Incidence and prognostic value of FLT3 internal tandem duplication and D835 mutations in acute myeloid leukemia

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Background and Objectives. Cytogenetics is the most important prognostic factor in acute myeloid leukemia (AML). However, a high proportion of patients show normal or intermediate-risk karyotypes. In these patients, other determinants could help to identify those with a higher risk of relapse. Recently, internal tandem duplications (ITD) and D835 mutations in FLT3 tyrosine kinase receptor have been shown to confer a bad prognosis in AML.

Design and Methods. We analyzed the incidence of these mutations in a total of 208 patients of different AML subsets and their prognostic relevance in non-promyelocytic *de novo* AML.

Results. FLT3 mutations were detected in 24% of *de novo* AML, 42% of acute promyelocytic leukemia (APL) and 17% of secondary AML. Four patients showed both ITD and D835 mutations. Ninety-four per cent of the patients with FLT3 alterations were classified into the intermediate-risk group. There was no association between the presence of FLT3 alterations and response to induction while the alterations were associated with a worse disease-free survival and event-free survival in both the overall and intermediate-risk patients.

Interpretation and Conclusions. Our data confirm that any of the mutations in FLT3 confer a bad prognosis in AML. Because of the high prevalence of these mutations within the intermediate-risk group, their detection could be useful to identify patients with a poor prognosis.

Key words: FLT3 mutations, acute myeloid leukemia, prognostic value, intermediate-risk cytogenetics.

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Although 60–80% of adult patients receiving conventional treatments for acute myeloid leukemia (AML) achieve complete remission (CR), disease relapse eventually occurs in the majority of cases.[1] During the last few years, several efforts have been made to identify prognostic factors that may predict treatment outcomes, thereby allowing bettering tailoring of post-remission therapy for patients. Currently, cytogenetics is considered the strongest prognostic factor and three major risk groups (low, intermediate, and high) have been distinguished according to AML karyotype at diagnosis.[2,3] However, the so-called intermediate risk group, which represents approximately two-thirds of cases, comprises a heterogeneous group of patients with apparently normal karyotypes or patients with a variety of other aberrations whose prognostic outcome is uncertain. Therefore, better identification of prognostic indicators in this group has been of particular interest in the past few years.

Recently, mutations in the FLT3 (fms-like tyrosine kinase receptor)[3] have been reported to be involved in leukemogenesis. This receptor belongs to the class III tyrosine kinase receptor family, and is expressed in the hematopoietic progenitor cells[4] as well as in a high percentage of AML and acute lymphoblastic leukemias (ALL).[5,6] Binding of its ligand (FL) causes dimerization and triggering of tyrosine kinase activity thus promoting stem cell proliferation.[7]

The most frequent FLT3 mutations found in AML and myelodysplastic syndromes (MDS) are internal tandem duplications (ITD) that have been reported in 20–30%[8–10] and 3–16%[9,10] of cases, respectively. These mutations affect the juxtamembrane domain and can be of variable length although all cases are in-frame mutations.[6–9] ITDs lead to dimerization and constitutive activation of the receptor in the absence of its ligand,[11] and recent studies with cultured cells or animal models point to a leukemogenic potential of such alterations.[12,13] In AML, FLT3 ITDs have been more frequently detected in patients with a normal karyotype and their presence has been associated with an adverse prognosis.[8–10] More recently, several mutations affecting aspartate 835 (D835) in the tyrosine kinase domain of the receptor have been found in about 7% of AML.[9,14,15] These mutations also seem to affect tyrosine kinase receptor activity.[15] Several studies have suggested that D835 mutations may also confer poor prognosis in AML,[9,15] although their clinical significance needs to be confirmed by more analyses.

The aim of the present study was to determine the

incidence of both ITD and D835 mutations in the FLT3 gene, in a series of 208 adult patients with AML and to evaluate their potential prognostic value.

