Heterogeneous Patterns of FLT3 Asp\textsuperscript{835} Mutations in Relapsed de Novo Acute Myeloid Leukemia: A Comparative Analysis of 120 Paired Diagnostic and Relapse Bone Marrow Samples

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\textbf{ABSTRACT}

\textbf{Purpose:} We analyzed Asp\textsuperscript{835} mutations of FLT3 on paired marrow samples at diagnosis and relapse from 120 adult patients with de novo acute myeloid leukemia (AML) to determine the role of FLT3 Asp\textsuperscript{835} mutation in the relapse of AML.

\textbf{Experimental Design:} Asp\textsuperscript{835} mutation was analyzed by DNA PCR amplification of exon 20 of FLT3 gene followed by EcoRV digestion. All of the mutations were confirmed by sequence analysis. Mutant to wild-type allelic ratio was determined by Genescan analysis. The Expand Long Template PCR System was used to determine the allelic location of internal tandem duplication of FLT3 (FLT3/ITD) and Asp\textsuperscript{835} mutations.

\textbf{Results:} Thirteen patients had Asp\textsuperscript{835} mutations at diagnosis, of them 8 lost the mutations at relapse, and the remaining 5 patients carrying Asp\textsuperscript{835} mutations at diagnosis relapsed with the identical mutation types. Another 6 patients acquired Asp\textsuperscript{835} mutations at relapse. Five samples harbored both FLT3/ITD and Asp\textsuperscript{835} mutations that were found on different alleles by cloning analysis in the 3 patients studied. There were no differences in WBC count, French-American-British subtype, percentage of marrow blasts, or circulating blasts between patients with and without Asp\textsuperscript{835} mutations, whereas the difference in the prevalence of Asp\textsuperscript{835} mutations among cytogenetic/molecular subgroups was statistically significant ($P = 0.025$).

\textbf{Conclusions:} The present study showed that patients with AML had heterogeneous patterns of FLT3 Asp\textsuperscript{835} mutations, either acquisition or loss of the mutations at relapse. Asp\textsuperscript{835} mutant clone may develop as a secondary event in a subset of patients with AML.

\textbf{INTRODUCTION}

The fms-like tyrosine kinase 3 (FLT3) gene encodes a class III receptor tyrosine kinase and plays an important role in hematopoiesis (1). Two distinct types of FLT3-activating mutations have been described in 30% of patients with acute myeloid leukemia (AML; Refs. 2–10). The majority of FLT3 mutations (20–25%) involved an internal tandem duplication (ITD) in the juxtamembrane domain of the FLT3 (FLT3/ITD; Ref. 2–8), and an additional 5–10% of patients carried a point mutation of Asp\textsuperscript{835} at exon 20 within the activation loop of the tyrosine kinase domain of FLT3 gene (6, 9–11). Both types of mutations induce factor-independent proliferation through constitutive tyrosine phosphorylation and enhance cell proliferation in the experimental system (9, 12–16).

\textit{FLT3/ITD} is the most common genetic lesion found in AML patients with normal karyotype and associated with a poor prognosis (2–8). The clinical relevance and prognostic significance of Asp\textsuperscript{835} mutations in AML is less clear. There have been only few systematic studies on molecular alterations associated with relapse of AML (17–20). We have recently demonstrated that a subset of relapsed AML reflected the selection and outgrowth of FLT3/ITD clone or evolution of a new clone harboring FLT3/ITD (19). The role of Asp\textsuperscript{835} mutations of FLT3 in the relapse of AML was less defined. Most of the previous studies on Asp\textsuperscript{835} mutation of FLT3 in adult AML were analyzed at initial diagnosis (6, 9–11). Up to now, only one study of Asp\textsuperscript{835} mutation in relapsed AML, which included a small number of patients, has been reported previously (20). Additional studies on a larger number of paired samples at both diagnosis and relapse from AML patients are warranted to clarify the role of Asp\textsuperscript{835} mutations at relapse.

