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# *FLT3*-activating mutations are associated with poor prognostic features in AML at diagnosis but they are not an independent prognostic factor

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*FLT3* gene alterations (internal tandem duplications – ITDs – and D835 mutations) are thought to be associated with poor-risk acute myeloid leukemia (AML). However, not all studies confirm this association, so it is still a matter of debate. Moreover, their association with other molecular abnormalities is less studied. We have investigated the presence of *FLT3*-ITD and D835 mutations in AML patients and their correlation with clinical and biological disease characteristics. The presence of ITD was analyzed in diagnostic samples of 176 AML patients and the D835 mutation in 135 of these patients. In all these patients, the presence of four well-known molecular abnormalities were also simultaneously characterized: *PML/RARα*, *AML1/ETO*, *CBFβ/MYH11* and *MLL* rearrangements. In all, 41 (23%) patients harbored *FLT3* mutations, with 34 (19.3%) of them positive for the ITD, and seven (5%) positive for the D835 mutation. Of the acute promyelocytic leukemia (APL) patients, 16 (27%) showed *FLT3* mutations, more frequently in M3 hypogranular cases (62% versus 17%, $P=0.001$) and cases with the short (bcr3) *PML-RARα* isoform (69%, $P=0.002$). In contrast, *FLT3* was never altered in patients with inv(16), t(8;21) or 11q23 abnormalities. *FLT3* mutations were significantly associated with some negative prognostic features at diagnosis (leukocytosis, high blast-cell percentage, and elevated LDH values), but they were not associated with different disease-free or overall survival. Therefore, we confirm a high frequency of FLT3 mutations in APL and in adult AML without recurrent cytogenetic translocations. In addition, they were not found as independent prognostic factors although associated with several adverse features at diagnosis.
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## Introduction

The *FLT3* gene belongs to the receptor tyrosine kinase class III family, which plays a central role in hematopoiesis.[1] This gene is expressed in normal hematopoietic stem cells and in the majority of acute myeloid leukemia (AML) blast cells.[2] The *FLT3* ligand stimulates proliferation and induces inhibition of apoptosis in AML cells expressing functional FLT3. Therefore, alterations in the structure and signaling are involved in the genesis of cancer.[3] Recently, internal tandem duplications (ITD) in the juxtamembrane domain (JM) coding sequence of the *FLT3* gene have been found in 16.5% of pediatric AML patients, approximately 20% of adult AML patients and 34% of elderly AML patients.[4–6] More recently, point mutations in codon 835 of the *FLT3* gene, sited at the activation loop of the second tyrosine kinase domain (TKD), have been described in 7% of adult AML patients.[7,8] However, the correlation of *FLT3* abnormalities with other molecular abnormalities frequently observed in AML has not been systematically explored.[11,27]

Many clinical studies report that *FLT3*-ITD is associated with poor outcome[9–12] and adverse risk factors.[6,13] However, in a recent report that studies a high number of newly diagnosed AML patients ($n=871$), those with *FLT3*-ITD are included in the intermediate risk group category.[14] Moreover, some reports have found correlations with adverse prognostic characteristics but not with inferior clinical outcomes.[6,15,16] As far as D835 point mutations are concerned, little is known of their prevalence and clinical significance.[17,18]

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For this purpose, in the present study, we have examined *FLT3*-ITD and D835 mutations in a series of 176 newly diagnosed adult AML patients in order to determine not only the frequency of *FLT3* alterations and its prognostic significance, but also its correlation with four other molecular abnormalities commonly observed in AML. Moreover, we have also carried out an extensive literature review in order to compile the information on the prognostic value of *FLT3* in AML and its utility in the definition of well-defined risk categories.

