Volume : 4 | Issue : 7 | July 2015 • ISSN No 2277 - 8179

Research Paper

Detection of FLT3-ITD mutations and prognostic impact of NPM1 exon 12 mutations in adult CN-AML patients

Medical science

KEYWORDS : CN-AML, FLT3-ITD, NPM1,PCR,HRM

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ABSTRACT

Acute myeloid leukemia (AML) is a heterogeneous group of leukemias that arise in precursors of myeloid, erythroid, megakaryocytic, and monocytic cell lineages. These leukemias result from clonal transformation of hematopoietic precursors through the acquisition of chromosomal rearrangements and multiple gene mutations .In the past few years, mutations in genes, such as Fms-like tyrosine kinase 3 (FLT3) , the myeloid-lymphoid or mixed-lineage leukemia gene(MLL), CCAAT/enhancer binding protein alpha (CEBPA), and Nucleophosmin (NPM1), have been identified in Cytogenetically Normal-Acute Myeloid Leukemia (CN-AML), and the presence of such mutations carries prognostic information. We aimed to study the prevalence, association with FLT3-ITD mutations, and prognostic impact of NPM1, exon 12 mutations in 50adult AMLpatients with normal karyotype receiving induction chemotherapy (cytarabine and daunorubicin). We performed HRM assay to detect NPM1 mutation and conventional PCR for FLT3-ITD gene mutation analysis on 50 CN –AML patients. Twenty two (22/50)44% were NPM1 mutation positive only, (16/50) 32% were both NPM1 mutation and FLt3 positive and (18/50) 36% were FLT3-ITD positive only.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of leukemias that arise in precursors of myeloid, erythroid, megakaryocytic, and monocytic cell lineages. These leukemias result from clonal transformation of hematopoietic precursors through the acquisition of chromosomal rearrangements and multiple gene mutations (Rubnitz, 2010).AML is the most frequent hematological malignancy in adults, with an estimated worldwide annual incidence of 3 to 4 cases per 100,000 people (Riva*et al*, 2012) AML is characterized by both a predominance of immature forms and loss of normal hematopoiesis (Foucar, 2008).

AML is the most common type of acute leukemia in adults, accounting for 80% of new cases and its incidence increases after 45 years (Mittal, 2001).About 45% of acute leukemias have an abnormal karyotype with a recurrent chromosomal alteration, and about 15% have 3 or more cytogenetic abnormalities (Betz, 2010).

The FAB classification system was based on conventional, readily available morphologicandcytochemical characteristics (Walter, 2013), it doesn't always reflect the genetic or clinical diversity of the disease (Vardiman*et al*, 2002) .It divides AML into 8 subtypes (M0 to M7) (Abdul-Hamid, 2011).

The World Health Organization (WHO) classification of AML encompasses four major categories: AML with recurrent genetic abnormalities, AML with multilineage dysplasia, AML that is therapy related, and AML not otherwise categorized (Kassem*et al*., 2011).

The FLT3 gene (FMS-like tyrosine kinase receptor 3) is located on chromosome 13q12 and encodes a Type III membrane receptor kinase (Chang *et al*., 2010). After activation by its ligand (FL), FLT3 supports the proliferation and survival of hematopoietic progenitors (Filip*et al*, 2012).Activating FLT3 mutations are likely to contribute to the development of leukemia in humans (Gari*et al*., 2008). The internal tandem duplication mutation of the FMS-like tyrosine kinase receptor (FLT3-ITD) and point mutations activate the FLT3 kinase and its downstream signaling pathways, like RAS/MAP-kinase , AKT/PI-3 kinase, and STAT signaling pathways, known to give leukemic cells a proliferation and survival advantage (Pauwels*et al*.,2013)Approximately one quarter of patients with AML harbor an FLT3-ITD mutation (Fathi*et al*., 2012).

