

Alterations of the FLT3 gene in acute promyelocytic leukemia: association with diagnostic characteristics and analysis of clinical outcome in patients treated with the Italian AIDA protocol

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Alterations in the FLT3 gene, including internal tandem duplications (ITDs) and D835 mutations occur frequently in acute myelogenous leukemia. We investigated the prevalence and clinico-biological correlations of FLT3 ITDs and D835 mutations in 90 patients with acute promyelocytic leukemia (APL) receiving the AIDA protocol. Twenty patients in which both presentation and relapse material was available were analyzed sequentially. Thirty-three patients (37%) harbored the ITD, and seven (7.7%) the D835 mutation in blasts obtained at diagnosis. Presence of ITDs was strongly associated with high WBC count ($P = 0.0001$), M3 variant ($P = 0.0004$), and the short (BCR3) PML/RAR α isoform ($P = 0.003$). There was no difference in response to induction in the two ITD+ve and ITD-ve groups, while a trend towards inferior outcome was observed for ITD+ve cases when analyzing disease-free survival (DFS) and relapse risk (RR). These differences, however, did not reach statistical significance. Sequential studies showed variable patterns in diagnostic and relapse material, ie ITD (-ve/-ve, +ve/+ve, +ve/-ve, -ve/+ve) and D835 (-ve/-ve, +ve/-ve, -ve/+ve). Our results indicate that FLT3 alterations are associated in APL with more aggressive clinical features and suggest that these lesions may not play a major role in leukemia progression.

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Introduction

The FLT3 gene encodes a tyrosine kinase receptor involved in the proliferation and differentiation of hemopoietic stem cells. The receptor is a member of the class III receptor tyrosine kinases (RTKs) including FMS, PDGFR, and c-KIT that share similar overall structure with five immunoglobulin-like extracellular domains, a transmembrane region, a juxtamembrane (JM) domain, two intracellular tyrosine kinase domains divided by a kinase insert domain and a C-terminal region.^{1–3} Interaction of the RTKs with their specific ligands leads to receptor dimerization, stabilizing the catalytic domain with the activation loop (A-loop) in an open conformation. Receptor dimerization and the subsequent phosphorylation of tyrosine residues accompanies RTKs activation and is followed by induction of multiple intracellular signalling pathways leading to cell proliferation and activation.³ Amplification, overexpression, or somatic mutation of RTKs results in increased receptor signalling, causing tumorigenesis.⁴

Recently, Nakao *et al*⁵ reported the frequent occurrence in acute myeloid leukemia (AML) of somatic alteration in the FLT3 gene consisting of an internal tandem duplication (ITD) of the JM domain coding sequence. The length of duplicated DNA varies from approximately 18 to 108 nucleotides in individual AML cases, but the duplicated region is always main-

tained in frame and causes constitutive activation through spontaneous oligomerization of the receptor, independently from the presence of the ligand.⁶

Various successive studies showed that ITD is detectable in approximately 20% of *de novo* AMLs, thus representing the most frequent genetic aberration currently known in this disease.^{7–12} Interestingly, this alteration was more commonly detected in some particular subsets, such as acute promyelocytic leukemia (APL) and in AMLs with normal karyotype. Moreover, in non-APL AMLs, the presence of ITD was associated in most reported series with unfavourable outcome.^{8–12} In particular, a recent study conducted in 854 patients identified ITD as the most important factor predicting for relapse and disease-free survival in non-APL AML.¹² These correlations were not found in other studies,^{13,14} probably reflecting different therapeutic context, whereas in one study it was suggested that loss of a wild-type FLT3 allele, more than the ITD alteration, was associated with unfavorable outcome.¹⁴ Finally, a distinct type of alteration occurring in a downstream region of the same gene has been recently described.¹⁵ This alteration consists of a missense mutation in codon D835 within the A loop of the receptor that causes constitutive activation by triggering the A-loop into an active conformation. The D835 mutation was found in approximately 7–8% of AMLs, an incidence significantly lower than that of the ITD. Unlike the ITD, the presence of the D835 mutations has not been correlated with prognostic outcome in AML.¹⁵

