Analysis of Chromosomal Aberrations and FLT3 gene Mutations in Childhood Acute Myelogenous Leukemia Patients

Çocukluğ Çağı Akut Myeloid Lösemi Hastalarında Kromozomal Değişiklikler ve FLT3 Geni Mutasyonlarının Araştırılması

Ender Coşkunpinar¹, Sema Anak², Leyla Ağaoğlu², Ayşegül Ünüvar², Ömer Devecioğlu², Gönül Ayoğan³, Çetin Timur⁴, Ahmet Faik Öner⁵, Yıldız Yıldırım⁶, Tiraje Celkan⁷, Inci Yıldız⁷, Nazan Sarper⁸, Uğur Özbek¹

¹İstanbul University, Institute of Experimental Medical Research, Department of Genetics, İstanbul, Turkey
²İstanbul University, School of Medicine, Department of Pediatric Hematology-Oncology, İstanbul, Turkey
³Bakırköy Maternity and Children's Hospital, İstanbul, Turkey
⁴Göztepe Education and Research Hospital, Department of Pediatric Hematology, Istanbul, Turkey
⁵Yüzüncü Yıl University, School of Medicine, Department of Pediatrics, Van, Turkey
⁶Şişli Etfal Education and Research Hospital, Department of Pediatric Hematology, Istanbul, Turkey
⁷İstanbul University, Cerrahpaşa School of Medicine, Department of Pediatric Hematology-Oncology, İstanbul, Turkey
⁸Kocaeli University, School of Medicine, Department of Children’s Health and Diseases, Kocaeli, Turkey

Abstract

Objective: To identify the well-known common translocations and FLT3 mutations in childhood acute myelogenous leukemia (AML) patients in Turkey.

Material and Methods: The study included 50 newly diagnosed patients in which t(15;17), t(8;21), and inv(16) chromosomal translocations were identified using real-time PCR and FLT3 gene mutations were identified via direct PCR amplification PCR-RE analysis.

Results: In all, t(15;17) chromosomal aberrations were observed in 4 patients (8.0%), t(8;21) chromosomal aberrations were observed in 12 patients (24.0%), inv(16) chromosomal aberrations were observed in 3 patients (6.0%), and FLT3-ITD mutations were observed in 2 patients (4.0%); FLT3-D835 point mutation heterozygosity was observed in only 1 patient (2.0%) patient.

Conclusion: Despite of the known literature, a patient with FLT3-ITD and FLT3-D835 double mutation shows a better survival and this might be due to the complementation effect of the t(15;17) translocation. The reported mutation rate in this article (4%) of FLT3 gene seems to be one of the first results for Turkish population.

Key Words: Childhood AML, FLT3 gene mutations, ITD, D835 mutations, Chromosomal translocations
Introduction

Acute myelogenous leukemia (AML) is a malignant disease of myeloid stem cells linked to oncogenic fusion proteins, which is due to chromosome translocations and inversions. Numerous translocations have been described in AML, of which the most common are t(8;21), t(15;17), and inv(16). These recurring translocations are currently used as the basis for classification of AML [1]. As such, AML-associated fusion proteins function as aberrant transcriptional regulators, with the potential to interfere with normal myeloid cell differentiation [1-3]. FMS-like tyrosine kinase 3 (FLT3)—a new member of the receptor tyrosine kinase (RTK) III subfamily—was originally identified in hematopoietic stem/progenitor cells and is important for normal lymphohematopoietic stem cell function [4]. FLT3 is aberrantly expressed in the most of AML patients. The FLT3 gene is located on chromosome 13 (13q12) [5]. To date, 2 distinct types of FLT3 gene mutations have been identified in AML cases: 1. Internal tandem duplication (ITD) mutations, which occur within the juxtamembrane region of the gene; 2. Point mutations that occur at codon 835 (D835) within the kinase domain. FLT3 is aberrantly expressed in the most of AML patients. The FLT3 gene is located on chromosome 13 (13q12) [5]. To date, 2 distinct types of FLT3 gene mutations have been identified in AML cases: 1. Internal tandem duplication (ITD) mutations, which occur within the juxtamembrane region of the gene; 2. Point mutations that occur at codon 835 (D835) within the kinase domain. Both types of mutations constitutively activate FLT3 tyrosine kinase activity [6]. FLT3 gene mutations are strongly associated with leukocytosis and poor prognosis in AML patients [5,7,8]. Patients with either of these mutations have a higher risk of recurrence and a lower survival rate [8]. It was recently reported that the FLT3 gene mutant/normal ratio can be used as a marker for the selection of therapy [5-8]. The present study aimed to indentify the well-known common AML translocations and FLT3 mutations in childhood AML patients in Turkey.