The *NPM1*gene is located on chromosome 5q35 (Ammatuna*et al*., 2011) and encodesNucleophosmin/B23 which is a nucleolarphospho-protein (Yung, 2007), predominantly localized in the nucleolus (Verhaak*et al*., 2005), many of its functions require its continuous shuttling between cytoplasm, nucleoplasm, and nucleoli (Federici, Falini., 2013).

NPM1 mutation accounts for approximately 35% of all AML cases (Dang *et al*., 2013) and it represents the most frequent molecular aberration in it (Scholl *et al*., 2007) mainly; the NPM1 locus is lost or translocated, leading to the formation of fusion genes and proteins (Balatzenko*et al*., 2014) and involve a 4 bp insertion in a limited region of exon 12 (Laughlin *et al*., 2008).

We aimed to study the prevalence, association with FLT3-ITD mutations, and prognostic impact of NPM1, exon 12 mutations in 50 AML adults' patients with normal karyotype.

Patients and Methods

The study was conducted on 50newly diagnosed adult AML patients. All patients were presented at the Hematology Unit of the Alexandria Main University Hospital.Fifty apparently healthy volunteers of matched age and sex were included as control group to determine the presence or absence of FLT3-ITD and NMP1 mutations in normal individuals. Informed consent was obtained from all patients according to the Ethical Committee for Human Research in Alexandria Main University Hospital.

Diagnosis of AML was based on the FAB classification. All patients were subjected to full history taking, complete clinical examination, routine laboratory investigations including complete blood count (CBC), liver function tests, and renal function tests, and abdominal ultrasound to assess the condition of the liver and spleen.

Volume : 4 | Issue : 7 | July 2015 • ISSN No 2277 - 8179

Bone marrow aspirate (BM) examination, cytochemistry (myeloperoxidase stain) was done. Immunophenotyping by flowcytometrywas doneusing surface markers when needed for patients only. HRM assay was performed to detect NPM1 mutation, andDetection of FLT3-ITD mutation was done by genomic polymerase chain reaction (PCR) technique (Sheikhha*et al*., 2003, kiyoi*et al*., 1999).

Criteria for Treatment Outcomes

CBC and BM aspirates were performed on day 28 after receiving induction chemotherapy. Cases who died before treatment or their data were not available were excluded.

The conventional initial treatment of AML is induction chemotherapy with the 7+3 regimen, which consists of 7 days of continuous intravenous infusion of cytarabine 100mg/m2 and 3 days of daunorubicin 45-60 mg/m2. There is roughly a 64-70% chance of obtaining a complete remission with this regimen for all patients with AML. However, this success rate declines to roughly 46% in patients aged 56-65 years, 39% in patients aged 66-75 years, and only 33% in patients older than 75 years. (Ho , Butera., 2011).

For the individual patient with AML, the achievement of a hematological complete remission (CR) is a major decision point during induction treatment. It is not only used as an endpoint to evaluate the effect of induction treatments, but also plays a key role in the evaluation of treatment strategies. For the first time in 1990, a group of experts defined the criteria for CR (Chesonet *al*., 1990). CR was defined by the presence of less than 5% blasts in the bone marrow (BM) with more than $1x10^9$/l neutrophils and more than $100x10^9$/l platelets in the peripheral blood (PB). Patients with complete remission with incomplete platelet recoveryCRp achieved the above criteria with the exception of the platelet count remaining less than $100x10^9$/l.Partial remission (PR) was defined as achieving CR criteria in the peripheral blood with bone marrow blast reduction by ≥50% but remaining >5% (Ravandi*et al*., 2010). Infrequently, patients do not show fully recovered neutrophil and/or platelet counts before they proceed to the next chemotherapy cycle. The cellularity of the BM is directly influenced by the dose intensity of the induction treatment and also depends on the time interval following treatment (de Greef*et al*., 2004).

