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DOI: 10.4103/ijh.ijh_52_20

Comparison of differences in blood laboratory results between acute promyelocytic leukemia and acute promyelocytic leukemia+FLT3-internal tandem duplication patients

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Submission: 28-09-2020
Revised: 03-12-2020
Accepted: 12-12-2020
Published: 21-06-2021

Abstract:

BACKGROUND: Acute promyelocytic leukemia (APL) is a well-defined subtype of acute myeloic leukemia (AML) and known by t (15;17)(PML-RARA) mutation. About 20%–40% of AML patients indicate FMS-like tyrosine kinase 3 (FLT3) mutations. FLT3 mutations contain two famous mutations: FLT3-internal tandem duplication (ITD) and FLT3-tyrosine kinase II domain.

OBJECTIVES: Many studies have been done on FLT3-ITD. In these studies have been acknowledged that the FLT3-ITD mutation had a poor prognosis of on AML patients. This study was performed on two APL and APL + FLT3-ITD groups. This study aimed to compare the differences in blood laboratory assays between APL and APL + FLT3-ITD patients.

METHODS: This study contained 73 patients which divided into two groups: APL and FLT3-ITD + APL. The study methods included: cell counting and peripheral blood smear, extraction of mRNA and DNA, and cDNA synthesis electrophoresis.

RESULTS: This study was ruled out on patients involved with APL in GHAEM hospital Mashhad, Iran. All patients were diagnosed with t (15;17) (PML-RARA). The age range of patients was 7–63 years (mean: 30.86). Fifty-eight (79.5%) of patients (male: 22 and female: 36) were involved solely with APL and 15 (20.5%) of them (male: 10 and female: 5) were APL + FLT3-ITD mutation. Blood parameters that were analyzed included white blood cell, red blood cell, hemoglobin, hematocrit, mean cell volume, and platelet count. Each group of patients' population was categorized into high risk factors and low risk factors.

CONCLUSIONS: the consequence of the current study demonstrated that FLT3-ITD mutation had a bad effect on laboratory assays in patients involved with APL.

Keywords:

Acute promyelocytic leukemia, blood cells, Fms-like tyrosine kinase, internal tandem duplication, laboratory assays

Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia in adults. AML disorders are divided into various subgroups. The first classification of AML was French-American-British (FAB) that classified the AML into M0-M7 subgroups. FAB classification was depended on the predominant cell morphology in bone

How to cite this article: Kanesbi MM, Jarahi L, Ayatollahi H, Sheikhi M. Comparison of differences in blood laboratory results between acute promyelocytic leukemia and acute promyelocytic leukemia+FLT3-internal tandem duplication patients. Iraqi J Hematol 2021;10:34-40.

marrow aspiration or peripheral blood smear (PBS).[1] The new classification of blood disorders is presented by the World Health Organization (WHO). The WHO in due to reach an accurate diagnosis classified the AML in new categories such as cytogenetic assays, molecular pathology, and immunohistochemistry.[2] Acute promyelocytic leukemia (APL) is a well-known subtype of AML which occurs in 15%–20% of whole AML disorders.[3] About 7%–8% happening is because of *de novo* mutations.[4] APL is characterized by translocation between chromosomes 15 and 17 [t (15;17) (q22;q21)] that joint two genes on these chromosomes together and make the PML-RARA complex and the dominance of promyelocytic cells in the bone marrow and PBS.[1,5,6] This genetic disorder occurs in over 98% of APL patients.[5] PML-RARA chimeric protein blocks the differentiation process and causes the reduction of maturation procedure in progenitor cells.[7-10] The immunophenotype of the APL cells shows a heterogeneous CD13, strong expression of CD33 and CD34, negative for HLA-DR, and the absence of CD15.[11,12] One of the most frequent mutations that occur in AML is FMS-like tyrosine kinase 3 (FLT3). FLT3 gene is located on chromosome 13q12 that encodes tyrosine kinase receptor class III (TKRs). FLT3 expression has an important role in the differentiation and proliferation of hematopoietic stem cells (HSC).[13-16]. The structure of FLT3 includes five immunoglobulin-like regions termed: an extracellular domain, juxta-membrane domain, a transmembrane region, and two intracellular tyrosine kinase domain.[17,18] FLT3 has two well-known mutations: FLT3 with internal tandem duplication mutation (FLT3-ITD) and FLT3 with tyrosine kinase II domain mutation (FLT3-TKD).[3] Various studies have demonstrated that *de novo* FLT3-ITD mutation happens in 20% of AML cases.[4,9,12] The possibility of FLT3-ITD and FLT3-TKD occurrence in AML subtypes is 12%–38% and 5%–10%, respectively.[5,11,13] Many studies have shown that FLT3-ITD has a poor prognosis and a high possibility of recurrence in AML patients.[5,11,12] Adversely, some studies acclaimed that the effect of the FLT3-ITD mutation on laboratory outcomes are controversial yet.[4,8,11,13] However, the effect of FLT3-TKD is not so clear, and there is no particular prognosis.[11,19]

