Blending Data of FLT3-ITD Mutation and Nucleophosmin Gene Mutations Detection Showed Significant Results in Comparison to Separate Mutation Detection in 32 Iraqi Adult Acute Myeloid Leukemia Patients

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Abstract

Background: FLT3-ITD mutation and *NPM1* mutations dual detection has been proved to be significant in AML patients throughout the world.

Objectives: this study was designed to shed light on the significance of dual detection of FLT3-ITD mutation and *NPM1* mutations in Iraqi AML patients for the first time.

Design and settings: this is a prospective study at a general community hospital in Baghdad (Baghdad Teaching Hospital) during the period from October 2010 till November 2011.

Patients and Methods: thirty two adult AML patients were enrolled within the study, in addition to 33 healthy adult chosen randomly as control group. FLT3-ITD mutation was detected using conventional PCR, whereas *NPM1* mutations were detected using single strand confirmatory polymorphism-RT-PCR.

Results: about 47% of AML patients showed positive *NPM1* mutations and 21.88 had FLT3-ITD mutation. 42.86% of patients with FLT3-ITD mutation had in addition *NPM1* mutations. Patients who had both mutations constitute 20% of all *NPM1* mutated cases (3/15), (*P*=.097).The largest number of patients were found in group one (FLT3-ITD_ve/*NPM1*_ve),

40.6%, and the least number were of group four (FLT3-ITD+ve/*NPM1*+ve), 9.4%. Significantly the lowest mean age of adult AML patients was found in patients group two who had FLT3-ITD mutation only, (***P*=.021**).The mean WBC count and blast cell percent was highest in patients with FLT3-ITD mutation than other groups. The mean platelet count in patients who had *NPM1* mutations was significantly highest when compared to other groups, and it was significantly lowest in patients with FLT3-ITD mutations (**P=.03**). Finally, 3/3(100%) of patients harbor both *NPM1* & FLT3-ITD mutations had not achieved complete hematological remission whereas 5/12(41.7%) of patients with *NPM1* mutations only had achieved complete hematological remission, (*P*=.180).

Conclusions: FLT3-ITD mutations had been found in 20% *NPM1* mutated cases. Good prognostic parameters were found in patients who had *NPM1* mutations only, whereas bad prognostic parameters were found in patients who had FLT3-ITD mutation with or without *NPM1* mutations. Good prognostic implications of *NPM1* mutations status are superseded in FLT3-ITD positive cases which have a consistently poor prognosis.

Key words: FLT3-ITD, NPM1, Mutation, AML, Prognosis, Iraqi patients

Running title: FLT3-ITD Mutation and Nucleophosmin Gene Mutations Dual Detection in 32 Iraqi Adult Acute Myeloid Leukemia Patients

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Introduction

The pathogenesis of acute myelogenous leukemia is heterogeneous on the molecular level. Molecular alterations can be intertwined and result from integration of multiple anomalies in the genetic program of the leukemic cell.[1] About 50% of AML have no evident chromosomal abnormalities, but 85% of those cases have mutations in at least one gene specifically FLT3, *NPM1* and CEBPA genes.[2] These mutations are valuable in allowing more clear-cut prognostic estimates, the development of specific therapies and the molecular monitoring of disease.[3]

Nucleophosmin belongs to the Nucleoplasmin /Nucleophosmin family of nuclear chaperones. Its overexpression in hematopoietic stem cells, increases self-renewal repopulating ability and survival to DNA damage.[4] *NPM1* mutations are often the only genetic abnormality detected in AML blasts and are very stable during the course of the disease.[5] As Being stable mutations in AML, *NPM1* mutations prospectively represent a reliable marker for monitoring minimal residual disease.[6]

On the other hand, internal duplication of FLT3 is detected in one-third of patients with normal cytogenetic or in patients with t (15; 17) (APL). This mutation, if present, might be a predictor for relapse in AML.[7]

