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ORIGINAL ARTICLE

Detection of C-kit gene Mutation in Patients with Acute Myeloid Leukemia

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ABSTRACT

Background: *Acute Myelogenous Leukemia (AML) is a malignant disease of haematopoietic stem cells. C-kit is a Tyrosine Kinase Receptor class III (RTK) which is expressed on early hematopoietic progenitor cells and shares in hematopoietic stem cell proliferation, differentiation and survival. c-kit is a proto-oncogene, so activating c-kit mutations may contribute in the pathogenesis of leukemia in humans. Exon 11 of c-Kit gene is the frequent site for mutations in different kinds of tumors.* ***Objectives:*** *We aim to screen the mutation status of exon 11 of c-kit gene and further evaluation of these mutations as a prognostic marker in AML cases.* ***Methodology:*** *To determine the frequency of exon 11 mutations in 60 de novo AML cases, we have done polymerase chain reaction followed by direct DNA sequencing.* ***Results:*** *The c-kit mutations in exon 11 were detected in (14/60) 23.3% in AML cases. Two different missense mutations were detected. Those included Pro121Gln and another mutation, Pro171His. There was significant decrease in the rate of achievement of complete remission (CR) in the mutated group compared to the wild group (P=0.02). In addition, the one year survival (OS) was less in the mutated group compared to the wild group (P=0.02).* ***Conclusion:*** *Mutations in exon 11 of the c-kit gene can be involved in pathogenesis and is a useful predictive genetic marker in AML.*

INTRODUCTION

Acute myeloid leukemia (AML) is a variable group of malignant disorders characterized by blocked differentiation and unregulated proliferation of hematopoietic stem cells with subsequent increase of immature myeloid precursors (blasts) in bone marrow and peripheral blood[1].

Leukemia is the 12th most common type of neoplastic disease and 11th most common cause of cancer-related death worldwide[2]. In Egypt, it is estimated that leukemia causes a death rate of 5.55 per 100 000, therefore, Egypt is ranked as the 5th among countries of the Middle East and the 19th among all countries of the world regarding leukemia-related death[3].

Available data suggest the role of c-kit, a human receptor tyrosine kinase in the pathogenesis of leukemia. Early hematopoietic cells are dependent on c-Kit-mediated signals for their proliferation and survival, hence activating c-kit mutations may contribute in the pathogenesis of leukemia in humans [4]. High level of c-kit has been reported in 60% -80% of AML cases [5].

The c-Kit receptor is a transmembrane protein with an extracellular domain comprised of five immunoglobulin-like repeats, a single Trans Membrane domain, a juxta membrane domain, 2 intracellular Tyrosine Kinase domains and a Kinase Insert domain, and a C-terminal domain [6].

The genomic locus coding for the c-kit gene receptor has 21 exons. The c-kit gene mutations in exon 11 have been implicated in several malignant conditions including gastrointestinal stromal tumors [7], human germ cell tumors [8], and AML [9] and [10]. Prognostic value of c-Kit mutations in core binding protein in AML has been reported in different populations [11]. However, no study so far has reported the frequency of mutations in exon 11 of c-kit gene in AML in Egypt. We have screened the mutation status of exon 11 of c-kit gene and further evaluated these mutations as a prognostic genetic factor in AML cases.

METHODOLOGY

Subjects

We included 60 newly diagnosed AML cases presented to the Medical Oncology Department, Zagazig University, Egypt. Ethical approval was obtained from the institutional ethical committee of Faculty of Medicine, Zagazig University, Egypt. Written consents were obtained from all of the study participants. Clinical data were recorded. Leishman stained blood, bone marrow films were examined and the cases were classified according to the French American British (FAB) criteria [12]. All the 60 AML

cases were categorised as, M1 (n = 4), M2 (n = 28), M3 (n = 8), M4 (n = 14) and M5b (n = 6). Out of 60 AML cases, 30 were males and 30 were females with age ranging from 17years to 75 years. The mean age of cases was 41.7 ± 16.96 years with a median age 39.5 years.

Methods

DNA Extraction

From each patient, 2 ml of venous blood or 1 ml of bone marrow aspirate were obtained under complete aseptic conditions and put in sterile vacutainer containing EDTA as anticoagulant. Specimens were stored at -20^{o}C till used. Using a commercially available DNA extraction kit (QIAGEN, Germany), genomic DNA was extracted.