In the present study, we analyzed bone marrow (BM) samples collected sequentially at diagnosis and relapse from 120 patients to investigate the role of Asp\textsuperscript{835} mutations in the relapse of AML. To our knowledge, the present study is the largest series in which Asp\textsuperscript{835} mutations have been extensively analyzed in paired diagnostic and relapse samples.

\textbf{MATERIALS AND METHODS}

\textbf{Samples.} Beginning in April 1991, BM samples were taken, with informed consent, at diagnosis and during follow-up from 120 consecutively treated adult patients with \textit{de novo} AML. Each patient had matched diagnostic and first relapse samples available for analysis, 12 of them also had second
Relapse samples. Twenty complete remission samples from AML patients in which 15 patients carrying Asp835 mutations at diagnosis or relapse and an additional 20 control marrow samples obtained from patients who underwent lymphoma staging work-up were also studied. This study was approved by the Human Research Committee of Chang Gung Memorial Hospital. All relapse samples contained at least 25% BM blasts. The mononuclear cells from BM samples were enriched by Ficoll-Hypaque (1.077 g/ml; Amersham Pharmacia, Buckinghamshire, United Kingdom) density gradient centrifugation and cryopreserved in 10% DMSO and 20% fetal bovine serum at −70°C or in liquid nitrogen. Cytochemical study, immunophenotyping, cytogenetic analysis, Southern blot analysis of MLL rearrangement, and reverse transcription-PCR detection of common fusion transcripts were performed at diagnosis with the method described previously (19). The morphological subtypes were classified according to the French-American-British (FAB) criteria (21, 22). All patients were diagnosed and treated at Chang Gung Memorial Hospital, Taipei, Taiwan, where they received induction chemotherapy consisting of daunomycin and cytarabine (3 + 7 regimen). The postremission therapy consisted of high-dose cytarabine plus daunomycin, alternating with etoposide for 4–6 courses. Since 1994, patients with AML-M3 have received all-trans-retinoic acid in addition to chemotherapy described above. Eleven patients underwent stem cell transplantation.

Detection and Sequencing of Asp835 Mutation of FLT3.
Genomic DNAs were extracted from frozen BM mononuclear cells collected at both diagnosis and relapse by using a DNA extraction kit (Puregen Gentra System, Minneapolis, MN) according to the manufacturer’s instruction. The RFLP-mediated PCR assay was used to detect the mutations at codons 835 and 836 of FLT3 as described by Yamamoto et al. (9) with minor modification (23). Genomic DNA was PCR-amplified using the forward primer 5′-CCGCCAGGAACGTGCTTG-3′ and the reverse primer 5′-GCCATCCATTTTACAGGCAG-3′; these primers amplified the region containing exon 20 in which the Asp835 and Ile836 mutations can occur (9). A 25-μl aliquot of amplified products was digested with 10 units of EcoRV (New England Biolab, Beverly, MA) at 37°C overnight and then run on an 8% polacylamide gel. Undigested bands were excised, purified, and then sequenced. Faint aberrant PCR bands were individually cut from the gel, cloned into the pCR II-TOPO cloning vector (Invitrogen, Carlsbad, CA), and then sequenced.

Determination of Mutant to Wild-Type (WT) Allelic Ratio at Codon 835. The ratio was determined by Genescan analysis. The genomic PCR assay was performed again on samples positive for Asp835 mutations, except that the forward primer was labeled at the 5′-end with fluorescein-FAM (Proligo, Paris, France). PCR products (4 μl) were mixed with 5 μl of a solution of formamide (95%) and loading buffer (5% blue dextran, 25 mM EDTA) that contained 0.55 μl of Rox-1000 (Applied Biosystems, Foster City, CA). A 1.5-μl sample of this mixture was loaded onto a 5% Long Ranger-6 M urea gel (FMC, Rockland, ME; AppliChem, Darmstadt, Germany) with 1× Tris-borate EDTA (89 mM Tris, 89 mM borate, 2 mM EDTA) running buffer. Electrophoresis was performed at 200 W for 3.5 h and then analyzed by the automated ABI PRISM 377 DNA sequencer. The areas under the curves were quantified for both mutant and the WT alleles by use of Genescan 3.1 software (Applied Biosystems). The ratio of mutant to WT allele was calculated as the area under the curve for mutant (114 bp) divided by the area under the curve for WT (68 bp).