Various studies have assessed the twofold detection of FLT3-ITD mutations & *NPM1* mutation since these two mutations are the most frequent molecular abnormality in AML patients especially in those cases with normal cytogenetic.[8, 9, 10] Furthermore, detection of those mutual mutations can be used in classifying AML patients into different prognostic groups.8 The presence of *NPM1* mutations with a wild type FLT3 gene is a favorable marker for relapse free survival and over-all survival in normal karyotype AML and for complete remission after intensive induction therapy.[4] The current research is a completion of previous research that have focused on the detection of the frequency of these mutations (FLT3-ITD

and *NPM1*) in 32 Iraqi AML patients separately in order to have an insight on the prognostic impact of combined mutation detection (FLT3-ITD and *NPM1*) in those Iraqi AML patients. [11, 12]

Materials and Methods

Patients and controls

Thirty two adult patients with AML were diagnosed at Hematology Department / Teaching Laboratories, Baghdad teaching Hospital, Baghdad, Iraq during the period from October 2010 till November 2011. Patients' diagnosis was based on peripheral blood and bone marrow aspiration findings stained with leishman stain and SBB stain examined by two hematology specialists for assessment of FAB classification. All patients were assessed at admission and after first course of induction therapy. In addition, 33 healthy adult individuals served as healthy control group were included randomly in relation to age and sex in the current study. Extracted RNA from OCI/AML3 cell line was used as positive control for molecular analysis of *NPM1* mutations. Informed consent was obtained from each individual enrolled in this study and the research was approved by the ethical committee at the college of Medicine, Al-Nahrain University, Baghdad-Iraq.

From all patients, two mills of peripheral blood samples or bone marrow aspirate samples according to the availability were collected, whereas from control group, two ml of peripheral blood samples were collected in EDTA tube. Samples were divided as one ml for DNA analysis and one ml was equally divided into two eppendorff tubes each contain one ml trizol reagent mixed well and kept in deep freeze(-70^{o}C) until the day of analysis.

Detection of FLT3-ITD mutation

High molecular weight DNA was extracted according to the kit protocol (Promega) following instruction manual. All samples were analyzed for FLT3 mutation in exon 11using conventional PCR thermal cycler (Eppendorf Master Cycler, France). The whole juxta-membrane and the first part of tyrosine kinase-1 domain where most of the reported mutations are located were screened using exon 11 specific primers.[13] Fifty to 100 nano gram of DNA(5 µl)was amplified in a 50 µl reaction mixture containing 1.5 mM MgCl, 50 mM KCl, 200 µM each deoxy ribonucleotide triphosphate (dNTP), 2.5 units Taq polymerase, 40 picomol of each primer (Forward Primer 11F: 5'-CAATTTAGGTATGAAAGCC-3', Reverse Primer 12 R: 5'-CAAACTCTAAATTTTCTCT-3'). Amplification process consisted of 40 cycles of 30 sec at 94^{o}C for denaturation, 45 sec at 50^{o}C for annealing, 1 minute at 72^{o}C for extension and 1 cycle of 7 minutes at 72 ^{o}C for the final extension. [13] Twenty µl of the PCR product was electrophoresed on 2.5% agarose gel (Promega), using 100bp DNA ladder (Promega) as molecular weight marker and stained with ethedium bromide (Promega).

Interpretation of FLT3-ITD mutation results

The amplified DNA product of the wild type from the patients and healthy control was about 133 bp band, whereas the mutated type showed additional band > 133bp, (**Figure 1**). The presence of any PCR fragment longer than the wild-type allele was considered positive for FLT3-ITD.