The purpose of this study is to measure and compare the blood laboratory assays in APL and APL + FLT3-ITD patients to determine the outcome of the FLT3-ITD mutation on laboratory results in APL patients.

Methods

Patients

This research was a retrospective study performed on 73 patients who referred to "Cancer and Molecular Pathology Research Center" of GHAEM Hospital in Mashhad during the years 2016–2019. This study was approved by the Ethics committee of Mashhad University of Medical Sciences. The patients have been diagnosed by the hemato-oncology specialist, and then, the blood samples were referred to the pathology and hematology laboratory. The patients' sample can be either bone marrow aspiration or whole blood, and then, the smears were stained by Giemsa, myeloperoxidase, and acid phosphatase.

Sample acceptance values

(a) Diagnosis of APL with t (15;17) based on the WHO 2016 classification; (b) the sample should be sufficient and enough filled; and (c) patients with newly diagnosed or recurrent APL.

Sample rejecting values

(a) Clotted or hemolysis sample; (b) Dubious or uncertain results; (c) Patients without blood disorders; (d) Patients with non-myeloid disorders; and (e) Other FLT3 mutations such as FLT3-TKD.

Techniques

Complete blood count

Blood cell count was counted by hematology analyzer-SYSMEX. The following parameters were measured for all samples: white blood cells (WBCs), red blood cells (RBCs), hematocrit (Hct), hemoglobin (Hb), mean cell volume (MCV), and platelet count (PLT). Apparently, these samples were abnormal in analyzing by the analyzer and usually showed some errors due to the blasts or precursor cells presence so the blood smear should be checked by pathology specialist or hematology technician. The evaluation of PBS revealed an increased number of myeloid precursors with a predominance of promyelocytes along with some Auer rods. The other cells (RBCs and platelets) often had no significant abnormality in their morphology.

DNA extraction

In order to detect FLT3-ITD mutations, DNA extraction procedure was carried out using a commercial DENAzist Asia kit® according to manufacturer's instructions. The procedure was as follows: 20 µl of proteinase K was transferred to a 1.5-ml microfuge tube followed by 150 µl of ethylenediaminetetraacetic acid (EDTA)-blood sample and two buffers (150 µl buffer I and 200 µl buffer II). The sample was mixed promptly by a vortex, and then the mixture was incubated at 60°C for 15 min. After incubation, 300 µl of ethanol 96% was added with gentle mixing and lysate was transferred into a spin column in a collection tube. The spin column was centrifuged at 8000 rpm for 2 min. Then, 400 µl buffer III was added into the spin column, and it was centrifuged for another 1 min at 10,000 rpm. Eventually, 100 µl buffer IV (elution buffer) was added for DNA elution.