Detection of FLT3/ITD. The DNA PCR assay for the detection of FLT3/ITD, Genescan analysis to determine the ITD mutant level, and sequencing of the duplicated fragments of FLT3/ITD were performed as described previously (19).

Expand Long Template PCR Assay. To clarify whether the ITD and Asp835 mutations were on the same allele for samples carrying both FLT3/ITD and Asp835 mutations, allelic specific forward primers located at the junction regions of WT FLT3 and ITD (nt1810-1821 and nt1762-1770 for patient 1, nt1767-1780 and nt1742-1748 for patient 5, and nt1792-1804 and nt1748-1755 for patient 6, respectively, National Center for Biotechnology Information/GenBank under accession no. Z26652) were designed for each of the 3 patients studied. The primer sets of allelic specific forward primer and a common reverse primer of 5′-GCCATCCATTCTACAGGGCAG-3′ (nt2548-2567) at exon 20 could amplify the juxtamembrane domain containing ITD through TK2 domain. The Expand Long Template PCR assay was carried out according to the amplification of cDNA section of the manufacturer’s instruction (Roche, Mannheim, Germany). The PCR product was run on a 2% agarose gel, then cut from the gel, purified, and subjected to direct sequencing.

Sensitivity of RFLP-PCR Assay. A cloned DNA obtained from a sample carrying Asp835Tyr was serially diluted by mixing with the cloned DNA obtained from WT Asp835 of FLT3
1328 FLT3Asp<sup>835</sup> Mutations in Relapsed AML

Table 1 Clinical characteristics of acute myeloid leukemia patients with FLT3 Asp<sup>835</sup> mutations in paired diagnostic and relapse samples

<table>
<thead>
<tr>
<th>French-American-British subtype</th>
<th>Chromosome/leukemic cells</th>
<th>% bone marrow</th>
<th>Allelic ratio</th>
<th>FLT3/ITD</th>
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* Mutant to wild-type ratio.
* Also positive for FLT3/ITD.
* Values in parenthesis indicate the allelic ratio at second relapse.

MLL-PTD indicates partial tandem duplication of MLL gene, MLL-CBL indicates fusion transcript of MLL and CBL genes.24

RESULTS

Asp<sup>835</sup> Mutations at Diagnosis and Relapse. The BM samples from 120 adult patients with AML were studied at both diagnosis and relapse. Their ages at diagnosis ranged from 15 to 74 years with a median of 42 years; 65 were male. The first relapse occurred at a median of 12 months (range, 2.5–102 months) after the initial diagnosis. Asp<sup>835</sup> mutations were detected in 13 patients at diagnosis compared with 11 at first relapse (P = 0.830). Five patients had Asp<sup>835</sup> mutations at both diagnosis and relapse. Six patients acquired Asp<sup>835</sup> mutations and lost the mutations at first relapse. A total of 101 patients had exclusively the WT Asp<sup>835</sup> of FLT3 at both diagnosis and relapse. Of the 12 patients who had two relapses, Asp<sup>835</sup> mutation was detected at diagnosis and both two relapses in 1 patient, and Asp<sup>835</sup> mutation was present at both two relapses but absent at diagnosis in another patient; the remaining 10 patients did not have Asp<sup>835</sup> mutations at both diagnosis and two relapses. None of the 20 marrow samples obtained from control subjects had Asp<sup>835</sup> mutations, nor did the 15 samples of control subjects had Asp835 mutations, nor did the 15 samples of control subjects had Asp835 mutations, nor did the 15 samples of control subjects had Asp835 mutations, nor did the 15 samples of control subjects had Asp835 mutations.