Polymerase Chain Reaction

To screen for the c-Kit gene mutations, c-Kit gene exon 11 amplification was carried out via a polymerase chain reaction (PCR) using primers c-Kit-F (5-ATTATTAAAAGGTGATCTATTTTTC-3) and c-Kit-R (5-ACTGTTATGTGTACCCAAAAAG-3),[13]. PCR amplification was performed in a 25 ul reaction mixture, with 200 ng of extracted DNA (volume taken was calculated by dividing 200 by the concentration obtained by nanodrop), 1ul of 10pmol of both forward and reverse primers, 12.5 ul of the ready master mix containing (dNTPs, MgCL2, hot startTaq DNA polymerase and Taq buffer) purified distilled water was added to reach the total volume 25 ul. Detection of products was done by a 2 % agarose gel electrophoresis (Figure 1).

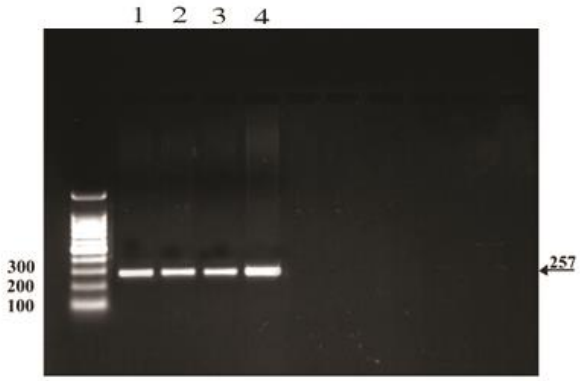

Fig. 1: PCR amplification products of c-Kit gene exon 11 at 257bp by agarose gel electrophoresis. 100bp DNA Ladder in the first lane, cases in the remaining lanes.

Sequencing

Amplicons were sequenced using an automated sequencer, ABI PRISM 3500 Genetic Analyzer (Applied Biosystem, USA). The sequencing results were examined at National Center for Biotechnology Information (NCBI), nucleotide BLAST (Basic Local Alignment Search Tool) web site for sequencing analysis services and for detection of c-Kit gene mutations (Figure2) and (Figure3).

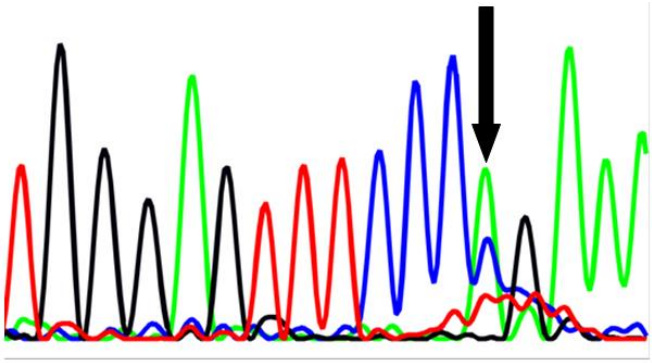

Fig. 2: Four-color/one-lane fluorescent sequencing showing missense mutation A→C (Pro121Gln).

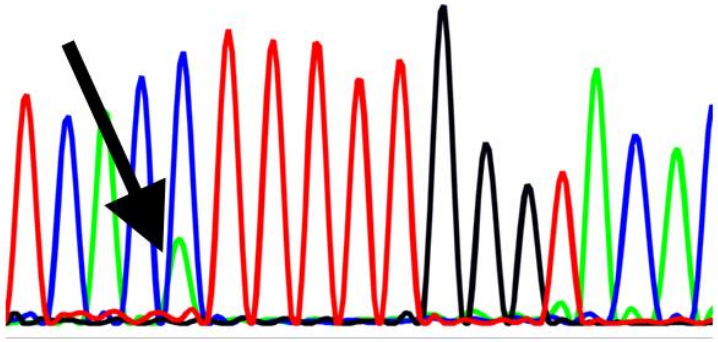

Fig. 3: Four-color/one-lane fluorescent sequencing showing hotspot mutation C→A (Pro171His).

In our study, two different missense mutations were reported. The missense mutation Pro121Gln was found in two AML-M4 cases. Another mutation Pro171His was found in hotspot region in four AML-M4 cases, two AML-M1 cases, four AML-M2 and two AML-M5b cases.

RESULTS

This study included 60 newly diagnosed AML patients. As regards clinical findings among patients, fatigue was the most frequently presented in 42 patients (70%), followed by fever 40 (66.7%), hepatomegaly 38 (63.3%), splenomegaly 26 (43.3%), purpura 22 (36.7%), bleeding 20 (33.3%), bone aches 10 (16.7%) and finally lymphadenopathy 8 (13.3%).