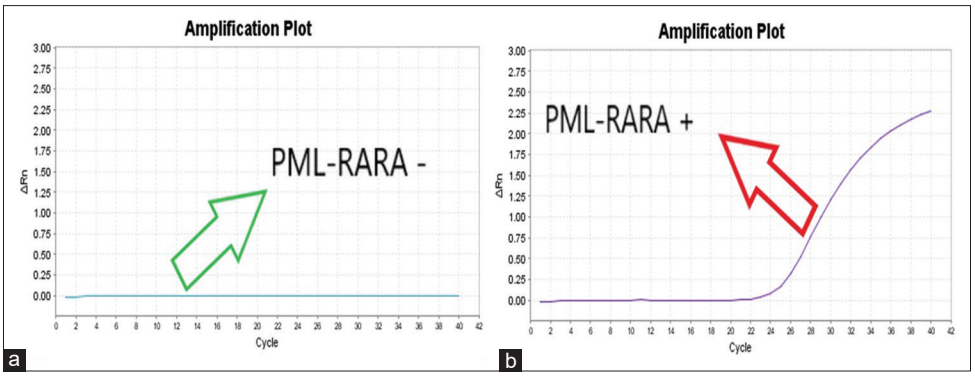

Figure 1: Graphical table of real-time polymerase chain reaction detection of PML/RARA fusion by ABI step one. (a) Amplification plot for a negative sample. (b) Amplification plot for a positive sample

RNA extraction and cDNA synthesis

RNA was extracted from peripheral blood or bone marrow included EDTA anticoagulant. Then, APL blood samples were referred to the cytogenetic department. RNA extraction was done by Yekta Tajhiz Azma kit order (Cat NO: YT9065). mRNA was converted to the cDNA and marker probes were added to cDNA. The cDNA synthesis process was performed by Yekta Tajhiz Azma kit protocol (Cat NO: YT4500). 0.1 ng-5 µl of target RNA and 1 µl of oligo (dT) 18 primer (50 µM) were mixed then this complex was combined with distilled water to make a 13.4 µl solution. After a short centrifuge, 4 µl of 5x buffer with 0.5 µl inhibitor of RNase (RNasin 40U/µl), 1 µl of dNTPs complex, 1 µl of M-MLV (Moloney Murine Leukemia Virus reverse transcriptase) were added. Finally, the mixture was incubated at 42°C for 60 min and 5 min at 70°C.

Real-time polymerase chain reaction

Bio-system step one plus real-time polymerase chain reaction (PCR) system (ABI-Step one) was used to detect the PML-RARA fusion on cDNA chain. The sequence of cDNA primers in sense gene chain was F: 5´-AGG CAG TTC A-3´ and in anti-sense gene chain was R: 5´-ATC TCA GGG A-3´ and the specified probe (Yekta Tajhiz Azma, Cat NO: YT2505) was 5´FAM-AGT GCC CAG CCC TCC CTC GC-TAMRA 3´. The real-time amplification program was 1. Holding section: 30 s in 95°C 2. Thermal cycling: 4 s in 95°C and then 32 s in 60°C. Afterward, a heightened curve demonstrates the mutated gene and a straight line show a normal gene sequence by ABI-Step one real-time PCR [Figure 1].

Detection of FLT3- internal tandem duplication mutation

For the detection of FLT3-ITD mutation, extracted DNA from the blood samples was multiplied by the PCR. The sequence of forward primer was F: 5´TGG TGT TTG TCT CCT CTT CAT TGT-3´ and reverse primer was R: 5´GTT GCG TTC ATC ACT TTT CCA A-3´. The cycling

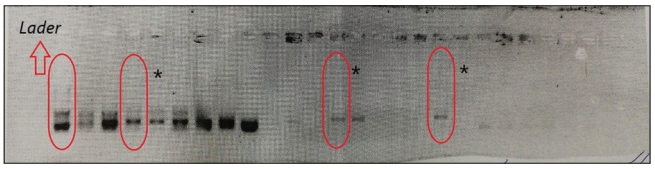

Figure 2: The result of proliferated DNA on Agarose Gel electrophoresis. Marked samples showed obvious and reliable bands. For other vague samples the whole process was repeated again to obtain accurate results