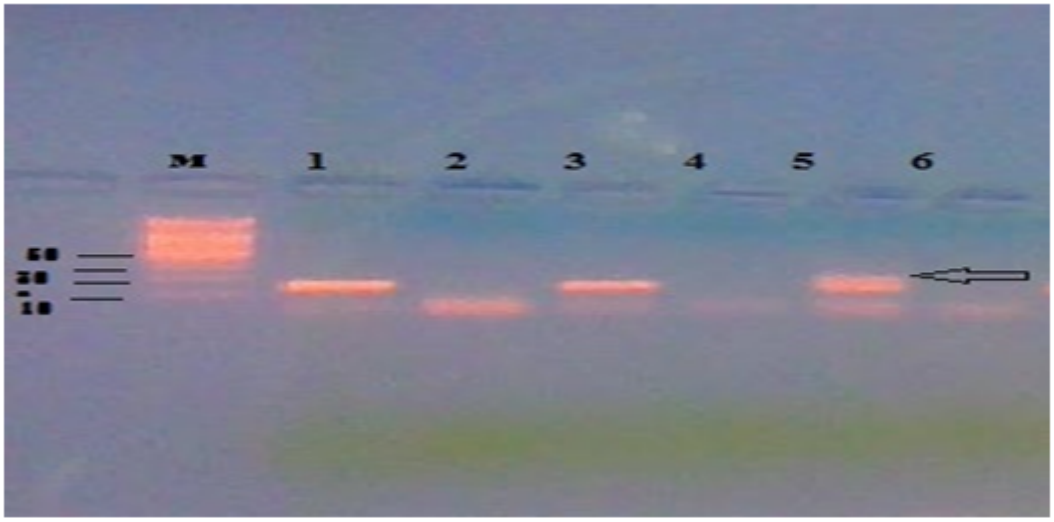

Figure 1: Detection of FLT3-ITD mutation using PCR in adult AML patients.

Lane 1: amplified product from healthy control showed a band about 133 bp. Lane 3: amplified product from AML patient showed wild type band about 133 bp. Lane 5: amplified product from AML patients showed two bands (wild about 133 bp and an extra mutated band, arrow). Lane 6: negative control. M: Molecular weight marker(DNA ladder). Gel electrophorecis was carried in 2.5% agarose gel.

NPM1 Mutations Detection

Total RNA was extracted from bone marrow cells or peripheral blood cells using bioZOL^{TM}-G RNA Isolation Kit (BioWORLD-US) following the instruction manual. Various types of NPM1 mutations were screened in exon 12 where all reported *NPM1* mutations specific for AML were located. Single Strand Confirmatory Polymorphism-Reverse transcriptase- Polymerase Chain Reaction (SSCP-RT-PCR) technique was used. A forward and backward primers "NPM-F, and NPM-R" were used that specifically amplify *NPM1* exon 12 only if the *NPM1* mutations that are specific for AML were expressed.[14]

Single step Accu power® RocketScript RT/PCR Premix Kit (BiONEER-Korea) was used for c DNA synthesis and amplification using Rocket Script ™ reverse transcriptase and Taq DNA polymerase in one tube following the manufacturer manual. To the 0.2 ml ready to use tube, approximately 1 µg of RNA and 5 pico moles of each primer (BiONEER,Korea) "NPM-F , 5_-ATCATCAACACCAAGATCA-_3 and NPM-R , 5_-

CATGTCTGACCACCGCTACT -_3 were added. The volume was completed to 50µl using nuclease free water. For the negative control tube, nuclease free water was added instead of the template RNA, whereas to the positive control tube 1 µg of RNA extracted from OCI-AML3 cell line was added. The reaction was performed under the conditions published earlier.14 PCR product was visualized by electrophoresis on 3% agarose gel (Promega, US).

Interpretation of NPM1 mutations results

All mutated cases showed hetero duplex formed from mutant allele and the wild type allele presented as two bands ,the first band approximately 550 bp, whereas the second band approximately 320 bp. This hetero duplex was absent in cases negative for *NPM1* mutation and healthy control group. OCI/AML3 cell line which was used as positive control showed hetero duplex formation on agarose gel electrophoresis, (**Figure 2**).