Statistical Analysis. Frequencies of Asp<sup>835</sup> mutations at diagnosis and relapse were compared with Fisher’s exact test. χ<sup>2</sup> analysis was used to compare data among different subgroups. The clinical variables and the presence of Asp<sup>835</sup> mutations were compared by t test. The correlation between the percentage of leukemic cells and the mutant to WT allelic ratio was analyzed by linear regression. Overall survival was estimated by the method of Kaplan and Meier and were compared using the log-rank test. All Ps were calculated by using two-sided tests. P ≤ 0.05 was considered as statistically significant. The significant analysis was performed by using a software of SPSS 8.0 for Windows (SPSS Ins. Chicago, IL.).
Asp$^{835}$Glu, 1 Asp$^{835}$His, and 1 Asp$^{835}$Tyr plus Asp$^{835}$Val in our AML patients with relapse.

Four patients (nos. 1, 3, 5, and 6) had concomitant FLT3/ITD and Asp$^{835}$ mutations in the diagnostic samples and another one (no. 16) had both FLT3 mutations in the relapse sample. Diagnostic RNA samples from 3 of the 4 patients that harbored both ITD and Asp$^{835}$ mutations were available for analysis with the Expand Long Template PCR System. The PCR products of 807 bp for patient 1, 829 bp for patient 5, and 822 bp for patient 6, respectively, were recovered, sequencing of these PCR products which contained the respective ITD fragments revealed that all of the nucleotides at codon 835 were exclusively WT GAT (Asp) but not mutant sequences of GTT (Val), GAA (Glu), or TAT (Tyr), indicating that the ITD and Asp$^{835}$ mutations were on different alleles.

**Comparison of Mutant to WT Allelic Ratio at Codon 835 of FLT3.** All 19 patients with Asp$^{835}$ mutations had DNA samples available for Genescan analysis to determine the allelic distribution (Table 1). The mutant to WT allelic ratio ranged from 0.01 to 2.48 at diagnosis, which was similar to those ranging between 0.11 and 2.08 at first relapse ($P = 0.905$). Of the 5 patients who had Asp$^{835}$ mutations at both stages, the allelic ratio was increased in 2 patients (nos. 10 and 13) and decreased in 3 patients (nos. 9, 11, and 12) at relapse. Linear regression analysis failed to show a correlation between the percentage of BM leukemic cells and the mutant to WT allelic ratio either at diagnosis ($P = 0.689$) or at first relapse ($P = 0.521$). For the 5 patients who had Asp$^{835}$ mutations at both diagnosis and relapse, the difference in the mutant to WT allelic ratio between diagnostic and relapse samples did not correlate with the difference in the percentage of BM leukemic cells at both stages ($P = 0.397$). The mutant to WT allelic ratio of the 2 patients at second relapse (nos. 9 and 14) were both increased as compared with those of first relapse.

**Clinical and Hematological Characteristics of Relapsed AML Patients with Asp$^{835}$ Mutations.** The clinical and hematological features of the 19 patients carrying Asp$^{835}$ mutations are shown in Table 1. A comparison of clinico-hematological features between patients with and without Asp$^{835}$ mutations was made. There were no significant differences in age, platelet count, or WBC count between patients with and without Asp$^{835}$ mutations, either at diagnosis or relapse. The percentage of BM leukemic cells at diagnosis in patients with Asp$^{835}$ mutations was $73.1 \pm 5.0\%$ (mean $\pm$ SE) compared with $72.9 \pm 1.9\%$ for patients without mutations ($P = 0.982$). There was also no difference in the percentage of BM leukemic cells at first relapse between patients with and without Asp$^{835}$ mutations ($P = 0.699$). Similarly, no correlation was found between presence of Asp$^{835}$ mutations and the percentage of circulating leukemic cells, either at diagnosis ($P = 0.808$) or at relapse ($P = 0.999$).

