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Clinical impact of change of *FLT3* mutation status in acute myeloid leukemia patients

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***FMS*-like tyrosine kinase 3 (*FLT3*) is one of the most frequently mutated genes in acute myeloid leukemia and is associated with worse clinical outcome. Changes in *FLT3* mutation status can occur during the course of disease, but the clinical impact of a change is unclear. We retrospectively reviewed 3555 acute myeloid leukemia patients, who have been assessed for *FLT3* mutation at our institution between May 2002 and January 2011. We found that 42 (6.2%) out of 680 patients with *FLT3* mutation experienced a change of *FLT3* mutation status. In all, 36 patients with wild-type *FLT3* at the time of initial diagnosis gained mutation (Negative/Positive) and six initially *FLT3*-mutated patients became wild type during their following relapses (Positive/Negative). The 5-year survival of these patients was similar to that of patients with persistently wild-type *FLT*3 (Negative/Negative; $P=0.464$), and significantly better than patients who had stable *FLT3* mutation during their disease course (Positive/Positive; $P<0.001$). However, after mutations became detectable in the Negative/Positive group, the forward survival of these patients tracked that of the Positive/Positive group after relapse ($P=0.761$). In addition, we did not find a significant difference in survival between patients with internal tandem duplications and those with point mutations in the tyrosine kinase domain of the *FLT3* gene. These results suggest that *FLT3* mutations are unstable and that there is potential clinical value in continuously monitoring *FLT3* mutation status.**
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The *FMS*-like tyrosine kinase 3 (*FLT3*) gene is located on chromosome 13q12 and encodes a membrane-bound receptor tyrosine kinase that has an important role in hematopoiesis.[1–3] *FLT3* is one of the most frequently mutated genes in hematological malignancies, present in over 30% of adults with acute myeloid leukemia.[4–8] The most common type of *FLT3* mutation is internal tandem duplications (*FLT3-ITD*s) in the juxtamembrane domain of the receptor, which have been found in 15–35% of adult acute myeloid leukemia patients.[4,5,9,10] Point mutations in the heavily converted areas of the intracellular tyrosine kinase domain (TKD), most commonly the nucleotide substitution of aspirate 835 (*FLT3-D835*), occur in 5–10% of adult acute myeloid leukemia patients.[3,7,8,11] Although most patients have only one type of the *FLT3* mutation, 1–3% of acute myeloid leukemia patients have both *FLT3-ITD* and *FLT-D835*.[8,11,12] *FLT3-ITD* and *FLT-D835* cause constitutive activation of *FLT3*, leading to aberrant activation of multiple downstream pathways, such as phosphatidyl-inositol 3–kinase, mitogen-activated protein kinase, and signal transducer and activator of transcription 5.[13]

Patients with acute myeloid leukemia associated with *FLT3* mutation usually present as *de novo* disease with high peripheral leukocyte count, high bone marrow blast count, and normal cytogenetics.[14] *FLT3-ITD* is an independent predictor of poor prognosis and is associated with increased relapse risk after chemotherapy, and decreased disease-free

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survival and overall survival.[6,8,10,15,16] The clinical significance of *FLT3-D835* is still unclear. Some studies have shown that *FLT3-D835* is associated with shorter disease-free and overall survival.[7,14,17,18] Other studies did not identify differences in these parameters.[8,14] Conflicting data may be due to small patient numbers, different treatment regimens, and patient selection.

The high frequency of *FLT3* mutations in acute myeloid leukemia has made the *FLT3* gene a promising target for developing therapeutic agents. There are several classes of *FLT3* inhibitors currently in development or in clinical trials with varying degrees of potency and selectivity for the target.[19] *FLT3* inhibitors offer a potential paradigm shift in the standard therapy of acute myeloid leukemia patients. Some studies have even reported potential benefits of administering *FLT3* inhibitors to acute myeloid leukemia patients without detectable *FLT3* mutations.[20] One potential explanation is that *FLT3* mutations are unstable, and that patients may acquire or lose mutations along the disease course.

