Cytogenetic and FMS-Like Tyrosine Kinase 3 Mutation Analyses in Acute Promyelocytic Leukemia Patients

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ABSTRACT

Background: The secondary genetic changes other than the promyelocytic leukemia-retinoic acid receptor (PML-RARA) fusion gene may contribute to the acute promyelocytic leukemogenesis. Chromosomal alterations and mutation of FLT3 (FMS-like tyrosine kinase 3) tyrosine kinase receptor are the frequent genetic alterations in acute myeloid leukemia. However, the prognostic significance of FLT3 mutations in acute promyelocytic leukemia (APL) is not firmly established. **Methods:** In this study, the chromosomal abnormalities were analyzed by bone marrow cytogenetic in 45 APL patients and FLT3 internal tandem duplications (ITD) screening by fragment length analysis and FLT3 D835 mutation by melting curve analysis were screened in 23 APL samples. **Results:** Cytogenetic study showed 14.3% trisomy 8 and 17.1% chromosomal abnormalities other than t(15;17). About 13% of the patients had FLT3 ITD, and 26% had D835 point mutation. FLT3 ITD mutation was associated with higher white blood cell count at presentation and poor prognosis. **Conclusion:** The PML-RARA translocation alone may not be sufficient to induce leukemia. Therefore, we assume that FLT3 mutations and the other genetic and chromosomal alterations may cooperate with PML-RARA in the development of APL disease. *Iran. Biomed. J. 16 (1): 10-17, 2012*

Keywords: Chromosome aberrations, FMS-like tyrosine kinase 3, Acute promyelocytic leukemia

INTRODUCTION

Most cases of acute promyelocytic leukemia (APL) are characterized by t(15;17) (q22;q21), leading to formation of the promyelocytic leukemia-retinoic acid receptor (PML-RARA) fusion protein [1]. PML-RARA plays a critical role in determining disease phenotype, mediating the characteristic differentiation block through the repression of genes implicated in myelopoiesis [2, 3]. However, the studies indicate that secondary genetic changes other than the PML/RARA fusion gene are required for this leukemia to arise [4]. Although the precise nature of the cooperating genetic events in generating the APL phenotype remains uncertain, a number of potential candidate genes have been proposed to play a role in this process [4]. For instance, the product of reciprocal fusion gene product RARA-PML has been suggested to contribute to the leukemogenesis, thereby predisposing to the acquisition of additional oncogenic lesions [5]. There has also been considerable interest in the potential role of activating mutations of genes encoding receptor tyrosine kinases, which commonly accompany acute myelocytic leukemia (AML)-associated translocations including t(15;17), giving rise to the proposition that they could provide a common class of cooperating mutation in the development of the disease [4]. FMS-like tyrosine kinase 3 (FLT3) is a receptor tyrosine kinases expressed on hematopoietic progenitors. It is closely related to FMS proto-oncogene protein and is commonly mutated in acute myeloid leukemia Mutation of the FLT3 gene is common in AML [5, 6]. Interestingly, transgenic mice coexpressing PML-RAR with FLT3[W51] (constitutively activated form of murine FLT3), FLT3 internal tandem duplications (ITD), or K-Ras (K12D) develop APL with a short latency and a high penetrance [7-9].

Numerous mutations have been identified in FLT3 gene. The majority, present in approximately 25% of

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patients, are ITD that lead to in-frame insertions within the juxtamembrane region of the receptor. Less frequent are mutations involving the region encoding the activation loop; most commonly affected codons are aspartate 835 and isoleucine 836 (D835/I836) which have been reported in approximately 8% of patients with AML [10, 11]. In vitro studies have revealed that both classes of mutation lead to constitutive activation of the receptor. Studies on AML have found that the presence of an ITD to be an adverse prognostic indicator predicting for higher incidence of relapse; however, the significance and biologic characteristics of FLT3 activation loop mutations remain uncertain and, for reasons that are unclear, they do not appear to predict for poor outcome. The frequency of FLT3 ITD varies dramatically across cytogenetically and molecularly defined subsets of AML, and they are particularly prevalent in patients with t(15;17) [12-14]. Although previous studies considering patients with APL have highlighted an association between ITD and elevated white blood cell (WBC) count, hypogranular variant (M3v)morphology, and the short (bcr3) isoform of PML-RARA, the prognostic significance of FLT3 mutations in APL has not been firmly established [14-19]. This is important because it has a potential bearing on treatment stratification in this disease and is highly pertinent given the recent clinical interest in drugs targeting FLT3.In vitro studies of small-molecule FLT3 inhibitors have shown that they can suppress proliferation in ITD-expressing cell lines and in primary AML blast cells and can prolong survival in mouse models of ITD-induced disease [20-22]. Phase 1/2 trials of FLT3 inhibitors as single agents have led to partial hematologic responses in a proportion of patients with refractory, relapsed, or poor-risk AML. Here, we aim to study FLT3 mutations and other additional chromosomal alterations in APL patients.

