

## Original Article

## Detection of KIT and FLT3 Mutations in Acute Myeloid Leukemia with Different Subtypes

Farhad Zaker PhD\*, Mohammad Mohammadzadeh MSc\*, Mohammad Mohammadi MSc\*\*

**Background:** Mutations in KIT and fms-like tyrosine kinase 3 genes lead to uncontrolled proliferation of leukemic cells with a poor prognosis. Since, data concerning the incidence and associations with patients characteristics vary amongst different studies, the aim of the present study is to identify and quantify the frequency of mutations in Iranian patients suffering from acute myeloid leukemia.

**Methods:** Internal tandem duplication and D835 mutations in the fms-like tyrosine kinase 3 gene of acute myeloid leukemia patients were studied through polymerase chain reaction and polymerase chain reaction-RFLP analysis. Amplified products for a point mutation in D816 for KIT have also been identified through the polymerase chain reaction-RFLP technique. The mutations in exon 8 of KIT were detected by using the PCR and the Conformational Sensitive Gel Electrophoresis techniques, and amplified products have been confirmed by sequencing techniques.

**Results:** Internal tandem duplication and D835 mutations in the fms-like tyrosine kinase 3 gene occurred in 18% and 6% of AML patients, respectively. Frequencies of mutation were 1.4% and 4.7% in exon 8 and D816 of the KIT gene in acute myeloid leukemia patients. These results were substantially different for various subclasses of French-American-British classification.

**Conclusion:** This study revealed that approximately 30% of acute myeloid leukemia patients have either KIT or fms-like tyrosine kinase 3 genetic mutations. The presence of fms-like tyrosine kinase 3 was significantly associated with M3 morphology and mutations of KIT were significantly associated with M2 and M4 subtypes.

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**Keywords:** Acute Myeloid Leukemia • CSGE • mutations • PCR

### Introduction

Amongst various factors, mutations for cell differentiation and proliferation are considered to be effective factors in the development of acute myeloid leukemia (AML). fms-like tyrosine kinase 3 (FLT3) and KIT genes belong to the family of tyrosine kinase class III receptors that induce signals for cell proliferation. Mutations of these genes, however, result in

autonomously leukemic cell proliferation and an unfavorable prognosis.<sup>1-3</sup>

A significant over expression of the FLT3 gene (70 – 100%) occurs in both AML and acute lymphoblastic leukemia (ALL)<sup>4-6</sup> and point mutations in both the D835 and internal tandem duplication (ITD) of this gene occur as main mutations. These mutations lead to a steady and continual activation of the FLT3 tyrosine kinase receptor without ligand stimulation.<sup>7,8</sup> The ITD mutation is the most frequent abnormality that occurs in 20 – 25% of AML patients and the D835 point mutation has been identified in 6 – 10% of patients.<sup>9-12</sup> The augmented activation of KIT with SCF causes cell proliferation whereby in AML, an abnormal increase of proliferation occurs in two ways; first in AML, mutations of D816 (in exon 17) or exon 8 KIT lead to autonomous activation of KIT, and also through over expression of KIT in

**Authors' affiliations:** \*Oncopathology Research Center, Cellular and Molecular Research Center, Iran University of Medical Sciences, \*\*Research Center, Iranian Blood Transfusion Organization, Tehran, Iran.

**Corresponding author and reprints:** Farhad Zaker PhD, Cellular and Molecular Research Center, Iran University of Medical Sciences, Hemmat Freeway, Tehran, Iran, P.O. Box: 14155-6183 Tel: +98-912-376-2258, Fax: +98-218-805-4355, E-mail: farhadz20@yahoo.co.uk

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AML when KIT is expressed in 80 – 90% of blast cells.<sup>13,14</sup> Generally KIT mutations have been reported in less than 5% of AML cases.<sup>15</sup> The poor prognosis in AML patients with mutations in the KIT and FLT3 genes, has led to the development of drugs inhibiting these mutations.<sup>16-18</sup> Due to insufficient studies of the mutations in Iran, the diagnosis and frequency of these mutations with different subtypes in AML patients is an important concern.

## Materials and Methods

### Patients

Blood samples from 212 adult AML patients with various French-American-British (FAB) classifications were obtained from Iranian hematologic and blood transfusion centers between 2006 and 2007. The diagnosis of AML and the assignment of FAB classification were based on morphology and immunophenotype.