Detection of FLT3-ITD by Genomic PCR Technique

Genomic DNA was extracted from either peripheral blood (PB) leucocytes or bone marrow (BM) cells using GFX genomic blood purification kit (Biosciences, Amersham, UK). Extracted DNA was then amplified by PCR using primers to amplify exons 14 and 15 (previously known as exons 11 and 12) which are sites for FLT3-ITD mutation, 14F (5'-GCA ATT TAG GTA TGA AAG CCA GC-3'), and 15T (5'-CTT TCA GCA TTT TGA CGG CAA CC-3') as described previously (Sheikhha*et al*., 2003,Kiyoi *et al*., 1999). The total reaction volume of 50 UL contained 300-500 ng DNA and 20 pmol of each primer. 2X PCR Master Mix (Ferments Life Science) were used. It was composed of dNTPs (dATP, dCTP, dGTP, and dTTP), 0.4mM of $Mgcl_2$ (4 mM), and 0.05 units/ml of Taq polymerase in reaction buffer. Samples were amplified.

NPM1High Resolution Melting Analysis

The PCR and melting analysis for NPM1mutation was performed on the Rotor-Gene 6000, a real-time PCR machine with HRM capability and a 96/384-well capacity.

All samples were tested in duplicate. At least 5 different normal controls for each gene were included in each run. Approximately 10 ng of DNA was amplified in a total volume of 10 µL containing 400 nMofeach of the relevant forward and reverse primer, NP Mex12F:TGATGTCTATGAAGTGTTGTGGTTCC NPMex12R:CT CTGCATTATAAAAAGGACAGCCAG

The conditions were 95°C (5 min) and a touchdown of 10 cycles of 95°C (10 sec), 65°C–55°C (10 sec, 1°C/step), and 72°C (30 sec) and a further 45 cycles. The melting program was 95°C (1 min), 45°C (1 min), and then 65°C–95°C (5 sec, 1°C/sec). Thirty acquisitions were collected per °C. Upon completion of the run (approximately 2 hours), analysis was performed using the software supplied with Rotor-Gene 6000. The melting curves were normalized and temperature was shifted to allow samples to be directly compared. Difference plots were generated by selecting a negative control as the baseline and the fluorescence of all other samples was plotted relative to this sample. Significant differences in fluorescence were indicative of mutations.

Statistical Analysis of the Data

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean, standard deviation, and median. Comparison between different groups regarding categorical variables was done using chi-square test. When more than 20% of the cells had expected count less than 5, correction for chi-square was conducted using Fisher's exact test or Monte Carlo correction. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test, and D'Agstino test; also Histogram and QQ plot were used for vision test. If it reveals normal data distribution, parametric tests were applied. If the data were abnormally distributed, nonparametric tests were used. For normally distributed data, comparisons between different categories were done using independent *t*-test or F-test (ANOVA). For abnormal and ordinal data, comparisons between different categories were done using Mann Whitney test or Kruskal Wallis test. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

Results

Clinical characteristics

A total of 50 newly diagnosed AML patients (17-70 years of age) were examined for the presence of FLT3-ITD and NPM1 mutations; of them 25 (56%) were males and the other 22 (44%) were females (Table 1). All the control subjects were negative for the studied genes. Results show that 36 % of patients were FLT3 positive and 44 % were NPM1 positive. Median values and range of Hb, WBC, platelet and %blasts counts for all patients are shown.

Table 1-Clinical characteristics of 50 CN-AML patients

Clinical characteristics	All patients (n=50)
Sex-no (%)	
Female Male	22 (44.0%) 25 (56.0%)
Median age – year(range)	34.50(17.0-70.0)
Median WBC count- $x10^9$/L (range)	35.25 (3.70-159.0)
Median Hb level - g/dL (range)	7.90 (4.70-12.60)
Median Plt count - $x10^9$/L (range)	45.0 (8.0-1060.0)
Median PB blasts - % (range)	54.50 (9.0-95.0)
Median BM blasts - % (range)	66.0 (10.0-97.0)
FAB subtype - no. (%)	
M0	1 (2.0%)
M1	8(16.0%)
M2	14(28.0%)
M4	9(18.0%)
M5	16(32.0%)
M6	2(4.0%)

Keys: WBC – white blood cell; Hb – haemoglobin; Plt – platelet; PB – peripheral -blood; BM – bone marrow; FAB - French-American-British.