MATERIALS AND METHODS

Cytogenetic analysis/detection of promyelocytic leukemia-retinoic acid receptor fusion. A total of 23APL patients diagnosed according to the French-American-British criteria including 19 men and 4 women with a median age of 35, median WBC count of 11,000, and median platelet of 79.6×10^9 /L were enrolled in this study. Cells from the diagnostic bone marrow samples were cultured for 24 hours according to routine cytogenetic protocols. Chromosome analysis was performed on 20 metaphase cells of 45 APL patients. Karyotypes were performed according to the International System for Human Cytogenetic Nomenclature (2005). All 45 patients were confirmed by conventional cytogenetic and reverse transcriptase-

polymerase chain reaction (RT-PCR) to have t(15;17) or PML-RARA fusion transcript.

Analysis of the FMS-like tyrosine kinase 3 internal tandem duplications and D835 mutations. Total RNA was extracted from Ficoll-Hypaque isolated leukemic blasts using RNeasy Kit (Qiagen, Valencia, CA, USA), and 1 µg total RNA was converted into cDNA by reverse transcription using random hexamer primers using QuantiTect Reverse Transcription Kit (Qiagen, USA) as recommended by manufacture. PCR-based amplification of cDNA was carried out for detection of ITD of exon 14 (formerly exon 11).Two µl of cDNA was amplified in a total volume of 20 µl reaction mixture containing Taq PCR Master Mix (Qiagen, USA), PCR grade water (Qiagen, USA) and 0.25 pM of each primer to yield a product of 365bp from wild type allele. We used the following two oligonucleotide primers: 6R, 5′ATCCTAGTACCTTCCCAAACTC-3′and 5F, 5′F AM-TCGAGCAGTACTCTAAACATG-3′. The PCR condition was one cycle of 5 min at 94°C, followed by 34 cycles of 1 min at 94°C, 1 min at 60°C and 1min at 72°C; a final extension of 10 min at 72°C. The samples were analyzed for the presence of ITD using gene scan fragment length analysis. Separation was done with a fluorescence capillary electrophoresis system ABI PRISM 3100 DNA analyzer. After PCR, 1 µl PCR product was mixed with 13.5 µl H_2O (molecular biology grade water, Qiagen, USA) containing 0.5 µl GeneScan-500 ROX Size Standard (Applied Biosystems). PCR fragment was denatured at 95°C for 3 min and then separated on an ABI PRISM 3100 Bioanalyzer. Evaluation of the data was accomplished with the Genescan Analysis Software (Gene Mapper 3.5). Analysis for TKD mutations was performed by Light Cycler based melting curve analysis with the forward primer 17F: 5′-CCGCCAGGAACGTGCTTG-3′; reverse primer 17R: 5′-ATGCCAGGGTAAGGAT TCACACC-3′and hybridization probes, 835A CAGGCAGACGGGCAT TGCCCCTG-Flou and Red 705-GT TGGAATCACT CATGATATCTCGAG-P. The PCR reaction was carried out in a 20 µl reaction volume contained with 2 µL cDNA, 0.5 µM each of forward and reverse primer, 0.75 µM hybridization probes, 4 mM $MgCl_2$ and 2 µL LightCycler-FastStart DNA Master Hybridization Probe Mix (Roche Diagnostics, Mannheim, Germany). Amplification was performed with 45 cycles using 65°C annealing temperature. Final melting curve analysis was started at 40°C up to 95°C with slope of 0.2°C/s and continuous detection with channel F2/F1. Data were analyzed using the LightCycler 3.0 software (Roche Diagnostics, USA) and the second derivative maximum method by melting curve analysis mode. As melting is sequence dependent, monitoring the precise melting behavior by observing the change in

fluorescence allows the detection of variant sequences. In addition, sequence variants in the DNA such as mutations give rise to heteroduplexes that form earlier melting products.

