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ORIGINAL ARTICLE

Fms-Like Tyrosine Kinase 3 (Internal Tandem Duplication) Gene Mutation in Acute Myeloid Leukemia Patients And Its Association With Cd34.

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ABSTRACT

Background: Acute myeloid leukemia (AML) is a heterogeneous disorder characterized by clonal expansion of myeloid progenitors in the bone marrow and peripheral blood **Objectives:** This study aimed to assess the role of FMS- like tyrosine kinase 3- Internal Tandem Duplication (FLT3 -ITD) gene mutation in AML patients and to find out the impact of the CD34 expression in these cases.

Subjects & Method: 30 adult newly diagnosed AML patients were included in this study. They were 20 males and 10 females, their ages ranged from 18-60 years with median age of 45.6 years for molecular detection of FLT3-ITD gene mutation by PCR technique.

Results: FAB subtypes of the cases revealed that the most prevalent FAB subtype was M5, M2, M4 presented by (50%, 30%, 20%), respectively. Patients were classified into favorable (6.6%), intermediate (80%) and unfavorable (13.3%) according to cytogenetic abnormalities. Mean value of CD34% (± SD) among cases was 45.53±25.32 with range (5-91), while it was 42.52±29.9 with range (5-91) among FLT3-ITD positive cases and 44.21±22.67 with range (16-88) among FLT3-ITD negative cases with no significant statistical relation between FLT3-ITD gene and CD34, P (0.672). CD34 positivity among FLT3-ITD positive cases showed 9 cases (75%) CD34% positive and 3 cases (25%) CD34% negative while among FLT3-ITD negative cases there were 15 cases (83.3%) CD34% positive and 3 cases (16.7%) CD34% negative, with no significant statistical relation. As regard the outcome, among FLT3-ITD positive cases 5 cases (41.7%) were relapsed and 7 cases (58.3%) died. On the other hand among FLT3-ITD negative cases one case (5.6%) was relapsed and 11 cases (61.1%) died, There is significant elevation in the relapsed cases among the FLT3-ITD positive cases in relation to the negative group with (P = 0.025), while there is no significant relation between FLT3-ITD gene and death with (P = 0.879). Kaplen Meier survival analysis in respect to FLT3-ITD shows that patients with FLT3-ITD gene negative had better median overall survival with (p <0.05) FLT3-ITD positivity contributes to a short CR duration and lower CR rate.

Conclusion: The bad prognosis of FLT3-ITD mutation is the most effective factor in determination of the line of treatment due to high incidence of relapse in FLT3-ITD positive cases.

Key words: Acute myeloid leukemia, FMS-like tyrosine kinase 3, CD34..

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by the accumulation of immature myeloid progenitor cells in the bone marrow, compromising of normal blood cell production and ultimately resulting in bone marrow failure. [(1)].

The growth and differentiation of hematopoietic cells is governed by the concerted action of growth factors and their receptors. One of these receptors, FMS-like tyrosine kinase 3 (FLT3), also called stem cell kinase 1 (STK1) or fetal liver kinase 2 (FLK2), belongs to the group of class 3 receptor tyrosine kinases, together with other growth factor receptors such as c-Kit, PDGF-R, and c-FMS [(2)].

FMS-like tyrosine kinase 3 (FLT3), a member of the type III receptor tyrosine kinase family is expressed in about 90% of leukemic blasts of patients with acute myeloid leukemia (AML). The human gene for FLT3 is found in the chromosomal region 13q12.2, where it expands through approximately 97.3 Kb and gives rise to a 3842-bp transcript. FLT3 mutations occur in approximately one-third of patients with AML. Constitutive activation of the FLT3 receptor tyrosine kinase, either by internal tandem duplication (ITD) of the juxtamembrane region or by point mutations in the second tyrosine kinase domain (TKD), has been described in patients with acute myelogenous leukemia (AML). In-frame duplications of 3 to >400 base pairs (bp), known as internal tandem duplications (ITDs), are the most common, occurring in up to 30% of adult patients with de novo AML [(3)].

In acute myeloid leukemia (AML), further prognostic determinants are required in addition to cytogenetics to predict patients at increased risk of relapse. Studies have indicated that an internal tandem duplication (ITD) in the FLT3 gene may adversely affect clinical outcome [(4)].

