Laboratory Assays in Patients with Acute Promyelocytic Leukemia and FLT3-TKD Mutations

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Abstract

Acute Promyelocytic Leukemia (APL) is a well-characterized subtype of Acute Myeloid Leukemia (AML). The majority of patients with acute promyelocytic leukemia harbor the reciprocal translocation of t(15;17)(PML-RARα). Recent studies have shown that FMS-Like tyrosine kinase receptor-3 (FLT-3) mutations occur in approximately one-third of AML patients. Two major categories of clinically significant FLT3 activating mutations have been identified: Internal tandem duplication (ITD) mutations within the juxtamembrane domain of FLT3 gene and missense point mutations in the tyrosine kinase domain. Several studies have demonstrated a poor overall survival with FLT3-ITD mutation which is found in approximately 20-40% of AML patients. In contrast, the prognostic significance of FLT3-TKD alone is still unclear, but some studies indicate that it also confers a poor prognosis in AML patients. The current study was performed on 66 [Male: 27(41%); Female: 39(59%)] APL patients to analyze and compare hematological parameters between APL patients with wild-type FLT3 (FLT3-WT) [87% (n=58)] and APL patients with mutant FLT3-TKD [12% (n=8)]. Quantification of PML-RARα transcripts was performed by Real-time quantitative PCR. FLT3-TKD mutation was detected by Polyacrylamide Gel Electrophoresis and CBC values were measured with a SYSMEX automated hematology analyzer. The participants of both cohorts were stratified into high-risk and low-risk groups based on their hematological parameters. The findings indicated that APL patients with FLT3-TKD mutation had laboratory features with more favorable outcome compared with patients with wild-type FLT3.

Keywords: Acute promyelocytic leukemia; FLT3-TKD mutation; Blood parameters

Introduction

Acute Promyelocytic Leukemia (APL, M3 by the French-American-British (FAB) classification) is a distinctive subset of acute myeloid leukemia (AML) [1-8]. The reciprocal translocation of t (15; 17) (PML-RARα) accounts for over 95% of all acute promyelocytic leukemia cases [1].

FMS-Like tyrosine kinase receptor-3 (FLT-3) is one of the most frequently mutated genes in acute myeloid leukemia (Approximately 20-40% of all AML cases). The human FLT-3 gene resides on chromosome 13q12 and encompasses 24 exons [6,7]. It encodes a 993-amino acid protein which belongs to the receptor tyrosine kinase class III family. FLT-3 is primarily expressed by various hematopoietic immature cells, including CD34+ stem cells and committed lymphoid and myeloid progenitors, thus playing a crucial role in normal processes of hematopoiesis [8-12].

Two major types of FLT-3 activating mutations have been described: the most common internal tandem duplications (ITDs), present in 30-40% of AML and 15-40% of APL patients, and missense point mutations within tyrosine kinase domain (TKD), present in 7% of AML and 10-20% of APL cases [9,13]. The D835Y point substitution is the most commonly identified TKD mutation and constitutes up to 50% of mutations [9,11,12,14]. Other less frequent point mutations such as K663Q, Y842C, V952A, and those in the juxtamembrane domain also have been reported [9,12]. Several studies indicated that the presence of an FLT3-ITD in AML patients is associated with poor prognosis and impaired overall survival. In contrast, the prognostic impact of an FLT3-TKD on the Patients with AML remains controversial and needs further investigations [2-4,7,12].

In the present study, which included two groups of APL patients with wild-type FLT-3 and mutant FLT3-TKD, we evaluated the impacts of FLT3-TKD mutation on complete blood count parameters.

Methods and Materials

Patients

This research was a retrospective study performed on 66 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. Peripheral blood or bone marrow smears of all patients were stained by Giemsa, Myeloperoxidase and acid phosphatase. All smears were assessed by two accomplished pathologists. The inclusion criteria were as follows: (1) Patients who were diagnosed with acute promyelocytic leukemia according to FAB classification and concurrent t (15; 17) according to WHO 2016 classification; (2) Either newly diagnosed or recurrent APL cases; (3) Patients with sufficient samples for further analyses. The research exclusion criteria were as follows: (1) Cases diagnosed with other myeloid malignancies; (2) Patients without t (15; 17) and those who harbored other FLT-3 mutations other than FLT3-TKD, (3) Clotted or hemolysis samples.

