Evaluation of FLT3 mutations in Patients with Acute Promyelocytic Leukemia

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Abstract

Introduction: The secondary genetic changes other than the PML-RARA fusion gene may contribute to the acute promyelocytic leukemogenesis. Chromosomal alterations and mutation of FLT3 tyrosine kinase receptor are the frequent genetic alterations in acute myeloid leukemia (AML). However, the prognostic significance of FLT3 mutations in acute promyelocytic leukemia (APL) is not firmly established.

Patients & Methods: FLT3 ITD screening by fragment length analysis and FLT3 D835 mutation by melting curve analysis in 23 APL samples was screened in this study.

Results: About13% of the patients had FLT3 internal tandem duplications (ITDs), and 26% had D835 point mutation. FLT3 ITD mutation was associated with higher white blood cell (WBC) count at presentation and poor prognosis.

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

Keywords: Acute promyelocytic leukemia; FLT3 tyrosine kinase; Internal tandem duplication

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, 2) PML-RARA plays a critical role in determining disease phenotype, mediating the characteristic differentiation block through the repression of genes implicated in myelopoiesis.(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 events implicated in generating the full disease phenotype remains uncertain, a number of potential candidates have been proposed to play a role in this process.(5) These include the reciprocal fusion gene product RARA-PML, which is expressed in approximately 75% of patients and has been postulated contribute to leukemogenesis by promoting genomic instability, thereby predisposing to the acquisition of additional oncogenic lesions.(6) There has also been considerable interest in the potential role of activating mutations of genes encoding receptor tyrosine kinases (RTKs), 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 an RTK expressed on hematopoietic progenitors. 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 ITD, or K-Ras (K12D) develop APL with a short latency and a high penetrance.(7- 11)

Numerous mutations have been identified in FLT3 gene. The majority, present in approximately 25% of patients, are internal tandem duplications (ITDs) 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.(12, 13) 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 ITDs varies dramatically across cytogenetically and molecularly defined subsets of AML, and they are particularly prevalent in patients with t(15;17).(14-16) Although previous studies considering patients with APL have highlighted an association between ITDs 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.(16- 21) 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.(22-24) 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.

Aim: We aim to study FLT3 mutations as additional chromosomal alterations in APL patients.

Patients and methods

A total of 23 APL patients diagnosed according to the French American British (FAB) criteria including 19 men and 4 women with a median age of 35, median WBC count of 11000, median platelet of 79.6 $\times 10^9$/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. Karyotypes were performed according to the International System for Human Cytogenetic Nomenclature (ISCN) 2009. All patients were confirmed by conventional cytogenetics and reverse transcriptase–polymerase chain reaction (RT-PCR) to have t(15;17) or PML-RARA fusion transcript.

Analysis of the Flt3 ITD and D835 mutations: Total RNA was extracted from Ficoll-Hypaque isolated leukemic blasts using RNeasy kit (Qiagen, Valencia, CA), and 1 µg total RNA was converted into cDNA by reverse transcription using random hexamer primers using Quantitect reverse transcription kit (Qiagen) as recommended by manufacture. PCR-based amplification of cDNA was carried out for detection of ITD of exon14 (formerly exon11). Two µl of cDNA was amplified in a total volume of 20 µl reaction mixture containing Taq PCR master mix (Qiagen), PCR grade water (Qiagen) 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´FAM-TCGAGCAGTACTCTAAACATG-3´.