The frequency of Asp$^{835}$ mutations at diagnosis according to FAB subtype was 0 of 5 in M0, 2 of 19 (10.5%) in M1, 7 of 66 (10.6%) in M2, 2 of 7 (28.6%) in M3, 0 of 11 in M4, 0 of 5 in M5a, 1 of 2 (50%) in M5b, and 1 of 5 (20%) in M6. At relapse, the distribution of FAB subtype in patients harboring Asp$^{835}$ mutations was 0 in 5 M0, 1 in 15 M1, 4 in 75 M2, 3 in 7 M3, 1 in 12 M4, 0 in 4 M5a, and 2 in 2 M5b. None had M6 at relapse. No difference was found in the incidence of Asp$^{835}$ mutations with respect to FAB subtype at diagnosis ($P = 0.576$), whereas the distribution of FAB subtype in relapsed AML was significantly different ($P = 0.008$). Cytogenetic or molecular analysis was performed in 99 patients at diagnosis, and one did not have metaphases for analysis; Asp$^{835}$ mutations were found in 5 of the 42 patients (11.9%) with normal karyotypes, in 2 of the 7 (28.6%) with t(15;17)/PML-RAR$, in 1 of the 5 (20%) with inv(16)/CBFB$\beta$-MYH11, in 2 of the 3 with partial tandem duplication of the MLL gene (MLL-PTD), and in 1 patient with $MLL-CBL$ (24). Asp$^{835}$ mutation was absent in all samples from 21 patients with t(8;21)/AML1-ETO, 7 patients with 11q23 translocations, including 4 with t(6;11), 2 with t(9;11), and 1 with t(11;19)/MLL-ELL, 3 with $-7/-7q$., and 9 with other abnormalities. The difference in the prevalence of Asp$^{835}$ mutation in various cytogenetic or molecular subgroups at diagnosis was statistically significant ($P = 0.025$). Most patients did not have cytogenetic analysis at relapse, but molecular analysis with reverse transcription-PCR assay was performed in the 44 patients who had specific fusion transcripts, including AML1-ETO, MLL rearrangements, PML-RARA$, or CBFB$\beta$-MYH11 at diagnosis. All these patients relapsed with the same fusion genes. Nine patients had normal karyotypes at both
FLT3-Asp835 Mutations in Relapsed AML

Asp835 mutations, as with ITD mutations, cause constitutive activations associated with leukemia. The observations of Asp 835 and mutation-negative patients (Fig. 3, unpublished results) showed that there is no significant difference in overall survival between mutation-positive and mutation-negative patients (Fig. 3, P = 0.1712).

DISCUSSION

FLT3 Asp835 mutations have been studied in patients with newly diagnosed AML by several groups (6, 9–11); however, only one small series of relapsed AML has been examined for these mutations (20). In that study, 2 of 44 (4.5%) patients had Asp835 mutations and another 2 acquired the mutations but none lost it at relapse. In the present study, we found that 6 of 120 AML patients acquired Asp835 mutations at relapse, and 8 patients carrying Asp835 mutations at diagnosis did not have detectable Asp835 mutations at relapse. None of the 15 remission samples had Asp835 mutations that were present at diagnosis or relapse, indicating that Asp835 mutations were somatic mutations associated with leukemia. The observations of Asp835 mutations being present only at diagnosis but not at relapse in a subset of AML patients suggest that chemotherapy was able to eradicate the leukemic clone carrying the Asp835 mutations, but when the leukemia relapsed, the Asp835 mutations did not recur.

We have recently demonstrated that patients with relapsed AML had a significantly higher prevalence of FLT3/ITD than those at diagnosis (19). On the basis of the observation that Asp835 mutations, as with ITD mutations, cause constitutive activation of the receptor FLT3, induce autophosphorylation, and enhance proliferation of 32D cells (9), it is thus conceivable that the incidence of Asp835 mutations would be increased during the progression of AML. In contrast, we did not observe an increased frequency of Asp835 mutations at relapse as compared with that at diagnosis. We found that the mutant to WT allelic ratio in AML patients carrying Asp835 mutations varied considerably. Our assay system allowed us to detect as little as 0.1% of Asp835 mutant allele within a population. The present data illustrated that Asp835 mutations may occur in only a small fraction of leukemic cells in a given sample, which contained abundant blasts. Similar observations were described in FLT3/ITD mutations (19, 20) or mutations of N-ras and p53 in AML (17, 25, 26). On the other hand, 2 patients had a mutant to WT allelic ratio of 2:1, suggesting a loss of the WT allele or biallelic mutations. The present result showed that no difference was observed in the mutant to WT allelic ratio between diagnosis and relapse samples. Of the five paired samples with Asp835 mutations at both stages, the changes of mutant to WT allelic ratio were heterogeneous, and there was no correlation between the ratio and the percentage of BM blast cells. Taken together, the findings of no increase in frequency of Asp835 mutations or in the mutant level along with loss or acquisition of mutant clone at relapse in some patients indicated that Asp835 mutations are a secondary event in a subset of patients with AML.

