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ORIGINAL ARTICLE

Prognostic significance of the *FLT3* ITD mutation in patients with normal-karyotype acute myeloid leukemia in relapse

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Background
Fms-like tyrosine kinase 3 internal tandem duplication (*FLT3* ITD) mutation is related to poor prognosis in normal-karyotype acute myeloid leukemia (AML). However, the prognostic significance of the mutation at relapse has not been adequately investigated. We investigated the prognostic significance of the *FLT3* ITD mutation at relapse in normal-karyotype AML patients.

Methods
We analyzed 69 normal-karyotype AML patients, in whom paired bone marrow samples taken at initial diagnosis and subsequent relapse were analyzed for the *FLT3* ITD mutation at the Asan Medical Center between 1995 and 2009.

Results
Forty patients showed a persistent wild-type genotype, 11 showed the *FLT3* ITD mutation at diagnosis and relapse, and 9 lost and another 9 acquired the mutation at relapse. The mutation status at relapse affected the overall survival (OS), with the mutation group showing shorter OS and survival after relapse than the wild-type group did ($P<0.001$ and $P<0.001$, respectively), despite having received more frequent stem cell transplantation after relapse than the wild-type group did. However, no difference was detected in the OS and survival after relapse with regard to the mutation status at diagnosis.

Conclusion
The patients with *FLT3* ITD mutation at relapse showed poorer prognoses than those without the mutation. However, mutation status at diagnosis did not affect the outcome. These results suggest that, in normal-karyotype AML patients with relapse, the prognostic significance of *FLT3* ITD mutation at relapse is greater than that of the mutation status at diagnosis.

Key Words AML, Prognosis, *FLT3* ITD, Relapse, Normal karyotype

INTRODUCTION

The Fms-like tyrosine kinase 3 (*FLT3*) gene encodes a class III receptor tyrosine kinase that plays important roles in cellular proliferation and differentiation [1-8]. To date, 2 types of *FLT3* mutations have been found to induce autophosphorylation through ligand-independent *FLT3* dimerization, leading to uncontrolled hematologic progenitor cell proliferation and malignancy. One such mutation is an internal tandem duplication (ITD) of the region between exon 14 and 15 encoding the juxtamembrane domain; the other, in exon 20, is a point mutation in aspartic acid residue 835 (D835) within the activation loop of the second tyrosine kinase domain [9, 10]. The *FLT3* ITD mutation is found in approximately 30% of newly diagnosed acute myeloid leukemia (AML) patients, and an association of the mutation with poor prognostic indicators, such as leukocytosis, higher marrow blast counts, shorter overall survival (OS) and event-free survival (EFS), and higher relapse rates, has been firmly established in those with newly diagnosed AML, especially in the intermediate cytogenetic risk group [11-16].

The *FLT3* D835 mutation occurs less frequently than the *FLT3* ITD mutation does, and the prognostic significance of this mutation in newly diagnosed AML patients is less clear, with previous studies reporting conflicting outcomes [17-19]. In addition to the *FLT3* ITD mutation, mutations in the *nucleophosmin1* (*NPM1*) gene are the most frequent genetic alterations, being found in approximately 50% of patients with normal-karyotype AML, and known to be associated with better response to induction therapy and a more favorable outcome in the absence of *FLT3* ITD mutations [20].

Thus far, the prognostic impact of the *FLT3* ITD mutation at relapse has not been adequately evaluated in patients with normal-karyotype AML. Therefore, we analyzed *FLT3* ITD mutation status at diagnosis and relapse in patients with normal-karyotype AML relapse, and investigated the prognostic significance of *FLT3* ITD mutation at relapse.

MATERIALS AND METHODS

1. Selection of patients and assessment of clinical variables

Paired bone marrow samples collected at diagnosis and relapse from 69 patients newly diagnosed with normal-karyotype AML between 1995 and 2009 at Asan Medical Center were analyzed for *FLT3* ITD mutation status. Data for clinical variables, including CBC, blast counts in peripheral blood (PB) and bone marrow (BM), karyotype at initial diagnosis and relapse, and implementation of stem cell transplantation (SCT) were obtained from all patients. The dates of initial diagnosis, first complete remission (CR), first relapse, SCT performance, and death were established by performing a retrospective review of the patients' medical records. CR was defined as the presence of less than 5% blasts with more than 20% cellularity in a standardized BM aspirate after the first or second course of induction chemotherapy. Relapse was defined as more than 5% leukemic blasts in BM aspirates in patients who had previously achieved CR state. OS was defined as the time from diagnosis to death or last follow-up, and survival after relapse was defined as the time from first relapse to death or last follow-up.

