

Comparison of Delta- PCR and Conventional Fragment Analysis for the Detection of FLT3-ITD Mutations in Paired Diagnosis-Relapse Samples of Patients with Acute Myeloid Leukemia

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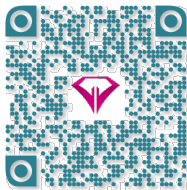
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ABSTRACT

Background & Objective: FLT3-ITD mutation detection has been an integral part of diagnostic work ups focused on acute myeloid leukemia. However, some studies have indicated that the mutation is unstable during the various stages of the disease. The purpose of this study was to evaluate the stability of this marker in paired diagnosis-relapse samples using Delta-PCR method.

Materials & Methods: In this retrospective study, paired diagnosis-relapse bone marrow or peripheral blood samples from 180 adult AML patients were analyzed for FLT3-ITD mutations using conventional fragment analysis and Delta-PCR methods. A dilutional experiment of DNA derived from a FLT3-ITD mutated patient in normal peripheral blood was performed in order to evaluate the sensitivity of each method.

Results: All samples were analyzed using both conventional fragment analysis and Delta-PCR methods. FLT3-ITD mutations were detected in 24 diagnostic samples (13.3%) and 28 relapse samples (15.5 %) through conventional fragment analysis. Three out of four patients who were FLT3-ITD positive in the relapse samples had a mutation in the diagnostic samples using the Delta-PCR method. On the other hand, at the time of diagnosis and relapse, the mutation test results were incompatible in only 3.6% of patients based on the results of the Delta-PCR method compared to 14.2 based on conventional fragment analysis. Our findings revealed that the sensitivity of Delta - PCR as related to FLT3-ITD detection was 0.2 %. Compared to the conventional fragment analysis, with a sensitivity of 2%, Delta - PCR shows greater sensitivity and specificity.

Conclusion: The conventional testing of the FLT3-ITD mutation by fragment analysis did not detect a significant proportion (11%) of FLT3-ITD positive samples in AML patients. Delta PCR increased the sensitivity and specificity relative to the conventional method. The detection of FLT3-ITD mutation through Delta PCR is important in order to detect minor clones at diagnosis or during the monitoring of AML patients.

Keywords: Acute myeloid leukemia, Delta-PCR, FMS-like tyrosine kinase, internal tandem duplication



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Introduction

Despite As the most common leukemia among adults, acute myeloid leukemia (AML) is a type of cancer affecting the myeloid line of blood cells, in which the rapid growth of abnormal cells causes different problems within the normal production of blood cells in the bone marrow (1-3). Nowadays, various types of genetic mutations have been found to be associated with the development and/or relapse of disease (4-6). Moreover, these genetic mutations have

been shown to affect the clinical implications and survival rate among patients (7, 8). Mutations in FMS-like tyrosine kinase (FLT3), which is a member of the class III receptor tyrosine kinase family, have shown to occur in 20-30% of all AML cases (9). The main role of FLT3 as a cell-surface receptor is to maintain the survival, proliferation and differentiation of hematopoietic progenitor cells. FLT3-ITD mutations have shown to be responsible for the continuous

activation of FLT3 and as a result, the uncontrolled proliferation and apoptosis resistance of the myeloid cells (10). Based on previous reports, there has been a direct correlation between the presence of ITD mutations in FLT3 and poor prognosis in AML patients (11). Furthermore, an increased rate of disease relapse and a lower rate of survival has been reported in AML cases with FLT3-ITD mutations (12-14).

Detection of FLT3-ITD mutations in AML patients may provide potential targets for treatment using novel therapeutic methods targeting FLT3 receptor tyrosine kinase inhibitors. Moreover, FLT3-ITD mutations may be considered as a useful marker for monitoring minimal residual disease (MRD) in AML patients (15).

Conventional PCR using fluorescent PCR followed by fragment analysis is the routine test used in the detection of FLT3-LTD mutations in AML cases (16). However, this method suffers from low sensitivity and specificity. Based on some reports, it is not able to discriminate minor clones of FLT3-ITD mutations in some FLT3-ITD mutated patients (17). This can be due to the burden of the disease, which is lower than the detection limit of the method. As a result, using an efficient methodology is of utmost importance for the sensitive and specific detection of mutations (18).