Change of *FLT3* status (gain or loss) at time of initial diagnosis and subsequently, usually at time of relapse, has been observed repeatedly in a small fraction of the patients in earlier studies.[10,16,21–29] However, owing to a lack of a sufficient number of patients, no study has been able to provide statistical significant correlations between *FLT3* status changes and disease progression. The goal of this study is to examine the clinical features and outcome of a large cohort of acute myeloid leukemia patients in whom *FLT3* mutation status changed. In a retrospective review of 3555 acute myeloid leukemia patients, we identified 42 (6.2%) with a change in *FLT3* status. Our findings suggest that acquiring a *FLT3* mutation during the course of disease has a negative impact on patient survival and may justify changes in clinical management.

Materials and methods

Case Selection

We retrospectively reviewed all clinical and laboratory data of all adult acute myeloid leukemia patients assessed for *FLT3* mutation at the University of Texas M. D. Anderson Cancer Center between May 2002 and January 2011. All cases were diagnosed and classified as acute myeloid leukemia according to World Health Organization criteria. In all patients, *FLT3* mutation testing was performed on bone marrow aspirate specimens obtained at initial diagnosis and at time of multiple follow-up visits. To assess the impact of a change in *FLT3* mutation status on patient outcome, the study cohort was divided into the following four groups based on the mutation-testing results at the time of initial diagnosis and at follow-up: 1) a Negative/Negative group of patients who were negative for *FLT3* mutations at the time of initial diagnosis and remained negative at time of all follow-up visits, including during clearly documented hematological relapses; 2) a Positive/Positive group of patients who were positive for *FLT3* mutations at the time of initial diagnosis and remained positive at time of all follow-up visits when their bone marrow blast count was at least 5% (ie, above the sensitivity of our *FLT3* assay); 3) a Negative/Positive group of patients who were negative for *FLT3* mutation at the time of initial diagnosis but acquired mutation in at least one of their follow-up tests (ie, gained *FLT3* mutation during persistent disease after treatment or at relapse); and 4) a Positive/Negative group of patients who were positive for *FLT3* mutations at the time of initial diagnosis but became wild type in all follow-up test, including during documented relapse of acute myeloid leukemia (ie, lost *FLT3* mutation). Patients who had received stem cell transplantation or *FLT3* inhibitor treatments were excluded. The patients' clinical information, complete blood count, morphological findings in bone marrow, and cytogenetics results were obtained from the medical records. This study was approved by the institutional review board and conducted in accordance with the Declaration of Helsinki.

Cytogenetic and Molecular Analyses

Conventional cytogenetic analysis was performed on bone marrow aspirate samples in all cases, as described previously.[30] Karyotypes were reported using the International System for Human Cytogenetic Nomenclature.

Genomic DNA was extracted from fresh bone marrow aspirate samples for *FLT3* mutation analysis using the Autopure extractor (QIAGEN/Gentra, Valencia, CA, USA). *FLT3-ITD* and *FLT3-D835* mutations were screened using polymerase chain reaction (PCR) followed by capillary electrophoresis on an Applied Biosystems Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), as previously described.[31] For *FLT3-D835* point mutation analysis, PCR products were digested with *Eco*RV before capillary electrophoresis. Sequences were analyzed and mutant allelic ratios were calculated using GeneScan software (Applied Biosystems). The specimens were also analyzed for other genes commonly mutated in acute myeloid leukemia, including *NPM1*, *KRAS*, and *NRAS*. These genes were assessed by PCR followed by capillary electrophoresis or direct sequencing (Sanger sequencing or pyrosequencing), according to previously described protocols.[31,32] The sensitivity of the *NPM1* and *FLT3* assays is approximately 2.5%. The sensitivity of Sanger sequencing is approximately 20% and for pyrosequencing, 5–10%.