Results of *NPM1* and *FLT3*-ITD mutation status

Positive and negative counts of both genes and the induction response to chemotherapy along with FAB types are shown in Table 9; were M5 was the highest among all FABs subtypes 16(32%). Of 22 patients , 2(9.1%) achieved complete remission

after induction chemotherapy , there was a difference in CR rates of mutated NPM1$^+$ and unmutated NPM1$^-$ patients but not reaching significant level.

Table 2- Results of *NPM1* and *FLT3*-ITD mutation status of CN-AML patients

	No.	%
FLT3-ITD		
Wild type	32	64.0
Mutant	18	36.0
NPMI		
Wilde type	28	56.0
Mutant	22	44.0
Induction Response (n = 22)		
Complete remission	2	9.1
Non evaluate	5	22.7
Partial remission	2	9.1
Induction death	3	13.6
Resistant disease	10	45.5

Clinical characteristics of 50 CN-AML patients

NPM1 mutations and patient characteristics (WBC count, platelet count and % Blasts)

Median WBC counts (x10^9/L) of NPM1 wild type and mutant positive patients were 21.30 and 69.40 respectively. The incidence of mutations was significantly higher in patients with high WBC count (P =0.004). Median platelet counts (x10^9/L) of NPM1 wild type and mutant-positive patients were 31.0 and 54.0respectively. No significant association of the mutation with high platelet count was seen (P = 0.319). Median Absolute blasts in PB (x10^9/L) for NPM1 wild type and mutant-positive patients were 13.39 and 35.48, respectively. Higher of mutants was seen in patients with high absolute blasts but this was statistically insignificant (P = 0.007). All these results are given in Table 3.

Table 3-WBC count, Platelet count and %Blasts in WT and NPM1 mutant-positive Patients.

	NPM1		p
	Wild type(n = 28)	Mutant (n = 22)	
Platelets (x10^9/L)			
Min. – Max.	8.0 – 1060.0	13.0 – 211.0	0.319
Median	31.0	54.0	
WBCS (x10^9/L)			
Min. – Max.	3.70 – 147.0	15.10 – 159.0	0.004*
Median	21.30	69.40	
Blast (PB) (%)			
Min. – Max.	9.0 – 94.0	18.0 – 95.0	0.233
Median	47.0	64.0	
Absolute blasts in PB (x10^9/L)			
Min. – Max.	1.0 – 124.95	4.46 – 80.10	0.007*
Median	13.39	35.48	
Blast (BM) (%)			
Min. – Max.	10.0 – 95.0	28.0 – 97.0	0.544
Median	71.0	62.50	

p: p value for comparing between wild type NPM and mutant NPM1, *: Statistically significant at p ≤ 0.05

Clinical characteristics of 50 CN-AML patients, divided by *FLT3-ITD1*$^+$ and *FLT3-ITD*$^-$

FLT3-ITD mutations and patient characteristics (WBC count, platelet count and % Blasts)

Median WBC counts (x10^9/L) in FLT3-ITD wild type and a mutant-positive patient was 21.30 and 78.60 respectively. A significantly higher incidence of FLT3-ITD mutations was seen in patients with high WBC count (P =0.004). Median platelet counts (x10^9/L) of FLT3-ITD wild type and mutant-positive patients were 38.0 and 55.0respectively. No significant association of the mutations with platelet count was seen in these samples (P= 0.485). Median values of Absolute blasts in PB (x10^9/L) of FLT3-ITD wild type and mutant-positive patients were 13.39 and 37.04 respectively. The mutations were found to be significantly associated with high absolute blast (P = 0.008). The incidence of FLT3-ITD was higher in those with elevated peripheral blasts. All these results are given in Table 4.