The PCR condition was one cycle of 5 min at 94°C was followed by 34cycles of 1min 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 internal tandem duplications using gene scan fragment length analysis .Separation was done with a fluorescence capillary electrophoresis system ABI PRISM 3100 DNA analyzer. After PCR, one µl PCR product was mixed with 13.5 µl H2O (molecular biology grade water, Qiagen) containing 0.5 µl GeneScan-500 ROX size standard (Applied Biosystem). PCR fragment were denatured 3min at 95°C 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 LightCycler based melting curve analysis with forward primer 17F: 5´-CCGCCAGGAACGTGCTTG-3´, reverse primer 17R: 5´-ATGCCAGGGTAAGGATTCACACC-3´ and hybridization probes, 835A CAGGCAGACGGGCATTGCCCCTG-Flou and Red705-GTTGGAATCACTCATGATATCTCGAG-P. (Leukemia Diagnostic lab, Klinikum Grosshadern, Munich, Germany). The PCR reaction was carried out in a 20µl reaction volume contained with 2 µL cDNA, 0.5 µM of forward and reverse primer, 0.75 µM Hybridization-Probes, 4 mM MgCl2 and 2 µL LightCycler- FastStart DNA Master Hybridization mix (Roche Diagnostics, Mannheim, Germany).

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
1	M	20	3.7	70	57	-	-
2	M	16	8.7	65	20	-	-
3	M	41	7.7	70	22	-	-
4	F	47	2.6	85	17	-	-
5	M	49	1	60	149	-	-
6	F	46	1.6	75	14	-	+
7	M	49	19	80	569	+	-
8	M	38	2.5	85	110	-	-
9	M	22	9.7	90	34	-	+
10	M	40	6.4	95	46	-	+
11	F	40	46.6	90	57	-	-
12	M	20	1.8	45	109	-	-
13	M	40	6	60	30	-	-
14	M	18	24	75	5	-	-
15	F	57	7.8	90	34	-	+
16	M	16	37.4	80	32	+	-
17	M	37	1.1	80	75	-	-
18	M	21	5.8	90	23	-	+
19	M	27	12.9	60	6	+	+
20	M	60	28.8	90	27	-	-
21	M	23	4	45	400	-	-
22	M	40	6	90	31	-	-
23	M	37	0.7	70	43	-	-
24	M	37	7		43		
25	M	23	4.8		8		
26	M	51	11.9		44		
27	M	26	0.8		280		
28	F	24	1.1		10		
29	F	55	7.7		12		
30	F	44	1		28		
31	M	34	1.9		32		
32	F	29	20.1		35		
33	F	32	5.7		35		
34	M	62	2.1		77		
35	F	26	1.5		22		
36	F	15	5.8		19		
37	M	30	1.2		32		
38	M	22	11.4		36		
39	F	55	1.1		35		
40	M	37	0.7		45		
41	M	23	4		400		
42	M		28.8		27		
43	M	27	12.9		6		
44	M	19	3.3		17		
45	M	55	1.5		13		

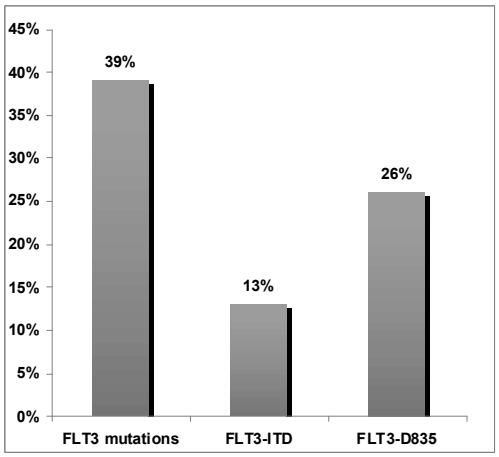

Figure- 1. The Percentage of FLT3 mutations in APL patients.

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/sec and continuous detection with channel F2/F1. Data were analyzed using the LightCycler 3.0 software (Roche Diagnostics) 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, Hb and platelet count with FLT3 status were evaluated by chi square test. Overal Survival were analyzed using Kaplan-Meier estimates. The patients were followed up from the first day of diagnosis to the date they were last known to be alive.

Result

Patient's characteristic: Patients clinical data including WBCs count, platelet count, blast percentage in the bone marrow, Sex, age, mutational status of the FLT3 gene, are shown in Table-1.