Statistical Analysis

Categorical variables were compared using the Chi-square test. Patient survivals were estimated by the

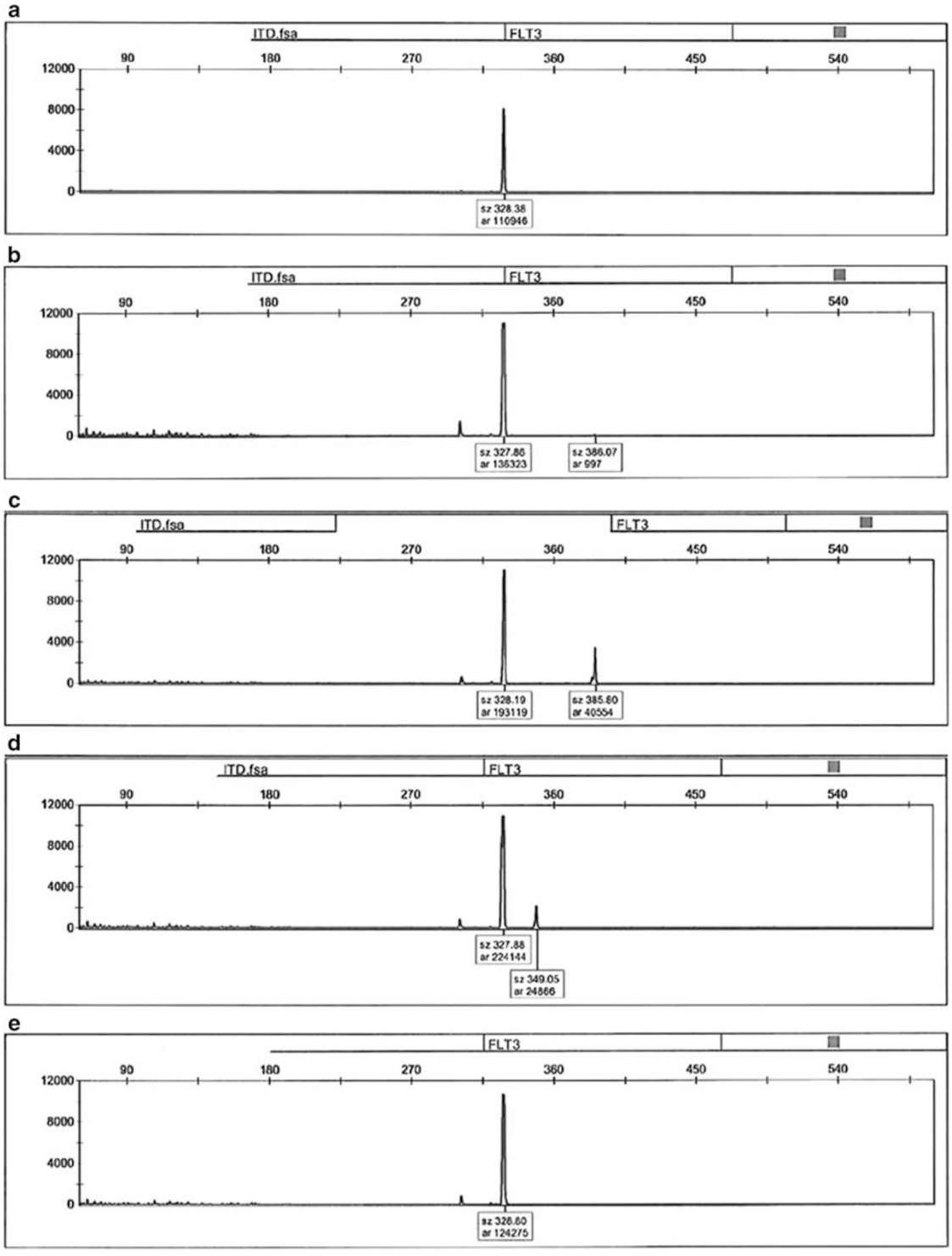

Figure 1 Representative examples of GeneScan results of *FLT3* mutation status changes in acute myeloid leukemia patients. A patient diagnosed acute myeloid leukemia with 30% bone marrow blasts and wild-type *FLT3* (**a**) went into complete remission after treatment, but relapsed 2 months later with 7% blasts and a detectable *FLT3-ITD* mutation (**b**). The patient became resistant to therapy and a bone marrow biopsy 3 months later showed 78% blasts and more prominent *FLT3-ITD* mutation (**c**). Another patient diagnosed acute myeloid leukemia with 46% bone marrow blasts and *FLT3-ITD* mutation (**d**) went into complete remission after treatment. This patient relapsed 5 months later with 23% blasts and no detectable *FLT3* mutation (**e**).

Kaplan–Meier method, and differences among groups were determined by the log-rank test.

Results

FLT3 Mutation Status Changes in a Subset of Acute Myeloid Leukemia Patients

During our review period of May 2002 to January 2011, a total of 3555 patients were diagnosed with acute myeloid leukemia at our institution and had multiple bone marrow samples tested for *FLT3* mutation. Among these patients, 2875 (80.9%) never tested positive for *FLT3* mutation throughout their clinical course, whereas 680 (19.1%) tested positive for *FLT3* mutation at least once. These mutated cases included 541 (79.6%) with *FLT3-ITD* and 139 (20.4%) with *FLT3-D835*.

In most patients with *FLT3* mutation ($n=638$; 93.8%), mutation was detected at time of initial diagnosis and the mutation persisted in all follow-up specimens with 5% or more blasts. In 42 (6.2%) patients who changed their mutation status, 36 (5.3%) were negative for *FLT3* mutation at initial diagnosis but mutations were later detectable in follow-up samples, including 18 patients with *FLT3-ITD* alone, 11 patients with *FLT3- D835* alone, and 7 patients with both types of mutations. These patients