Immunophenotyping has previously been demonstrated as an independent factor for risk stratification of AML among surface antigens studied, CD34 antigen which exhibited poor prognosis in adult patients with AML. However, it is still unclear whether CD34 expression on the blasts has independent prognostic value for patients with FLT3 +AML [(5)]. So the aim of the study is to assess the role of FLT3 (ITD) gene mutation in AML patients and to find out the impact of the CD34 expression in these cases.

SUBJECTS AND METHODS

Site of study: This study was conducted in Clinical Pathology and Medical Oncology Departments, Faculty of Medicine, Zagazig University Hospitals during the period from November 2017 to November 2018.

Sample size: A comprehensive sample of thirty subjects were included in this study. Adult patients with newly diagnosed AML. They were 20 males and 10 females. Their ages ranged from 18-60 years with median age of 45.6 years. Written informed consent was obtained from all participants, the study was approved by the research ethical committee of Faculty of Medicine, Zagazig University. The study was done according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Inclusion criteria: Age > 16 years old and patient diagnosed as de novo AML before receiving induction chemotherapy.

Exclusion criteria: Age < 16 or >60 years old, previously treated AML patients, promyelocytic leukemia (M3) or other malignancy.

Treatment plan: Patients were treated by an induction regimen 3&7 regimen consisting of continuous infusion cytarabine (100 mg/ m2) daily for 7 consecutive days combined with 3 days of doxorubicin (30 mg/m2). Patients with 60 years or poor performance status were treated by 2+5 (cytarabine 100 mg/m2 daily for 5 combined with 3 days of doxorubicin 25 mg/m2) regimen or low dose cytarabine 10 mg/m2 / 12 hours for 14 days.

Methods:Clinical assessment: by complete history taking, including: Age, sex, symptoms of anemia, fever, bleeding tendency, bone aches and history of previous treatment, together with the onset and duration of the clinical course and clinical examination was done particularly for fever, purpura, bruising, gum bleeding, lymphadenopathy and organomegally.

Routine laboratory Investigations including Complete blood count (CBC): Was done on automated cell counter, model XS 500i (Sysmex, Japan), together with examination of Leishman stained peripheral blood smears for differential leucocytic count. Liver, kidney functions tests and Lactate dehydrogenase were done by using cobas 8000 autoanalyser (Roche diagnostics, Germany). Bone marrow aspiration and examination: using Leishman and cytochemical stained smears was done.

Specific laboratory investigations including: Immunophenotyping by flowcytometry: using Bectron Dickenson Facs Calibar device to detect the following markers (MPO, CD13, CD33, HLA-DR, TdT, CD14, CD64, CD34, CD3, CD20 and CD22). Cytogenetic analysis: karyotyping by G banding technique using image analyser Imstar (Paris, France), karyotyping was done according to International System for Human Cytogenetic Nomenclature (ISCN). Molecular detection of FLT3-ITD gene mutation included the following steps: Firstly; Genomic DNA was extracted from peripheral blood leucocytes of EDTA anticoagulant blood by using GeneJET genomic DNA purification kit (Mini Kit) contains the proteinase K solution, lysis solution, wash buffer I, wash buffer II, elution buffer and collection tubes.

Then; DNA was amplified by primers designed to detect the gene mutation using DreamTaq Green PCR Master Mix (2X) and primers were provided as lyophilized agents with forward primer 5′-GCA ATT TAG GTA TGA AAG CCA GC-3′ and reverse primer 5′-CTT TCA GCA TTT TGA CGG CAA CC-3 and the thermal cycle was obtained by thermal cycler Gene Amp, PCR system 2400 (Perkin Elmer, USA). Finally; PCR amplification products were then detected by electrophoresis on 2% agarose gel containing ethidium bromide and were visualized by ultraviolet light transillumination as shown in **(figure 1)**

Statistical analysis: Data collected throughout history, basic clinical examination, laboratory investigations and outcome measures coded, entered and analyzed using Microsoft Excel software. Data were then imported into Statistical Package for the Social Sciences (SPSS version 20.0) (Statistical Package for the Social Sciences) software for analysis. According to the type of data qualitative represent as number and percentage , quantitative continues group represent by mean ± SD , the following tests were used to test differences for significance;. difference and association of qualitative variable by Chi square test (X^2) . Differences between quantitative independent groups by t test or Mann Whitney, paired by sign test. Chi-Square testX^2 was used to test the association variables for categorical data.