To our knowledge, only few studies have investigated the frequency and the clinico-biological correlations of FLT3 alterations in APL. In particular, Kiyoi *et al*¹⁶ reported a significant association of the ITD with hyperleukocytosis and high LDH level in APL, but found no correlation with response to therapy and outcome in patients treated with all-*trans* retinoic acid (ATRA) and chemotherapy. In the other two studies, APLs were analyzed in conjunction with the other AMLs and no specific investigation on clinico-biological correlations were reported.^{12,15}

In this study, we analyzed the frequency and clinico-biological correlations of ITD and D835 mutations in a large series of APL patients who received uniform therapy according to the Italian AIDA (ATRA plus idarubicin) protocol. Moreover, we analyzed the FLT3 gene status in sequential samples from 20 APL patients in which both presentation and relapse material was available.

Patients and methods

Patients

One hundred and two patients with newly diagnosed APL were observed and treated with the AIDA protocol¹⁷ at the Department of Human Biotechnology and Haematology of the

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University La Sapienza of Rome during the period April 1993–October 2001. Leukemic RNA and/or DNA were available for 90 of these patients and were used for the present study. With very few exceptions, nucleic acids were obtained from bone marrow cells isolated at diagnosis which contained >70% and most commonly >85% infiltration by leukemic blasts. APL was morphologically diagnosed according to the FAB classification and confirmed in all cases at the genetic level by RT-PCR detection of the PML/RAR α hybrid as previously described.¹⁸ The following clinical characteristics at diagnosis were analyzed: age, sex, FAB classification, peripheral WBC count, platelet count, haemoglobin level, karyotype and PML/RAR α isoform. In 20 cases who underwent disease relapse after front-line therapy, both diagnostic and relapse material was available for the study of FLT3 gene status.

Analysis of the *Flt3* ITD and D835 mutations

For the screening of the ITD, we analyzed total RNA in all cases. In selected cases, both DNA and RNA from the same patient were analyzed in parallel (see Results). Total RNA was extracted from Ficoll–Hypaque isolated leukemic blasts using the method of Chomczynsky and Sacchi.¹⁹ RNA was reverse-transcribed using random examers primers as previously described in the BIOMED-1 Concerted Action protocol.²⁰ Two μ l of cDNA were amplified in a total volume of 50 μ l of the reaction mixture containing 200 μ M of each dNTP, 1 \times PCR buffer, 1.5 U of Taq-Gold DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA) and 30 pmol of each primer. Pre-heating of the mixture at 94°C for 5 min was followed by 35 cycles of 45 s at 56°C, 30 s at 72°C, and 30 s at 94°C. A final extension of 10 min was carried out at 72°C on a Gene Amp PCR System 2400 (Perkin Elmer, Emeryville, CA, USA). With the aim of simultaneously analyzing both ITD and D835, we adopted a multiplex PCR strategy using the following four oligonucleotide primers: R5, 5'-tgtcgagcagtactctaaaca-3' and R6, 5'-atcctagtacctccaaactc-3' which amplify the entire transmembrane domain and the JM domain of the gene;⁵ and 17F: 5'-ccgccaggaacgtgcttg-3' and 17R: 5'-gcagcctcattgcccc-3' which explore the region containing exon 17 where the D835 mutation is located.¹⁵ For genomic amplifications of ITD the primers used were 11F, 5'-caatttaggtatgaaagcc-3' and 11R, 5'-caaactctaatttctct-3'.⁵

A schematic representation of the FLT3 gene with location of the above oligonucleotide primers is shown in Figure 1.

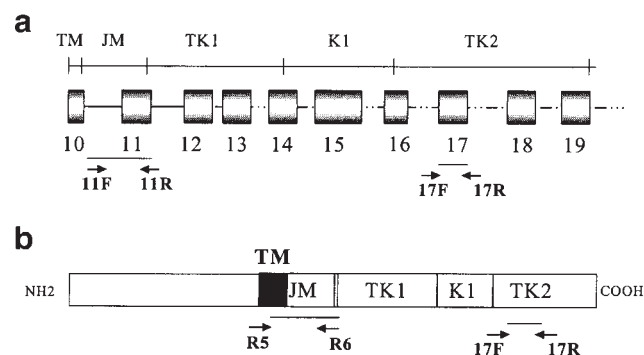


Figure 1 Schematic representation of the FLT3 gene with location of primers used for amplification of ITD and D835. (a) Genomic organization of the downstream part of the FLT3 gene and positions of primers for amplification of ITD and D835 mutations. (b) Structure of full length cDNA of FLT3 and position of primers for multiplex RT-PCR. TM, transmembrane domain; JM, juxtamembrane domain; TK, tyrosine kinase domain; K1, kinase insertion domain.