## Materials and methods

### *Patients*

A total of 176 newly diagnosed AML patients referred to our reference laboratory at the University Hospital of Salamanca (Spain) were retrospectively analyzed for the presence of *FLT3*-ITD and 135 of them for D835 mutations. All of these patients were also systematically evaluated to assess the presence of *PML/RARα*, *AML1/ETO* and *CBFβ/MYH11* fusion transcripts as well as *MLL* rearrangements. Intensive chemotherapy treatment with curative intention was administered in 154 patients. All of them were treated according to the Spanish PETHEMA protocol (AML-95, APL-96).[19,20]

### *Molecular analysis*

Total RNA was extracted from BM and/or PB samples by the guanidinium thiocyanate/phenol chloroform method. The RNA integrity was assessed in all cases by RT-PCR amplification of the ubiquitous Abelson control gene (*ABL*).

*Detection of FLT3 mutations* *FLT3*-ITD was examined by RT-PCR amplification of the JM domain from exon 14 to 15 with the primers R5 5′-TGTCGAGCAGTACTCTAAACA-3′ and R6 5′-ATCCTAGTACCTTCCCAAACTC-3′.[2] PCR products were separated on 1.5% agarose gels stained on ethidium bromide. In the absence of ITD, a 366 bp fragment indicates the size of the wild type, whereas additional upper bands are detectable in cases harboring the ITD. To detect *FLT3*-D835 point mutations, we used the restriction fragment length polymorphism-mediated (RFLP) PCR assay, since D835 and I836 codons are encoded by the nucleotide sequence GATATC, which forms the *Eco*RV restriction site. We amplified exon 20 of the *FLT3* TK domain by using the primers 17F 5′-CCGCCAGGAACGTGCTTG-3′,[7] and 17RC 5′-GCAGACGGGCATTGCCCC-3′.[21] *FLT3*-D835 amplified products were then subjected to digestion with *Eco*RV (Roche Diagnostics GmbH) and electrophoresed on a 3% agarose gel. As it eliminates the *Eco*RV recognition site, the D835 results in an undigested product of 114 bp, which corresponds to the mutated allele, 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 cut out from the gel, purified and reamplified. The PCR product was sequenced in both directions with the Big Dye Terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA, USA).

*Detection of fusion transcripts* The presence of the *PML/RARα*, *AML1/ETO* and *CBFβ/MYH11* fusion transcripts was analyzed by RT-PCR as previously described,[22] according to the primers, protocols and criteria of the European 'BIOMED-1 Concerted Action'.[23] *MLL* rearrangements were studied by Southern blot analysis with methods previously reported[22] with the exception of 16 patients in whom high molecular weight DNA of sufficient quality was not available.

### *Statistical methods*

The association between variables was analyzed by the $\chi^2$ and Fisher's exact tests and Student's *t*-test. The probability of disease-free survival (DFS) and overall survival (OS) was estimated according to the Kaplan–Meier method. Survival was calculated from the date of diagnosis to death or last follow-up and DFS was calculated from the date when complete remission (CR) was achieved to relapse, death or last follow-up. These analyses were performed using the SPSS statistical software (SPSS Inc., Chicago, IL, USA).

## Results

### *Patients and molecular analysis of FLT3*

In all, 176 patients were included in the study. Out of the total, 60 harbored the t(15;17) translocation, while seven had the inv(16) and two the t(8;21). Three cases had 11q23 rearrangements while the remaining 104 patients lacked any of these alterations.

*FLT3*-ITD was identified in 34 of the 176 (19.3%) patients analyzed. The frequency of *FLT3*-ITDs differed significantly between molecular subgroups ($P<0.05$): of the 60 patients with t(15;17), 15 (25%) exhibited the ITD (eight typical M3 and seven microgranular M3 variants [M3v]). In contrast, *FLT3*-ITD was never observed in patients with inv(16), t(8;21) or 11q23 alterations. Of the 104 remaining patients negative for the translocations and rearrangements described above, 19 (18%) were ITD-positive. On agarose gel electrophoresis, only one of the 176 (0.5%) patients displayed a strong mutant band and complete loss of both wild-type alleles. Table 1 shows the clinical and biological features at diagnosis of these patients according to the *FLT3* status and to the presence or absence of t(15;17) translocation.

