented from the patients' records and carried out at the Medical City complex's Hematology Centre in Baghdad City.

FLT3-ITD Gene Detection Method

Thermofisher™ DNA extraction kit (USA) was utilized to isolate genomic DNA (gDNA) from the samples. To break up the cells and digest them, 200µl of the genomic lysis buffer solution and 20µl of the proteinase K solution were added. Following a series of washing steps, gDNA samples were extracted using spin columns that were included with the kit. We used a spectrophotometric nanodrop instrument (Biometrica™, Taiwan) to evaluate the purity of the gDNA. PCR was used to amplify exons 13–15 of the FLT3-ITD gene, and the product was highly purified with an appropriate quantity of gDNA using a thermal cycler (SimpliAmp™, Singapore).

A 20µl total volume PCR reaction is made up of 10µl of PCR Master Mix (2X), 1µl each of forward and reverse primers (Oligomer Biotechnology©, Turkey), and 8µl of a combination of nuclease-free distilled water and purified extracted gDNA material, mixed until the gDNA achieves the necessary dilution of 25 ng. The following primer sequences are used: FLT3-ITD reverse primer: 5'-TC CTA GTA CCT TCC CAA ACT-3', and FLT3-ITD forward primer: 5'-GT CGA GCA GTA CTC TAA ACA-3'. Following the manufacturer instructions, the thermal cycler plan was set. See Table 1.

Table 1. Thermal cycler program for genomic DNA amplification:

Process description	Temperature (^{0}C)	Time(minutes)	Cycles
Initial Denaturation	95	10:00	1
Denaturation	95	00:45	45
Annealing	60	00:45	
Extension	72	00:45	
Final Extension	72	10:00	1
Hold	12	∞	1

PCR products for each sample were mixed to create the PCR pools. A 2% agarose gel electrophoresis was used to qualify the PCR pool products. NucleoFast® 96 PCR kit (MACHEREY-NAGEL GmbH) was used to purify the high-quality samples.

Utilizing the NexteraXT DNA Library Preparation Kit (Illumina Inc., USA), library preparation was carried out. At both ends of the amplicons, a particular sequence of oligonucleotides was ligated to serve as an index and an adaptor. Sequencing of the samples was done using the Miseq system (Illumina Inc., USA) using 150 base pair paired-end readings for each fragment.

Raw reading data was collected in FASTq format. The data were examined using Integrated Genomics Viewer (IGV) version 2.3 software (Broad Institute). The BWA algorithm MEM (0.7.17) was used to align raw reads to Human Genome 19 [17]. Two distinct methods were utilized to make the variant calls: GATK Haplotype Caller and GATK Unified Genotyper, which worked in conjunction to provide complementary results [18].

Statistical Analysis

The IBM-SPSS 26.0 (IMB Inc., Armonk, NY, USA) Windows version was used for statistical analysis. For the determination of statistical significance differences between quantitative numerical variables, we used the T-test for independent samples, and the results of these variables were expressed in mean and standard deviation. The Pearson chi-square test was used for qualitative nominal variables, which were expressed in numbers or frequencies. Events of statistical significance have a P-value less than 0.05.

RESULTS

Fifty adult patients with newly diagnosed AML made up the research population. The mean age of participants was 47.64 ± 17.110 years. Male patients represent the majority of those enrolled in this study (58%), with a male-to-female ratio of 1.38:1 (29 men to 21 women). The data analysis of FLT3-ITD mutation on IGV software is illustrated in Fig. 1. The FLT3-ITD gene mutation in the study population group was identified in 8 (16%) patients. Female predominance was noticed in the FLT3-ITD mutation, with an incidence of 62% (P = 0.200). FLT3-ITD-mutated AML patients were found to be insignificantly younger than those without mutation (P = 0.314). A detailed FLT3-ITD mutation characteristic concerning the clinical presentation is illustrated in Table 2.