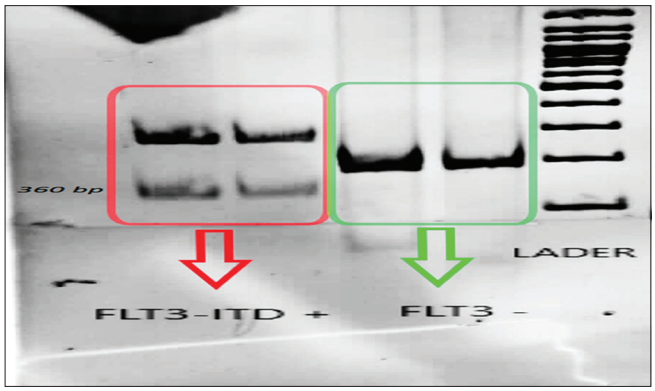

Figure 3: Positive and negative FLT3-internal tandem duplication mutation by polyacrylamide gel electrophoresis

program of the PCR was: holding in 94°C for 5 min, 30 s in 94°C, 30 s in 61°C, 30 s in 72°C (the last three steps were repeated 40 cycles), and at the end, it held in 72°C for 5 min. After that, the proliferated DNA was loaded on the 2% agarose (Ladder: YTA 50 bp DNA) electrophoresis gel to that the final product is sufficient. The agarose gel electrophoresis voltage system was set on 100–110 volt. After 20–25 min, the gel was ready for examination under the ultraviolet (UV) light. We reached a single and clean DNA banding on the agarose gel [Figure 2].

The final diagnosis of FLT3-ITD is on the 5% polyacrylamide electrophoresis gel. The samples that were clear on agarose gel would mix with 7 µl of loader solution and then loaded on the polyacrylamide gel. The polyacrylamide gel electrophoresis voltage system was set on 250–260 volt for 1.5–2 h. The gel was stained