Table 4-WBC count, platelet count and % blasts in FLT3-ITD WT and mutant-positive patients

	FLT3-ITD		p
	Wild type(n = 32)	Mutant (n = 18)	
Platelets (x10^9/L)			
Min. – Max.	8.0 – 1060.0	13.0 – 149.0	0.485
Median	38.0	55.0	
WBCS (x10^9/L)			
Min. – Max.	3.70 – 147.0	17.80 – 159.0	0.004*
Median	21.30	78.60	
Blast (PB) (%)			
Min. – Max.	9.0 – 94.0	18.0 – 95.0	0.257
Median	49.0	64.0	
Absolute blasts in PB (x10^9/L)			
Min. – Max.	1.0 – 124.95	4.46 – 80.10	0.008*
Median	13.39	37.04	
Blast (BM) (%)			
Min. – Max.	10.0 – 95.0	32.0 – 97.0	0.537
Median	71.0	612.50	

p: p value for comparing between wild type FLT3-ITD and mutant FLT3-ITD, *: Statistically significant at p ≤ 0.05

Clinical characteristics of 50 CN-AML patients, divided by *NPM1*/*FLT3*-ITD mutation status

Median WBC counts (x10^9/L) in NPM1$^+$/FLT3-ITD$^+$, NPM1$^+$/FLT3-ITD$^-$, and NPM1$^-$/FLT3-ITD$^-$ was 78.60, 40.30 and 21.30 respectively. A significantly higher incidence of both NPM1$^+$/FLT3-ITD$^+$ mutations was seen in patients with high WBC count (P = 0.011). Median platelet counts (x10^9/L) of both NPM1$^+$/FLT3-ITD$^+$ was 55.0, in NPM1$^+$/FLT3-ITD$^-$ 48.50 and lower in NPM1$^-$/FLT3-ITD$^-$ 31.0. No significant association of the mutations with platelet count was seen in these samples (P = 0.586). Median values of absolute blasts of NPM1$^+$/FLT3-ITD$^+$, NPM1$^+$/FLT3-ITD$^-$, and NPM1$^-$/FLT3-ITD$^-$ was 37.04, 17.50, and 13.39 respectively. The mutations in both genes were found to be significantly associated with high blast percentage (P=0.021).

Table 5- WBC count, platelet count and % blasts in NPM1/FLT3-ITD WT and mutant-positive patients

	NPM1/FLT3-ITD			p
	NPM1$^+$/FLT3-ITD$^+$ (n = 18)	NPM1$^+$/FLT3-ITD$^-$ (n = 4)	NPM1$^-$/FLT3-ITD$^-$ (n = 28)	
Platelets (x10^9/L)				
Min. – Max.	13.0 – 149.0	33.0 – 211.0	8.0 – 1060.0	0.586
Median	55.0	48.50	31.0	
WBCS (x10^9/L)				
Min. – Max.	17.80 – 159.0	15.10 – 116.40	3.70 – 147.0	0.011*
Median	78.60	40.30	21.30	
Blast (PB) (%)				
Min. – Max.	18.0 – 95.0	20.0 – 88.0	9.0 – 94.0	0.478
Median	22.37	61.50	47.0	
Absolute blasts in PB (x10^9/L)				
Min. – Max.	4.46 – 80.10	9.11 – 54.56	1.0 – 124.95	0.021*
Median	37.04	17.50	13.39	
Blast (BM) (%)				
Min. – Max.	32.0 – 97.0	28.0 – 90.0	10.0 – 95.0	0.816
Median	62.50	69.50	71.0	

p: p value for comparing between NPM1/FLT3-ITD categories

*: Statistically significant at p ≤ 0.05

Distributions of mutations according to sex

Percentages of FLT3-ITD and NPM1 mutations were higher in male than in female patients but these were not statistically

Volume : 4 | Issue : 7 | July 2015 • ISSN No 2277 - 8179

significant (Table 6). In female patients, the percentage of wild type of NPM1 and FLT3-ITD was 53.6 % and 46.9 % respectively- and a mutated sample was 31.8 % and 38.9% respectively. In male patients however, the percentage of mutated samples of NPM1 and FLT3-ITD was 68.2% and 61.1% was higher than that of the wild type ones 46.4% and 53.1 % but this difference was not statistically significant.