Design and Methods

Patients

Two hundred and eight patients with AML recruited from five Spanish institutions were retrospectively analyzed for the presence of FLT3 mutations. They included 166 patients with non-promyelocytic *de novo* AML, 19 patients newly diagnosed as having acute promyelocytic leukemia (APL) and 23 with secondary AML (sAML). Nine cases of sAML were therapy-related and 14 were secondary to MDS. The prognostic impact of FLT3 alterations was analyzed only in the group of patients with *de novo* AML.

Treatment

One hundred and forty-two of 166 patients were enrolled into different intensive chemotherapy trials in which induction chemotherapy consisted of a combination of anthracycline plus cytarabine, with or without etoposide. As post-remission therapy, 50 patients followed a chemotherapy program, 35 patients received autologous bone marrow or peripheral blood stem cell transplantation, and 24 patients underwent allogeneic bone marrow transplantation.

Definitions

Cytogenetic risk groups were defined as follows: *high risk*: –5/del(5q), –7/del(7q), abn 3q, complex aberrations (≥ 3 independent aberrations), t(9;22) and t(6;9); *low risk*: t(8;21) and inv(16); *intermediate risk*: all other karyotypic aberrations or a normal karyotype.

Complete remission (CR) and hematologic relapse were defined according to the National Cancer Institute criteria.[16] Event-free survival (EFS) was defined as time from diagnosis to resistance, relapse or death from any cause. Disease-free survival (DFS) was calculated from the day CR was achieved to the time of relapse or death.

Patients' samples

DNA was obtained from the mononuclear cells of bone marrow or peripheral blood samples using the salting out procedure described by Miller *et al*.[17] RNA was obtained by the phenol-chloroform procedure of Chomczynski and Sacchi.[18] Then 0.5 μg of RNA were reverse transcribed into cDNA in a 25 μL reaction volume using the TaqMan Gold RT-PCR Kit (PE Applied Biosystems, Branchburg, New Jersey, USA).

Detection of ITD

ITDs were investigated by polymerase chain reaction (PCR) with the primers described by Nakao *et al*.[6] Primers 11F-11R and R5-R6, which amplify the juxtamembrane domain of the receptor, were used to amplify DNA and cDNA, respectively. The amplification reaction was performed in a final volume of 50 μL with 2 μL of DNA or cDNA, 0.6 μM of each primer, 2 mM of $MgCl_2$ and 2 U of DNA polymerase (Promega). After 2 min of initial denaturation at 95°C, 30 cycles of 45 sec at 94°C, 1 min at 58°C, and 2 min at 72°C were performed. The amplified products were finally electrophoresed on a 1.5% agarose gel stained with ethidium bromide.

Detection of D835 mutations

A fragment of 114 bp, corresponding to exon 17, was amplified using the primers 17F and 17R in DNA samples.[15] When analyzing cDNA, we used the reverse primer 17RC (5'–GCAGACGGGCATTGCCCC–3'). The amplification reaction was carried out using 2 min of initial denaturation at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C. The amplified product was then subjected to digestion with EcoR V restriction endonuclease (Promega). In the presence of a wild-type exon 17 the amplified fragment was digested into two fragments of 68 and 46 bp, easily distinguishable upon electrophoresis on a 2.5% agarose gel. Mutations affecting either D835 or I836 amino acids led to the detection of the undigested product of 114 bp, in addition to the two 68 and 46 bp fragments corresponding to the digestion of the wild-type allele.

In selected cases, the presence of a D835 mutation was confirmed by sequencing of the amplified products. In these cases the undigested band was purified from the agarose gel and re-amplified. The PCR product was sequenced using Big Dye Terminator cycle sequencing chemistry (Applied Biosystems). Sequences were compared with the wild-type sequence registered in Genbank [accession #XM_166272 (mRNA) and NT_033997 (DNA)].

Statistical methods

χ^2 and Fisher's exact tests were used to analyze differences in the distribution of variables among subsets of patients. For comparison, unadjusted time-to-event analyses were performed using the Kaplan-Meier estimate,[19] log-rank tests and their generalizations.[20–22]

All survival estimates are reported plus or minus (±) 1 standard error. The median duration of follow-up of patients who remain alive was 26 months (range, 13–126 months). The patients' follow-up was updated as of May 2002.