**Table 1** Diagnostic characteristics of AML patients according to the *FLT3* status

| *Characteristic* | *Non-APL/t(15;17) negative (n=116)* | | | *APL/t(15;17) positive (n=60)* | | |
|---|---|---|---|---|---|---|
| | *Wild-type* FLT3 (n=91) | *Mutated* FLT3 (n=25) | P | *Wild-type* FLT3 (n=44) | *Mutated* FLT3 (n=16) | P |
| Mean age (years) | 59±20 | 60±18 | NS | 46±21 | 34±12 | 0.008 |
| Male sex (%) | 54 | 48 | NS | 73 | 69 | NS |
| WBC count ($10^9$/l) | 28±50 | 66±55 | 0.003 | 9.6±18 | 42±51 | 0.023 |
| BM blasts (%) | 67±21 | 81±13 | 0.0001 | 79±16 | 86±11 | NS |
| PB blasts (%) | 40±30 | 63±30 | 0.001 | 36±32 | 74±22 | 0.0001 |
| LDH (U/l) | 895±762 | 1346±747 | 0.018 | 674±380 | 964±384 | 0.023 |
| Platelets ($\times 10^9$/l) | 70±61 | 65±62 | NS | 40±40 | 20±12 | 0.005 |
| Hemoglobin (g/dl) | 8.9±2.4 | 9.6±2.5 | NS | 8.7±2.3 | 9.0±2.3 | NS |
| *FAB subtype* | | | | | | |
| M0 | 9 (10%) | 0 | | | | |
| M1 | 15 (16%) | 8 (32%) | | | | |
| M2 | 23 (25%) | 10 (40%) | | | | |
| M3 | 4 (4%) | 0 | | 39 (89%) | 8 (50%) | |
| M3v | 4 (4%) | 1(4%) | NS | 5 (11%) | 8 (50%) | 0.001 |
| M4 | 20 (22%) | 1(4%) | | | | |
| M5 | 12 (13%) | 5(20%) | | | | |
| M6 | 3 (3%) | 0 | | | | |
| M7 | 1 (1%) | 0 | | | | |
| *PML-RARα isoform* | | | | | | |
| Bcr1 | | | | 31 | 4 | |
| Bcr2 | | | | 4 | 1 | 0.002 |
| Bcr3 | | | | 9 | 11 | |

WBC: white blood cell; BM: bone marrow; PB: peripheral blood; LDH: lactate dehydrogenase; APL: acute promyelocytic leukemia; NS: not significant.

D835 mutations could be analyzed in only 135 out of the 176 patients because of lack of more RNA samples. Mutations were found in only seven (5%) cases: one was positive for the t(15;17) with an M3v morphology, while the other six positive cases did not have any fusion transcript. None of the patients had both *FLT3*-ITD and D835 mutations.

## *FLT3 status and leukemia characteristics*

Owing to the distinct clinical and prognostic behavior of APL and non-APL patients, cases were analyzed separately in order to evaluate their clinical outcome in relation to the presence of *FLT3* mutations: non-APL or t(15;17)-negative patients ($n=116$) and APL or t(15;17)-positive patients ($n=60$) (Table 1). Statistical analyses demonstrated that the D835 mutation did not seem to confer distinct characteristics or a more aggressive clinical course than ITDs in our patients, as described in other series.[8,17] Thus, the seven D835-positive patients were included in the ITD-positive group for the analysis of presenting features and prognostic outcome. Among the non-APL cases, the presence of ITD/D835 mutations did not correlate with patient gender, age, hemoglobin, platelet count or FAB morphology, whereas it did show strong association with increased WBC counts ($P=0.003$), high percentage of BM and PB blasts ($P<0.001$), and high serum LDH level ($P=0.018$). In APL cases, *FLT3* mutations were related to younger patient age ($P=0.008$), elevated WBC counts ($P=0.023$), high percentage of PB blasts cells ($P=0.0001$), high serum LDH level ($P=0.023$), decreased platelets counts ($P=0.005$) and M3 variant morphology (62 *versus* 17%, $P=0.001$). FLT3 mutations also correlated with the short (bcr3) PML-RARα isoform (bcr3: 69%, bcr1: 25% and bcr2: 6%, $P=0.002$) (Table 1).