2. Cytogenetic analysis

Cytogenetic analyses were performed on at least 20 metaphases using paired diagnosis and relapse BM samples. According to the classification system proposed by the US Southwest Oncology Group (SWOG), cytogenetic abnormalities at relapse were grouped into 4 categories [21]: (1) good-prognosis: inv(16)/t(16;16)/del(16q) or t(8;21) lacking del(9q) and complex karyotypes; (2) intermediate-prognosis: normal karyotype, +8, +6, Y, and del(12p); (3) poor-prognosis: 5/del (5q), 7/del(7q), abn(3q), t(6;9) and complex karyotypes; (4) unknown-prognosis: all the other above-mentioned abnormalities.

3. Patient treatment

All patients received standard induction chemotherapy with cytarabine and daunorubicin. This regimen included continuous intravenous infusion of 200 mg/m^2/day (100 mg/m^2/day for patients aged >60 years) of cytarabine on days 1-7 and 45 mg/m^2/day of daunorubicin on days 1-3. Patients who failed to achieve CR, but attained partial remission (PR) received a second identical cycle of induction chemotherapy. At relapse, the patients received the same regimen of induction chemotherapy used following the initial diagnosis. Depending on patient age and availability of a suitable donor, the patients received autologous or allogeneic SCT on the first CR status or later. Patients with *FLT3* ITD mutation at initial diagnosis were preferentially invited to receive autologous or allogeneic SCT at the first CR status. However, there was no difference in the induction chemotherapy regimen according to *FLT3* ITD mutation status at initial diagnosis or relapse.

4. Detection of *FLT3* ITD and *NPM1* mutations

FLT3 ITD and *NPM1* mutations were identified using PCR-capillary electrophoresis methods. Genomic DNA was extracted from BM mononuclear cells using the Qiagen DNA purification kit (Qiagen, Hilden, Germany) and stored at -20°C. Primer sets were designed to detect the *FLT3* ITD and *NPM1* 4 base pair insertion mutations. Additionally, a specific set of *HBG* primers was used to ascertain DNA integrity and validate the precision of PCR amplification. The sequences of the primers specific to *FLT3*, *NPM1*, and *HBG* were as follows. *FLT3* primers: forward, 5'-FAM-AGCA ATT TAG GTA TGA AAG CCA GCTA-3'; reverse, 5'-CTT TCA GCA TTT TGA CGG CAA CC-3'. *NPM1* primers: forward, 5'-GTT TCT TTT TTT TTT TTT CCA GGC TAT TCA AG-3', reverse, 5'-HEX-CAC GGT AGG GAA AGT TCT CAC TCT GC-3'. *HBG* primers: forward, 5'-HEX-CCA GAA GAG CCA AGG ACA GGT ACG-3', reverse, 5'-AGA TCC CCA AAG GAC TCA AAG AAC C-3'. The 20 μL multiplex PCR reaction solution consisted of 200 ng of genomic DNA, 20 pmol of each primer, 25 mmol/L $MgCl_2$, 2.5 mmol/L dNTP, 2 μL of 10× PCR buffer, 0.2 μL of Qiagen HotTaq DNA polymerase and water. The PCR conditions were as follows: the mixture was heated to 95°C for 15 min for initial denaturation, and then 35 cycles of 95°C for 20 sec, 60°C for 40 sec, and 72°C for 40 sec were performed, followed by a final extension phase of 72°C for 30 min. The PCR products were then diluted 1:30 and analyzed by capillary electrophoresis using ABI 310 genetic analyzer (Applied Biosystems Inc., Foster city, CA, USA) and GeneScan Analysis software (version 1.2, Applied Biosystems Inc., Foster City, CA, USA). The data is represented as a ratio of mutant cells:wild-type cells. The lowest detection limit of this protocol was 5%, which had been determined by previous serial dilution experiments [22]. For the specimens, in which *NPM1* mutation was detected, additional direct sequencing was performed to confirm the mutation subtype.

5. Analysis of clinical variables related to *FLT3* ITD paired mutation status at diagnosis and relapse

An analysis of clinical variables related to *FLT3* ITD muta-

tion status at diagnosis and relapse was performed. Results were grouped into 4 categories: (1) persistent wild type: NN (negative at diagnosis and relapse); (2) acquired mutation: NP (negative at diagnosis and positive at relapse); (3) loss of mutation: PN (positive at diagnosis and negative at relapse); and (4) persistent mutation: PP (positive at diagnosis and relapse). The clinical variables described above were compared individually between NN and PN, NN and NP, NN and PP, and PN and PP groups. Two additional comparisons were made: between patients positive for *FLT3* ITD mutation at relapse (NP+PP) and those negative for *FLT3* ITD mutation at relapse (NN+PN), and between patients positive for *FLT3* ITD mutation at diagnosis (PN+PP) and those negative for *FLT3* ITD mutation at diagnosis (NN+NP). Additionally, we compared the prognosis in patients without *FLT3* ITD mutation stratifying according to *NPM1* mutation status at diagnosis or relapse to identify the prognostic impact of *NPM1* mutations. We also compared the prognosis of patients on the basis of *FLT3* ITD mutant:wild-type ratio in patients with the mutation at diagnosis or relapse in order to evaluate the effect of the amount of mutation on the prognosis.