Sequence analysis showed that the distribution of Asp835 mutation types was similar at both diagnosis and relapse, with Asp835 Tyr being the most common type as reported by others (6, 9–11). We did not observe a deletion or point mutation at codon 836 of FLT3, which has been described by other investigators (6, 11). All of the five paired samples harbored the mutations at both stages exhibited identical types. One patient had two types of Asp835 mutations, cloning analysis revealed that the two mutants derived from two different alleles, but we could not preclude the possibility of Asp835 Tyr and Asp835 Val mutants occurring in separate leukemia clones in the same patient.

We compared the clinical and hematological features of patients with and without Asp835 mutations, and there were no difference in age, platelet count, or WBC count between patients with and without Asp835 mutations either at diagnosis or at relapse. Likewise, there was no correlation between the percentage of blasts in BM or peripheral blood and the Asp835 mutation status at both stages of the disease. No significant correlation was observed between FAB subtype and presence of Asp835 mutations at diagnosis, but we observed a higher frequency of Asp835 mutations in the small number of patients with relapsed AML-M3 (3 of 7) and AML-M5b (2 of 2). The frequency of Asp835 mutations was significantly different among the cytogenetic subgroups, and Asp835 mutations occurred more frequently in patients with MLL-PTD or t(15;17)/PML-RARa than in any of patients with t(8;21)/AML1-ETO, t(7;7)/q-, or common 11q23 translocations.

The coexistence of Asp835 mutation with PML-RARa or CBFbeta-MYH11 in our AML patients also supported the two-hit model of leukemogenesis (27–29). It was of particular interest to note that 5 (nos. 1–3, 5 and 6 in Table 1) of the 8 patients who lost Asp835 mutations at relapse possessed a mutant clone of FLT3/ITD at relapse; moreover, the mutant level of FLT3/ITD in 4 of them was higher at relapse as compared with that at diagnosis (Table 1). It appears that the leukemia clone harboring the Asp835 mutation was outgrown by the more aggressive clone carrying FLT3/ITD at relapse. Our data suggest that a substituted FLT3/ITD clone provides a proliferating advantage than that provided by Asp835 mutant clone. It is also notable that all of the 5 patients carrying Asp835 mutations at both stages did not
have FLT3/ITD, and their remission samples were absent for the mutations, suggesting that Asp\(^{835}\) mutations play a role in the leukemogenesis and progression of AML in these patients. For those without FLT3 activation mutations, it suggests likely that other genetic alterations, which confer a proliferative and survival signal to the cells, may be present (29, 30). Ras mutation is a potential target of activation mutation in the light of FLT3 mutations inducing transformation of murine hematopoietic cells lined mediated by Ras and STAT5 pathways (14). We have examined the N-ras mutations by DNA PCR amplifying exons 1 and 2 followed by direct sequencing in 14 patients who did not have Asp\(^{835}\) mutations at relapse, but we failed to find N-ras mutations in these patients (data not shown). It is conceivable that additional mutations are required for the progression of AML.

In the present study, the prognostic impact of Asp\(^{835}\) mutations was analyzed, we found no significant association between outcome and Asp\(^{835}\) mutation status. However, because we specially looked at a selected cohort of AML patients who later relapsed, the current data did not represent the real prognostic significance of Asp\(^{835}\) mutations in AML patients that should also include those who did not have relapses. In addition, our results showed that Asp\(^{835}\) mutations might lose at relapse; therefore, Asp\(^{835}\) mutation is not a suitable marker for monitoring minimal residual disease or detecting early relapse in patients harboring these mutations at diagnosis.

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