Concerning the distribution of FLT3-ITD among FAB classification subtypes, the study did not find any FLT3 mutations in the M7 subtype. The

mutation was mostly found in patients with M5 37.5%, followed by M1 25%, and lastly by M0, M3, and M4 with a frequency of 12.5% evenly for each subtype (P = 0.300). Furthermore, the most common presenting symptom in AML patients was fever (40%), followed by pallor (28%), whereas the most common presenting symptom in FLT3-ITD mutated patients was fever (50%), followed by bleeding tendency (37.5%), with no specific relation to other patients without FLT3-ITD mutation (P = 0.529, P = 0.248, respectively). Moreover, the most common presenting sign in AML patients was splenomegaly (34%). Splenomegaly was also the most presented sign in FLT3-ITD mutated patients (62.5%) with no specific relation to other patients without the FLT3-ITD mutation (P = 0.063).

Table 2. Relation of FLT3-ITD mutation to clinical presentation of AML disease.

Clinical presentation		FLT3-ITD negative		FLT3-ITD positive		Total AML	%	P-value
		No.	%	No.	%			
Gender	Male	26	61.9	3	37	29	58	0.200
	Female	16	38.1	5	62	21	42	
Age/Years		48.71±17.499		42±14.579		50	100	0.314
		FLT3-ITD negative		FLT3-ITD positive		All AML patients		
FAB classification	Subtypes	No.	%	No.	%	Total	%	0.300
	M0	2	4.76	1	12.5	3	6	
	M1	2	4.76	2	25	4	8	
	M2	5	11.9	0	0	5	10	
	M3	12	28.5	1	12.5	13	26	
	M4	10	23.8	1	12.5	12	24	
	M5	9	21.42	3	37.5	12	18	
	M6	2	4.76	0	0	٢	4	
		FLT3-ITD negative		FLT3-ITD positive		All AML patients		
Clinical symptoms & signs		No.	%	No.	%	Total	%	
Fever		16	38.1	4	50	20	40	0.529
Pallor		12	28.6	2	25	14	28	0.837
Bleeding tendency		8	19	3	37.5	11	22	0.248
Lymphadenopathy		9	21.4	2	25	11	22	-
Joint pain		9	21.4	2	25	11	22	0.823
Weight loss		11	26.2	2	25	13	26	0.944
Skin rash		3	7.1	0	0	3	6	0.363
Hepatomegaly		14	33.3	2	25	16	32	0.643
Splenomegaly		12	28.6	5	62.5	17	34	0.063
Total		42		8		50	100	-

AML; Acute Myeloid Leukemia; FAB: French-American-British; No.: Number; %: Percentage.

Regarding the relationship of the hematological parameters between the patients harboring FLT3-ITD and other AML patients without mutation that enrolled in the current study (Table 3), the means of white blood cell count, hemoglobin level, platelet count, and peripheral blood blast percentage in patients harboring FLT3-ITD mutation were insignificantly higher than other patients without mutation (P = 0.861, P =0.256, P = 0.954, and P = 0.411, respectively).

Table 3. Hematological characteristics of FLT3-ITD mutation in patients with AML

Hematological parameters	FLT3-ITD negative	FLT3-ITD positive	P-value
Mean Hemoglobin level mg/dL	8.45±2.123	9.37±1.856	0.256
Mean White blood cell count × 10^9/L	38.89±51.704	42.26±36.521	0.861
Mean Platelet count × 10^9/L	59.07±44.100	60.12±60.567	0.954
Mean Peripheral blood blast %	50.00±27.934	59.25±34.254	0.411
Total	42	8	-