As regards laboratory findings among patients, the median WBC count was 31.75 $X10^9$/L (1.7-250.5 $X10^9$/L), the median of peripheral blood blasts was 67% (5 – 90%) and of bone marrow blasts was 66.5% (25 – 93%).

In this study, normal cytogenetic was detected in 26 (43.3%) patients, while abnormal cytogenetic was detected in 32(53.4%) patients. According to the risk stratification; the favorable, intermediate were 26 (43.3%) and 6 (10%) respectively. Among the favorable group, 8 (13.3%) had t (15;17), 10 (16.7%) had t(8;21) and 8 (13.3%) had Inv (16). While among intermediate group 4 (6.7%) had Tri 11 and 2 (3.3%) had Del (20)q11. Failed cases were 2 (3.3%).

In this study, we found a significant increase in the age of the patients in the mutated group in comparison to the wild group with mean age 54.57 ± 12.02 and 37.78 ± 16.38 years respectively (P=0.001). As regards to sex, males in the mutated group (12/14) were significantly more than in the wild group (18/46) (P=0.005), data not shown.

Table 1: Comparison between the wild and mutated groups as regards laboratory findings.

Variable	Wild group (No.=46)	Mutated group (No.=14)	Test	*P*
WBC (x 10^9/L)				
Mean ± SD	47.97 ± 47.38	63.33 ± 33.38	**MW**	**0.03**
Median	29.6	62	**2.65**	**S**
Range	1.7 – 250.5	5.2 – 106		
HB (gm/dl)				
Mean ± SD	8.11 ± 1.33	6.77 ± 1.74	**t**	**0.003**
Median	8.11	6.77	**3.06**	**HS**
Range	5.7 – 10.8	4.6 – 9.6		
Platelets (x 10^9/L)				
Mean ± SD	66 ± 68.65	68.29 ± 49.25	MW	0.35
Median	32	67	0.92	NS
Range	3 – 233	13 – 170		
PB blasts (%)				
Mean ± SD	61 ± 14.44	70.86 ± 15.75	**t**	**0.02**
Median	60	71	**2.41**	**S**
Range	5 - 90	48 – 86		
BM blasts (%)				
Mean ± SD	61.83 ± 10.57	69.13 ± 13.16	**t**	**0.04**
Median	61	69	**2.13**	**S**
Range	25 – 93	53– 86		
ESR (mm/hr)				
Mean ± SD	95.59 ± 34.83	128.5 ± 22.22	**MW**	**0.001**
Median	97.5	117	**3.22**	**HS**
Range	23.5- 150	105 - 160		
LDH: (IU/L)				
Mean ± SD	1497 ± 940.29	1782 ± 2277	MW	0.12
Median	1127	8.14	1.54	NS
Range	436.2- 4200	395 - 6982		

MW: Mann Whitney t: t-test

The majority of mutations detected in this study were found in AML cases with high count of WBC and blast cells, high ESR and low HB level (Table 1).

Table 2: Comparison between the wild and mutated patient groups as regards cytogenetic risk categories.

Variable	Wild group (No.=46)		Mutated group (No.=14)		Test	P
	No.	%	No.	%		
Cytogenetics						
Failed	2	4.4	0	0	**F**	**<0.05**
Normal	22	47.8	4	28.6		**S**
Abnormal	22	47.8	10	71.4		
Risk						
Favorable	18	39.1	8	57.1	**F**	**<0.05**
Intermediate	4	8.7	2	14.3		**S**
Favorable						
t(15;17)	8	17.4	0	0		
t(8;21)	6	13.0	4	28.6	**F**	0.60
Inv (16**)**	4	8.7	4	28.6		NS
Intermediate						
Tri 11	2	4.3	2	14.3	**F**	0.40
Del(20)q11	2	4.3	0	0		NS

Table 2 shows the abnormal cytogenetic pattern in the mutated group, (10/14) 71.4% was significantly more than that in the wild group, (22/46) There was a statistically significant difference between mutated and wild groups regarding risk stratification (P<0.05). No difference was detected between both groups in favorable or intermediate classification.