Computations were performed using 4F and 1L programs from the BMDP statistical library (BMDP Statistical Software Inc, Los Angeles, CA, USA).

Results

Incidence of FLT3 mutations

Alterations in the FLT3 gene were detected in 52 patients out of the 208 cases analyzed. These aberrations included ITD in 32 cases (eight of them with more than one ITD), D835 mutations in 16 cases and both types of alteration (ITD + D835) in four patients. Forty of 166 *de novo* AML patients (24%) had mutations in FLT3 (24 ITD, 12 D835 and four ITD + D835), while eight of 19 APL patients (42%) had FLT3 alterations (six ITD and two D835). Finally, four of 23 sAML (17%) carried some type of mutation in the FLT3 gene (two ITD and two D835).

Sequencing of 10 samples with D835 mutation led to the identification of four different mutations (Table 1), the most frequent of which was D835Y (five cases), followed by D835H mutation (three cases). One patient carried a D835V mutation, and another the D835A mutation, which had not previously been described.

FLT3 alterations and correlation with presenting features in de novo AML patients (excluding APL)

Details of clinical characteristics at diagnosis of the 166 *de novo* AML patients are given in Table 2. The presence of FLT3 ITD was clearly associated with hyperleukocytosis ($p < 0.0001$), intermediate-risk cytogenetics ($p = 0.03$), and particularly with normal karyotype ($p = 0.04$). No clinical variables were associated with D835 mutations. The four patients with both ITD and D835 mutations had white blood cell counts greater than 50×10^9/L and normal karyotypes. Finally, five out of seven patients with more than one ITD alteration also had hyperleukocytosis and a normal karyotype at diagnosis.

Treatment outcome according to FLT3 status in de novo non-promyelocytic AML patients

FLT3 alterations had no influence on response to induction (Table 3). The three-year Kaplan-Meier estimate of DFS for the whole series was 40±5%. FLT3 alterations were associated with a shortened DFS (ITD-positive 41±12%, D835-positive 14±12%, ITD/D835-negative 43±6%) and EFS (ITD-positive 28±9%, D835-positive 11±9%, ITD/D835-negative 43±6%), as shown in Figures 1A and 1B. In the intermediate cytogenetic group, patients with a mutated FLT3 had a shorter EFS (ITD-positive 30±10%, D835-positive 17±11%, ITD/D835-negative 36±6%) and DFS (ITD-positive 39±12%, D835-positive 22±14%, ITD/D835-negative 44±7%), as shown in Figures 2A and 2B. DFS in FLT3-positive AML patients was 17±14%, 38±20% and 47±19%

Table 1. Mutations found affecting Asp 835 in FLT3.

Mutation	*n*	*Amino acid change*
GAT→TAT	5	Asp→Tyr
GAT→CAT	3	Asp→His
GAT→GTT	1	Asp→Val
GAT→GCT	1	Asp→Ala

Table 2. Clinical characteristics of *de novo* AML patients.

Characteristics	*Wild-type FLT3* *n*	*(%)*	*Mutated FLT3* *n*	*(%)*	*p*
Cases	126		40		
Age (years)					
≤20	18	(14.3)	4	(10.0)	
21-50	39	(31.0)	18	(45.0)	ns
>50	69	(54.8)	18	(45.0)	
Gender					
Male	63	(50.0)	20	(50.0)	ns
Female	63	(50.0)	20	(50.0)	
WBC ($\times 10^9$/L)					
≤10	62	(49.2)	4	(10.0)	
10-50	39	(31.0)	17	(42.5)	<0.0001
>50	25	(19.8)	19	(47.5)	
Hemoglobin (g/dL)					
≤10	78	(61.9)	29	(72.5)	ns
>10	48	(38.1)	11	(27.5)	
Platelets ($\times 10^9$/L)					
≤50	55	(44.0)	21	(52.5)	ns
>50	70	(56.0)	19	(47.5)	
FAB subtype					
M0	5	(4.0)	2	(5.0)	
M1	33	(26.2)	14	(35.0)	
M2	44	(34.9)	7	(17.5)	
M4	17	(13.5)	9	(22.5)	ns
M5	18	(14.3)	6	(15.0)	
M6	4	(3.2)	0	(0.0)	
M7	2	(1.6)	0	(0.0)	
Unclassified	3	(2.4)	2	(5.0)	
Cytogenetic risk group					
Low	14	(12.6)	0	(0.0)	
Intermediate	81	(73.0)	31	(93.9)	0.03
High	16	(14.4)	2	(6.1)	

Table 3. Clinical outcome of *de novo* AML patients.