Materials and Methods

Patients

The study included 50 newly diagnosed childhood AML patients (28 male and 22 female) that presented for molecular diagnosis to Istanbul University, Institute of Experimental Medicine, Istanbul, Turkey, between October 2007 and July 2008. The Istanbul University, School of Medicine Ethics Committee approved the study protocol (project No. 1850/2007) and informed consent was provided by the patients’ parents. Diagnostic bone marrow samples were divided into 2 parts; 2 x 10^7 cells were preserved in RTL buffer (cat. No. 79216, Qiagen, Germany) at –80 °C until RNA isolation and the remainder of the samples were used for DNA isolation, according to the manufacturer’s instructions (cat. No. 11796828001 Roche Applied Sciences, Germany).

Determination of t(15;17), t(8;21) and inv(16) chromosome abnormalities

Total RNA was extracted from bone marrow specimens using a QIAamp RNA Blood Mini Kit (cat. No. 52304, Qia-gen, GmbH, D-40724 Hilden, Germany), according to the manufacturer’s instructions. cDNA was synthesized from 1 μg of total RNA, as previously described [7]. The quality of the obtained cDNA was evaluated via β-globin PCR performed using the following primers: forward: 5’ GAA GAG CCA ACG ACA GGT AC 3’; reverse: 5’ CAA CTT CAT CCA CGT TCA CC 3’. Chromosomal abnormalities [t(15;17), t(8; 21), and inv(16)] were identified via real-time PCR, using the LightMix primer/probe set (cat. No. 40-0135-16 cat. No. 40-0196-16 cat. No. 40-0229-16 TIB Molbiol GmbH, Berlin, Germany), and the Light Cycler FastStart Özet

Amaç: Bu çalışmada Türk çocukluğ çağ AML hastalarında sık görülen kromozomal translokasyonların ve FLT3 mutasyonlarının belirlenmesi amaçlanıdı.

Gereç ve Yöntemler: Yeni tanı almış 50 hastada real time PCR yöntemi ile t(15;17), t(8;21), inv(16) kromozomal translokasyonlarının varlığı ve PCR yöntemi ile FLT3 geni ITD tipi mutasyonlar ve D835 nokta mutasyonları varlığı araştırıldı.

Bulgular: Olguların 4’ünde t(15;17) (8.0%), 12’sinde t(8;21) (24%), 3’ünde inv(16) (6.0%) kromozomal düzensizlikleri, 2 olguda FLT3-ITD mutasyonu (4.0%), 1 olguda FLT3-D835 nokta mutasyonu (2.0%) varlığı tespit edildi. t(15;17) pozitif AML M3’li bir olguda hem FLT3-ITD hem de FLT3-D835 mutasyonlarını bakımından heterozigotluk tespit edildi.

Sonuç: Literatürden farklı olarak, bir hastamızın daha uzun sağkalımının t(15;17) translokasyonunun mutasyonu bireylerde iyi yönde düzeltici etkisinin neden olabileceğini düşünülmektedir. FLT3 geninde görülen bu mutasyon oranı (4%) Türk toplumun için ilk sonuçlardan biridir.