Detection of FLT3-D835 mutation by melting curve analysis: Screening for FLT3 D835 mutation status was performed using a melting curve based LightCycler assay (Roche Diagnostics, Mannheim, Germany) with forward primer 17F; reverse primer17R 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 (Figure-3). From 23 APL patients analyzed, six patients were positive for FLT3 D835 mutation.

From 23 APL patients analysed, 8 (39%) of patients were positive for FLT3 mutations; 3 (13%) had ITD mutations and 6 (26%) had D835 mutations.

By using Chi square test no significant association was found between D835 and patients presenting features e.g. WBC, Hb and platelet count. No association was found between D835 mutation with hyperleukocytosis, nor with other blast characteristics at diagnosis. However, the presence

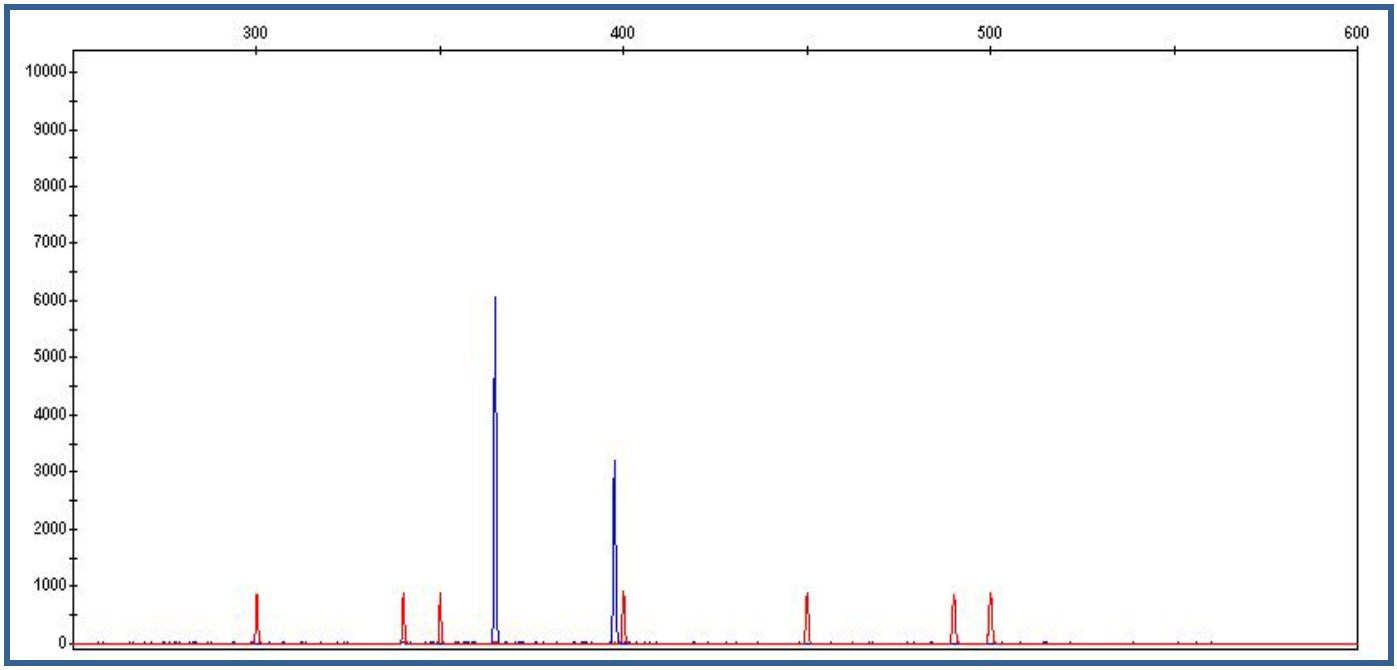

Figure 2. Detection of FLT3 internal tandem duplications by capillary electrophoresis-based fragment analysis. CE pherograms; x axis represents size of the PCR products in bases, y axis represents relative fluorescence intensity. The blue peaks represent the FAM-labeled fragments from the ITD portion 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.