Table 3: Comparison between the wild and mutated groups as regards response to treatment:

Variable	Wild group (No.=46)		Mutated group (No.=14)		χ^2	P
	No.	%	No.	%		
Treatment results:						
CR	37	80.4	7	50	5.08	**0.02**
NR	9	19.6	7	50		**S**
Odds ratio (95% CI)	**4.11 (1.15 to 14.73)**					**0.03 S**
Hazard ratio (95% CI)	**2.5 (1.02 to 4.35)**					**0.04 S**

NR: no response χ^2: chi square CR: complete remission

In the wild group, (37/46) 80.4% achieved CR, while (9/46) 19.6% failed to respond to treatment. On the other hand, in the mutated group, (7/14) 50% achieved CR, while (7/14) 50% did not respond to treatment. We reported a significant decrease in the rate of achievement of CR in the mutated group compared to the wild group (P=0.02) (Table 3).

As regards odds ratio, in a cumulative manner, the mutated group was 4.11 times more likely for CR achievement failure than the wild group, this was statistically significant (P=0.03). While as regards hazard ratio, at any point of time, the mutated group was 2.5 times more likely for CR achievement failure than the wild group, this was also statistically significant (P=0.04) (Table 3).

Table 4: Comparison between the wild and mutated patient groups as regards treatment outcome during follow up period.

Variable	Wild group (No.=37)		Mutated group (No.=7)		Test	P
	No.	%	No.	%		
Follow up:						
Remission	34	91.9	4	57.1	F	0.042
Relapsed	3	8.1	3	42.9		S

In table 4, the wild group, (34/37) 91.9% remained in CR, while only (3/37) 8.1% of those who achieved CR went into relapse. In the mutated group, (4/7) 57.1% remained in CR, while (3/7) 42.9% of those who achieved CR had been relapsed A significant increase in the relapse rate in the mutated group was reported (P=0.042).

Table 5: Comparison between the wild and mutated patient groups as regards one year OS.

variable	Wild group (No.=46) %	Mutated group (No.=14) %	Logrank test	P
Follow up				
4 months	89.1	78.6		
8 months	82.6	50		
12 months	80.4	50	5.76	**0.02 S**
Median	Not reached	5 months		
Mean	10.43 months	7.79 months		
Odds ratio (95% CI)	4.65 (1.75 to 13.2)		---	**0.03 S**
Hazard ratio (95% CI)	3.17 (1.08 to 7.63)		---	**0.02 S**

Table (5) show one year OS of the wild and mutated groups. The mean time of OS was statistically significant shorter in the mutated group (P=0.02). As regards odds ratio, in a cumulative manner, the mutated group was 4.6 times more expected to have shorter OS than the wild group, with a statistically significant increase (P=0.03). While as regards hazard ratio, at any point of time, the mutated group was 3.17 times more likely to have shorter OS than the wild group, with also a statistically significant increase (P=0.02) (Figure 4).

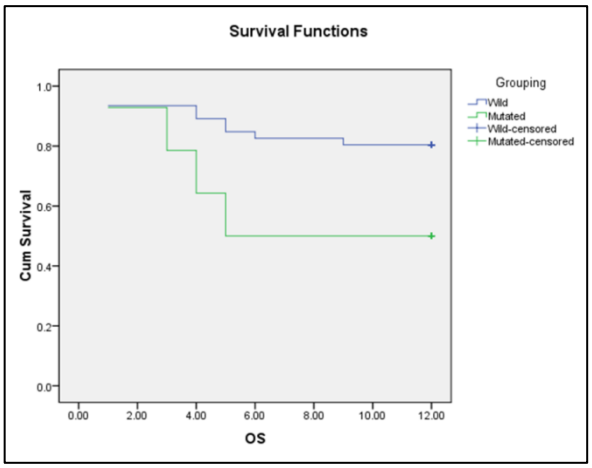

Fig. 4: Comparison between the wild and mutated groups as regards one year OS.

DISCUSSION

To our knowledge, this study is the first one to report mutations in exon 11 region of the c-kit gene in AML cases in Egypt and to evaluate these mutations as a prognostic marker in AML cases. Due to their location in juxta membrane domain, exon 11 mutations might lead to over-expression of KIT in AML cases.

Age and sex of patients included in this study was comparable to the study of Hussian et al. [13] who demonstrated that mean value ± SD of the age of their patients was 36.4± 6.08 and male to female ratio was 34/26. Patients included in Wakita et al. [10] study had a median age of 50.3 years ranging from 31–72 years and male to female ratio was 18/8. This difference might be due to different population or small sample size.

As regard cytogenetic findings, our results were detected in 36.7%, comparable to large studies from different geographic regions, cytogenetic abnormalities identified in 55.2%, 41.8% and 2.2%, of cases[7,14,15] respectively. The differences among these studies may be caused by genetic differences among studied populations.