Anahtar Sözcükler: Çocukluğ çağ akut myeloid lösemi, FLT3 gen mutasyonları, Internal tandem duplikasyon-ITD, D835 mutasyonları, Kromozomal translokasyonlar
Detection of FLT3-ITD mutations

FLT3-ITD mutations were indentified via PCR. The forward primer was in exon 14 (14F 5'-GCAATTTAG-GTATGAAAGCCAGC-3') and the reverse primer was in exon 15 (15R 5'-CTTTCACAGCTTTGACGGCAACC-3'), as described by Wang et al. [4]. Amplification was performed in a reaction volume of 50 µL with 100 ng µL⁻¹ of DNA, 10 pmol of each primer, 10 mmol dNTP, and 2.5 U of Ex-Taq DNA polymerase (cat. No. RR001A Takara, Japan) in the buffer (10 mmol L⁻¹ of TrispHCl [pH 8.3], 50 mmol L⁻¹ of KCl, and 1.5 mmol L⁻¹ of MgCl₂). The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for 30 cycles, and elongation for 10 min at 72 °C. Amplification products were analyzed on 3% agarose gel stained with ethidium bromide and samples with the specific PCR products (329bp) were considered as positive for FLT3-ITD mutations. The specific amplicons were purified using the QIAEX II Gel Extraction Kit (cat. No. 20021, Qiagen, Hilden, Germany), according to the manufacturer's instructions, and directly sequenced for confirmation of PCR.

Detection of FLT3-D835 mutations

FLY3-D835 mutations were identified using the PCR-RFLP method. The primers employed were 20F 5'-CGA-CAGAAGCGTCTGTTCG-3' and 20R 5'-GCAGCCTCACCATT-GCCCA-3', as described by Wang et al. [4]. At codon 835 an aspartate amino acid is encoded, providing a recognition site for restriction enzyme EcoRV; as such, mutants can be detected via the loss of this enzyme restriction site. The PCR setup was as described above. The specific products were detected on agarose gel, followed by EcoRV (cat. No. 50-720-3590 Takara, Japan) digestion at 37 °C for 4 h. Restriction products were detected on a 3.5% agarose gels and undigested PCR product indicated the presence of the mutation (Figure). The results were confirmed via direct sequencing.

Statistical analysis

Clinical and laboratory characteristics at diagnosis were statistically correlated (age, sex, WBC count, hemoglobin level, PLT count, blast cells rate) with t(15;17), t(8;21), inv(16) chromosomal aberrations, and FLT3 mutations (Table 1). Fisher’s exact test and Pearson's chi-square test were performed using SPSS v.12.0. P values less than 0.05 were considered statistically significant.

Results

Diagnoses—based on French-American-British (FAB) classification—were as follows: M0 (n = 3); M1 (n = 9); M2 (n = 15); M3 (n = 12); M4 (n = 3); M5 (n = 5); M7 (n = 1). Additionally, 1 patient was lacking clinical data and could not be classified, and another patient that died following BMT and couldn’t be classified was thought to

Figure. a. PCR amplification of the FLT3-ITD region (lane M: size marker; lanes 1-10: normal samples). b. PCR amplification of the FLT3-ITD region (lane M: size marker; lanes 1-3: normal samples; lane 4: negative control; lane 5: FLT3/ITD-positive case). c. PCR amplification of FLT3-D835 (lane M: size marker; lanes 1-6: normal samples; lane 7: negative control). d. D835 mutation detection (lane M: size marker; lane 1: FLT3-D835-positive case; lane 2: wild type; lane 3: EcoRV undigested sample).
Table 1: Characteristics of childhood AML patients carrying FLT3 gene mutations and/or chromosomal aberrations.