Delta polymerase chain reaction (PCR) assays have been shown to be more sensitive than conventional testing for the detection of FLT3-ITD mutations (19). In this study, we compared the sensitivities of the conventional method with Delta PCR testing for the detection of FLT3-ITD in paired samples from the diagnosis and relapse of AML patients. The purpose of this study was to evaluate the stability of this marker in different stages of the disease using a sensitive and specific Delta-PCR method.

Materials and methods

Patients and sampling

In this study, paired diagnosis-relapse bone marrow and/or peripheral samples from 182 adult acute myeloid leukemia patients who were admitted to the

Hematology, Oncology and Stem Cell Transplantation Research Center of Shariati Hospital were enrolled in our study. Consent forms were obtained from the patients before the sample collection (ethics code: IR.TUMS.SPH.REC.1396.3051). The demographic data was collected from their medical files. For the 180 patients at diagnosis and all of the study patients at relapse, DNA was extracted from freshly isolated bone marrow/peripheral blood mononuclear cells using a standard salting-out technique. In 22 patients, the diagnosis sample DNA was extracted from bone marrow smears slides. Briefly, the blood smear was scraped off with a clean scalpel and placed in a sterile microfuge tube. DNA extraction was performed with a final suspension volume of 20 μ L using the QIAamp DNA Mini Kit (Qiagen). Conventional fragment analysis was performed on all samples, as previously reported (16).

Delta-PCR and fragment analysis

Delta-PCR is considered to be a sensitive technique with high specificity for the detection of FLT3-ITD mutations. In this technique, 3 primers including one standard forward primer and two reverse primers (external primer labeled with 6-FAM, and internal reverse primer labeled with HEX) were used (19). In terms of locating the targets, the reverse primers differed by 19 base pairs (bp) (delta) (Table 1). In delta-PCR, the internal primer is designed in such a way as to act as a confirmation probe. Positive ITD mutations were detected by the presence of a pair of products with a delta of 19 ± 1 bp larger than the products from the wild type allele (160-bp green peak and 179-bp blue peak). Delta-PCR was performed according to Beierl et al. (19) with a few modifications. Briefly, PCR was performed in a total volume of 20 μ L, containing 1 μ L of DNA sample (20-40 ng), 0.7 μ L of forward primer, 0.5 μ L of each reverse primer (internal and external primers), and 10 μ L of master mix (Ampliqon, Denmark). The PCR cycles included a denaturation step (95°C for 4 min), 35 cycles (95°C for 30 seconds, 62°C for 30 seconds and 72°C for 45 seconds), and a final extension step in 72°C for 15 min.

Table 1. Nucleotide sequences in the Delta-PCR.

Primer type	Sequence (5'-3')	Labling	Length (bp)	Product size (bp) $\Delta = 19$ bp
FLT3-F	GCAATTTAGGTATGAAAGCCAGC		23	-
FLT3-R1	CAATGGAAAAGAAATGCTGCAG	Fam	22	182
FLT3-R2	CAGAAACATTTGGCACATTCCA	Hex	22	163
Amelogenin-F	CCCTGGGCTCTGTAAAGAATAGTG		24	106 bp
Amelogenin-R	ATCAGAGCTTAAACTGGGAAGCTG		24	