Statistical methods. The correlation between sex, WBC, hemoglobin and platelet count with FLT3 status were evaluated by chi square test. Overall survival was analyzed using Kaplan-Meier estimates. The patients were followed up from the first day of diagnosis to the last date up to 30 months.

RESULTS

Patient's characteristic. Patients' clinical data including WBC count, platelet count, blast percentage in the bone marrow, sex, age, mutational status of the FLT3 gene, and karyotype in 45 patients with APL are shown in Table 1.

Cytogenetic results. The cytogenetic study of 10 samples was not determined because of the poor quality of metaphases. However, all patients were positive for PML-RARA fusion transcripts. From 35 patients analyzed by cytogenetic study, 24 (68.6%) patients showed an apparently balanced t(15;17); 5 cases (14%) showed t(15;17)/trisomy 8; and 8 patients (22.88%) showed t(15;17)/chromosomal abnormalities other than t(15;17) (Fig. 1A) including i(17q), t(11;15), del 16(q22), t(3;6)(q12;p25), del17(q21), +18, del7 (q31) and t(10;18) (Table 1). By using the primers 5F and 6R we were able to amplify a specific 365 bps of fluorescein-labeled fragment as wild type allele and the duplicated alleles that have a shift in the pick. By subtracting the size of FLT3 wild type allele from FLT3 ITD allele we can get the size of the inserted nucleotides in exon 14 of FLT3 gene. From 23 APL patients analyzed by this technique, 3 patients were positive for FLT3 ITD mutation (Fig. 2).

Mutation by melting curve analysis. Screening for FLT3 D835 mutation status was performed using a melting curve based on LightCycler assay (Roche Diagnostics, Mannheim, Germany) with forward primer 17 F; reverse primer17 R and hybridization probes 835A and 835S, spanning the mutated region. Sequence variants in the DNA such as mutations give rise to heteroduplexes that form earlier melting products allowing detection of mutations. All cases that revealed an aberrant melting curve were considered positive for FLT3 D835 mutation (Fig. 3). From 23 APL patients analyzed, 6 patients were positive for FLT3 D835 mutation, 8 (39%) were positive for FLT3 mutations, 3 (13%) had ITD mutations and 6 (26%) had D835 mutations (Fig. 1B).
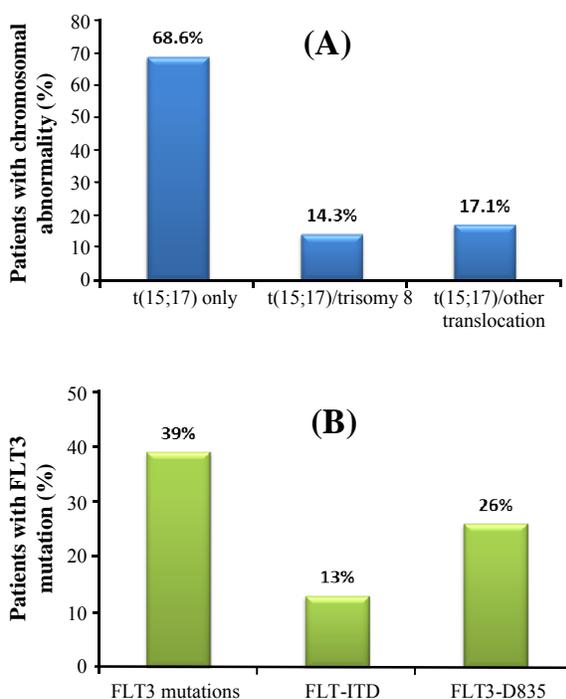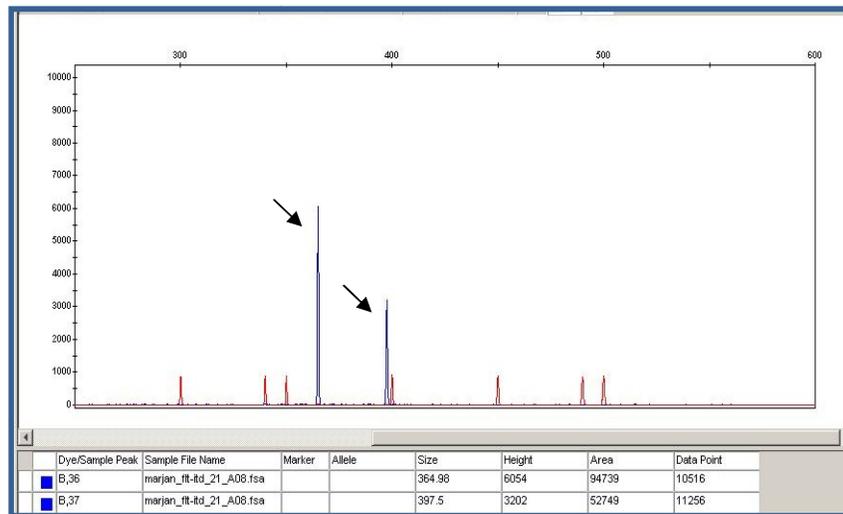