The amplified products were digested with *EcoRV* (Amersham International, UK) and electrophoresed on a 3% agarose gel containing ethidium bromide. Because it eliminates the *EcoRV* recognition site, the D835 results in undigested DNA of the mutated allele in cases harboring the mutation. By contrast, the higher molecular weight fragment amplified to investigate the ITD does not contain *EcoRV* recognition sites and is not therefore cut by the enzyme (Figure 2).²

Statistical methods

The distributions of categorical and continuous variables were compared between *Flt3*+ve and *Flt3*-ve groups. Pearson chi-square statistic or, if applicable, Fisher's exact test was used to test differences in the distribution of variables. Unadjusted time-to-event analyses were performed using the Kaplan–Meier estimate, log-rank tests and their generalizations.^{22–24} Relapse-free and disease-free survival (RFS and DFS) were calculated from the day of CR achievement. Relapse and death in CR were considered the 'events', whichever occurred first, to analyze when applicable as censored data in RFS and DFS. Computations were performed using 4F and 1L programs from the BMDP statistical library (BMDP Statistical Software, Los Angeles, CA, USA).

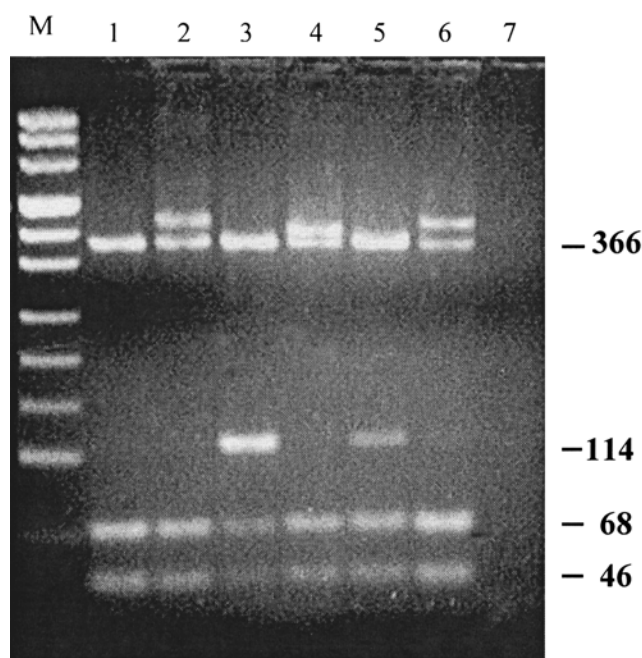


Figure 2 Results of multiplex RT-PCR for the simultaneous detection of both ITD and D835 in some representative APL cases. Lanes 1–6 show the results obtained in six APL patients at diagnosis. The upper part of the ethidium bromide-stained agarose gel shows the amplification products of the FLT3 where ITD are clustered. The 366-bp fragment indicates the size of the wild type in the absence of ITD, whereas additional upper bands are detectable in cases harboring the ITD (lanes 2, 4, 6). The lower part of the gel shows, for the same patients, the results of *EcoRV*-digested amplification products of exon 17 where the D835 mutations occur. Cases with wild-type exon 17 have undergone complete digestion of the 114-bp fragment resulting in two 68-bp and 41-bp bands (lanes 1, 2, 3, 6). Because it eliminates the *EcoRV* recognition site, the D835 mutation results in undigested DNA for the mutated allele in cases harboring the mutation (lanes 3, 5). Lane 7, negative control (all reagents plus water). M, molecular weight marker (pUC Mix No. SM0301).