Table 6- Distribution of the different mutations according to sex.

Mutation Type	Wild type (%)	Mutant (%)	p
FLT3/ITD			
Male	17(53.1%)	11(61.1%)	0.585
Female	15(46.9%)	**7**(38.9%)	
NPM1			
Male	13(46.4%)	15(68.2%)	0.124
Female	15(53.6%)	7(31.8%)	

Table 7 shows the relation between NPM1/FLT3-ITD sex; it also shows that 61.1% of the results were males with $FLT3^+$& $NPM1^+$ while the rest were females. 100% of the samples were male $FLT3^-$& $NPM1^+$ and 53.6% were females $FLT3^-$& $NPM1^-$ and the rest were males, but there was no statistical difference among them.

Table 7- Distribution of the different mutations according to sex

	NPM1/FLT3-ITD						p
	$NPM1^+/FLT3^-$ (n = 18)		$NPM1^+/FLT3^+$ (n = 4)		$NPM1^-/FLT3^-$ (n = 28)		
	No	%	No	%			
Sex							
Male	11	61.1	4	100.0	13	46.4	^{MC}p = 0.117
Female	7	38.9	0	0.0	15	53.6	

Distributions of mutations according to age

FLT3-ITD mutations and age

Median age of FLT3-ITD wild type and mutant-positive patients was 34 and 34.5, respectively. Three age groups were made as are shown in Table 8.

Table 8- Incidence of FLT3-ITD mutations in different age groups.

	FLT3-ITD				p
	Wild type (n = 32)		Mutant (n = 18)		
	No	%	No	%	
Age					
Min. – Max.	17.0 – 70.0		19.0 – 60.0		1.000
Median	34.0		34.50		

NPM1 mutations and age

Median age of NPM1 wild type and mutant-positive patients was 32 and 36.5 respectively.

Table 9- Incidence of NPM1 mutations in different age groups.

	NPM1				p
	Wild type (n =28)		mutant (n = 22)		
	No	%	No	%	
Age					
Min. – Max.	17.0 – 70.0		19.0 – 60.0		0.732
Median	32.0		36.50		

Distributions of mutations according to FAB types

FLT3-ITD mutations and FAB types

The number and percentage of FLT3-ITD wild type and mutant-positive patients in each FAB type were calculated; M4 and M5 were the highest among all FABs (27.8%) (Table 10).

Table 10-Incidence of FLT3-ITD mutants in each FAB type.

	FLT3-ITD				p
	Wild type (n =32)		Mutant (n = 18)		
	No	%	No	%	
FAB subtype					
M0	1	3.1	0	0.0	0.552
M1	4	12.5	4	22.2	
M2	10	31.3	4	22.2	
M3	0	0.0	0	0.0	
M4	4	12.5	5	27.8	
M5	11	34.4	5	27.8	
M6	2	6.3	0	0.0	

NPM1 mutations and FAB types

In M4 and M5 the incidence of mutations was high but again this was not statistically significant, and there was no mutations in M0, M3 (results were excluded) and M6 (Table 11).

Table 11- Incidence of NPM1 mutations in different FAB types

	NPM1				p
	Wild type (n =28)		Mutant (n = 22)		
	No	%	No	%	
FAB subtype					
M0	1	3.6	0	0.0	0.920
M1	4	14.3	4	18.2	
M2	9	32.1	5	22.7	
M3	0	0.0	0	0.0	
M4	3	10.7	6	27.3	
M5	9	32.1	7	31.8	
M6	2	7.1	0	0.0	

The induction response to chemotherapy

The induction response to chemotherapy along with FAB types, sex and age were show a significant association in median's ages of patients was seen in these samples (p=0.022). After the chemotherapy was administrated, a CR occurred in all FAB subtypes except M4. No significant association of the induction response with blood profile was seen among these samples.