were designated as the Negative/Positive group. Another 6 (0.9%) patients were positive for *FLT3* mutation at the time of initial diagnosis but subsequently became wild type for *FLT3* in all follow-up, including 5 patients with *FLT3-ITD* alone, and 1 patient with both *FLT3-ITD* and *FLT3-D835*. These patients were designated as the Positive/Negative group. Representative results demonstrating the change of mutation status are shown in Figure 1.

FLT3 Status Changes and Clinical Characteristics

We compared the clinical characteristics and molecular profiles of the 42 patients with a change in *FLT3* status to a random selection of 57 patients with persistently negative *FLT3* mutation test results (Negative/Negative) as well as 49 randomly selected patients who persistently retained *FLT3* mutation in follow-up sample (Positive/Positive). The results are summarized in Table 1.

No statistically significant differences were found with respect to age and gender among these groups. Significantly higher bone marrow blast counts were observed in the *FLT3*-mutated groups (Positive/Positive, Negative/Positive, and Positive/Negative), compared with the Negative/Negative group ($P=0.001$), consistent with earlier studies.[6,8] The frequency of *FLT3-ITD versus FLT3-D835* was similar among the *FLT3*-mutated groups ($P=0.135$). The mutant allelic ratio was also similar among these groups ($P=0.676$ and 0.450 for *FLT3-ITD* and *FLT3-D835*, respectively).

As expected, *NPM1* mutations were more frequently seen in *FLT3*-mutated groups compared with the Negative/Negative group ($P=0.005$), due to the strong association between these two mutations. *RAS* mutation rates were similar among all groups (7–14%) except the Positive/Negative group, in which a higher rate (33%) was observed. However, due to the small sample size, the difference was not statistically significant ($P=0.339$). Additionally, a higher frequency of cytogenetic clonal evolutions was found in patients with *FLT3* mutations ($P=0.006$), especially in patients in the Negative/Positive group.