Results

The ITD was detected in 33 of 90 (37%) cases as a higher molecular weight band compared to the 366 bp wild-type fragment. Eight of these mutated cases were subject to re-amplification and direct sequencing analysis which confirmed the presence of ITDs ranging from 22 to 66 repeated nucleotides. In one of these eight cases, an insertion was found in addition to the ITD. All inserted or duplicated sequences were in frame. Three of the 33 (9%) ITD+ve cases showed more than one ITD, with two higher molecular weight bands in addition to the wild-type one. No cases had absence of expression of the wild-type allele. Eighteen of the 90 cases (13 ITD+ve, 5 ITD-ve) were also studied on genomic DNA and the results were in all cases concordant with those obtained amplifying RNA. The D835 mutation was detected in 7 (7.7%) cases. Four of these cases were sequenced and revealed GAT→TAT (three cases) or GAT→GAG (one case) mutations, resulting in Asp→Tyr and Asp→Glu, respectively. All the D835 mutations were found in cases without ITD. All seven cases were confirmed as harboring the D835 mutation following analysis on genomic DNA. The results of multiplex RT-PCR for the simultaneous detection of both ITD and D835 in some representative cases are shown in Figure 2.

Clinical and biologic features of APL patients at diagnosis according to FLT3 are shown in Table 1. Because the D835 mutation did not appear to confer distinct features nor more aggressive clinical course, the seven patients with D835 mutation were included in the ITD-ve group for the analysis

Table 1 Diagnostic characteristics of patients according to ITD

Characteristic	ITD-ve n = 57 (63%) n (%)	ITD+ve n = 33 (37%) n (%)	P
Age			
<20	10 (17)	2 (6)	0.13
21-50	25 (44)	20 (61)	
>70	22 (39)	11 (33)	
Gender			
Male	27 (47)	16 (49)	NS
Female	30 (53)	17 (51)	
WBC (×10 ⁹ /l)			
<10	50 (88)	16 (49)	0.0001
10-50	7 (12)	11 (33)	
>50	0	6 (18)	
Hemoglobin (g/dl)			
<10	37 (65)	23 (70)	NS
>10	20 (35)	10 (30)	
Platelets (×10 ⁹ /l)			
<40	37 (65)	26 (79)	0.17
>40	20 (35)	7 (21)	
FAB subtype			
M3	50 (88)	18 (55)	0.0004
M3v	7 (12)	15 (45)	
Cytogenetics			
t(15;17)	51 (90)	33 (100)	0.13
t(15;17) + others	6 (10)	0	
BCR			
BCR1	35 (62)	10 (30)	0.003
BCR2	3 (5)	0	
BCR3	19 (33)	23 (70)	
Relapse risk ^a			
Low	19 (33)	3 (9)	0.0001
Intermediate	31 (54)	13 (39)	
High	7 (12)	17 (52)	

^aAccording to Sanz *et al.*²⁵

Table 2 Patient outcome according to presence or absence of FLT3 gene ITD

Characteristic	ITD-ve n = 57 (63%) n (%)	ITD+ve n = 33 (37%) n (%)	P
Respost to induction			
CR	55 (96)	32 (97)	
Death	1 (2)	1 (3)	
NE ^a	1 (2)		
DFS at 8 years	66 ± 7%	55 ± 9%	0.13
RFS at 8 years	71 ± 7%	61 ± 9%	0.12

^aNot evaluable (patient refused treatment).
 DFS, disease-free survival; RR, relapse risk.

of presenting features and prognostic outcome. Presence of the ITD mutation was not correlated to patient gender, hemoglobin, platelet count or age, whereas it showed strong association with high WBC count ($P = 0.0001$), M3 variant morphology ($P = 0.0004$), and the short (BCR3) PML/RAR α isoform ($P = 0.003$).

The relationships between the presence or absence of ITD and clinical outcome are given in Table 2. There was no difference in response to induction therapy with ATRA and idarubicin (AIDA) in the two ITD+ve and ITD-ve groups. A trend towards inferior results was observed for ITD+ve cases when analyzing disease-free survival (DFS) and relapse-free survival (RFS). This difference, however, did not reach statistical significance. In the ITD+ve and ITD-ve groups, the 8-year DFS was 55 (9%) and 66 (7%) ($P = 0.13$), and the 8-year RFS was 61 (9%) and 71 (7%) ($P = 0.2$).