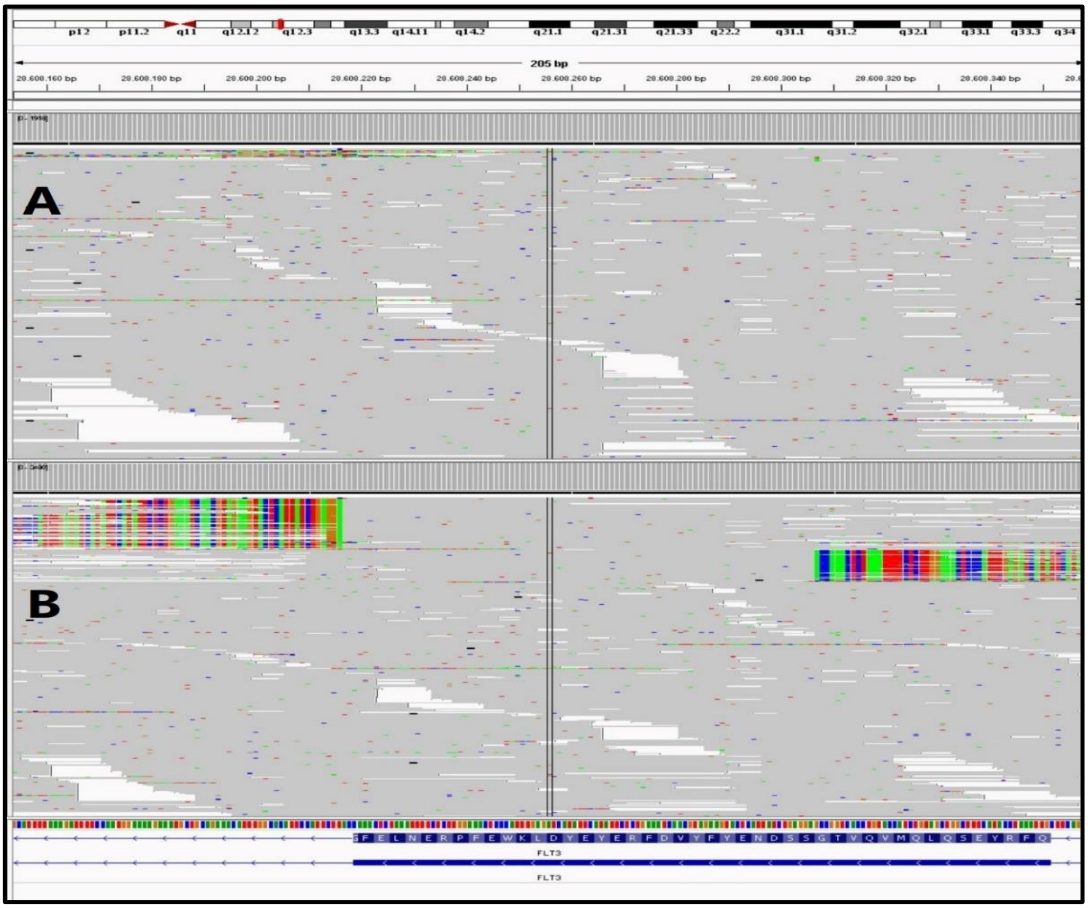

Figure 1. Integrative Genomics Viewer (IGV) images of the next-generation sequencing (NGS) data of the FLT3-ITD variant A: FLT3-ITD mutation is negative; B: FLT3-ITD mutation is positive.

DISCUSSION

About 13 to 35 percent of all patients with AML disease might develop a genetic aberration known as FLT3-ITD, which has a substantial impact on the clinical prognosis for patients afflicted with acute myeloid leukemia. The outcome of the FLT3-ITD mutation impact on these patients is dismal [19].

Leukocytosis and a higher proportion of blast cells in the bone marrow and peripheral blood of AML patients are strongly related to the FLT3 mutation [20].

In the current study, the mean age of patients with AML was 47.64 ± 17.110 years, 58% of the AML patients were males with a male-to-female ratio of 1.38:1, and these results agreed with most of the other regional Iraqi studies in Baghdad City [21-24].