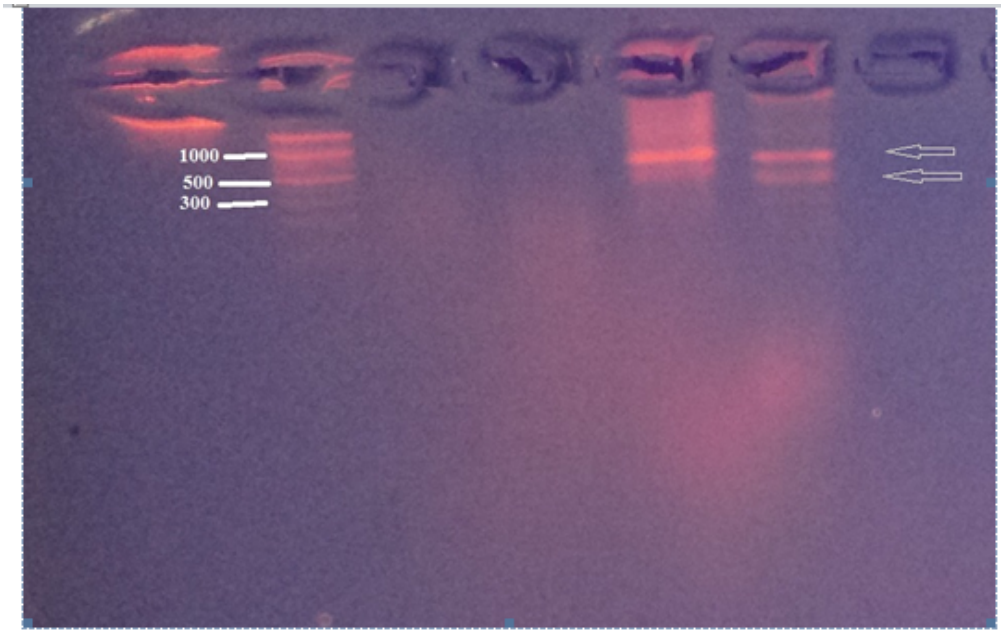

Figure 2: Detection of *NPM1* mutations using single strand confirmatory polymorphism RT-PCR in AML patients. Lane 4: amplified product from positive control (OCI/AML3 cell line)showed hetero duplex formation from wild and mutant alleles of *NPM1* gene appears as 2 bands (about 550 & 320 bp, arrows). Lane 3: amplified product from AML patient showed hetero douplex formation of wild and mutant alleles of *NPM1* gene as in positive control. Lane 1 & 2: amplified product from a healthy control and wild type AML patient respectively, showed absence of hetero douplex formation. Lane 5: negative control. M: Molecular weight marker(DNA ladder). Electrophoresis was carried in 3% agarose gel at (4V/cm) for 120 min.

Statistical Methods

SPSS program (Statistical Package for Social Sciences) version 16 and Microsoft Office Excel 2007 were used for analysis of data. Numeric data were expressed as mean+ SE, frequency was used to express discrete data. Student t-test was used to analyze numeric data while Chi-square was used to analyze discrete data. Values were considered statically significant when $P < 0.05$.

Results

Out of 32 AML patients, 15 (46.88%) patients showed positive *NPM1* gene mutations. Moreover, seven out of those 32 (21.88%) AML patients had FLT3-ITD mutation. Three out of those seven (42.86%) with FLT3-ITD mutation had in addition *NPM1* gene mutations. Those three patients who had FLT3-ITD and *NPM1* gene mutations constitute 20% of all *NPM1* gene mutated cases (3/15), (*P*=.097), (**Table 1**).

Table 1: Relation of FLT3 -ITD with NPM 1 Mutations in Adult AML Patients

Mutations Type	FLT3-ITD _ve	FLT3-ITD +ve	Total	P
*NPM1*wild	13	4	17	0.097
NPM1 mutated	12	3	15	

The AML patients were categorized into four groups as following:

1. FLT3-ITD_ve/*NPM1*_ve, 13 patients (40.6%); **2.** FLT3-ITD+ve/*NPM1*_ve, 4 patients (12.5%); **3.** FLT3-ITD_ve/*NPM1*+ve, 12 patients (37.5%); **4.** FLT3-ITD+ve/*NPM1*+ve, 3 patients (9.4%).