Fig. 1. Chromosomal abnormality and FLT3 mutations in APL patients. **(A)** Percentage of the patients with chromosomal aberrations; **(B)** Frequency of FLT3 mutation in APL patients. Two patients from the trisomy 8 group had translocations other than t(15;17).

By using Chi square test, no significant association was found between D835 and patients presenting features e.g. WBC, hemoglobin and platelet count. No association was found between D835 mutation and hyperleukocytosis, and also other blast characteristics at diagnosis. However, the presence of FLT3 ITD mutation was associated with higher presenting WBC count.

Impact of FMS-like tyrosine kinase 3 mutation status on outcome. Patients were monitored during the treatment and after achievement of complete remission. We analyzed overall survival according to the FLT3 mutations status in 23 patients from the time of diagnosis. The 30-months Kaplan-Meier estimate for overall survival was 50% and 87% for patients with and without FLT3 mutations, respectively, with a median overall survival time of 9 months for patients with FLT3 mutation and 19 months for patients without mutations (Log rank test: $P = 0.043$, Fig. 4). There was a significant difference in death rate according to FLT3-ITD mutation but not D835 mutation. Of 3 patients with FLT3-ITD mutation, 2 patients were expired within early days of induction remission period.

Table 1. APL patient's clinical data, FLT3 mutation status and karyotype.