| Case No. | SEX | AGE (months) | WBC count (x10^9 L\(^{-1}\)) | Hb (g dL\(^{-1}\)) | PLT count (x10^9 L\(^{-1}\)) | BM Blast (%) | T(15;17) | T(8;21) | Inv(16) | ITD | D835 | CD34 (%) | FAB Classification |
|----------|-----|--------------|-------------------------------|-------------------|-----------------------------|--------------|---------|---------|---------|------|-------|----------|---------------------|
| 1        | M   | 20           | 15.920                        | 8.90              | 15.000                      | 44           | Negative| Negative| Positive| Negative| WT     | 47.4       | M0                  |
| 2        | M   | 1            | 132.000                       | NA                | NA                          | NA           | Negative| Negative| Positive| Negative| WT     | NA         | NA                  |
| 3        | M   | 132          | 1.200                         | 5.90              | 32.000                      | 60           | Positive| Positive| Negative| Negative| WT     | NA         | M3                  |
| 4        | F   | 120          | 4.100                         | 7.00              | 99.000                      | 40           | Negative| Positive| Negative| Negative| WT     | 1.38       | M1                  |
| 5        | F   | 120          | 1.100                         | 7.90              | 31.000                      | 100          | Positive| Positive| Negative| Negative| WT     | 4.0        | M3                  |
| 6        | F   | 180          | 3.600                         | 11.20             | 104.000                     | 22           | Negative| Positive| Negative| Negative| WT     | 45.2       | M2                  |
| 7        | F   | 132          | 5.200                         | 6.90              | 43.000                      | 45           | Negative| Positive| Negative| Negative| WT     | 33.8       | M2                  |
| 8        | F   | 60           | 8.500                         | 11.00             | 71.000                      | 53           | Negative| Positive| Negative| Negative| WT     | 15.9       | M2                  |
| 9        | M   | 96           | 12.100                        | 7.40              | 22.000                      | 48           | Negative| Positive| Negative| Negative| WT     | 44.2       | M2                  |
| 10       | M   | 144          | 10.710                        | 10.20             | 29.000                      | 47           | Negative| Positive| Negative| Negative| WT     | 69.5       | M2                  |
| 11       | M   | 84           | 23.700                        | 10.70             | 86.000                      | 72           | Positive| Negative| Negative| Positive| WT     | 8.81       | M3                  |
| 12       | M   | 120          | 9.800                         | 7.40              | 28.000                      | 61           | Negative| Positive| Negative| Negative| WT     | 86.5       | M2                  |
| 13       | M   | 24           | 5.450                         | 7.70              | 61.000                      | 37           | Negative| Positive| Negative| Negative| WT     | 1.76       | M1                  |
| 14       | M   | 180          | 1.300                         | 8.90              | 291.000                     | 81           | Negative| Positive| Negative| Negative| WT     | NA         | M1                  |
| 15       | M   | 204          | 1.400                         | 12.80             | 25.000                      | 51           | Negative| Positive| Negative| Negative| WT     | 70.7       | M2                  |
| 16       | F   | 168          | 5.500                         | 3.60              | 6.000                       | 48           | Negative| Positive| Negative| Negative| WT     | 58.6       | M2                  |
| 17       | M   | 14           | 74.600                        | 8.10              | 79.000                      | 80           | Negative| Positive| Negative| Positive| WT     | 31.7       | M5                  |
| 18       | F   | 84           | 11.900                        | 7.20              | 45.000                      | 49           | Negative| Positive| Negative| Negative| WT     | NA         | M2                  |
| 19       | F   | 54           | 1.800                         | 8.20              | 17.000                      | 94           | Positive| Negative| Negative| Negative| WT     | 1.73       | M3                  |
| 20       | F   | 72           | 27.000                        | 8.30              | 38.000                      | 48           | Negative| Positive| Negative| Negative| WT     | 48.8       | M2                  |
| 21       | M   | 144          | 3.730                         | 11.50             | 24.000                      | 73           | Negative| Positive| Negative| Negative| WT     | NA         | M2                  |

WBC: White blood cell, Hb: hemoglobin, PLT: platelet, BM: bone marrow, FAB: French-American-British; WT: wild type; HET: heterozygous; NA: not available.
be M0 or M7. Median age of the patients was 8.42 ± 5.24 years (range: 0-18 years). The median white blood cell (WBC) count was 30,394.17 ± 57,255.86 mL⁻¹ (range: 890-260,000 mL⁻¹), the median platelet (PLT) count was 83,851.06 ± 76,349.87 L⁻¹ (range: 2 x 10⁹-291 x 10⁹ L⁻¹), the median hemoglobin level was 3.4 ± 2.67 g dL⁻¹ (range: 3.4-14.3 g dL⁻¹), and the median bone marrow blast rate was 60.86% ± 22.9% (range: 12%-100%).