### Molecular studies

DNA extraction was performed on all blood samples by the proteinase K and phenol methods.<sup>19</sup>

### Analysis of ITD of the FLT3 gene and D835 mutations

FLT3-ITD and FLT3-D835 mutations were amplified using PCR (Corbett Research) and products were electrophoresed on an 8% polyacrylamide gel as previously described by Gilliland et al.<sup>12</sup> To detect ITD, exons 11 and 12 were amplified by PCR using primers 11F (5'-GCA ATT TAG GTA TGA AAG CCA GC-3'), and 12R (5'-CTT TCA GCA TTT TGA CGG CAA CC-3'). To detect D835, exon 17 was amplified by PCR using primers 17F (5'-CCG CCA GGA ACG TGC TTG-3'), and 17R (5'-GCA GCC TCA CAT TGC CCC-3'). Finally, PCR products were digested with the EcoRV enzyme.<sup>20,21</sup>

### KIT mutational analysis

The Conformational Sensitive Gel Electrophoresis (CSGE) method was used to screen for mutations in exon 8 of the KIT gene as described previously by Gari et al.<sup>22</sup> Briefly, PCR was performed using primers 8F (5'-TTCTGCCCTTTGAACTTGCT-3') and 8R (5'-AAAGCCACATGGCTAGAAAA-3') as previously described by Rapley et al.<sup>23</sup> PCR products were denatured by heating at 95°C for 5 minutes and then incubated at 65°C for 30 minutes. These

heteroduplexed products were then electrophoresed on a 10% polyacrylamide gel consisting of 99:1 acrylamide:BAP (bis-acrolypiperazine), 10% ethylene glycol, 15% formamide and 0.5x TTE buffer (tris, taurine, and EDTA). Samples displaying abnormal CSGE profiles were directly sequenced by an automated sequencing machine to confirm the presence of the mutation. To detect KIT-D816, exon 17 amplified by PCR using primers 17F (5'-TGT ATT CAC AGA GAC TTG GCA-3') and 17R (5'-TAA TTA GAA TCA TTC TTG ACG-3'). Then, PCR products were digested with AatII enzyme as described by Looijenga et al.<sup>24</sup>

## Results

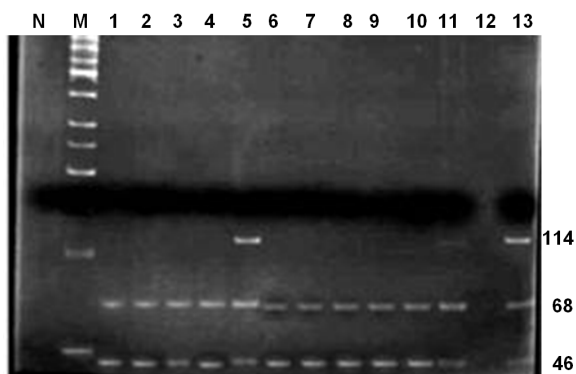
The median age of onset for AML was 47±12 (range from 18 – 75) years, and blood samples from 126 males and 86 females were included in the study.

### Mutational analysis of FLT3

The ITD mutation was observed in 18% of AML patients. In Figure 1, lanes 1 and 3 represented patient specimens with an AML M3 subtype that had a mutation. Lanes 9 and 14 represented AML patients with M2 and M4 subtypes. In all cases, small bands (329 bp) have represented normal cell clones and larger bands were mutated leukemic cell clones. To detect a D835 mutation, exon 17 of FLT3 has been amplified by PCR and then digested with ECORV enzyme (Figure 2). The 114 bp products were shown to be separated into 68 bp and 46 bp with no mutation in the sequences. Patients 5 and 13 produced different bands in which the upper band with 114 bp has a changed enzyme restriction site due to a mutation and the lower band with 68 bp and 46 bp represented genes of a normal cell clone



**Figure 1.** ITD mutations in different subgroups of AML patients (N=negative control, M=marker)

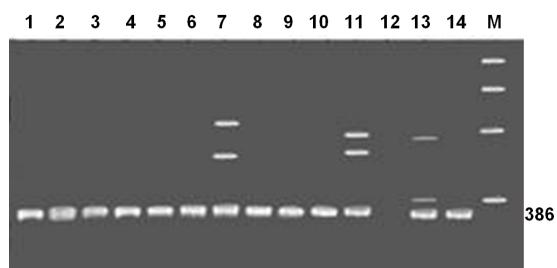


**Figure 2.** D835 mutations in different subgroups of AML patients (N=negative control, M=marker)

in the AML patient. Overall, the present study indicated that in 18% of the ITD mutations; 16% have been found in the M3 subclass and the remainder belong to both M2 and M4 subclasses. Furthermore, of the 6% with mutations in D835; 4% were located in the M3 subclass and the rest in both M2 and M5 subclasses.

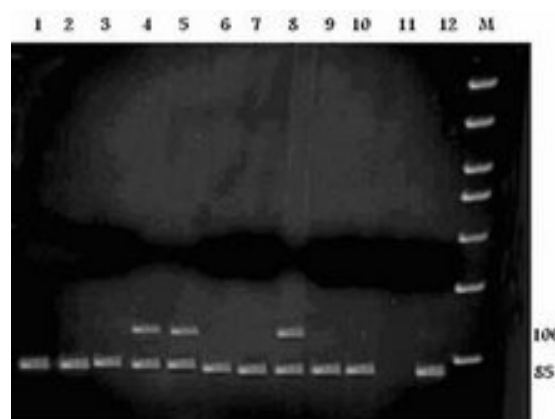
### Mutational analysis of KIT

Of the 212 AML patients investigated in this study, three patients' specimens had mutations in exon 8. Figure 3 demonstrates lanes 7, 11, and 13 that relate to patients M4, M2 and M4, each of them had 3 different bands. The remainder show one band which represented a wild type with 386 bp. In addition, genomic sequencing confirmed insertion and deletion sequences in exon 8 in two patients' samples but the third sample was failed. These new mutations have been submitted and documented in the Gene Bank (FJ177639 and FJ189474). In one case, the fragment GACAGGCT has been deleted and TGGCA was inserted. In the other case, however, only the GACAGGCTT fragment has been inserted without any deletion. Of all patients studied, samples from ten patients were shown to have a point mutation at D816. The AatII enzyme was capable of identifying the sequence present in the wild type of