6. Multivariate analysis of OS and survival after relapse with respect to variables, including *FLT3* ITD mutation status at diagnosis and relapse

Multivariate analysis of OS and survival after relapse with respect to *FLT3* ITD mutation status at diagnosis and relapse was performed. For the analysis at diagnosis, WBC counts and blast percentage in PB and BM, which were found to be significantly different among *FLT3* ITD mutation groups in univariate analysis, were used to adjust the relevant outcomes. For the analysis at relapse, duration of CR and SCT performance rates after relapse, which showed significant differences among *FLT3* ITD mutation groups in univariate analysis, were used to adjust the dependent measures.

7. Statistical analysis

Pearson's chi-squared or Fisher's exact tests (for small numbers) were used to compare categorical variables. Mann-Whitney *U* test was used to compare continuous variables. Estimations of OS and survival after relapse were performed using Kaplan-Meier methods and compared using log-rank test. Multivariate analyses of OS and survival after relapse were performed using Cox's proportional hazards model. For all analyses, tests were two-tailed, and *P*-values $\leq$ 0.05 were considered statistically significant. All calculations were performed using SPSS 13.0.1 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

1. Overall patient characteristics

All patients achieved first CR after a median interval of 32.0 days and relapsed within a median of 7.6 months from the first CR. A second CR was achieved in 26 patients (37.7%), of whom 8 (30.8%) relapsed again after the second round of induction chemotherapy. Fifty-five patients (79.7%) died after relapse with a median interval of 3.8 months. The *FLT3* ITD mutation was detected in 20 patients (29.0%) at diagnosis and relapse, with median mutant:wild-type ratio as 0.3 (range, 0.05-4.36) and 0.3 (range, 0.05-9.94) at diagnosis and relapse, respectively. The *NPM1* mutation was detected in 19 (27.5%) and 13 (18.8%) patients at diagnosis and relapse, with median mutant:wild-type ratio as 0.55 (range, 0.16-0.86) and 0.55 (range, 0.07-0.75) at diagnosis and relapse, respectively. Among the 19 patients who showed *NPM1* mutation at diagnosis, 16 (84.2%) had *NPM1* type-A mutation. Among the 13 patients who showed *NPM1* mutation at relapse, 10 (76.9%) had *NPM1* type-A mutation. The remaining 3 patients had *NPM1* type-D mutation at diagnosis and relapse. Cases showing a change of *NPM1* mutation type from diagnosis to relapse were not found.

2. Comparison of clinical variables and prognosis between patients with different paired *FLT3* ITD mutation status

Tables 1 and 2 show comparisons of the clinical variables and prognosis between patients with different *FLT3* ITD mutation status at diagnosis and relapse. SCT performance rates at the first CR ranged from 25.0% to 44.4%, which is variable but this variation was not driven by *FLT3* ITD mutation status at diagnosis and relapse. However, SCT performance rates after the first relapse were significantly different. Patients with acquired or persistent *FLT3* ITD mutation at relapse more frequently underwent SCT than those with persistent negative status did (55.6% or 63.6% vs. 20.0%; *P*=0.043, 0.009, respectively). There was no prognostic impact of being in the poor cytogenetic risk group at relapse (OS: 20.6 vs. 22.0 months, *P*=0.950; duration of CR: 7.5 vs. 8.9 months, *P*=0.669; survival after relapse: 10.2 vs. 14.3 months, *P*=0.214).

The PP group showed a significantly shorter median duration of CR (6.9 months) than the NN group (10.3 months; *P*=0.045); the PP group also showed a significantly higher median WBC count (44.1×10^9/L vs. 8.8×10^9/L; *P*=0.046) than the NN group, but these variables did not differ between the PP and PN groups. The PN group showed significantly higher median WBC counts (66.1×10^9/L vs. 8.8×10^9/L; *P*=0.011) and blast percentages in PB (85.0% vs. 38.5%, *P*=0.006) and in BM (87.0% vs. 60.2%, *P*=0.001) than the NN group. The median OS was significantly shorter in the NP (9.2 months; *P*=0.002) and the PP (12.1 months; *P*=0.003) groups than in the NN group (19.5 months). The median survival after relapse was also significantly shorter in the NP (5.3 months; *P*=0.017) and the PP (4.9 months; *P*=0.024) groups than in the NN group (10.3 months). The median OS and survival after relapse in the PP group was significantly shorter than that in the PN group (45.5 months and 43.5 months; *P*=0.025 and 0.028, respectively).