Case no	Sex	Age	WBC (× 109/L)	Blast (%)	Plt (× 109/L)	FLT3 -ITD	FLT3 D835	Karyotype
1	M	20	3.7	70	57	-	-	
2	M	16	8.7	65	20	-	-	46,XY,t(15;17)(q22;q21)[20]
3	M	41	7.7	70	22	-	-	46,XY,15q+,iso(17q)[8]/46,XY,iso(17q)[1]/46,XY[1]
4	F	47	2.6	85	17	-	-	
5	M	49	1.0	60	149	-	-	
6	F	46	1.6	75	14	-	+	46,XY,t(15;17)(q22;q21)[20]
7	M	49	19.0	80	569	+	-	
8	M	38	2.5	85	110	-	-	46,XY,t(15;17)(q22;q21)[20]
9	M	22	9.7	90	34	-	+	47,XY,t(15;17)(q22;q21),+8,der(15)t(11,15)(q13,p13)[7]/46,XY[13]
10	M	40	6.4	95	46	-	+	
11	F	40	46.6	90	57	-	-	46,XY,t(15;17)(q22;q21)[21]/46,XY[48]
12	M	20	1.8	45	109	-	-	
13	M	40	6.0	60	30	-	-	46,XY,t(15;17)(q22;q21),t(10;18)(p11.2;q11.2)[25]
14	M	18	24.0	75	5	-	-	
15	F	57	7.8	90	34	-	+	46,XY,t(15;17)(q22;q21)[20]
16	M	16	37.4	80	32	+	-	46,XY,t(15;17)(q22;q21)[14]/46,XY[36]
17	M	37	1.1	80	75	-	-	46,XY,t(15;17)(q22;q21)[3]/46,XY[47]
18	M	21	5.8	90	23	-	+	46,XY,t(15;17)(q22;q21)[14]/46,XY[10]
19	M	27	12.9	60	6	+	+	47,XY,t(15;17)(q22;q21),+8[8]/46,XY[3]
20	M	60	28.8	90	27	-	-	46,XY,t(15;17)(22;q21)[3]/46,XY[22]
21	M	23	4	45	400	-	-	
22	M	40	6	90	31	-	-	46,XY,t(15;17)(22;q21)[47]/46,XY[3]
23	M	37	0.7	70	43	-	-	46,XY,t(15;17)(22;q21)[47]/46,XY[3]
24	M	37	7	80	43			46,XY,t(15;17)(q22;q21)[14]/46,XY[36]
25	M	23	4.8		8			46,XY,t(15;17)(22;q21)[2]/46,XY,idem,del(7q)(q31)[13]/46,XY,idem,+8,+18[5]
26	M	51	11.9		44			46,XY,t(15;17)(22;q21)[9]/46,XYt(15;17)(22;q21),del16(q22)[4]
27	M	26	0.8		280			46,XY,der(6) t(3;6)(q12;p25) t(15;17)(24;q21)[2]/46,XY,idem [15]/46,XY,idem,der(17)(del(17)?t(15;17)(22;q21))[5]/46,XY[2]
28	F	24	1.1		10			46,XY,t(15;17)(22;q21)[8]/46,XY[12]
29	F	55	7.7		12			46,XY,t(15;17)(22;q21)[10]
30	F	44	1.0		28			46,XY,t(15;17)(22;q21)[15]/46,XY[5]
31	M	34	1.9		32			46,XY,+8, t(15;17)(22;q21)[18]/46,XY[3]
32	F	29	20.1		35			46,XY,t(15;17)(22;q21)[10]
33	F	32	5.7		35			46,XY,t(15;17)(22;q21)[13]/46,XY[7]
34	M	62	2.1		77			46,XY,t(15;17)(22;q21)[22]/46,XY[8]
35	F	26	1.5		22			46,XY,t(15;17)(22;q21)[4]/46,XY[7]
36	F	15	5.8		19			46,XY,t(15;17)(22;q21)[20]
37	M	30	1.2		32			46,XY,t(15;17)(22;q21)[2]/46,XY,der(15) t(15;17)(22;q21)[1]/ 46, XY,del(17)(q21)[1]/ 46, XY[1]
38	M	22	11.4		36			46,XY,t(15;17)(22;q21)[34]/del (2)[1]
39	F	55	1.1		35			46,XY,t(15;17)(22;q21)[30]/46,XY[1]
40	M	37	0.7		45			46,XY,t(15;17)(22;q21)[47]/46,XY[3]
41	M	23	4.0		400			46,XY,t(15;17)(22;q21)[3]/46,XY[12]
42	M		28.8		27			46,XY,+8, t(15;17)(22;q21)[8]/46,XY[3]
43	M	27	12.9		6			46,XY,t(15;17)(22;q21)[9]/46,XY[10]
44	M	19	3.3		17			46,XY,t(15;17)(22;q21)[12]/46,XY[13]
45	M	55	1.5		13			

|	Dye/Sample Peak	Sample File Name	Marker	Allele	Size	Height	Area	Data Point
■	B,36	marjan_flt-itd_21_A08.fsa			364.98	6054	94739	10516
■	B,37	marjan_flt-itd_21_A08.fsa			397.5	3202	52749	11256

Fig. 2. Detection of FLT3 internal tandem duplications by capillary electrophoresis-based fragment analysis. Capillary electrophoresis pherograms; x axis represents size of the PCR products in bases, y axis represents relative fluorescence intensity. The arrows represent the fluorescein-labeled fragments from the ITD portion of the assay while the red peaks represent internal size standard. The distance between the wild type peak and the FLT3-ITD peak defines the length of the insertion.