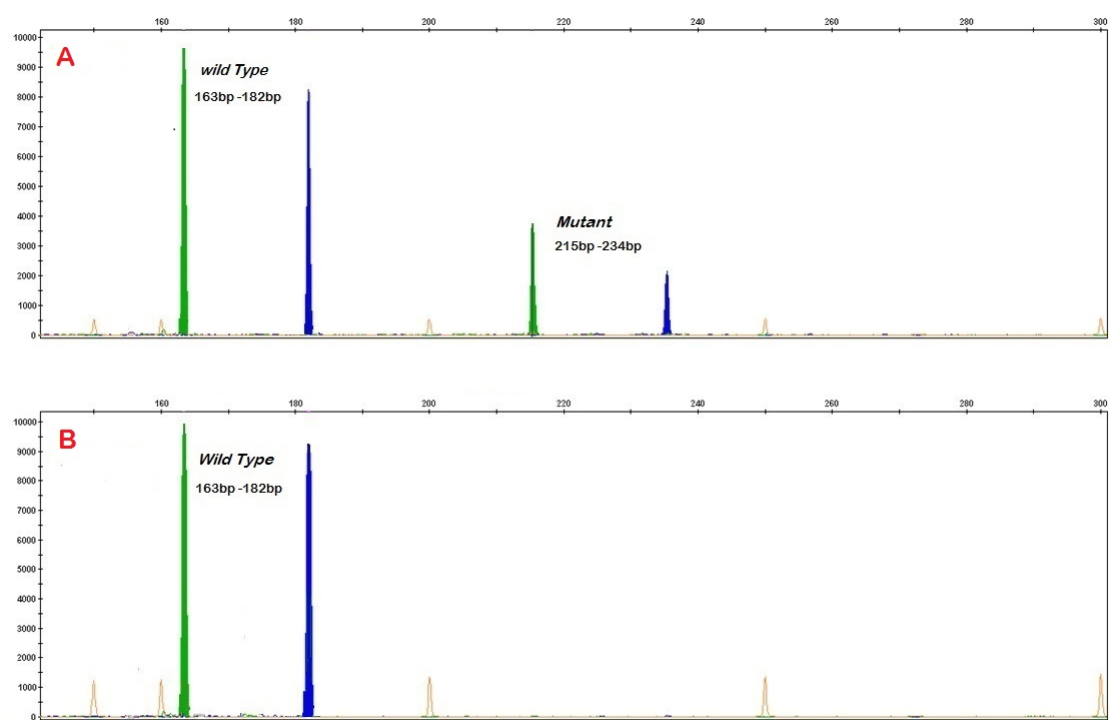


Fig 1. Fragment analysis of mutant and wild type alleles of FLT3 gene with ITD mutation via delta-PCR method. In patients with FLT3-ITD mutation 4 peaks (2 peaks were related to wild type allele and 2 peaks were related to mutant allele (A) For patients with WT FLT3, 2 peaks (163 bp and 182 bp) were observed (B).

For the fragment analysis, the reactions were prepared with a final volume of 10 μ l, containing 0.5 μ l of PCR product, 0.5 μ l of a 106 bp amplicon of amelogenin-labeled TAMRA (as a reference peak), 0.4 μ l GeneScan™ 500 LIZ® Size Standard (Applied Biosystem), and 9.1 μ l of Hi-Di™ Formamide. The reaction tubes were placed in a thermocycler at 95°C for 5 min and then they were immediately placed on ice. After 3 minutes of cooling, the samples were transferred to a plate to perform electrophoresis (Figure 1). ABI Genetic Analyzer 3130 was used for the electrophoresis and fragment separation with two injection times for each sample (10 and 60 seconds). The injection time was 60 seconds for all of the negative samples to ensure that the samples were negative for FLT3-ITD mutation.

The obtained data were analyzed using Gene Mapper software. By dividing the peak height of the ITD product to that of the wild type product, the allelic ratio was calculated. If more than one ITD product existed; peak heights of ITD were added and divided by peak height of wild type. The following formula was employed to determine the allelic ratio when 60 seconds of injection time was used and the wild type FLT3 peak was off-scale (19):

Assessing the sensitivity of delta-PCR

The sensitivity of both mutation detection methods was gauged using different dilutions (1/10, 1/50, 1/100, 1/500 and 1/1000) of a FLT3-ITD mutated sample with allele ratio 50% into a normal peripheral blood sample.

Statistical analysis

For comparison of continuous variables in groups, based on FLT3 mutation status, Mann-Whitney test was used. The chi-square test was employed to determine statistically significant differences in FAB subtype frequencies between WT FLT3 and FLT3-ITD groups.

Results

Among The experiment was performed on a total of 180 paired diagnosis-relapse samples; 105 (58.3%) males and 74 (41.1%) females. Table 2 shows patient characteristics (median and range) of all patients, FLT3 wild type (FLT3 WT) and FLT3-ITD patients.