**Figure 3.** Exon 8 mutations in different subgroups of AML patients (M=marker)

KIT and hence the 106 bp PCR products were spliced into 85 bp and 21 bp, which this enzymatic DNA analysis would not occur if a mutation was present at D816. While the 85bp band was detectable, the 21bp band ran off the gel. In Figure 4, the D816 mutation has occurred in columns 4, 5, and 8. Due to sequence alteration at the breaking point, the 106 bp nucleotides have not been digested. In general, out of 212 AML patients, 3 (1.4%) contain exon 8 mutations and 10 (4.7%) have D816 point mutations in the KIT gene. One of the M2 samples had mutations in both exon 8 and D816. These mutations were typically located within the M2 and M4 subclasses, whereas only one case of M1 had a D816 mutation.



**Figure 4.** D816 mutations in different subgroups of AML patients (M=marker)

### Discussion

Mutations of both the KIT and FLT3 genes have a great impact on leukemia pathogenesis, most specifically on patients who suffer from AML. Mutations also lead to uncontrolled proliferation of leukemic cells. Despite the pathogenic effect of these mutations, they are not the sole cause of acute leukemia, which may require other genomic alterations related to cell differentiation. These mutations are usually associated with a poor prognosis.<sup>25-28</sup>

In this study of adults with AML, a total of 24% mutations were observed in the FLT3 gene of which 18% were ITD that mostly occurred in the M3 subclass which was characterized by the T.<sup>15-17</sup> No positive cases of ITD mutation were observed in patient specimens within the M0, M1, and M5 subclasses. Evidence to date has suggested that PML/RARA gene fusion is not sufficient for

leukomogenesis. Other factors such as mutations of FLT3 could also confer a proliferative advantage hence halting the differentiation process.<sup>20,26,27</sup> Various studies have reported a high occurrence of ITD in 385 of 1595 of adult patients (24%) with AML and another study has shown that ITD mutations occur in 20 to 30% of AML cases and D835 occurs in 7 to 10% of patients.<sup>21,29</sup> ITD has mostly been observed within AML M3 samples and the least occurrence within AML M2 samples.<sup>20,29</sup> However, other studies have reported an irregular occurrence of mutations among the subclasses.<sup>25,28</sup> In this study, most FLT3 mutations have been observed within M3 subclasses. The second most abundant FLT3 mutation occurred in D835 of this gene which has been identified within 6% of our patient samples, but it was not detected in people with M1, M0, and M4 subclasses. The D835 substitution has been reported in 30 out of 429 cases (7%) of AML, in 1 out of 29 cases (3%) of MDS and in 1 out of 36 cases (3%) of ALL.<sup>21,30</sup>

There are a few reports about the involvement of c-KIT mutations (exon 8 and 17) in AML patients, of which these mutations are not a rare event in core binding factor (CBF) leukemia.<sup>3,25</sup> In AML patients that were investigated in this study, the mutation of exon 8 KIT gene has occurred in 1.2% of M2 cases, 3.7% of M4 cases, and in 1.4% of total cases. Most mutations and alteration in sequences were detected in the KIT gene when exon 17 in this gene was analyzed. This mutation is a point mutation in which the aspartic amino acid in the 816 (D816) locus is substituted with other amino acids, leading to loop activation.<sup>31,32</sup> In the present work, the D816 mutation occurred in 3.7% of the M1, 9.8% of M2, and 1.8% of the M4 subclasses as well as 4.7% of total cases with AML, whereas the frequency of mutations in the previous study were 0.9%, 3.1%, 1.8%, and 1.7%, respectively.<sup>33</sup> Therefore the frequency of the D816 mutation has been shown to be more than reported in the past. Ethnicity may strongly influence the frequency of the reported mutated gene. Out of all the studied patients, one patient with an M2 subtype had both exon 8 and D816 mutations, hence harboring 5.6% of the KIT mutation.

## Conclusion

In this study we demonstrated that FLT3 mutations are frequent molecular abnormalities in AML patients with an incidence of 24%. The

presence of ITD was significantly associated with M3 morphology. Mutations of c-KIT resulted in 5.6% of AML (one patient had both mutations together) that was significantly associated with M2 and M4 subtypes. These data show that approximately one third of AML patients had mutations in the tyrosine kinase receptor. Although our data do not support its value as a prognostic factor in AML patients, further investigation is required.

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