Table 12-Relation between NPM1/FLT3-ITD with demographic data and FAB subtype

	Induction Response										p
	Complete remission (n=2)		Non evaluate (n=5)		Partial remission (n=2)		Induction death (n=3)		Resistant disease (n=10)		
	No	%	No	%	No	%	No	%	No	%	
Sex											
Male	2	100.0	2	40.0	2	100.0	2	66.7	7	70.0	^{MC}p = 0.609
Female	0	0.0	3	60.0	0	0.0	1	33.3	3	30.0	
Age											
Min.–Max.	54.0 – 54.0		37.0 – 54.0		26.0 – 36.0		33.0 – 60.0		19.0 – 54.0		0.022*
Median	54.0		45.0		31.0		45.0		25.50		
FAB subtype											
M0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0.984
M1	0	0.0	1	20.0	1	50.0	0	0.0	2	20.0	
M2	0	0.0	0	0.0	0	0.0	1	33.3	4	40.0	
M4	2	100.0	2	40.0	0	0.0	1	33.3	1	10.0	
M5	0	0.0	2	40.0	1	50.0	1	33.3	3	30.0	
M6	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	

p: p value for comparing between induction response categories, *: Statistically significant at p ≤ 0.05MC: Monte Carlo test

Table 13-Relation between NPM1/FLT3-ITD with some lab investigations

	Induction Response					Test of sig.	p
	Complete remission (n=2)	Non evaluate (n=5)	Partial remission (n=2)	Induction death (n=3)	Resistant disease (n=10)		
Hb (g/dl)							
Min. – Max.	8.90 – 8.90	4.80-12.60	7.90 – 10.0	5.80 – 9.40	6.50 – 11.20	F =0.387	0.815
Median	8.90	10.0	8.95	8.40	7.90		
Platelets ($x10^9$/L)							
Min. – Max.	61.0 – 61.0	44.0 – 81.0	13.0 – 71.0	47.0 – 113.0	13.0 – 211.0	$^{KW}\chi^2$= 0.545	0.909
Median	61.0	56.0	42.0	54.0	40.0		
WBCS ($x10^9$/L)							
Min. – Max.	90.10 – 90.10	35.70 – 159.0	34.80 – 88.40	80.40– 116.40	15.10 – 89.0	$^{KW}\chi^2$= 2.683	0.443
Median	90.10	88.20	61.60	113.50	35.15		
Blast (PB) (%)							
Min. – Max.	51.0 – 51.0	39.0 – 95.0	72.0 – 93.0	20.0 – 70.0	18.0 – 93.0	$^{KW}\chi^2$=3.192	0.363
Median	51.0	42.0	82.50	46.0	72.0		
Absolute-blasts in PB ($x10^9$/L)							
Min. – Max.	45.95 – 45.95	33.92 – 62.01	4.46 – 63.36	23.82 – 56.28	4.46 – 80.10	$^{KW}\chi^2$= 0.403	0.940
Median	45.95	37.04	33.91	51.98	12.46		
Blast (BM) (%)							
Min. – Max.	35.0 – 35.0	50.0 – 95.0	76.0 – 96.0	28.0 – 97.0	55.0 – 91.0	$^{KW}\chi^2$= 4.005	0.261
Median	35.0	54.0	86.0	32.0	80.0		

p: p value for comparing between induction response categories

F: F test (ANOVA)