All of the samples were analyzed using both conventional fragment analyses and Delta-PCR methods. FLT3-ITD mutations were detected in 24 diagnostic samples (13.3%) and 28 relapse samples (15.5 %) through conventional fragment analysis. This means that the results of the mutation examination were incompatible at both the time of diagnosis and at the time of relapse of the disease in 14.2 percent of patients based on the results of the conventional fragment analysis. Of the 24 patients with FLT3-ITD mutations at diagnosis, 23 patients showed the same major mutated clone at relapse and in one patient, a minor clone was predominant in the relapse sample. Three out of four patients who were FLT3-ITD positive in the relapse samples had a mutation in the diagnostic samples according to the Delta-PCR method. On the

other hand, at the time of diagnosis and relapse, the mutation test results were incompatible in only 3.6% of patients based on the results of the Delta-PCR method. The allele ratios in these patients were below the detection limit of conventional fragment analysis (0.2, 0.3, and 0.5). In only one patient, the diagnosis sample was negative according to both methods, but the patient relapsed with a FLT3-ITD mutation (Table 3).

With an injection time of 10 seconds, the conventional fragment analysis and delta-PCR sensitivity were 1/100(1%). By increasing the injection time of the genetic analyzer system from 10 seconds to 60 seconds, the sensitivity of delta-PCR changed from 1/100 to 1/500(0.2%)(Figure 2).

Using delta-PCR, the size of the inserted segment was calculated in the patients. The size of the segment was varied from 15 to 180 bp (medium 54). In total, among the 28 patients with FLT3-ITD at relapse, ten patients (35.7%) had more than one inserted segment, of which seven patients had two inserted segments, two patients had three inserted segments and one patient had five inserted segments. Compared to fragment analysis, delta-PCR detected a higher number of FLT3-ITD mutations per patient compared to conventional fragment analysis (43 vs 39 inserted segments), indicating the higher sensitivity of this method for the detection of subclonal mutations.

$$\text{allelic ratio} = \frac{\text{reference peak (10sec injection)}}{\text{WT FLT3 (10sec injection)}} \times \frac{\text{FLT3 - ITD peak (60 sec injection)}}{\text{referenc peak (60 sec injection)}}$$

Table 2. Patient characteristics (Median and range) of all patients, FLT3 WT and FLT3-ITD patients

	Total (n=180)	FLT3 WT(n=153)	FLT3-ITD (n=27)	P value
Age (Years)				
Median	41.00	41	40.50	
Range	15-79	150-250000	1700-294000	0.8
WBC count (x10 ⁹ /lit)				
Median	10400	5600	25000	
Range	150-294000	150-250000	1700-294000	0.001
Platelet count (x10 ⁹ /lit)				
Median	41000	40000	50000	
Range	2000-499000	2000-499000	4000-375000	0.77
Hb(g/dl)				
Median	8.5	8.4	8.9	
Range	4-14.2	4.9-14	4-14.2	0.6
FAB Types				
AML-M0	3	3(100)	0(0)	
AML-M1	24	22(91.7)	2(8.3%)	
AML-M2	55	50(90.9)	5(9.1)	
AML-M3	2	2(100)	0(0)	
AML-M4	66	52(78.8)	14(21.2)	
AML-M5	18	12(66.7)	6(33.3)	
AML-M6	4	4(100)	0(0)	
AML-M7	1	1(100)	0(0)	
Unknown	7	7(100)	0(0)	0.15

Table 3. Comparison of Delta PCR assay and conventional fragment analysis methods

	No. with conventional fragment analysis method result of (at diagnosis):			No. with conventional fragment analysis method result of (at relapse):		
Delta PCR assay results	Positive	Negative	Total	Positive	Negative	Total
Positive	24	3	27	28	-	28
Negative	-	153	153	-	152	152
Total	24	156	180	28	152	179

Discussion

The occurrence of FLT3-ITD mutation in AML patients is about 25%. This mutation is more prevalent in adults than in children (20). Patients with this mutation do not have a good prognosis, and for most of them, there is an urgent need for receiving allogeneic bone marrow transplant (21). Disease relapse can occur in patients who received transplantation. Recently, it has been shown that the FLT3-ITD mutation could be a good marker in identifying MRD, in addition to being a beneficial guide for using FLT3 inhibitors in AML patients (22-24). In a recent study, Wan and colleagues showed the predictive value of pretransplant FLT3-ITD levels in 84 patients who underwent allo-HCT during their first remission (25).