FLT3-ITD gene mutation in the present study was found in 8 out of 50 AML patients with an incidence of 16%, and it was similar to other regional studies with an incidence of 17.4% and 14.54%, respectively [24,25]. However, other regional study in Baghdad City reported a lower incidence of FLT3-ITD gene mutation with an incidence of 1.88% in patients with AML [26]; this may be explained by using different methods for gene detection and the diversity of distribution of the FLT3-ITD mutation among cases in the different studies. FLT3-ITD mutation incidence was in concurrence with studies in other countries that reported the

incidence of FLT3-ITD gene mutations, which were 16.1%, 17.8%, 18.3%, and 18.6% in Malaysian, Turkish, Japanese, and German AML patients, respectively [27-30]. However, other studies conducted in different countries showed a higher incidence of FLT3-ITD gene mutation among AML patients, with an incidence of 24%, 25.9%, 25%, and 28% in Syrian, Iranian, Turkish, and Chinese patients, respectively [8,31-33]; this rise in the FLT3-ITD mutation incidence may be due to geographical and ethnic diversities.

According to this study, AML patients harboring FLT3-ITD mutation were found to be insignificantly younger than those without the mutation (P = 0.314); this concept was consistent with other previous studies [25,34]. This could potentially be attributed to the interplay between the FLT3 mutation and additional genetic or environmental factors that influence the early onset and advancement of AML. The magnitude of these effects or frequencies may vary across age groups [35]. Moreover, females have a higher chance of forming the FLT3-ITD mutation, and this matches with the other applied researches [24,27,30].

The most prevalent symptom and sign in FLT3-ITD-mutated AML patients were fever and splenomegaly. However, this study showed that the existence of the FLT3-ITD mutation had no significant effect on the clinical picture of patients with AML disease, which is parallel to Dhahir et al. study [25].

In terms of the FAB classification, this study found that FLT3-ITD mutations were most common in the M5 subtype (37.5%), followed by the M1 subtype (25%). According to studies conducted by Hamed et al. and Sarojam et al., patients with FLT3-ITD mutations exhibited FAB M5 as the most frequent subtype, with a frequency of 50% and 25.4%, respectively. M2 subtype was the next frequent subtype, with a frequency of 20% and 23.9%, respectively [24,36]. The diversity of the studies' sample sizes and ethnic composition may help to explain these differences.

This research found that the peripheral blood of mutant FLT3-ITD patients had an insignificant rise in the means of blast cell percentage and total leucocyte count when compared to FLT3-ITD non-mutated patients (P = 0.411, P = 0.861, respectively). These findings are also consistent with the Dhahir et al. study [25]. However, Sarojam et al. showed that the rise of the peripheral blast cell percentage and total leucocyte count in FLT3-ITD mutated patients reach the significant level (P = 0.012, P < 0.01, respectively) [36]. This might be attributed to the ethnic diversity or could be a result of variances in testing techniques.

CONCLUSION

The study reports that among adult AML patients from Iraq, the incidence of the FLT3-ITD mutation was 16%, and since it was not significantly linked with a higher total leucocyte count or peripheral blood blast cell percent. There is no specific relationship established between the occurrence of the FLT3-ITD mutation and gender, age, hematological parameters, FAB classification, or the clinical picture of the AML disease.

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Conflict of Interest

The authors state that they have no conflicts of interest to disclose.

Authors Contribution

This work has been written and approved by all the authors.

ETHICAL DECLARATIONS

Ethics Approval and Consent to Participate

The research is subjected to the Ethical Committee the Ninevah Health Directorate Training and Development Center, Ministry of Health in Iraq. All participants in the current study obtained informed consent following Helsinki's declaration of ethical standards in 1975, which was revised in 2008.

Consent for Publication

The authors declare that there is no conflict of interest to disclose.

Informed Consent

All participants received informed and written consent was taken before the establishment of the study.

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Study Registration

Not required.

Authors' Contributions

All the authors are responsible for writing and approving this manuscript.

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