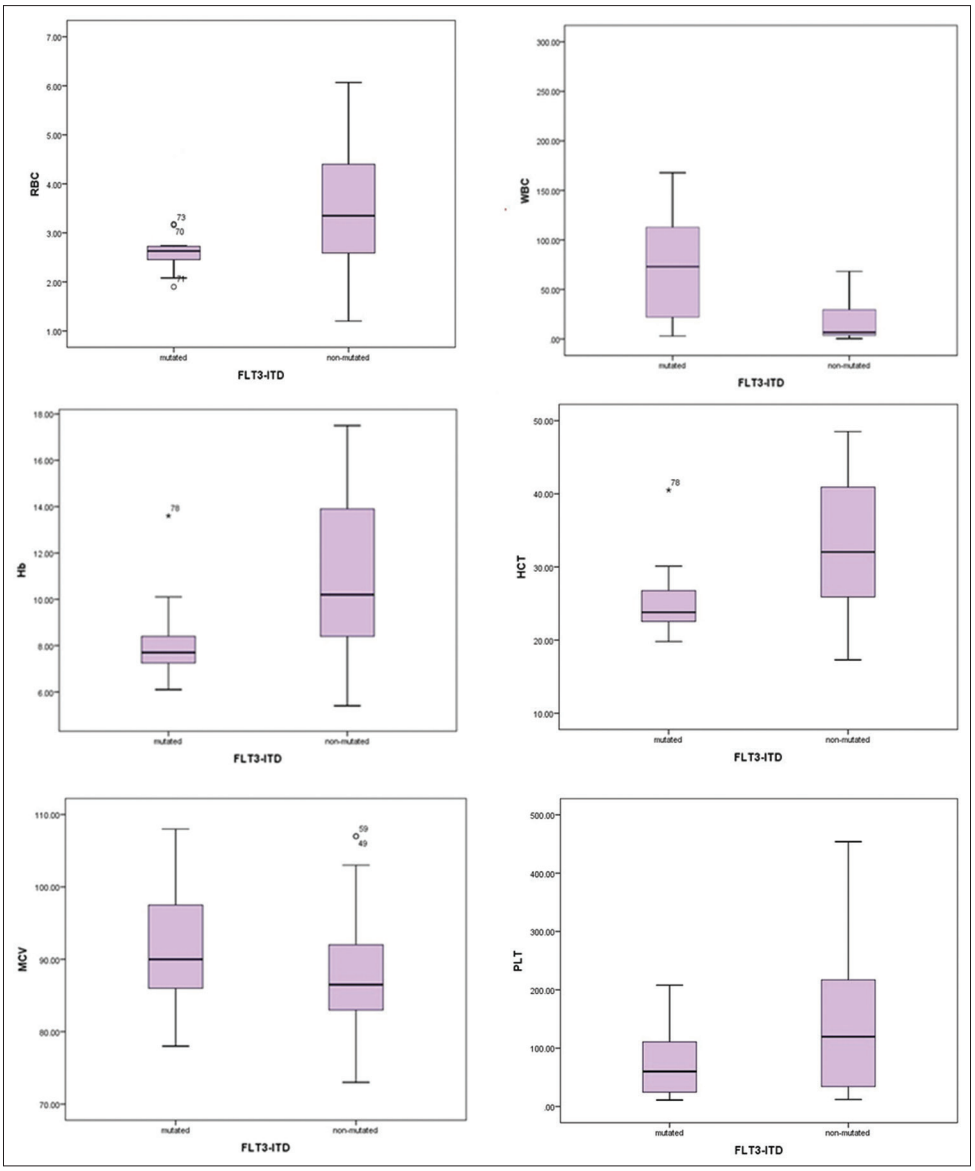

Figure 4: Box-Plot charts to compare the results of two groups of patients: APL+ FLT3-ITD patients and non FLT3-ITD APL patients. In all figures the Box No.1 show the dispersion related hematological index in APL+FLT3-ITD mutation population and Box No.2 is about the dispersion in APL population

in ethidium bromide for 5–6 min. The gel was ready to be analyzed under the UV light. Two-banded samples on polyacrylamide gel had FLT3-ITD mutation and wild-type samples were one banded [Figure 3].

Statistical analysis

The statistical analysis was done by the *RevMan version 5.3. Copenhagen: The Nordic Cochrane Centre*. The normality of parameters was checked by nonparametric tests in two independent parameters (Kolmogorov–Smirnov test). No parameters were normal. The comparing between quantitative parameters (blood parameters) and qualitative parameters (FLT3-ITD mutation) was checked by Mann–Whitney U-test. $P < 0.05$ was considered statistically meaningful.

Results

This study was done on 73 APL patients with age range 7–63 (mean: 32) years old. There were 32 (43.8%) male patients and 41 (56.2%) female patients. Hundred percent of patients had t (15;17)(PML-RARA). Fifty-eight (79.5%) patients involved just with APL and 15 (20.5%) of whole patients had APL + FLT3-ITD mutation. Although many scientific articles revealed that FLT3-ITD is a bad factor in acute myeloid leukemia malignancies, but some studies are dubious about FL3-ITD consequence. Patients' blood results in Tables 1 and 2 demonstrate the potency of FLT3-ITD mutation to make a poor outcome in laboratory blood tests results more than APL without FLT3-ITD mutationalso shown in Figure 4. In Table 2, patients were divided into low and high risk groups.