DISCUSSION

The current hypothesis of leukemogenesis is the two-hit model first presented by Gilliland in 2002 [10]. This hypothesis implies that two separate mutations with different consequences need to be present for AML to develop. The first group of mutations gives a proliferative and/or survival advantage to the cell, referred to as class I mutations. FLT3-ITD and FLT3 (Asp835) are Class I mutations. The second group of mutations, the class II mutations impairs differentiation and apoptosis, including PML/RARA. Detection of ITD mutation and its correlation with the expression of PML-RARA fusion transcript may provide insight into the underlying pathogenic mechanism of APL formation. In the present study, we used fragment length analysis for detection of FLT3-ITD mutation and melting curve analysis for screening FLT3-D835 mutation. The detection of ITD mutation has been reported some difficulties in some clinical samples. As in some clinical samples with ITD mutation, very small amounts of ITD mutant alleles relative to wild type cannot be separated by agarose gel electrophoresis or ITD bands are very faint and would have been difficult to interpret clinically. Using capillary electrophoresis detection allowed small amounts of ITD mutant products to be sensitively and
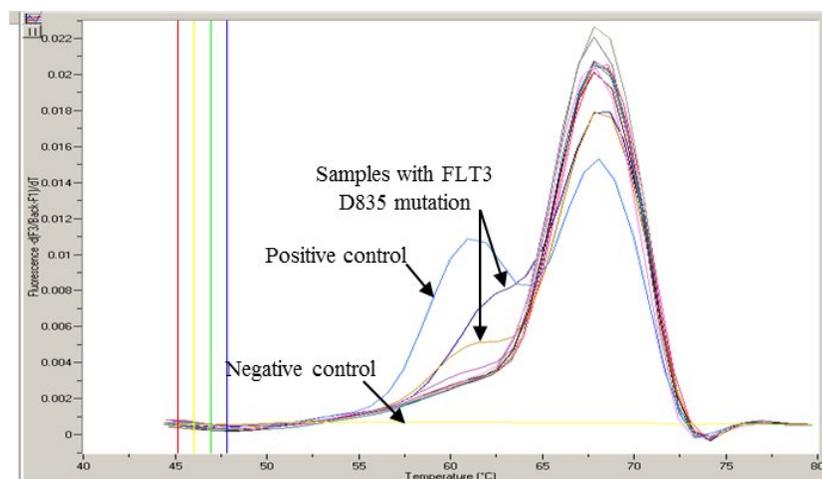
Fig. 3. Derivative (-dF/dT versus T) melting curves of the normalized fluorescence data. The wild-type sample has a single peak, whereas the D835 mutant samples have either a low temperature shoulder or two peaks.
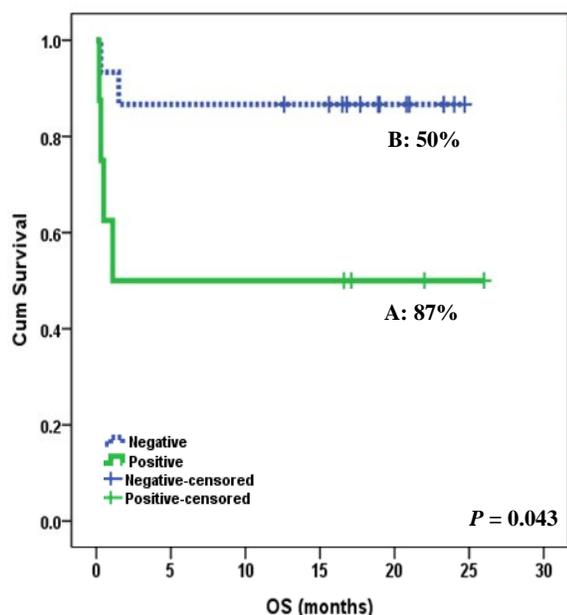
Fig. 4. Impact of FLT3 mutation status in outcome of APL patients. Patients with (**A**) and without (**B**) FLT3 mutation.