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Hematological analysis

Samples were analyzed using a SYSMEX automated hematology analyzer. The following parameters were measured for all samples: White Blood Cells (WBC), Red Blood Cells (RBC), Hematocrit (Hct), Hemoglobin (Hb), Mean Cell Volume (MCV), and Platelet count (PLT). Manual blood smears were prepared and examined by a pathologist to investigate abnormal test results that the automated analyzer flagged as unreliable. The evaluation of peripheral blood smears revealed an increased number of myeloid precursors with a predominance of promyelocytes along with some Auer rods.

DNA extraction: In order to detect FLT3-TKD mutations, DNA extraction procedure was carried out using a commercial DNAzist 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 EDTA-blood sample and two buffers (150 µl buffer I, and 200 µl buffer II). The sample was mixed promptly by a vortex, then the mixture was incubated at 60°C for 15 minutes. After incubation, 300 µl of ethanol 96% was added with gentle mixing and the lysate was transferred into a spin column in a collection tube. The spin column was centrifuged at 8,000 rpm for 2 minutes. Next, 400 µl Buffer III was added into the spin column and it was centrifuged for another 1 minute at 10,000 rpm. Finally, 100 µl Buffer IV (elution Buffer) was added for DNA elution.

RNA extraction and cDNA synthesis: Extraction of total RNA from frozen Bone marrow (BM) or peripheral blood (PB) mononuclear cells collected at diagnosis and kept at -80°C, was carried out using Yekta Tajhiz Azma Kit (CAT No: YT9065) according to manufacturer’s instructions. The synthesis of complementary DNA (cDNA) was performed by using a cDNA synthesis kit (Yekta Tajhiz Azma; CAT No: YT4500) according to the kit protocol. To synthesize cDNA; 5µl of RNA was mixed with 1 µl of oligo (dt) 18 primer (50 µM), and the resulting mixture was brought to a final volume of 13.4 µl by adding DEPC treated water. After a short centrifuge, 4 µl of 5x buffer, 0.5 µl inhibitor of RNase (RNasin 40U/µl), 1 µl of dNTP mix (10 mM) and 1 µl of M-MLV (Moloney Murine Leukemia Virus reverse transcriptase) were added. Finally, the mixture was incubated for 60 min at 42°C and 5 minutes at 70°C.

Real time PCR (RT-PCR): The Applied Bio-system Step One plus Real-Time PCR Systems (ABI-Step one) was employed to detect PML/RARA Fusion Transcript. Sequences of the forward primer, the reverse primer, and the probe were respectively as follows: F: 5´-AGG CAG TTC A-3´, 5´-ATC TCA GGG A-3´ and 5´FAM-AGT GCC CAG CCC TCC CTC GC-TAMRA 3´. The Real time PCR reaction was prepared at a final volume of 20 µl, using 10 µl of TaqMan master mix, 1 µl of each primer, 1 µl of probe, 2 µl of cDNA, and 7 µl of sterilized water. The target cDNA was amplified under the following cycling conditions: 95°C for 30 sec, 95°C for 4 seconds, and 60°C for 32 sec. Comparative CT method was used to determine PML/RAR α fusion transcripts (Figure 1).

![Amplification Plot A](image1.png)

**Figure 1**: Graphical representation of Real-time Polymerase chain reaction (PCR) detection of PML/RARA fusion transcript by ABI Step One. ∆Rn is plotted against PCR cycle number. (A) Amplification plot for PML-RAR α negative samples. (B) Amplification plot for PML-RARA positive samples.
Detection of FLT-3 TKD mutations: For detection of FLT-3 TKD mutation, genomic DNA was amplified by using F: 5′-CCG CCA GGA ACG TGC TTG-3′ as the forward primer and R: 5′-GCA GCC TCA CAT TGC CCC-3′ as the reverse primer. The cycling conditions for PCR were as follows: 94°C for 5 min, 94°C for 30 sec, 61°C for 30 sec, and 72°C for 30 sec; the last three steps were repeated 40 cycles. Agarose gel electrophoresis was employed to check that the final product is specific. PCR products were separated by 2% Agarose gel electrophoresis and visualized with the UV light. The 50 bp Marker (DNA Ladder) is used as molecular weight standard for agarose gel electrophoresis. The Agarose gel electrophoresis voltage system was set on 100-110 volt. After 20-25 min the gel was ready for examination under the UV light. We observed a single and quite clean DNA band in each lane of electrophoresis (Figure 2).