The relationship between the presence or absence of ITD/D835 mutations and clinical outcome are given in Table 2. In the non-APL group, CR rates were slightly lower in those patients with *FLT3* mutations (50 *versus* 68%). However, these differences were not statistically significant. Moreover, upon analyzing the 5-year probabilities for OS and DFS, the outcome of *FLT3* mutated cases was similar to that of wild-type cases (OS: 19 *versus* 16%, DFS: 36 *versus* 29%, $P>0.05$ for both) (Figures 1a and b). Similarly, among the APL patients, *FLT3* mutations were not associated with poor prognosis, showing a similar CR rate (88 *versus* 72%, $P>0.05$), OS (65 *versus* 53% at 5 years, $P>0.05$) and DFS (79 *versus* 68% at five years, $P>0.05$) for patients with or without *FLT3* mutations, respectively (Figures 1c and d).

## Discussion

We have analyzed the incidence of *FLT3* alterations (ITD and D835 point mutation) in a series of newly diagnosed AML patients and we have evaluated its possible association with other genetic lesions and

**Table 2** Clinical outcome of AML patients according to the *FLT3* status

| *Characteristic* | t(15;17) *negative* (n = 116) | | | t(15;17) *positive* (n = 60) | | |
|---|---|---|---|---|---|---|
| | *Wild-type* FLT3 (n = 91) | *Mutated* FLT3 (n = 25) | P | *Wild-type* FLT3 (n = 44) | *Mutated* FLT3 (n = 16) | P |
| CR rate (%) | 50/73 (68) | 11/22 (50) | NS | 31/43 (72) | 14/16 (88) | NS |
| Treatment failure (%) | 13.7 | 31.8 | NS | 4.7 | 0 | NS |
| Early deaths (%) | 17.8 | 18.2 | NS | 23.3 | 12.5 | NS |
| *OS at 5 years* (%) | 16 | 19 | | 53 | 65 | |
| Median (years) | 0.9 | 0.46 | NS | NR | NR | NS |
| Deaths | 59 | 15 | | 17 | 5 | |
| *DFS at 5 years* (%) | 29 | 36 | | 68 | 79 | |
| Median (years) | 1.4 | 3.4 | NS | NR | NR | NS |
| Relapses | 27 | 6 | | 7 | 3 | |

CR: complete remission; OS: overall survival; DFS: disease-free survival; NS indicates not significant; NR indicates not reached.