Patients in the Positive/Positive group had a significant lower complete remission rate than those of the Negative/Negative group ($P=0.003$). The other two groups of patients with changed

Table 1 Clinopathological characteristics of acute myeloid leukemia patients according to *FLT3* mutation statuses at diagnosis and follow-ups

	FLT3 mutation statuses at diagnosis and follow-ups				P-*value*
	Negative/Negative n = *57*	*Positive/Positive* n = *49*	*Negative/Positive* n = *36*	*Positive/Negative* n = *6*	
Age, median (range), year	65 (22–84)	65 (24–91)	64 (22–79)	62 (18–78)	0.971
Gender, male/female	32/25	24/25	17/19	4/2	0.218
Bone marrow blast count, median (range), %	32 (20–89)	59 (20–94)	51 (20–90)	80 (48–96)	0.001
FLT3 mutations:					
Mutation type:					0.235
ITD only, *n*	—	34	18	5	
D835 only, *n*	—	11	11	0	
ITD and D835, *n*	—	4	7	1	
Mutant allelic ratio at first detection:					
ITD, median (range)	—	0.44 (0.01–27.26)	0.36 (0.01–1.14)	0.15 (0.01–0.81)	0.676
D835, median (range)		0.41 (0.06–1.33)	0.19 (0.04–1.72)	0.72 (0.72–0.72)	0.450
Cytogenetic clonal evolution, *n*/*N* (%)	8/52 (15)	15/44 (34)	16/36 (44)	0 (0)	0.006
NPM1 mutated, *n* (%)	1 (2)	9 (18)	4 (11)	0 (0)	0.005
RAS mutated, *n* (%)	4 (7)	7 (14)	5 (13)	2 (33)	0.339
Complete remission rate, %	81	68	94	100	0.003

FLT3 mutation status showed complete remission rates closer to that of the Negative/Negative group. However, the higher complete remission rates in these two groups may be biased due to the fact that our patient selection criteria excluded patients without follow-up testing, and most of the patients who did not reach a complete remission died before they were re-tested for *FLT3*. As a result, the majority of these two groups were patients who went into complete remission and then relapsed.

FLT3 Status Changes Impact Patient Survival

The Kaplan–Meier curves plotting the overall survival among the four study groups are shown in Figure 2a. Patients who gained mutations (Negative/Positive) had an overall survival similar to that of the Negative/Negative group ($P=0.464$), and was significant better than that of the Positive/Positive group ($P<0.001$). The best survival appeared to be the six patients in the Positive/Negative group who lost *FLT3* mutation after first complete remission, although admittedly the sample size is too small to accurately predict their behavior. However, when examining survival time of the Negative/Positive patients after they turned positive for *FLT3* mutations, their survival curve tracked that of the Positive/Positive group after their first relapse (Figure 2b; $P=0.761$), and was significantly worse than that of the Negative/Negative group ($P<0.001$). In the Negative/Positive group, the median lag time from initial diagnosis without *FLT3* mutation to testing positive for mutation was 15 months, ranging from 2 to 45 months. The survival, therefore, became dramatically worse for these patients after gaining *FLT3* mutation and all but one patient died shortly after *FLT3* mutation was acquired.

We further compared the survival of patients with *FLT3-ITD* and those with *FLT3-D835* mutation in the Positive/Positive and Negative/Positive groups (Figure 3). No significant difference was found between acute myeloid leukemia patients with these two types of mutations in either overall survival or survival time after first relapse in Positive/Positive group or after *FLT3* mutation becoming positive in Negative/Positive group.