The U.S. Food and Drug Administration has recently approved Gilteritinib (Xospata®), a dual FLT3/AXL inhibitor, for the treatment of patients with relapsed or refractory acute myeloid leukemia (AML) with a Fms-like tyrosine kinase 3 (FLT3) gene mutation (26). About one-third of AML patients have a genetic fusion in MRD monitoring. NPM1 and FLT3-ITD mutations are the most frequent molecular aberrations in AML patients. The instability of the FLT3-ITD mutations in the various stages of the disease is one of the barriers to using these mutations to determine the MRD in the patients. Although the results of several studies indicate the instability of FLT3-ITD mutations during the disease evolution, the same studies have noticed that the low sensitivity of the method of mutation detection may be one of the reasons for the lack of identification of minor clones that may act as a dominant clone during the disease relapse (27). Levis et al. introduced a highly sensitive PCR/NGS-based method for evaluating the response to FLT3 inhibitors and monitoring MRD in FLT3-ITD mutated patients. They showed that there was a correlation between FLT3-ITD variant allele frequency $\leq 10^{-2}$ and overall survival in patients treated with gilteritinib (23).

The conventional fragment analysis method showed a limited sensitivity (1-5%) and specificity in detection of FLT3-ITD mutations (28-30). Delta-PCR is a novel technique, which can be used for measuring MRD in these patients. In the present study, we tested 180 paired diagnosis and relapse samples collected from AML patients regardless of the presence of FLT3-ITD status at diagnosis. In this study, we examined the performance of conventional fragment analysis assay for detecting FLT3-ITD mutations relative to Delta PCR method.

In 2013, Beierl et al. used delta-PCR for evaluation of bone marrow and peripheral blood samples from 31 AML patients. According to the authors, Delta-PCR with an accurate design could be an ideal choice for efficient and sensitive detection of different mutations, like insertions, deletions. This method is able to detect ITD mutations with higher sensitivity and specificity and therefore, can be used for measuring the MRD in AML patients.

In our study, three patients with a positive FLT3-ITD mutation at the time of relapse were negative for the mutation at the time of disease diagnosis. When the same patients were examined using delta-PCR, all three patients were positive for the mutations. In only one patient, the diagnosis sample was negative in both methods, but the patient relapsed with a FLT3-ITD mutation. This can indicate the stability of the FLT3-ITD marker in our study as conventional fragment analysis and delta-PCR was 86.6 and 96.6 respectively. In other words, one of the reasons for the instability of the mutation in different studies may be the low sensitivity of the mutation detection methods used.

In a retrospective study, Zuffa and et al. used amplicon-based ultra-deep-sequencing (UDS) and they succeeded in detecting the presence of a mutation in all five samples that were positive at the time of relapse or disease progression. They also lacked mutations at the time of diagnosis (31).

In the present study, the size and number of the inserted segments were checked in all patients. A higher number of ITDs per patient was detected due to the higher sensitivity of Delta-PCR. While in previous studies, up to ten FLT3-ITDs were detected in one in AML patients (19), the highest number of FLT3-ITDs per patient in our study was five. Among the ten patients who had more than one insertion (35.7%), seven patients were identified with two, two with three and one with five insertion segments. The presence of more than one inserted segment with different abundances has also been reported by other studies. The size of the inserted segment varied from 6 to 210 bp in Koszarska's et al. experiment and 22 patients out of 69 with FLT3-ITD mutations had more than one mutant amplicon (31.9 %) (32). However, in another experiment by Kim et al, among the 73 patients with a FLT3-ITD mutation, only two patients had more than one insertion (2.7%) (30).

Conclusion

In conclusion, our results emphasize that in the absence of other specific markers, delta-PCR is a sensitive, specific, and cost-effective method for detecting the minor clones of FLT3-ITD mutations. This includes the monitoring of MRD in AML patients with FLT3-ITD mutations. In this experiment, delta-PCR was able to detect the ITD mutation in all of the previously documented samples. Moreover, due to the simultaneous amplification of two bands, the specificity of this method is higher than the standard fragment analysis method that is performed through the use of only one pair of primers.

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Conflict of Interest

Authors declared no conflict of interests.

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