Characteristics	*Wild-type FLT3* *n*	*(%)*	*Mutated FLT3* *n*	*(%)*	*p*
Induction response					
CR	81	(77.9)	28	(73.7)	
Failure					
Resistance	15	(14.4)	6	(15.8)	ns
Death	8	(7.7)	4	(10.5)	
Post-induction therapy					
Chemotherapy only	37	(45.7)	13	(46.4)	
Autologous SCT	28	(34.6)	7	(25.0)	ns
Allogeneic SCT	16	(19.8)	8	(28.6)	

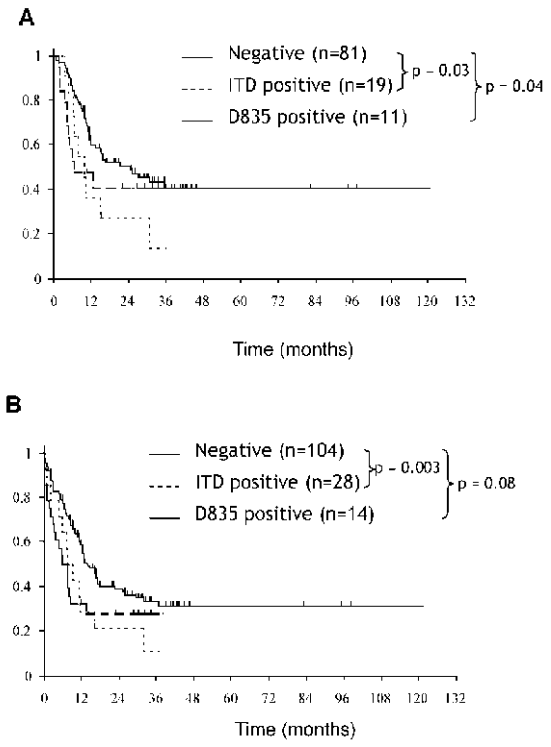

Figure 1. Kaplan-Meier analysis of the non-promyelocytic *de novo* AML patients according to the presence of a normal FLT3 receptor, ITD or D835 mutations. (A) DFS and (B) EFS.

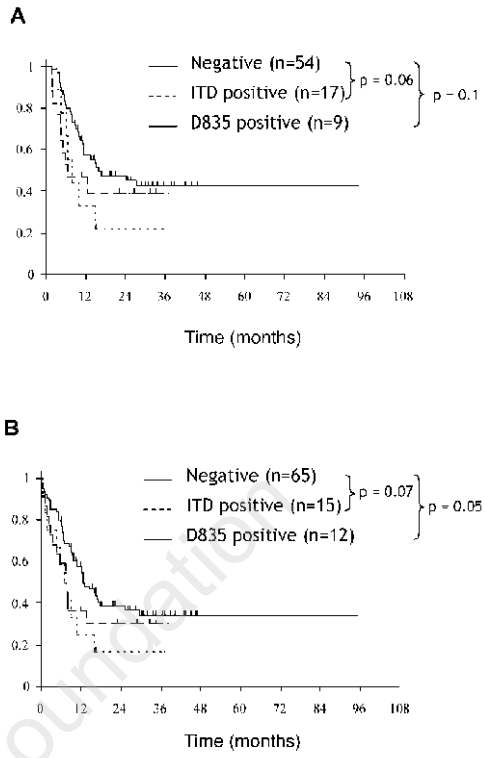

Figure 2. Kaplan-Meier analysis of the intermediate-risk group with normal or mutated FLT3. (A) DFS and (B) EFS.

for patients treated with chemotherapy, auto- and allograft, respectively, while it was 27±9%, 54±37% and 60±15% in FLT3-negative patients. All these differences showed a trend, but were not statistically significant ($p = 0.15$, $p = 0.07$ and $p = 0.16$, respectively).