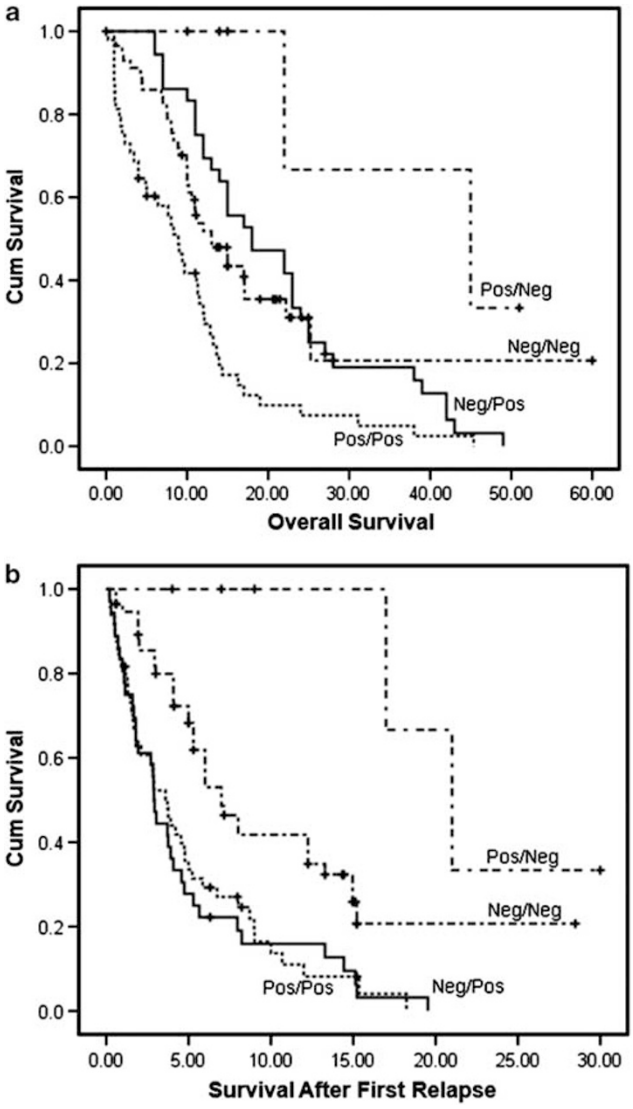

Figure 2 Survival of acute myeloid leukemia patients according to *FLT3* mutation statuses at diagnosis and follow-ups. (**a**) Overall survival of all patients in the four groups ($P<0.001$). (**b**). Survival after first relapse or after *FLT3* mutation detected in the Negative/Positive group ($P<0.001$).

Discussion

It is well established that *FLT3* mutation is an important prognostic factor in patients with acute myeloid leukemia. Therefore, accurate assessment of *FLT3* mutation status is crucial to risk stratification, clinical management, and treatment selection for acute myeloid leukemia patients. Several studies have observed *FLT3* mutation status changes in small subsets of acute myeloid leukemia patients and have implied the prognostic importance.[10,16,21–29] In aggregate, these data suggest that gain of *FLT3* mutations may be associated with worse prognosis.[24,27,29,33] These patients also seem not to benefit from intensifying chemotherapy.[34–36] The number of patients in these studies, however, are truly small. We present the first study focused on a large group of such patients, thereby making it possible to statistically analyze clinical characteristics and the prognostic impact of a change in *FLT3* status.

In this study, approximately 6% of acute myeloid leukemia patients had a demonstrated change in *FLT3* status during their disease course, similar to reports in earlier studies.[10,16,21–29] One previous study reported that *FLT3-ITD* is more often acquired than *FLT3-TKD* at time of relapse, 8% and 2%, respectively, whereas *FLT3-TKD* is more often lost