The largest number of patients 13/32(40.6%) were found in group one (FLT3-ITD_ve/*NPM1*_ve), followed by group three (FLT3-ITD_ve/*NPM1*+ve), 12/32(37.5%), then group two (FLT3-ITD+ve/*NPM1*_ve), 4/32(12.5%), and the least number of patients were of group four (FLT3-ITD+ve/*NPM1*+ve), 3/32(9.4%).

The lowest mean age of adult AML patients was found in patients group two, 24.3(8.4) years who had FLT3-ITD mutation only, whereas highest mean age was detected in patients group three, 42.3 (20.9) years who had *NPM1* mutations only, (***P*=.021**), (**Table 2**). Male gender was predominant in all patients groups except in patients group three (*NMP1* +ve /FLT3-ITD-ve) were mainly female gender, (*P*=.493). D*e novo* and new cases was detected more in group three, 10/12(83%) where only *NPM1* mutations present than in other groups, (*P*=.350, *P*=.841), (**Table 2**).

Furthermore, the mean WBC count was highest in patients group two, 61.9(46.4) x 10^9/L in which there was only FLT3-ITD mutation than other groups , whereas the lowest mean WBC count was found in patients group three, 32.8(28.8) x 10^9/L when only *NPM1* mutations was detected, (*P*=.197). Moreover, the mean platelet count in patients group three, 96.7(90) x 10^9/L was significantly highest when compared to other groups, and it was significantly lowest in patients group two, 32.3(15.5) x 10^9/L where FLT3-ITD mutation was present only (***P*=.03**), (**Table 2**). On the other hand, no significant difference was found in the mean hematocrit percent among all groups, (*P*=.655), (**Table 2**). Additionally, the mean peripheral blast cell percent was highest in patients group two, 82 (11.2) % in which only FLT3-ITD mutation was detected, but lowest mean peripheral blast cell percent was found in patients group three, 57.8 (27.8) % where only *NPM1* mutation were detected , (*P*=.288), (**Table 2**). Similarly, the mean bone marrow blast cells percent was highest in patients group two, 85.8(10.2) % that had only FLT3-ITD mutation in contrast to patients group three,

70.2(24.4)% that had *NPM1* mutations only where the lowest mean bone marrow blast cells percent was found ,(*P*=.324), (**Table 2**).

Table 2: Correlation of adult AML patients groups with their clinical presentation

Clinical Presentation	**FLT3-ve / *NPM1* _ve**	**FLT3+-ve / *NPM1* _ve**	**FLT3_ve/ *NPM1*+ve**	**FLT3+ve/ *NPM1*+ve**	***P***
Age /years Mean (SD)	37.15 (22.1)	24.25(8.4)	42.33(20.9)	41.00(18.5)	**.021***
Gender					
Male	7	3	4	2	.493
Female	6	1	8	1	
AML Diagnosis					
New	6	3	10	2	.350
Relapse	7	1	2	1	
Type of Cases					
De novo	12	4	10	3	.841
Secondary	1	0	2	0	
WBC x 10^9/L Mean (SD)	35.5 (24.2)	61.9(46.4)	32.8(28.8)	43.3 (69)	.197
Platelet x 10^9/L Mean (SD)	72.5(26.9)	32.3(15.5)	96.7(90)	46.7(52.2)	**.036***
Hematocrit % Mean (SD)	25.5(3.8)	24 (5.5)	25.5 (5.5)	27.3 (5.5)	.655
Peripheral blast % Mean (SD)	66.5(26.2)	82 (11.2)	57.8 (27.7)	63 (23.4)	.288
BMA blast % Mean (SD)	72.5(26.9)	85.8(10.2)	70.2(24.4)	85.3 (8.4)	.324
Response to IT					
Remission	4	3	5	0	.180
Failure	9	1	6	2	
Death	0	0	1	1	
Total	13	4	12	3	32

***Significant, BMA: Bone Marrow Aspirate, IT: Induction Therapy**

Finally, all patients in group four 3/3(100%) who harbored both *NPM1* & FLT3-ITD mutations had not achieved complete hematological remission which was on the contrary to patients group three who had only *NPM1* mutations, 5/12(41.7%) had achieved complete hematological remission followed by patients group one, 4/12(33%) who had no mutations, and the highest rate of remission was detected in patients group two 3/4(75%)(*P*=.180), (**Table 2**).