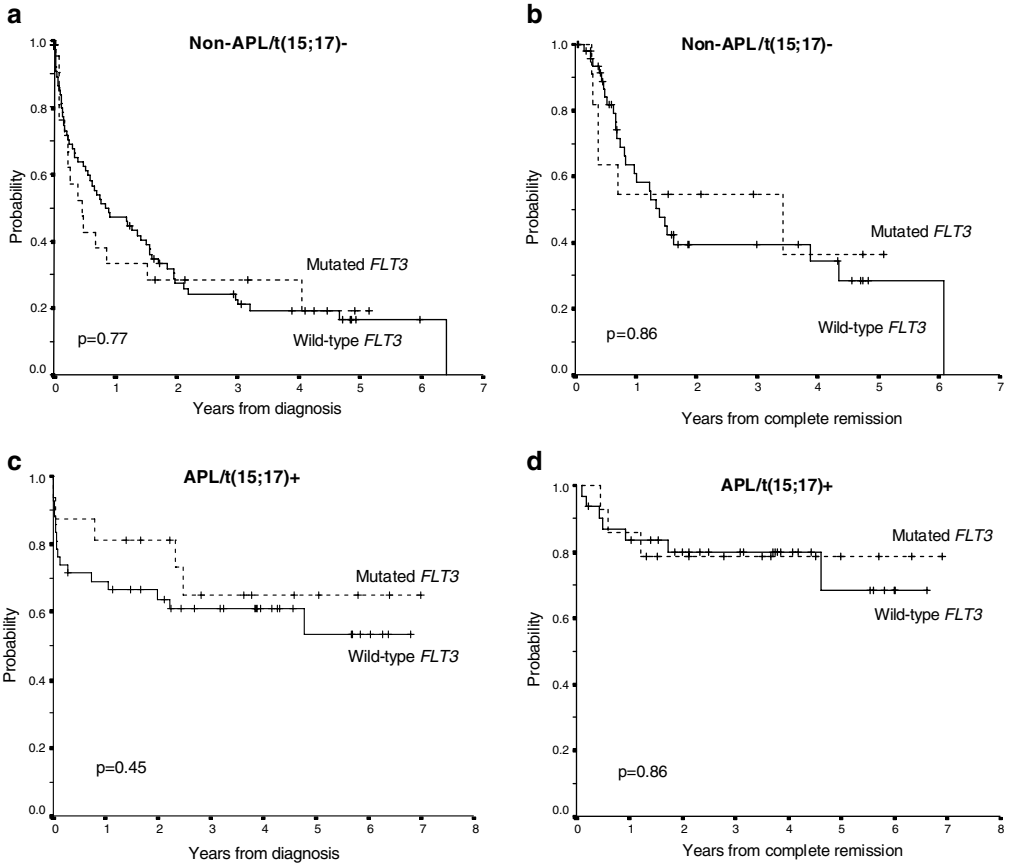


**Figure 1** Kaplan–Meier analyses of AML patients according to the *FLT3* status. (a) OS of non-APL patients. (b) DFS of non-APL patients. (c) OS of APL patients. (d) DFS of APL patients. Statistical differences were evaluated by the log-rank test.

prognostic relevance. The incidence of *FLT3*-ITD and D835 point mutations observed in our study (19.3 and 5%, respectively) is similar to that previously reported in adult AML patients (Table 3). None of the patients showed a combination of both *FLT3*/ITD and D835 mutations. Overall, a total of 23.3% of adult AML patients possessed an *FLT3*-activating mutation.

Interestingly, the frequency of *FLT3*-ITDs was significantly different between AML molecular subgroups. Thus, they were more frequent in APL t(15;17)-positive patients (27%), being as high as 62% (8/13) of cases with M3 variant morphology. This association between the ITD and APL morphology has been previously reported,[14,24] although the effect has not been underlined up to now. Similarly, those cases lacking any well-known AML translocation, had a frequency of *FLT3* mutations of 22%. By contrast, we were unable to detect *FLT3*-ITD or D835 mutations in