FAB classification and clinical features of the 50 childhood AML patients are summarized in Table 2. In all, 4 patients were positive for t(15;17), 12 were positive for t(8;21), and 3 were positive for inv(16) (Table 3). The 4 t(15;17)-positive patients were classified as M3, and 9 of the 12 t(8;21)-positive patients were M2, 2 were M1, and 1 was M4. Among the inv(16)-positive patients, 1 was M0, 1 was M1, and 1 was M5. None of the patients were classified as M6; therefore, statistical evaluation of the FAB M6 patients was excluded. In total, 2 patients had FLT3 gene mutations, 1 of which was classified as AML-M3 and interestingly the FLT3-D835 mutation was not a deletion, but a point mutation (g.IVS20 +49 A>G) that also changed the EcoRV restriction site. Both of these changes were previously described and are known to increase expression of FLT3 [1,5,6].

The hemoglobin level in the patients with FLT3-ITD mutations was significantly lower than in the patients without the mutation. In the present study there was a correlation between FAB M2 classification and t(8;21) positivity (P = 0.005), and between FAB M3 classification and t(15;17) positivity (P = 0.009), which is in agreement with previous reports. In addition inv(16) was positive in the FAB M0, M1, and M5 patients with P values of P = 0.001, P = 0.003, P = 0.002 respectively. None of the FAB M4 patients were positive. Bone marrow blast rates below and higher than 80% were compared with t(8;21) positivity and found that patients with t(8;21) had higher blast rates than non translocated ones (P = 0.049).

**Discussion**

In addition to observation of the standard clinical features and laboratory analysis, the diagnosis of AML requires additional procedures, including pathological examination, immunophenotyping, cytogenetics examination, and molecular diagnostics. Identification of the specific cytogenetic abnormality is important for selection of appropriate therapy and prognostic analysis [1,9]. Numerous translocations have been described in AML, of which the most frequent are t(15;17), t(8;21), and inv(16), accounting for 20%-30% of all chromosomal aberrations [1,10-12]. These aberrations, depending on their structure, lead to expression of a chimeric protein with new functions [8,13]. Prognosis is considered good in cases of(15;17)
associated with AML-M3, t(8;21), inv(16) associated with AML-M2, and inv(16) associated with AML-M4 [3,14,15]. It is known that the blast level in AML-M2 patients is 30%-90% [16-18].

In the present study the bone marrow blast rate in 11 of the 12 FAB M2 patients with t(8;21) translocation was over 80%, which shows that the bone marrow blast level in childhood AML patients classified as FAB M2 is high. Additionally, inv(16) was strongly correlated with FAB classification (P = 0.043). The 3 inv(16)-positive AML patients were classified as follows: M0 (n = 1); M1 (n = 1); M5 (n = 1). According to the literature, inv(16) occurs more frequently in patients classified as FAB M4 [19] and AML-M4 occurs more frequently in patients classified as FAB M4, primarily in patients aged ≥50 years [17,18]. The presence of inv(16) is indicative of a good prognosis in FAB M4 patients, but data concerning the relationship between inv(16) and other AML FAB classifications are lacking. In the present study inv(16) was not observed in any of the FAB M4 patients. The present findings are in agreement with those reported by Dash et al. and the American National Cancer Institute AML guideline [14,20]. The FLT3 gene is expressed primarily in hematopoietic stem cells [5,21,22]. Moreover, human leukemia and lymphoma cell lines also express FLT3 protein [22]. The 2 most common mutations of the FLT3 gene were FLT3-ITD and FLT3-D835. Among the 50 childhood AML patients in the present study, only 2 had FLT3-ITD mutations, of which 1 also had FLT3-D835 point mutation. The incidence of FLT3-ITD mutation in childhood AML patients (5-16%) is lower than that in adult AML patients (20-25%) [22,25]. Meshinchi et al. reported that the incidence of FLT3-TKD (tyrosine kinase domain) mutations in childhood AML patients was 6.7% [27], versus 7% reported by Karabacak et al. [23]. The known FLT3 gene mutation rate in adult AML patients is 20%-25% [22,25]; however, Liang et al. [24] reported a mutation rate in childhood AML patients of 11.3%, and Kondo et al. [22], Iwai et al. [25], and Krstovski et al. [26] reported rates of 5.3%-16.5%.