Discussion

The fact that molecular alterations in AML patients are so heterogeneous makes combined detection for different mutations in AML patients more significant. The most frequent mutation in AML is internal tandem duplications (ITDs) in the juxta-membrane domain and mutations in the second tyrosine kinase domain of the Fms-like tyrosine kinase 3 (*FLT3*) genes ; however, such mutations alone appear to be inadequate for leukemic transformation. [15] whereas, previous researches in Iraq have highlighted the frequency of FLT3-ITD mutation and *NPM1* mutations in adult AML individually, [11, 12] the current research arises as a continuation for previous observations to explore the significance of combined mutations detection (FLT3-ITD and *NPM1*) in those adult AML patients.

Out of 32 AML patients, 15 (46.88%) patients showed positive *NPM1* gene mutations. Moreover, seven out of those 32 (21.88%) AML patients had FLT3-ITD mutation. Three out of those seven (42.86%) with FLT3-ITD mutation had in addition *NPM1* gene mutations. Similarly, Pazhakh *et al*. had found that among AML patients with FLT3/ITD mutations 35% had mutant *NPM1* gene. [16] Those three patients who had FLT3-ITD and *NPM1* gene mutations constitute 3/15(20%) of all *NPM1* gene mutated cases, (*P*=.097),

(**Table 1**). In agreement with this result, Brown *et al*. had reported that FLT3-ITD mutations occur in 19% of *NPM1* mutated cases. [14]

It has been proposed that FLT3-ITD mutation and *NPM1* mutations are secondary events from a primary process that predisposes a myeloid stem/progenitor cell to errors in DNA replication. Supporting this possibility is the fact that many of the *NPM1* mutations, including the most common type mutant 'A', in which there are a 4-base pair mutation are similar to mutation that occurs in FLT3 –ITD. [17]

In order to determine the prognostic effect of interaction between *NPM1* and FLT3 ITD mutations, the adult patients were categorized into four groups. The largest number of patients, 13/32(40.6%) were found in group one (FLT3-ITD_ve/*NPM1*_ve), followed by group three (FLT3-ITD_ve/*NPM1*+ve), 12/32(37.5%), then group two (FLT3-ITD+ve/*NPM1*_ve), 5/32(12.5%), and the least number of patients were of group four (FLT3-ITD+ve/*NPM1*+ve), 4/32(9.4%). Similar to this finding Angela *et al.* had reported the highest percent of patients were those negative for both mutations, followed by those harboring *NPM1* mutation only, and the lowest percent was in patients carrying both mutations. [18]

Significantly the lowest mean age of adult AML patients was found in patients group two 24.3(8.4) years who had FLT3-ITD mutation only, whereas highest mean age was detected in patients group three 42.3 (20.9) years who had *NPM1* mutations only, (***P*=.021**), (**Table 2**). This result was in agreement with Thiede *et al*., study. [18] Male gender was over represented in all patients groups except in patients group three (*NMP1* +ve /FLT3-ITD-ve) where female gender was predominant, (*P*=.493). This finding was in accordance to Thieda *et al* study. [19] Moreover, the presence of *de novo* and new cases was detected more in group three 10/12(83%) where only *NPM1* mutations present than in other groups, (*P*=.350, *P*=.841), (**Table 2**), which was correspondingly reported by other studies. [19, 20]