Table 1: Quartiles of characteristics of patients with APL and APL+FLT3-ITD mutation and both together

Parameters	Non-FLT3 APL (median)	*P*	APL+FLT3-ITD (median)	*P*	Total (median)
Age (years)	7-63 (31.00)	0.22	26-55 (37.66)	0.22	7-63 (31.00)
Sex (male-female)	22-36	-	10-5	-	32-41 (73)
WBC (×103/µl)	0.4-253 (7.50)	<53 (7	3-167 (73.00)	<0.001	0.4-253 (9.70)
25%	3.70		18.70		4.75
50%	7.50		73.00		9.70
75%	31.65		119.50		49.10
RBC (×106/µl)	1.2-6.07 (3.50)	0.002	1.9-4.41 (2.63)	0.004	1.2-6.07 (3.01)
25%	2.68		2.44		2.56
50%	3.35		2.63		3.01
75%	4.37		2.74		4.18
Hb (g/dl)	5.4-17.5 (10.20)	<7.5 (	6.1-13.6 (7.70)	0.001	5.4-17.5 (9.30)
25%	8.40		7.10		7.75
50%	10.20		7.70		9.30
75%	13.82		8.50		13.30
Hct (L/L)	17.30-48.50 (32.05)	<8.50	19.80-40.50 (23.80)	0.003	17.3-48.5 (28.50)
25%	25.90		22.00		23.65
50%	32.05		23.80		28.50
75%	40.60		27.00		40.25
MCV (fl)	73-108 (86.50)	0.001	78-108 (90.00)	0.093	73-108 (87.00)
25%	83.00		86.00		83.65
50%	86.50		90.00		87.00
75%	92.00		98.00		92.30
PLT (×103/µl)	11-454 (123.00)	<54 (1	11-208 (60.00)	0.047	11-454 (96.00)
25%	37.75		21.00		33.00
50%	123.50		60.00		96.00
75%	228.00		124.00		211.00

WBC: White blood cell, RBC: Red blood cell, Hb: Hemoglobin, Hct: Hematocrit, MCV: Mean cell volume, PLT: Platelet

Table 2: Critical border of patient characteristics dividing in low risk and high risk groups

Parameters	Non-FLT3 APL (mean)	*P*	APL+FLT3-ITD (mean)	*P*	Total (mean)
Age (year)	7-63 (30.86)	0.22	26-55 (37.66)	0.22	7-63 (32.02)
Sex (male-female)	22-6	-	10-5		32-41 (73)
WBC (×103/µl)	0.4-253 (24.15)	<53 (2	3-167 (75.84)	<67 (7	0.4-253 (34.77)
<10, *n* (%)	36 (62.1)		2 (13.3)		38 (52.1)
≥10, *n* (%)	22 (37.9)		13 (86.7)		35 (47.9)
RBC (×106/µl)	1.2-6.07 (3.46)	0.002	1.9-4.41 (2.69)	0.004	1.2-6.07 (3.30)
<4, *n* (%)	39 (67.2)		14 (93)		57 (70.4)
≥4, *n* (%)	19 (32.8)		1 (7)		24 (29.6)
Hb (g/dl)	5.4-17.5 (10.73)	<7.5 (	6.1-13.6 (8.20)	0.001	5.4-17.5 (10.21)
<11, *n* (%)	33 (56.9)		14 (93)		51 (63)
≥11, *n* (%)	25 (43.1)		1 (7)		30 (37)
Hct (L/L)	17.3-48.5 (32.69)	<32.69	19.8-40.5 (25.62)	0.003	17.3-48.5 (31.23)
<35, *n* (%)	35 (60.3)		14 (93)		53 (65.4)
≥35, *n* (%)	23 (39.7)		1 (7)		28 (34.6)
MCV (fl)	73-108 (87.92)	0.001	78-108 (91.47)	0.093	73-108 (88.65)
<92, *n* (%)	46 (79.3)		9 (60)		55 (75.3)
≥92, *n* (%)	12 (20.7)		6 (40)		18 (24.7)
PLT (×103/µl)	11-454 (147.81)	<54 (1	11-208 (72.00)	0.047	11-454 (132.23)
<150, *n* (%)	32 (55.2)		14 (93)		51 (63)
≥150, *n* (%)	26 (44.8)		1 (7)		30 (37)