RESULT

Our patients were classified into favorable (6.6%), intermediate (80%) and unfavorable (13.3%) according to cytogenetic abnormalities [6] **(table 1)**. In this study FAB subtypes of the cases revealed that the most prevalent FAB subtype was M5 which was represented by 15 cases (50%). M2 and M4 represented by 9 and 6 cases with a percentage of (30% and 20%) respectively. Laboratory data of the studied cases showed mean value of TLC (± SD) 52.35±37.39 with range (11-157),mean value of HB (± SD) was 8.16±1.42 with range (4.4-10.7), mean value of Platelets (± SD) was 46.37±49.29 with range (5-188), mean value of BM blast% (± SD) was 63.77±17.63 with range (26-95), mean value of CD34% (± SD) was 45.53±25.32 with range (5-91),mean value of ESR (± SD) was 84.8±31.81 with range (21-140),mean value of LDH (± SD) was 724.96±361.7 with range (250-1840) **(Table 2).**

Mean value of CD34% (± SD) among cases was 45.53±25.32 with median 35.85 and range (5-91). The relation between FLT3-ITD gene and CD34 marker positivity is represented in **(table 3).** The mean value (± SD) of CD34% expression among FLT3-ITD positive cases was 42.52±29.9 with median 30.85 and range (5-91) and the mean value (± SD) of CD34% expression among FLT3-ITD negative cases was 44.21±22.67 with median 39.3and range (16-88). There is non-significant relation between FLT3-ITD gene and CD34 marker with P value (0.672). As regard the relation between FLT3-ITD gene and CD34 positivity among FLT3-ITD positive cases there were 9 cases (75%) CD34% positive and 3 cases (25%) CD34% negative while among FLT3-ITD negative cases there were 15 cases (83.3%) CD34% positive and 3 cases (16.7%) CD34% negative, there is no significant relation between FLT3-ITD gene and CD34 positivity with P value (0.6) **(Table 4)**, Among FLT3-ITD positive cases 5 cases (41.7%) underwent relapse, 7 cases (58.3%) did not, 7 cases (58.3%) died and 5 cases (41.7%) are alive on the other hand among FLT3-ITD negative cases one case (5.6%) underwent relapse, 17 cases (94.4%) did not, 11 cases (61.1%) died and 7 cases (38.9%) are alive, There is significant elevation in the relapse cases among the FLT3-ITD positive cases in relation to the negative group with P value (0.025) while there is no significant relation between FLT3-ITD gene and death with p value (0.879) **(Table 5) (Figure 2)**.

Table (1): Cytogenetic risk categories among AML patients:

Cytogenetics:	AML patients (No=30)	
	No	**%**
Normal	**22**	**73.3**
Abnormal	**8**	**26.7**
Risk		
Favorable	**2**	**6.6**
t (8,21)	2	6.6
Intermediate	**24**	**80**
Normal	22	73.3
Absent Y	2	6.6
Adverse:	**4**	**13.3**
t (9,22)	2	6.6
del 7	2	6.6

Table (2): Laboratory findings of the studied cases:

Variable	
TLC 10^3/dl:	
Mean ± SD	52.35±37.39
Median	53
Range	11-157
HB g/dl:	
Mean ± SD	8.16±1.42
Median	8.4
Range	4.4-10.7
Platelets 10^3/dl:	
Mean ± SD	46.37±49.29
Median	28
Range	5-188
BM blast%:	
Mean ± SD	63.77±17.63
Median	65.5
Range	26-95
CD34%:	
Mean ± SD	45.53±25.32
Median	35.85
Range	5-91
CD34% positivity:	
Positive	24 cases (80%)
Negative	6 cases (20%)
ESR mm/h:	
Mean ± SD	84.8±31.81
Median	84
Range	21-140
LDH IU/L:	
Mean ± SD	724.96±361.7
Median	682
Range	250-1840