All four patients with the simultaneous presence of ITD and D835 mutations died, one of them during induction and the other three of disease relapse at two, four and six months post-remission.

Discussion

This study shows that FLT3 alterations are strongly associated with hyperleukocytosis and intermediate-risk cytogenetics, particularly in those patients with a normal karyotype. In addition, in this study we found FLT3 mutation to be an unfavorable prognostic factor, shortening both DFS and EFS. With regard to the overall incidence of ITD mutations in patients with *de novo* AML, APL and sAML, our findings are in agreement with those reported by other groups.[8–10,14,15] Similarly, we confirm a strong association between t(15;17) and the presence of mutations in FLT3 (32% ITD and 11% D835). Although some investigators have suggested that an altered PML protein might contribute to impair genetic stability in APL cells thereby promoting a high incidence of FLT3 mutations,[23] the reasons underlying this association are still unclear. As previously reported, we found that the presence of ITD was strongly associated with hyperleukocytosis in AML.[8–10] However, such an association did not reach statistical significance for D835 mutations. This could be explained by the lower number of cases analyzed, but may also be because of an increase in the activity of the receptor due to the effect of these mutations.[24] Further studies are needed to explain how D835 mutations affect the activity of FLT3 and whether there are different effects among the mutation types. Interestingly, the vast majority of patients harboring FLT3 mutations (94%) belonged to the intermediate-risk group according to cytogenetics, mainly due to a high association of these mutations with a normal karyotype. This finding is particularly relevant in clinical terms, as the presence of these mutations also correlated with adverse DFS and EFS, therefore allowing the segregation of this vast group of AML patients with normal karyotype into two categories with distinct responses

to treatment. An additional contribution of our study is the evaluation of the prognostic impact of the less frequent D835 mutation of FLT3; the prognostic significance of this mutation has been analyzed in only two studies reported to date.[9,15] While our results suggest that the D835 mutation is associated with a poorer prognosis, in agreement with the finding reported by Thiede *et al*.,[9] further studies including a higher number of patients are needed to strengthen this observation. The high frequency of FLT3 alterations in AML has fostered investigations into molecularly targeted treatment and development of agents directed against this receptor, such as herbimycin A or CEP-701.[25,26] These new drugs could selectively act against leukemic cells with activated FLT3, not unlike all trans retinoic acid, which is specific for the fusion gene products of PML-RARα,[27] and imatinib mesylate (formerly STI571), which reversibly inhibits the enzymatic activity of BCR/ABL.[28] Recent findings indicate that other factors could also lead to constitutive activation of FLT3.[29] These observations, together with the recent identification of an additional mutation affecting the tyrosine-kinase domain,[30] should foster more extensive studies into the function of normal and mutated FLT3 receptors and highlight the importance of identifying new mutations in this and other receptors expressed in AML.

In summary, our data suggest that FLT3 mutations are frequently observed in AML and are associated with a poor prognosis. In the intermediate-risk cytogenetic group, FLT3 mutations might allow the identification of a subset of patients with a higher risk of relapse, who could potentially benefit from more intensive treatments.

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Contributions

IM, GM, PB, EB, ER: conception and design of the study, analysis and interpretation of the data; JR, PF, PL, AM, JC, AT: drafting of the study and critical revision of it; MAS: final approval of the version to be published. Primary responsibility for the paper: PB, MAS; tables 1-3: IM; figures 1-2 and statistical analysis: GM.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received July 22, 2002; accepted November 13, 2002. In the following paragraphs, the Editor-in-Chief summarizes the peer-review process and its outcomes.

What is already known on this topic

An internal tandem duplication of the FLT3 gene and additional mutations are found in about one third of patients with acute myeloid leukemia and are generally associated with a poor prognosis.

What this study adds

This study confirms recent independent reports, indicating that FLT3 mutations are frequently observed in acute myeloid leukemia and are associated with a poor prognosis.

Caveats

This is a retrospective study.