**Table 3** Comparative results of different studies on the incidence and prognostic value of *FLT3* alterations in AML

| *Reference* | *Cases* | *AML characteristics* | *Mutations (incidence)* | *Clinical outcome (P)* | *Multivariate analysis* |
|---|---|---|---|---|---|
| Sheikhha *et al.*[17] | 80 | Adult AML | ITD: 8/80 (10%)<br><br>D835: 6/80 (7.5%) | Higher RR<br>Lower OS (0.006)<br>Lower DFS (0.047)<br>No differences | NP |
| Rombouts *et al.*[9] | 81 | Adult AML | ITD: 18/81 (22%) | Lower CR (0.03)<br>Lower EFS (0.003)<br>Increased RR (0.01) | NP |
| Whitman *et al.*[3] | 82 | Adult AML<br>Normal citog. <60 years | ITD: 23/82 (28%)<br><br>Loss of wt-allele:<br>8/23 (35%) | Lower DFS (0.03)<br>No difference in OS<br>Lower DFS (0.0017)<br>Lower OS (0.0014)<br>No difference in CR | Independent prognostic factor for DFS and OS |
| Noguera *et al.*[24] | 90 | Adult M3 | ITD: 33/90 (37%)<br>D835: 7/90 (8%) | No difference in CR<br>No difference in DFS<br>No difference in RFS | NP |
| Meshinchi *et al.*[4] | 91 | Pediatric AML | ITD: 15/91 (17%) | Lower CR (0.005)<br>Lower OS (0.02)<br>Lower EFS (0.002) | Independent prognostic factor for poor outcome |
| Kainz *et al.*[27] | 100 | Adult AML | ITD: 26/100 (26%) | No difference in CR<br>Lower OS (<0.003)<br>Lower DFS (<0.02)<br>No difference in RR | Independent prognostic factor for OS if cases with t(15;17) are excluded |
| Abu-Duhier *et al.*[10] | 106 | Adult AML | ITD: 14/106 (13%) | Lower OS (0.0002) | NP |
| Shih *et al.*[28] | 107 | Adult M3 | ITD: 22/107 (21%)<br>D835: 20/107 (19%) | No difference in CR<br>No difference in OS<br>No difference in EFS | NP |
| Stirewalt *et al.*[31] | 140 | Elderly AML >55 years | ITD: 47/140 (34%) | No difference in CR<br>No difference in RFS<br>No difference in OS | ITD is not an independent prognostic factor for OS and RFS |
| Boissel *et al.*[16] | 159 | Adult AML (Non M3)<br><65 years | ITD: 40/159 (25%) | No difference in CR<br>No difference in RFS<br>No difference in OS | ITD is not an independent prognostic factor for OS and RFS |
| Moreno *et al.*[21] | 166 | Adult AML (Non M3) | ITD: 28/166 (17%)<br>D835: 16/166 (10%) | No difference in CR<br>Lower DFS (0.04)<br>No difference in EFS | NP |

**Table 3** *Continued*

| *Reference* | *Cases* | *AML characteristics* | *Mutations (incidence)* | *Clinical outcome (P)* | *Multivariate analysis* |
|---|---|---|---|---|---|
| Kiyoi *et al.*[15] | 201 | Adult AML (Non M3) | ITD: 46/201 (23%) | Lower CR (0.005)<br>Lower OS (0.002)<br>Lower DFS (0.006) | Independent prognostic factor for poor outcome only for OS in younger patients (<60 years.) |
| Yamamoto *et al.*[7] | 201 | Adult AML (Non M3) | ITD: 46/201 (23%)<br>D835: 8/201 (4%) | Lower OS (0.004)<br>Lower DFS (0.023)<br>No difference in OS<br>All relapsed | NP |
| Fröhling *et al.*[12] | 224 | Adult AML Normal citog. <60 years | ITD: 71/224 (32%)<br>D835: 32/224 (14%) | Lower OS (0.0004)<br>Lower Remission duration (0.03) | Independent prognostic factor for remission duration and OS |
| Zwaan *et al.*[29] | 234 | Pediatric AML | ITD: 27/234 (11.5%) | Lower CR (0.01)<br>Lower OS (0.037)<br>Lower DFS (0.09)<br>Lower EFS (0.0046) | Independent prognostic factor for RR and EFS<br>A high mutant/wild ratio is an independent prognostic factor |
| Kottaridis *et al.*[11] | 792 | Adult *de novo* AML <60 years | ITD: 210/792 (27%) | Lower CR (0.05)<br>Higher RR (<0.001)<br>Lower DFS (<0.001)<br>Lower OS (<0.001)<br>Lower EFS (<0.001) | Independent prognostic factor for RR, DFS, OS, and EFS |
| Schnittger *et al.*[14] | 871 | Adult *de novo* AML | ITD: 213/871 (25%) | No difference in CR<br>No difference in OS<br>Lower EFS (0.0072)<br>No difference in DFS | ITD is not an independent prognostic factor for OS, EFS and DFS |
| Thiede *et al.*[25] | 979 | Adult AML | ITD: 200/979 (20%)<br>D835: 75/979 (8%) | No differences in CR<br>Lower DFS (0.03)<br>Higher RR (0.008)<br>Lower OS (0.015) | ITD and D835 are not independent prognostic factors for OS and DFS<br>A high mutant/wild ratio is an independent prognostic factor |
| Present paper | 116 | Adult AML (Non M3) | ITD + D835: 25/116 (22%) | No difference in CR<br>No difference in OS<br>No difference in DFS | NP |
| | 60 | M3 | ITD + D835: 16/60 (27%) | No difference in CR<br>No difference in OS<br>No difference in DFS | |