specifically identified [23]. FLT3 mutations are associated with an adverse prognosis, and are the strongest separate marker for disease relapse in AML [24]. Alteration of D835 also appears to result in constitutive activation of the FLT3 receptor and portends a worse disease-free survival in at least some studies. FLT3 ITD mutations have been reported to occur in 20 to 30% of patients with AML, 3% of patients with myelodysplastic syndrome, and 3% of patients with acute lymphocytic leukemia and D835 mutations in 7% of patients with AML [24]. In the present study, our APL patients showed 39% FLT3 mutations, from which we found FLT3 ITD in 13% and FLT3 D835 missense mutation in 26% of APL patients. D835 and ITD mutations appear to occur independently, but not exclusively of one another and the presence of concurrent D835 and ITD mutations has been reported before. We also observed one patient with FLT3 ITD and FLT3 D835 mutations in APL samples. FLT3 ITD is much more frequent in M3 subtype of AML from the other AML groups. In one study, the FLT3 ITS has been shown in 23% and FLT3 D835 in 26% of APL patients [25]. In other study [26], FLT3-ITD gene was detected in 37% of APL patients. It seems that ITD mutation in our patients group is lower than that initially reported by others, but D835 mutation is similar to the studies on APL cases [12, 19]. Distinct from ITD, no significant association was found between D835 and patient presenting features. Despite the fact that at the biological level the D835 alteration would similarly predict, as does the ITD, for

increased autonomous cell proliferation, no association was found with hyperleukocytosis, or with other blast characteristics at diagnosis. Moreover, as also shown in AML patients [24], the D835 alteration does not appear to carry prognostic impact in APL.

Multivariate analysis shows that WBC count is the most important prognostic factor in APL, and poorer outcomes in patients with high presenting WBC counts result from many factors, including higher rates of ID, particularly as a result of hemorrhage, higher incidence of retinoic acid syndrome, as we also observed in two APL patients with FLT3 ITD mutations who died by hemorrhage and sepsis. The basis for the higher WBC counts in patients with APL is not fully understood, but some studies have indicated that a significant proportion of such patients have FLT3 ITD [17, 18, 26]. In APL, the prognostic relevance of FLT3 mutations is less clear, although recent studies have explored the impact of FLT3 mutations on outcomes of APL [27]. In this study because of technical limitation, we could not analyze more APL patients, thus investigating the prognostic significance of FLT3 mutation status in APL patients became limited by our relatively small sample sizes. Although it was difficult to detect its actual effect on outcomes, we could observe a higher WBC count and more death rates in FLT3 ITD group.

In our cytogenetic results we found several other chromosomal aberrations in 31.4% of the APL patients in addition to the t(15;17). Additional chromosomal abnormalities such as i(17q), t(10;18), deletions and more complex karyotypes were found in 17.1% of the APL patients. The incidence of chromosome abnormalities in addition to t(15;17) in APL has been reported before to be around 25-40% in primary APL patients and even higher in relapse [28]. The t(15;17) chromosome translocation in APL is classified as a favorable cytogenetic feature among acute myeloid leukemia patients. However, the prognostic significance of chromosomal abnormalities in addition to t(15;17) remained uncertain in previous reports [29]. In this study, we also did not observed prognostic significance of chromosomal abnormalities in addition to t(15;17) in our APL patients. Although additional chromosomal abnormalities as a whole do not influence the prognosis of patients, some individual abnormalities have been proposed to be associated with poor outcome. For instant, an isochromosome for the long arm of the derivative chromosome 17 is probably associated with poor outcome [29]. Trisomy 8 is the most frequent numerical aberration in AML, occurring at a frequency of 10% to 15%. The clinical impact of additional copies of chromosome 8 on leukemic progression and response to therapy also remains controversial [30]. The biologic significance of the extra chromosome was based on the observation of increased copies of the *c-MYC* oncogene that is

localized to 8q. It has been suggested that increases in *c-MYC* were important to the course of the disease and that trisomy of chromosome 8 was an alternative means for achieving amplification of this gene [28].

As the PML-RARA is not sufficient to develop APL, we assume FLT3 mutations and additional chromosomal alterations found in this APL series may cooperate with PML-RARA for leukemia development.

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