Table (3): The relation between FLT3-ITD gene and CD34 percentage:

Variable	FLT3-ITD+ve No (12)	FLT3-ITD-ve No (18)	MW	P value
CD34%				
Mean± SD	42.52±29.9	44.21±22.67	98	0.672
Median	30.85	39.3		
Range	5-91	16-88		

MW is for Mann Whitney test

Table (4) shows the relation between FLT3-ITD gene and CD34 positivity

Variable	FLT3-ITD+ve No (12)		FLT3-ITD-ve No (18)		$\chi 2$	P value
CD34%	**No**	**%**	**No**	**%**		
Positive	**9**	**75%**	**15**	**83.3%**	**Fisher**	**0.6**
Negative	**3**	**25%**	**3**	**16.7%**	**test**	

NB: the cutoff of CD34% in blast cells for positivity is 10% [17]

Table (5) shows the outcomes of the studied cases in relation to FLT3-ITD gene

Variable	FLT3-ITD+ve No (12)		FLT3-ITD-ve No (18)		P value
	No.	**%**	**No.**	**%**	
Relapse:					
No Yes	7 5	58.3 41.7	17 1	94.4 5.6	**0.025** **(S)**
Death:					
Alive Dead	5 7	41.7 58.3	7 11	38.9 61.1	0.879

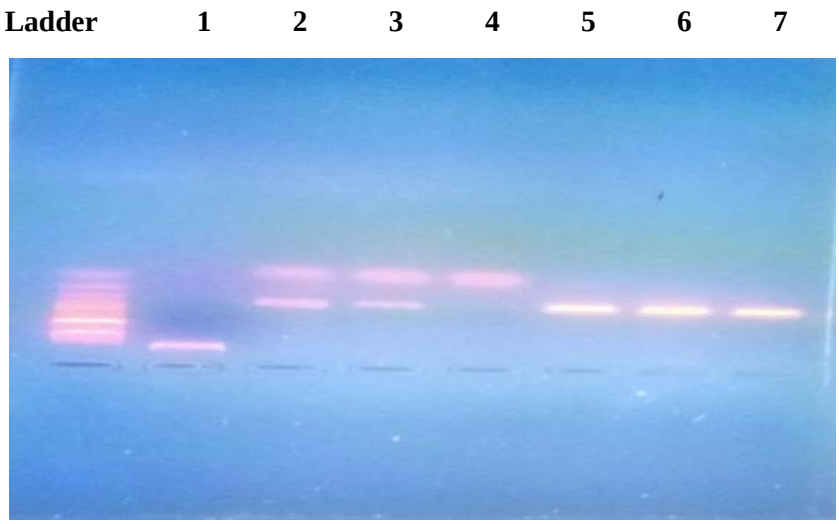

Figure (1) Gel electrophoresis of PCR technique of amplified FLT3-ITD gene mutation in AML patients. Ladder: 100bp, Lane 1: represent extracted DNA , Lane 2,3,4: represent positive FLT3-ITD , Lane 5,6,7: represent the 329bp fragments in absence of FLT3-ITD.

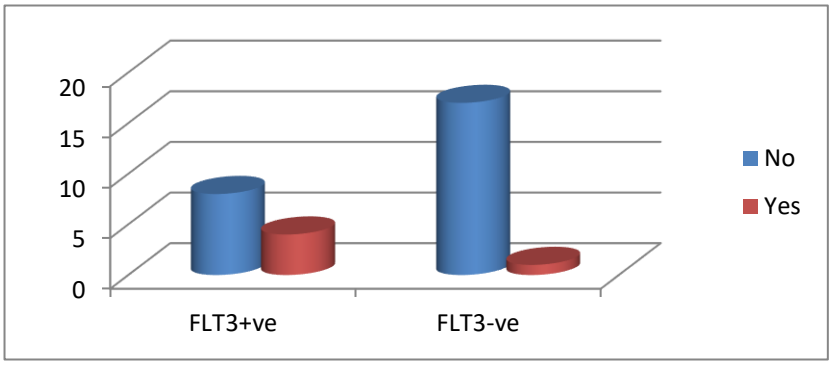

Fig (2) Relation between FLT3-ITD gene and relapse.