Patients with high risk factors usually show MDR and significant risk of disease recurrence. Patients with low risk factors often exhibit minimal risk of disease recurrence. WBC: White blood cell, RBC: Red blood cell, Hb: Hemoglobin, Hct: Hematocrit, MCV: Mean cell volume, PLT: Platelet, MDR: Multidrug resistance

The low risk factors include WBC <10 × 10^3/µl, RBC ≥4 × 10^3/µl, Hb ≥11 g/dl, Hct ≥35 L/L, MCV <92fl, and PLT ≥150 × 10^3/µl. The high risk factors include WBC ≥ 10 × 10^3/µl, RBC <4 × 10^6/µl, Hb <11 g/dl, Hct <35 L/L, MCV < 92fl, and PLT < 150 × 10^3/µl. The results show that the outcome of APL + FLT3-ITD

malignancies are involved with higher proliferation, increased renewability potency of HSC because of the FLT3-ITD ability to make more and more myeloid precursor cells, increased number of disease recurrence, intricate treatment procedure and significant change in blood parameters count as shown (i.e., conspicuous increase in WBC count whilst decrease in RBCs, HB, HCT, and PLTs).[14-16] 28.82 (49.7%) of APL and 12.46 (83.1%) of APL + FLT3-ITD mutation patients had high risk factors and 29.1 (50.3%) and 2.52 (16.8%) were low risk.

Discussion

APL is a subtype of acute myeloid leukemia and occurs because of t (15;17) in over 98% situations.[1,5,14] Other different mutations may occur with this malignancy. One of the major mutations in APL is FLT3-ITD. Many studies have shown that FLT3-ITD mutation has a poor prognosis on patient clinical situations and life longevity, but some studies mentioned that the consequence of APL + FLT3-ITD is controversial.[4,8,13] Souza Melo *et al*.'s study in 2014 was done on two groups of patients: APL (n = 26) and APL + FLT3-ITD (n = 8). The median amount in APL and APL + FLT3-ITD consequently was as follows: WBC (3.4 and 32.1 × 10^9/L), Hb (8 and 10 g/dl), and PLT (22.5 and 24 × 10^9/L).[5] A study of Gale *et al*. in 2005 on 203 APL patients whom 115 patients were negative for FLT3-ITD mutation and 69 of them were APL + FLT3-ITD. Blood cell characteristics contain median APL and APL + FLT3-ITD in two groups consequently were WBC (2.2 and 8.9 × 10^9/L) and PLT (23.5 and 23 × 10^9/L).[9] These studies have shown the poor outcome of FLT3-ITD mutation on blood parameters. This study is done on various blood characteristics described risk factors in two different phases: APL without FLT3-ITD mutation and APL + FLT3-ITD. The results showed that the presence of FLT3-ITD mutation causes: A: increase in WBC count and MCV index ratio to the non FLT3-ITD APL; B: Reduction in RBC/Hb/Hct and PLTs count ratio to the non-FLT3-ITD APL in most cases; C: Significant increase in the rate of high risk factors in patient with APL + FLT3-ITD (83.3%) and in APL patients were (54.5%). The complex APL + FLT3-ITD patients need more attention for treatment and FLT inhibitors and if necessary patients should receive blood component products for their cytopenias in RBC and PLT count and when WBC count is so high WBC apheresis is recommended to decline danger of leukostasis in patients. We assume that the increase in MCV index is due to the presence of blast and precursor cells in blood because these series of cells are larger than typical cells in the blood.

Acknowledgment

In the end, we appreciate Mashhad University of Medical Sciences (MUMS) and all individuals in Genetic department of GHAEM hospital for their patience and helpful ideas for this study.

Financial support and sponsorship

This study was financially supported by the Monetary Department of University.

Conflicts of interest

There are no conflicts of interest.

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