Regarding the correlation between patients groups and hematological parameters, the mean WBC count was highest in patients group two 61.9(46.4) x 10^9/L in which there was only FLT3-ITD mutation than other groups , whereas the lowest mean WBC count was found in patients group three 32.8(28.8) x 10^9/L when only *NPM1* mutations were detected, (*P*=.197). Thiede *et al*., had found higher WBC count in patients group four with both FLT3-ITD & *NPM1* mutations, followed by patients group two with FLT3-ITD mutation only. [19] Moreover, the mean platelet count in patients group three, 96.7(90) x 10^9/L was significantly highest when compared to other groups, and it was significantly lowest in patients group two, 32.3(15.5) x 10^9/L where FLT3-ITD mutation was present only (***P*=.03**), (**Table 2**). These findings might be explained by the capacity of *NPM1* mutated blast cell for thrombocytic differentiation. [21] On the other hand, no significant difference was found in the mean hematocrit percent among all groups, (*P*=.655), (**Table 2**), which was similarly reported by Thiede *et al*. [19] Furthermore, the mean peripheral and bone marrow blast cell percent, [82 (11.2) %, 85.8(10.2) %],respectively were highest in patients group two, in which only FLT3-ITD mutation was detected, but lowest mean peripheral and bone marrow blast cell percent was found in patients group three[57.8 (27.7) %; 70.2(24.4)%], respectively where only *NPM1* mutations were detected , (*P*=.288), (**Table 2**). In agreement to these findings, other studies had reported highest blast cell percent in patients who had FLT3-ITD mutation, [19, 20] those findings can be explained by the fact that FLT3 is a proto-oncogene with the capacity to enhance survival and proliferation of leukemic blast cells. [22]

Patients in group four who harbored both *NPM1* & FLT3-ITD mutations had the worst response to chemotherapy as 3/3(100%) had not achieved complete hematological remission which was on the contrary to patients group three who had only *NPM1* mutations where AML patients 5/12(41.7%) had achieved complete hematological remission followed by patients group one 4/12(33%) who had no mutations, and the best response was in patients

who had only FLT3-ITD mutations, 3/4 (75%), (*P*=.180), (**Table 2**). Other researchers had reported that the best response to treatment was detected in the patients who had only *NPM1* mutations and worst response was in patients had both mutations. [19, 20, 23, 24] It was documented that *NPM1* mutated AML has an increased sensitivity to chemotherapeutic agents secondary to its interaction with nuclear factor kaba B (NK-kB). [25]

To sum up, in those 32 Iraqi AML patients the presence of FLT3-ITD and NPM1 mutations together mutation might be associated with poorest prognosis in regards to other patients, this finding had been concurred by Schnittger *et al.* and Wertheim and Bagg, studies who had established that the favorable prognostic implications of *NPM1* mutations status are dominated in FLT3-ITD positive cases which have a uniformly poor prognosis. [7, 9]

In comparison to our previous findings, [11, 12] more significant findings had been obtained due to dual detection of FLT3-ITD and *NPM1* mutations in those AML patients that gave better prognostic representation.

Conclusions

FLT3-ITD mutations had been found in 20% of *NPM1* mutated cases. Good prognostic parameters (female gender, newly diagnosed *de novo* cases, lower mean WBC count , blast cell percent peripherally and in BMA, higher mean platelet count , and good response to induction therapy) were found in patients who had *NPM1* mutations only, whereas poor prognostic parameters (male gender, relapsed cases, secondary cases, higher mean WBC count, blast cell percent peripherally and in BMA , lower platelet count, and poor response to induction therapy) were found in patients had FLT3-ITD mutation with or without *NPM1* mutations. Promising good prognostic implications of *NPM1* mutations status are superseded in FLT3-ITD positive cases which have a consistently poor prognosis.

Conflict of interest: all authors declare no conflict of interest

Authors' contributions

EK, contributed to collection of data, performing the molecular studies, hematological assays, data analysis, drafting of the manuscript. SS, contributed to the concept and design, and revision of the manuscript. MA, contributed to part of the molecular studies and data analysis. The authors revised and approved the final submitted version of the manuscript.

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