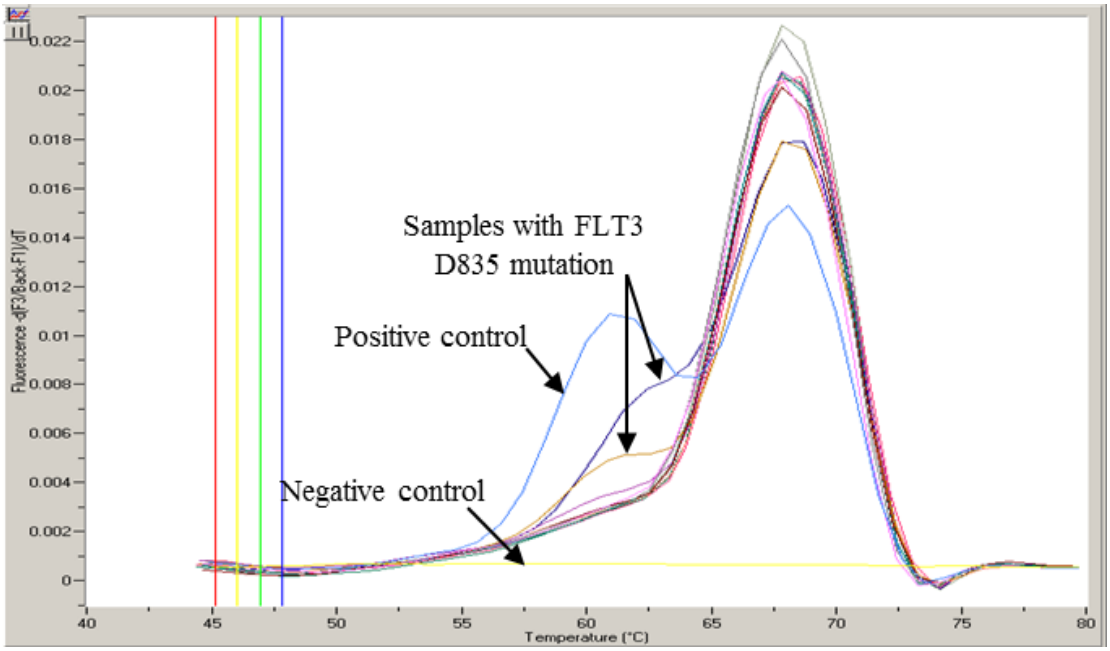

Figure 3. Derivative (-dF/dT versus T) melting curves of the normalized fluorescence data. The wild-type sample has a single peak, whereas the D835 mutant samples have either a low temperature shoulder or two peaks.

of FLT3 ITD mutation was associated with higher presenting WBC count.

Impact of FLT3 mutation status on outcome: Patients were monitored during the treatment and after achievement of complete remission. We analyzed overall survival (OS) according to the FLT3 mutations status in 23 patients from the time of diagnosis. The 30-months Kaplan-Meier estimate of OS was 50% and 87% for patients with and without FLT3 mutations respectively, with a median OS time of 9 months for patients with FLT3 mutation and 19 months for patients without mutations (Log rank test: P=0.043, Figure 4).

There was a significant difference in death rate according to FLT3-ITD mutation than D835 mutation. Of 3 patients with FLT3-ITD mutation, 2 patients were expired within early days of induction-remission period.

Discussion

The current hypothesis of leukemogenesis is the "two-hit" model first presented by Gilliland in 2001.