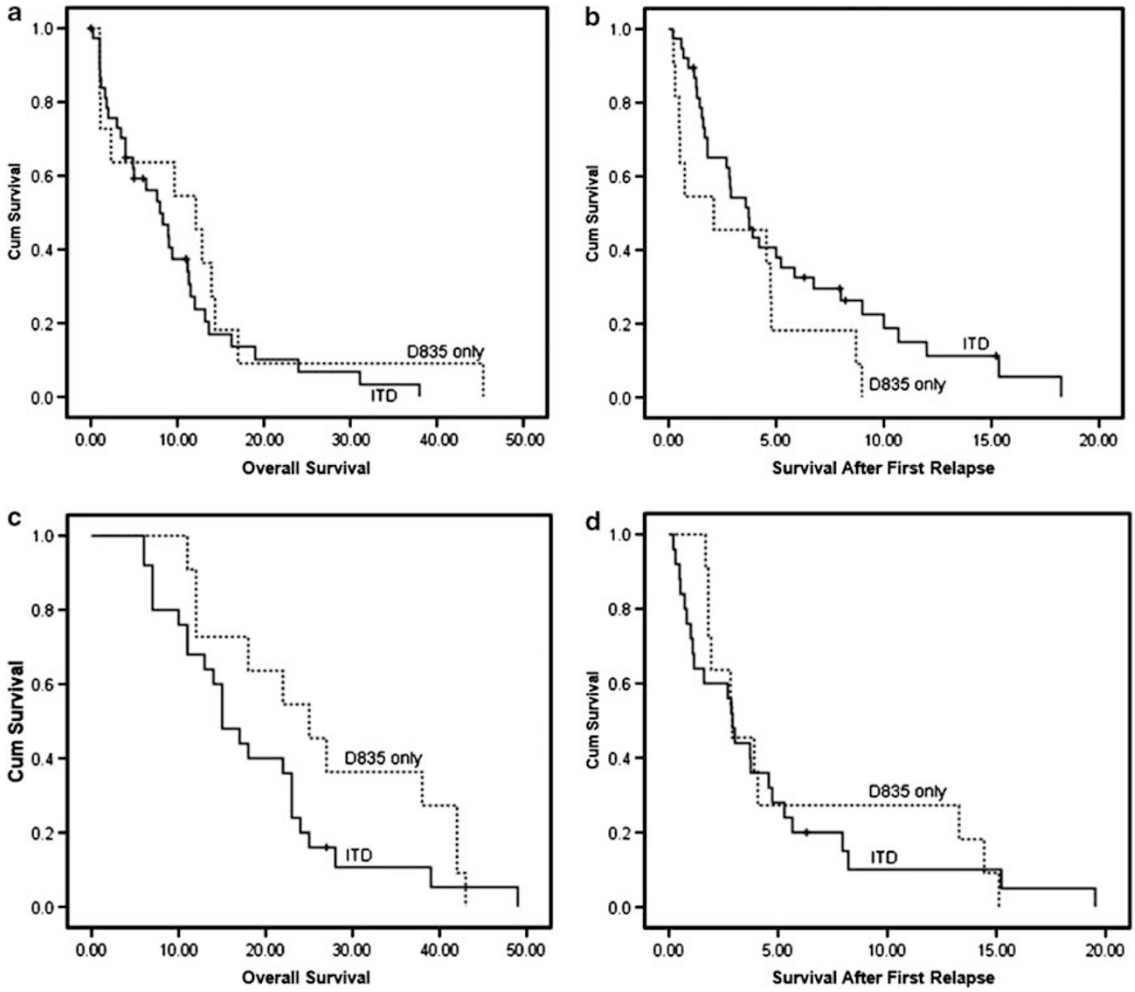

Figure 3 Comparaison of survival between acute myeloid leukemia patients with *FLT3-ITD* mutation and those with *FLT3-D835* mutation. (**a**) Overall survival in the Positive/Positive groups ($P=0.169$). (**b**) Survival after first relapse in the Positive/Positive groups ($P=0.780$). (**c**) Overall survival in the Negative/Positive groups ($P=0.086$). (**d**) Survival after *FLT3* mutations detected in the Negative/Positive groups ($P=0.368$).

than *FLT3-ITD*, 7% and 4%, respectively.[29] In our study, *FLT3* mutations were acquired far more often than lost, in a ratio of 6 to 1, during the disease course. In addition, *FLT3-ITD* was more often involved than *FLT3-TKD* in both patients with gain or loss of *FLT3* mutation.

We compared the clinical presentation of patients with a change in *FLT3* mutation status to randomly chosen groups of patients without change in *FLT3* status. There was no significant difference in age and gender distribution between these groups. However, patients who gained the mutations during follow-up (Negative/Positive group) presented with a higher average bone marrow blast count. The Negative/Positive group also had a higher frequency of *NPM1* mutations compared with the Negative/Negative group. Although the predictive power of these two findings is low, given the high frequency of a high blast count and *NPM1* mutation, and the relative low frequency of a change in *FLT3* status, these features in a patient presented with wild-type *FLT3* may serve as an early indicator for possible emergence of *FLT3* mutations.