$^{KW}\chi^2$: Chi square for Kruskal Wallis test

Discussion

FLT3-ITD and NPM1 mutations have been shown to be the most prevalent somatic alterations in AML. Our study was focused on 50 adult AML patients with normal karyotype. Mutation analysis for NPM1 by HRM technique and by conventional PCR for FLT3 gene mutation was performed. Neither NPM1 mutation nor FLT3-ITD was detected in the control group which is similar to previous studies (Falini*et al.* 2008, Alcalay*et al.* 2005, Facchetti*et al.*, 2009, and Döhner*et al.*, 2005)

Among results 18(36%) were positive for FLT3-ITD mutations and (44%) patients were positive for NPM mutation, which were similar to those reported in Germans (31%, 53%) (Döhner*et al.*, 2005; Schnittger*et al.*, 2002; Thiede*et al.*, 2006) and in Japanese (28.0%, 47.4%) (Suzuki *et al.*, 2005; Schlenk*et al.*, 2008).

The incidence of FLT3-ITD mutations was 36% which was slightly above Dunna*et al.* (2010) finding which was 20.4%.

NPM1 is nearly similar to an Egyptian study who found that nearly 47.9 % of patients were positive for NPM1 (Nafea*et al.*, 2011). Also it had been found similar to that reported by Falini et al. who discovered that NPM1 gene mutation targets 50 to 60% of adult CN-AML(Falini*et al.*,2005) and to another study conducted by using similar screening method (HRM) and found to be in 40 % of the patients(Tan *et al* , 2008).

NPM1 mutation is associated with wide morphologic spectrum and multiple lineage involvement. In our study, NPM1 mutation was detected in FAB subtypes from M1 to M5 (M3results were excluded). NPM1 mutation was significantly higher among FAB M5 and M6 patients. This is in accordance with Falini*et al.* (2005) and Schnittger*et al.* (2002) who stated that the incidence of NPM1 gene mutations is more common in AML with monocytic differentiation (M4 and M5 categories).In addition, there was no significant difference in age, sex, white blood cell count, haemoglobin level, platelet count, and peripheral blood blasts, which is different from what was reported so far by Balatzenko *et al.* (2014).NPM1-mutation-positive patients are more often females, with a normal karyotype, and usually present with high white blood cell (WBC) counts and higher percentages of bone marrow blasts, frequently with myelomonocytic or monocytic morphology, with absence or low expression of CD34, and with frequent FLT3 mutations.There was a significant difference in blast count observed between FLT3-ITD-positive subjects and FLT3- ITD-negative subjects (P=0.008). These findings were consistent with some previous reports (Fröhling*et al.*, 2002; Colovic*et al.*, 2007; Huang *et al.*, 2008; Schlenk*et al.*, 2008). Although the effect of FLT3-ITD on inducing leukemogenesis was not directly proved, the ligand-independent constitutive activation of FLT3 induced by ITD mutation could activate some downstream signal molecules including mitogenactivated protein (MAP) kinase, signal transducer and activator of transcription 5 (STAT5), and serine kinase Akt, which contribute to cell proliferation and survival advantages (Hayakawa *et al.*, 2000; Kiyoi*et al.*, 2002).There was no statistically significant relation between the age or sex of the patients and the FLT3-ITD positivity. This finding is similar to the published reports in adults AML (Thiede*et al.*, 2002, Kiyoi-*et al.*, 1999, Yamamoto *et al.*, 2001).About 40% of AML with mutated NPM1 carry an FLT3-ITD mutation. NPM1 mutations are found mainly in AML-NK (Falini, 2008).

As a conclusion our results show that the incidence of NPM1 mutation (44%) was higher than that ofFLT3-ITD(36%). In addition, there was no significant difference in age,sex,WBC count and PB blasts. While, the incidence of FLT3-ITD mutations was (36%) slightly above what was reported before (20.4%).

According to the response of the chemotherapy induction, a complete remission was achieved in (4%) of the patients and a significant association in medians of ages was shown (P=0.022).

Volume : 4 | Issue : 7 | July 2015 • ISSN No 2277 - 8179

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