The favorable risk group comprised 43.3% which was more than that found on the other studies[16,14,17]. while the intermediate risk group comprised 10% which was much lower than that reported by Hou et al.[7] (63.7%) and (61.3%) and Ostronoff et al.[17] due to difference in populations.

In the current study, exon 11 mutations were detected in 14 out of 60 AML cases (23.3%). This result is similar to another study which reported exon 11 mutations in 16% of AML cases[13].

Previous studies have reported several mutations in exon 11 in different tumors. Those mutations were more frequently detected than mutations in exons 8, 9, 13 and 17 of the c-kit gene[18,11], 65–92% of Gastrointestinal Stromal Tumors harbor KIT-activating mutations, the majority of which are localized in the juxta-membrane region involving exon 11.

We reported 2 different missense mutations in 14 AML cases. In our study the missense mutation Pro121Gln found in two AML-M4 cases. Another mutation Pro171His found in hotspot region in four AML-M4 cases, two AML-M1 cases, two AML-M2 and two AML-M5b cases.

One study, reported codon 582 mutations Trp582His and Trp582Tyr[19], while Trp582Try and Trp582Gln.were detected in another study[7]. In a different study, another substitution at the same codon was detected in which serine replaced tryptophan Trp582Ser.[13]

Also, mutations at codons Tyr578Phe, Tyr568Asp, and Arg588Phe, Arg588Tyr, Arg588Lys have been reported[19,20]; other studies documented other substitutions at Tyr568Ser, Tyr578Pro and Arg588Met[7,13].

Most of exon 11 mutations are located within the classic hotspot at the 50 end including codons 550–560. Another hotspot at the 30 end including codons 576–590 was documented [21]. Those mutations include missense mutations, frame deletions, and internal tandem duplications (mainly at the 30 end).

In this study, two novel missense mutations which has not been reported in any types of neoplasia were detected; Pro121Gln and Pro171His.

In the current study, we detected c-kit gene exon 11 mutations positioned between codons 550-591 obviously agrees with previous studies reporting mutations in different malignant conditions.

In this study, on comparison between the wild and mutated groups as regards laboratory findings, a significant increase in the TLC, PB and BM blasts and ESR was found in the mutated group versus the wild group (P=0.003). While, HB level was significantly lower in the mutated group (P0.001).

In agreement with our study, Carioli et al.[24] and Paschka et al.[25] also found higher WBC counts in association with KIT mutations. Also the majority of mutations detected in their study were found in AML cases with high count of WBC and Blast cells[13]. This might be associated with disease development and the prognosis. However, this finding was in contrast to other studies [11,22].

As regards response to treatment, a significant decrease in the rate of achievement of CR was found in the mutated group versus the wild group (P=0.02). In addition, the mean time of one year survival (OS) was shorter in the mutated group (P=0.02).

On comparing between the wild and mutated groups as regards treatment outcome during the follow up period, 91.9% of the wild group patients remained in CR, while only 8.1% of those who achieved CR went into relapse. While, in the mutated group, 57.1% remained in CR, while 42.9% of those who achieved CR had been relapsed.

Several studies found that KIT mutations didn't affect CR, but they were associated with a significantly higher risk of relapse**.** On the contrary, Chen et al.[27] reported that KIT mutations were found to adversely impact the CR of non-Caucasian patients but not that of Caucasian patients. This finding is consistent with the results of a study involved non-Caucasian patients[28]**.**

Also, Hou et al.[7] reported that KIT mutations increased the relapse risk and adversely affected the OS of non-Caucasian patients. The authors suggested the underlying reasons for these findings to be attributed to the social issues such as different accessibility of health care and compliance, or distinct ethnic genetic background.

Some studies have reported that KIT mutation is strongly associated with reduced overall survival (OS) and remission duration in AML patients [29,30]. Other studies found no obvious association [24,31]. The current data support the role of KIT mutational status as a prognostic marker as it can transform AML patients from favourable-risk AML to intermediate-risk AML [32]**.**

In conclusion, this study reports the presence of c-kit gene mutations in AML cases in Egypt. Those mutations may be involved in the pathogenesis.

Moreover, mutations in exon 11 of c-kit gene appear to be associated with decreased survival and failure to respond to treatment what makes these mutations represent valuable predictive marker in cases of AML.

Based on the results of the current study, larger studies are recommended involving more patients with longer periods of follow up for better evaluation of prognostic value of c-Kit mutations in AML patients.

Further studies are recommended to detect mutations in other c-Kit exons. Also therapeutic studies to assess the efficacy of c-kit inhibitors in AML patients with c-kit mutations are needed.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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