Importantly, our results show that a gain of *FLT3-ITD* mutation strongly correlates with disease progressions and worse prognosis. Patients in the Negative/Positive group initially enjoyed similar survival rate as patients in the Negative/Negative group, even after relapse, but this was no longer the case once a *FLT3* mutation became detectable. Affected patients showed dramatic exacerbations of their disease after relapse with *FLT3* mutation and commonly died shortly thereafter. The median survival of these patients after acquiring a *FLT3* mutation was very similar to that of patients in the Positive/Positive group after first relapse, often less than 5 months post first relapse.[34,36] Based on these results, we suggest that all acute myeloid leukemia patients be tested repeatedly for *FLT3* mutation over their disease course to monitor the mutation status.

The exact mechanism of the change in *FLT3* mutation status is not clear. One possibility is that

FLT3 mutations may emerge owing to the instability of the tumor genome.[24,25] The higher frequency of cytogenetic clonal evolution known to be associated with *FLT3* mutations found in this study potentially supports this possibility. A second possibility is that, leukemia cells with *FLT3* mutations may be present in bone marrow at time of initial diagnosis in a very small number that is below the level of detection of the assays to assess *FLT3*. These *FLT3*-mutated leukemia cells have a survival advantage over their wild-type counterparts and eventually become a dominant clone. There is evidence that conventional therapies for acute myeloid leukemia appear to give a selection advantage to leukemia cells depending on *FLT3* signaling, and resulting in increased expression of the mutant allele or loss of the wild-type allele at relapse.[37] This phenomenon may be related to clonal expansion after therapy. Findings in this study also show that emergence of *FLT3*-mutated leukemia cells in the Negative/Positive patient group correlated with a poor response to conventional chemotherapy, significantly impacting patient survival in a negative manner. Therefore, change in *FLT3* mutation status has practical implications for patient management, and is not simply an academic issue.

Conventional chemotherapy is not effective in eradicating *FLT3*-mutated leukemia cells. In our study, only 6 of 644 patients who were initially positive for *FLT3* mutations successfully eliminated these mutations during their disease course, presumably as a result of conventional therapy alone. Other studies also suggest that *FLT3* signaling may be a key survival mechanism in leukemia cells.[38] Overexpression of *FLT3* also has been detected in acute myeloid leukemia patients without *FLT3* mutations and it is associated with a poor overall survival.[39] These data argue that patients with *FLT3* mutations may require their own therapeutic regimens, including *FLT3* inhibitors, agents that target the aberrantly activated *FLT3* kinase. In fact, *FLT3* inhibitors have attracted broad attention as new therapeutic agents for acute myeloid leukemia, and there are multiple clinical trials that are currently in progress. Both small molecules and *FLT3*-directed antibodies are currently in trials.[19] It has been suggested that all acute myeloid leukemia patients might benefit from receiving *FLT3* inhibitor regimens starting from their initial diagnosis regardless of their mutation status.[20,37] Patients in the Negative/Positive group in our study would support this view.

In summary, this study demonstrates that a change of *FLT3* status occurs in a small subset of acute myeloid leukemia patients, approximately 6%. As a result of the large cohort of AML patients at our institution, we have reported on 42 patients who showed a change in *FLT3* status. Importantly, acquisition of *FLT3* mutation has a negative clinical impact on survival. The findings suggest that *FLT3* mutations are unstable and that acute myeloid leukemia cells are constantly evolving. Our data suggest that continuous testing for *FLT3* mutation throughout the disease course is important, and may be most important for patients that initially tested negative for *FLT3* mutation. Adding *FLT3* inhibitors to standard therapeutic regimens may be beneficial for all patients who suffer from acute myeloid leukemia.

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Disclosure/conflict of Interst

The authors declare no conflict of interest.

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