CR: complete remission; OS: overall survival; DFS: disease-free survival; RR: relapse rate; EFS: event-free survival; RFS: relapse-free survival; NP: not performed; NS: not significant.

AML cases harboring the inv(16), t(8;21) or 11q23 abnormalities. A similar low incidence in these molecular subgroups has been described in other reports.[14,16]

As far as the biological implications of *FLT3* abnormalities was concerned, we confirmed the association of such mutations with several classical risk factors such as high WBC counts, high blast percentage in BM (only in non-M3 cases) and PB, and increased LDH levels (Table 1). However, we were unable to detect an association between *FLT3* mutations and poor disease free or overall survival. Table 3 summarizes 19 series (including the present one) in which the prognostic value of *FLT3* abnormalities has been evaluated. Most series indicate that *FLT3*-ITDs confer a poor prognosis in adult AML, mainly in patients under the age of 60.[3,11,12,15,25] This adverse outcome for *FLT3*-ITDs has also been reported in pediatric AML patients, where it was associated with low CR rates and short survival.[4,26,29] However, some conflicting data has been observed regarding the independent prognostic implication of such mutations. In this way, although seven out of eleven studies that performed multivariate analysis demonstrated the value of *FLT3*-ITDs as a prognostic factor, there were four other reports that did not find this correlation. Thus, these latter series, generally dealing with a high number of newly diagnosed AML patients.[6,16,25] and including the largest published study on *FLT3*-ITDs,[14] did not find statistical significant differences in the remission rate and OS for the presence of *FLT3* mutations, and the patients were classified as having intermediate-risk instead of high-risk AML by Schnittger *et al*.[14] Moreover, the largest report to demonstrate that ITDs may have a significantly adverse effect on clinical outcome, includes a high number of patients with APL (159 out of 854) and many of these APL cases (36%) were *FLT3*-ITD-positive.[11] This contradicts the results obtained by other groups that did not find any prognostic significance for the ITDs in patients with APL, since this type of leukemia is associated with prolonged survival.[24,27,28] In addition, in elderly AML patients (>55 years), *FLT3* mutations are not associated with impaired clinical outcomes, since they did not influence the CR rate or survival.[6,30] However, the paper from Thiede *et al*.[25] points out that the quantitative determination of the ratio between the mutant and the wild-type *FLT3* alleles in ITD-positive patients, is an independent prognostic factor for poor outcome. Thus, the quantitative instead of the qualitative detection of *FLT3* alterations might be of a major prognostic significance. Taken together, these studies indicate that the prognostic significance of *FLT3* mutations is not entirely clear, and therefore, larger prospective analyses will be needed to clarify this matter of debate. Some of these discrepancies could be explained in part by differences in the treatment schemes used, or by the variability of leukemia types included in each series.[31]

In summary, we have observed that although *FLT3* mutations are associated with adverse clinical and biological disease characteristics, they are not an independent prognostic factor for AML. Moreover, we have found a positive correlation with the t(15;17) translocation, but a negative one with other well-defined AML molecular changes such as t(8;21), inv(16) or MLL gene rearrangements.

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