Our data is one of the first results FLT3-ITD mutation evaluation in Turkish pediatric AML patients and the mutation rate is 4.0%, which is much lower than the other study groups [23,27]. In the present study there wasn’t a correlation between FLT3 gene mutations, and age, gender, the WBC count, blast cell rate, or FAB classification. Although it was reported that the FLT3-ITD mutation rate increases with age Meshinchi et al. [27], Kondo et al. [22] and Wang et al. [4] and was not observed a significant difference between the mutation rate and the age in our results.

In the present study there wasn’t a significant difference between FLT3-ITD mutations and the WBC count. The WBC count in 95% of the present study’s patients was less than 50 x 10^9 L⁻¹, which is in agreement with the findings reported by Moreno et al. [28]. The most common feature of AML is chronic, severe anemia, which damages bone marrow. The present results show that there was a relationship between a low hemoglobin level and FLT3-ITD mutations, but not between a low hemoglobin level and FLT3-D835 mutation, which might have been due to the small number of patients withFLT3-D835 point mutation as well as the small study population. Pre-clinical studies highlighted the potential use of inhibitors against FLT3 kinase activity. Most of patients carrying FLT3 gene mutation have a 50% decrease in the peripheral blast count, along with inhibition of receptor autophosphorylation.

Herein we described the development of anemia in patients carrying FLT3 gene mutation. It is also known that FLT3 mutations are poor prognostic markers of AML. A 7-year-old male patient in the present study classified as AML-M3 had double FLT3 mutations and t(15;17) translocation. He was in remission for 1 year, and died after 19 months of diagnosis. While FLT3 mutation causes continuous tyrosine kinase activity, t(15;17) translocation deregulates RARα transcription factor and cell differentiation stops. Thus, AML occurs due to the combined effects of FLT3 gene mutations and t(15;17) translocation. To the best of our knowledge our 7-year-old male patient is the first reported case to have been in remission for approximately 1 year with double FLT3 mutation and translocation. This is also the first case with double FLT3 mutation and t(15;17) translocation that is reported in Turkish population. Most FLT3 gene mutations are reported as individual mutations and only a few cases of double mutation have been reported [5,22,29,30]; Moreno et al. reported 4 cases in 2003 [28] and Wang et al. reported 2 cases in 2005 [4]. All the reported double mutation patients died after induction therapy or relapsed in first months after diagnosis [28]. These results indicate that FLT3-ITD and FLT3-D835 mutations are markers of poor prognosis. In contrast to previous reports our 7-year-old patient survived longer, which might have been due to the combined effect of t(15;17) translocation (31,32); however, the quality of clinical response to FLT3 inhibitors has been minor, with many patients transiently responding with a decreased blast count (31,32). This finding needs to be confirmed by in vitro studies in which patients are followed-up for longer periods of time. It is not clear if FLT3 double mutation causes upregulation in tyrosine kinase activity or increases cell survival. AML occurs as a result of excessive prolifera-
tion and differentiation of myelogenous blasts. The present study is the first to perform detailed molecular analysis of Turkish childhood AML patients. The low FLT3 gene mutation rate (4%) seems to be unique to this study’s population. This result and the effects of double mutations need to be evaluated in larger patient groups.

We wish to thank the Research Council of Istanbul University for supporting this study.

Conflict of Interest Statement

The authors of this paper have no conflicts of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

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