The final PCR products with clear bands on agarose gel were digested for 16 hours at 37°C with EcoRV, then the samples were mixed with 7 µl of Loader solution and run onto the 5% Polyacrylamide gel. Electrophoresis was carried out at 260V for 2-2.5 hours. After the end of the electrophoresis, the gel was carefully stained in Ethidium bromide for 5-6 min and visualized under the UV light. Mutant FLT3-TKD was digested by the enzyme and appeared as three distinct bands at the end of the electrophoresis, the gel was carefully stained in Ethidium bromide for 5-6 min and visualized under the UV light. We observed a single and quite clean DNA band in each lane of electrophoresis (Figure 2).

Statistical analysis: The statistical analysis was performed using the SPSS ver.22. Continuous variables were analyzed with the Kolmogorov-Smirnov test for normal distribution between both groups with and without FLT-3 TKD mutation. The comparison between quantitative parameters (blood count parameters) and qualitative parameters (FLT3-TKD mutations) carried out using MANN-WHITNEY U test. P value less than 0.05 was considered statistically significant.

Results

This study was carried out on 66 patients with acute promyelocytic leukemia. Participants had an average age of 30.87 (range 7-63). Of all 66 patients, 27 (40.9%) were males and 39 (59.1%) were females. All of the patients harbored t (15; 17) (PML-RAR α). Of these 66 patients, 8 (12.13%) of patients had the FLT-TKD mutation. Not much research has been conducted on an FLT3-TKD mutation and its impacts on APL patients mainly due to the rarity of this mutation compared to an FLT3-ITD. Earlier studies on FLT3-TKD mutation and its effects on blood parameters and disease prognosis show conflicting results. The current study sought to further elucidate the impacts of FLT3-TKD mutation on hematological parameters in patients with acute promyelocytic leukemia. The assessment of changes in hematological parameters can be useful to better predict the disease prognosis. The results of patients' blood analysis in both groups were collected in Tables 1 and 2. In Table 1 hematological parameters were categorized as three quartiles (25%, 50%, and 75%). In Table 2 patients' results were classified into two high-risk and low-Risk groups. High-risk factors (patients with worse clinical status and probably higher rate of recurrence after therapy)
were WBC $\geq 10 \times 10^3/\mu l$, RBC $\geq 4 \times 10^6/\mu l$, Hb $\geq 11$ g/dl, Hct $\geq 35$ L/L, MCV $< 92$ fl and PLT $\geq 150 \times 10^3/\mu l$.

**Discussion**

The main purpose of this study was to investigate the possible association between FLT3-TKD mutation and subsequent changes in hematological parameters in a group of APL patients which could help us to better predict the prognostic impact of this mutation. While some earlier studies claim that FLT3-TKD mutation has a favorable prognosis in AML, the prognostic effect of this mutation in AML patients is still unclear. Guo H and colleagues (2016) evaluated 255 AML patients with various mutations. The frequency of FLT3-TKD mutations and APL patients were 4.0% (9/255) and 20.7% (53/255) respectively. The comparison of hematological parameters between patients with wild-type and mutant FLT3-TKD indicated the following results for WBC, PLT and Hb at 15.3-295.0; 10.73-295.0; 3.46, and at 19.46-23.58; 10.93-10.76; 0.027 respectively. The association between FLT3-TKD mutation and subsequent changes in hematological parameters in APL patients which could help us to better predict the prognostic impact of this mutation.

**Conclusion**

The range of WBC, PLT and Hb in both groups were (15.3-295.0; 0.9-146.0; p-Value=0.001), (6.4-12.4; 4.6-13.4; p-Value=0.33) and (12-146; 7-466; p-Value= 0.19) respectively. Whitman et al. found that the occurrence of hyper-leukocytosis and Leukostasis would be rare in FLT3-TKD, while these events are common in FLT3-ITD mutations. The higher rate of RBC count, Hb, and Hct demonstrated the low occurrence of anemia and lower MCV index showed the relatively small number of blasts cells in peripheral blood. The PLT count in FLT3-TKD+ patients was lower than wild-type patients (44.8% of patients with Wild-type FLT3-TKD had a platelet count upper than 150 $\times 10^9/L$, while 37.50% FLT3-TKD+ patients had a PLT count upper than 150 $\times 10^9/L$). In conclusion, we found that hematological parameters in APL patients with mutant FLT3-TKD suggested a more favorable outcome compared with the wild-type group.

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**References**