The results of the sequential evaluation of patients studied either at diagnosis or relapse are summarized in Table 3. In the group of 20 cases studied sequentially, five patients were analyzed at diagnosis and at the time of molecular relapse. Eight patients showed absence of FLT3 gene alterations both at diagnosis and relapse (three molecular and two hematologic relapses were included in this group). Six cases showed FLT3 alterations (four ITD and two D835) at diagnosis, but relapsed with wild-type FLT3, including two patients with molecular relapse. Four cases were ITD+ve both at diagnosis and relapse. One patient with wild-type FLT3 at presentation showed ITD+ve at relapse. Finally, one patient ITD+ve at presentation showed at relapse the D835 mutation and was ITD-ve.

Table 3 Sequential evaluation of ITD and D835 mutations

n	ITD		D835 mutation	
	Diagnosis	Relapse	Diagnosis	Relapse
(total = 20)				
8 ^a	-ve	-ve	-ve	-ve
4 ^b	+ve	-ve	-ve	-ve
2	-ve	-ve	+ve	-ve
4	+ve	+ve	-ve	-ve
1	-ve	+ve	-ve	-ve
1	+ve	-ve	-ve	+ve

^aThree patients in this group were studied at the time of molecular relapse.

^bTwo patients in this group were studied at the time of molecular relapse. In all other cases, hematologic relapses were studied.

SPOTLIGHT

Discussion

In this study, we detected ITD of the FLT3 gene in 37% of APL patients, a frequency considerably higher than that initially reported by Kiyoi *et al*,¹⁶ but similar to that more recently published in a large study on AML including 133 APL cases.¹² Such differences may reflect some selection bias, as in both above-mentioned series, as well as in our study, APL cases were retrospectively analyzed based on the availability of stored nucleic acids, which in turn may result in a higher prevalence of hyperleukocytic cases. This notwithstanding, our study further establish ITD as the most common genetic alteration currently known in APL besides the PML/RAR α rearrangement. Combining the D835, a total of 44.7% of APL patients here analyzed carried FLT3 alterations in their leukemic cells.

As to the D835 mutation, our reported prevalence in APL is in agreement with the study of Yamamoto *et al*.¹⁵ 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,¹⁵ 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. In light of these observations, we analyzed clinical outcome in the present series by including D835+ve/ITD-ve cases in the ITD-ve group.

In line with findings reported in other APL and AML studies,¹²⁻¹⁶ the presence of ITD was associated with significantly increased WBC counts, ie a well-established prognostic factor in APL.²⁵ In addition, we describe here for the first time a strong correlation between ITD and the BCR3 PML/RAR α isoform, a finding whose biological significance is unclear to us at present.

Distinct from the results reported in AML,⁸⁻¹² we did not find that the ITD has prognostic significance in APL, although a trend towards inferior outcome was observed in ITD+ve cases by analyzing disease-free survival and relapse risk. However, it is presumable that the low number of events recorded in the follow-up of APL patients receiving modern protocols 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 that we describe here in APL cases carrying the ITD, such as the BCR3 PML/RAR α isoform and the microgranular morphology (M3v), are notoriously linked to hyperleukocytosis in this leukemia.²⁶

Our observation in patients studied sequentially at diagnosis and relapse suggest that neither ITD nor D835 alterations have a major role in APL progression. In fact, extremely variable patterns were detected, with frequent observation of loss of FLT3 mutations at the time of disease recurrence. These results are in agreement with findings reported by Nakano *et al*²⁷ in other AMLs and suggest that clonal shift, rather than clonal progression, occurred in these cases. It is conceivable to hypothesize that both FLT3 mutations and disease relapse are independently associated with other as yet unknown factors determining genetic instability and ultimately associated with

leukemia progression. In this respect, however, the possibility exists that our methodologic approach for FLT3 analysis was not sensitive enough to detect FLT3 mutations at the time of molecular relapse. In fact, the sensitivity of PCR assay to detect PML/RAR α (approximately 10^{-4}) is considerably higher than that of the test used for FLT3 amplification (10^{-2}).

Finally, we note the clinical importance of detecting alterations in the FLT3 gene with high frequency in this leukemia, particularly in the perspective of the recently described possibility to specifically targeting this alteration for therapy. FLT3 inhibitors that are already being developed^{28,29} might be employed in APL patients with advanced disease carrying these alterations.

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