DISCUSSION

AML is the most common form of leukemia in adults accounting for approximately a third of all leukemias worldwide [(7)].

Despite the advancement in treatment options for AML, its prognosis is very variable, ranging from survival of few days to complete remission and cure. Many patients die either from complications of intensive chemotherapy, resistance to the current treatment options or they experience relapse after initial response to traditional chemotherapy [(8)].

FLT3 is a member of the class III "split kinase domain" family of receptor tyrosine kinases (RTKs) which also includes PDGFR, KIT, and FMS. [(9)].

FLT3 signaling activates intracellular pathways that promote proliferation and inhibition of apoptosis. The most common FLT3 mutation described in AML is the internal tandem duplication (ITD) mutation of the juxtamembrane segment, can be generated by the in frame insertion of 18 to more than 100 bp within the juxtamembrane region of FLT3. This mutation

leads to loss of the autoinhibition exerted by the juxtamembrane domain over the tyrosine kinase domain, generating a constitutively active FLT3 molecule. [(10)].

To achieve our aim, immunophenotyping and cytogenetic analysis were done for all the patients together with molecular detection of FLT3-ITD gene mutation by using PCR technique.

According to the FAB classification which was based on morphological, cytochemical testing and immunophenotyping, the most common encountered FAB subtype in this study was M5 (50%), followed by M2 (30%) and M4 (20%). These findings are in accordance with the study reported by Roland B and his colleagues who showed that M5 was one of the most commonly encountered subtypes of AML [(11)].

In the current study, cytogenetic abnormalities were detected in 26.6% of patients, while other patients were cytogenetically normal (CN-AML). These findings are in accordance with the study of Marilyn L. Slovak and his colleagues who reported that 62% of the patients in their study were CN-AML [(11)]. Our patients were classified into favorable (6.6%), intermediate (80%) and unfavorable (13.3%) according to cytogenetic abnormalities. The intermediate group was the dominant (80%), which is in agreement with Marilyn L. Slovak and his colleagues. with intermediate risk group 62%[(12)].

In this study, the median CD34% in the bone marrow blast cells was 35.85%, while Jordi Juncà and his colleagues reported CD34% median 53%[(13)].

In this study, Examination of FLT3-ITD gene mutation in AML patients revealed that (60%) of patients are FLT3-ITD negative, while (40%) are positive which is closely related to the study of Derek L. Stirewalt and his colleagues who reported (34%) FLT3-ITD positive cases in their study [(14)].

As regard CD34 positivity in relation to FLT3-ITD. (75%) of FLT3-ITD positive patients were CD34 positive and (83.3%) of FLT3-ITD negative patients were CD34 positive, on the other hand (62.5%) of CD34 positive patients did not show CR while (44.4%) of the subjects who showed CR underwent relapse, while (66.6%) of the CD34 negative patients showed CR and (25%) of them only underwent relapse.

On the other hand, the relation between FLT3-ITD gene mutation and CD34% in blast cells of bone marrow revealed that there is no significant relation between FLT3-ITD and CD34% (P value 0.672) which agrees with HarryDang and his colleagues and Hong-HuZhu and his colleagues who revealed that CD34 expression is a prognostic factor that is independent of FlT3-ITD for relapse, DFS and OS [(15)& (16)].

As regard, one-year DFS for the positive FLT3-ITD expression and Negative FLT3-ITD expression groups was estimated. Kaplan Meier analysis revealed that mean disease-free state was 3.8 months (1.7– 5.8) for the positive FLT3-ITD expression group and 6.02 months (3.6 – 8.3) for the Negative FLT3-ITD expression group which means no significant difference.

CONCLUSION

There are many factors that influence the prognosis of AML including presence of certain markers on the blast cells e.g.CD34 percentage, karyotyping analysis and molecular studies e.g.FLT3-ITD gene mutation, Although the relation between FLT3-ITD and CD34 in Acute myeloid leukemia patients proved to be insignificant in our study, CD34 percentage proved to be important independent prognostic factor in AML patients .The bad prognosis of FLT3-ITD is the most effective factor in determination of the line of treatment due to high incidence of relapse in FLT3-ITD positive cases.

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