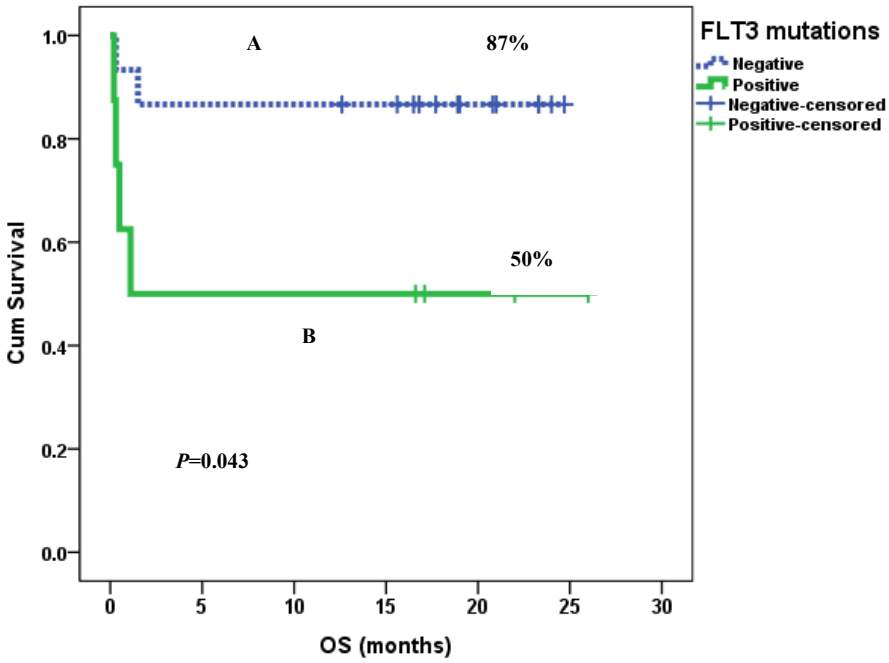

Figure 4. Impact of FLT3 mutation status in outcome of APL patients. A) Patients without FLT3 mutation; B)Patients with FLT3 Mutation.

This hypothesis implies that two separate mutations with different consequences need to be present for AML to develop. The first group of mutations are mutations giving 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, are mutations which impair 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(s) of APL formation. In 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 difficulty in some clinical samples. As in some clinical samples with ITD mutation, very small amounts of ITD mutant alleles relative to wild type can not be separated by agarose gel electrophoresis or ITD bands are very faint and would have been difficult to interpret clinically. Using CE capillary electrophoresis (CE) detection allowed small amounts of ITD mutant products to be sensitively and specifically identified.(25) Several molecular genetic aberrations have a prognostic impact in AML. The most important genetic aberration is in-frame internal tandem duplications (ITDs) of the receptor tyrosine kinase FLT3. FLT3 mutations are associated with an adverse prognosis, and is the strongest separate marker for disease relapse in AML.(26) 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 (MDS), and 3% of patients with acute lymphocytic leukemia and D835 mutations in 7% of patients with AML.(26) In present study our APL patients showed 39% FLT3 mutations, from which we found FLT3 ITD in13% 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[27]. In other study, 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.(28, 29) 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, nor with other blast characteristics at diagnosis. Moreover, as also

shown in AML patients,(26) the D835 alteration does not appear to carry prognostic impact in APL. It is conceivable that distinct levels of kinase activity may account for these clinical differences.(30)

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 high(er) 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.(21, 30-32) We also found higher WBC count and more death rate in FLT3 ITD group. In APL, the prognostic relevance of FLT3 mutations is less clear. Although previous studies have explored the impact of FLT3 mutations on outcomes of APL, these studies have been limited by a relatively small number of patients.(33) However, it is presumable that the low number of events recorded in the follow-up of APL patients receiving arsenic trioxide does not allow for significant differences to be found, and that the analysis of larger patient series may result in identification of more significant differences. On the other hand, it is likely that the FLT3 gene status remains strongly associated with initial leukocyte count and that the ITD alteration does not have independent value in prognostic assessment. In fact, other significant associations in APL cases carrying the ITD, such as the BCR3 PML/RAR isoform and the microgranular morphology (M3v), are notoriously linked to hyperleukocytosis in this leukemia.(34)

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.(Berger et al, 1991),(35). The t(15;17) chromosome translocation in acute promyelocytic leukemia 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.(36) In this study we also did not observed prognostic significance of As the PML-RARA is not sufficient to develop APL, we assume FLT3 mutations and additional chromosomal alterations in this APL series may cooperate with PML-RARA for leukemia development.

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