3. Comparison of clinical variables and prognosis between groups with different *FLT3* ITD mutation status at diagnosis and relapse

Table 3 shows 2 additional comparisons of clinical variables

Table 1. Comparison of clinical variables between patients with different paired *FLT3* ITD mutation status.

Clinical variables	Number of patients with *FLT3* ITD mutation status (% with each status)				*P*			
	NN	PN	NP	PP	NN vs. PN	NN vs. NP	NN vs. PP	PN vs. PP
Sex (M : F)	25 : 15	5 : 4	4 : 5	8 : 3	ns	ns	ns	ns
Age, median (range)	54.0 (6.0-79.0)	41.0 (27.0-70.0)	59.0 (5.0-73.0)	45.0 (23.0-73.0)	ns	ns	ns	ns
FAB classification								
M0	2 (5.0)	0	0	0				
M1	11 (27.5)	5 (55.6)	2 (22.2)	2 (18.2)				
M2	15 (37.5)	3 (33.3)	5 (55.6)	3 (27.3)				
M4	3 (7.5)	0	1 (11.1)	6 (54.5)				
M5	1 (2.5)	1 (11.1)	0	0				
M6	4 (10.0)	0	0	0				
M7	4 (10.0)	0	1 (11.1)	0				
Karyotype at relapse								
Good	0	0	0	0				
Intermediate	29 (72.5)	8 (88.9)	7 (77.8)	7 (63.6)				
Poor	5 (12.5)	1 (11.1)	0	1 (9.1)				
Unknown	6 (15.0)	0	2 (22.2)	3 (27.3)				
Clonal evolution								
No	27 (67.5)	8 (88.9)	7 (77.8)	7 (63.6)				
Yes	13 (32.5)	1 (11.1)	2 (22.2)	4 (36.4)				
SCT in 1st CR status								
No	30 (75.0)	5 (55.6)	6 (66.7)	7 (63.6)	ns	ns	ns	ns
Yes	10 (25.0)	4 (44.4)	3 (33.3)	4 (36.4)				
SCT after 1st relapse								
No	32 (80.0)	7 (77.8)	4 (44.4)	4 (36.4)	ns	0.043	0.009	ns
Yes	8 (20.0)	2 (22.2)	5 (55.6)	7 (63.6)				
NPM1 mutation status								
Persistent wild-type (NN)	35 (87.5)	6 (66.7)	6 (66.7)	3 (27.3)				
Lost at relapse (PN)	2 (5.0)	2 (22.2)	0	2 (18.2)				
Persistent mutation (PP)	3 (7.5)	1 (11.1)	3 (33.3)	6 (54.5)				
Laboratory findings at diagnosis, median								
Hb (g/dL)	8.1	8.4	9.0	9.3	ns	ns	ns	ns
WBC ($\times10^9$/L)	8.8	66.1	39.6	44.1	0.011	ns	0.046	ns
PLT ($\times10^9$/L)	56.0	29.0	96.0	50.5	ns	ns	ns	ns
PB blasts (%)	38.5	85.0	60.0	64.0	0.006	ns	ns	ns
BM blasts (%)	60.2	87.0	58.4	61.2	0.001	ns	ns	ns

Abbreviations: FAB, French-American-British classification; CR, complete remission; *FLT3* ITD, fms-like tyrosine kinase3 internal tandem duplication; NN, negative at diagnosis and relapse; PN, positive at diagnosis and negative at relapse; NP, negative at diagnosis and positive at relapse; PP, positive at diagnosis and relapse; ns, not significant; Hb, hemoglobin; WBC, white blood cell; PLT, platelet; PB, peripheral blood; BM, bone marrow; SCT, stem cell transplantation; *NPM1*, nucleophosmin.

Table 2. Comparison of prognosis between patients with different paired *FLT3* ITD mutation status.

Prognostic variables	*FLT3* ITD mutation status				*P*			
	NN	PN	NP	PP	NN vs. PN	NN vs. NP	NN vs. PP	PN vs. PP
Duration of CR, median (months)	10.3	6.6	5.4	6.9	ns	ns	0.045	ns
Overall survival, median (months)	19.5	45.5	9.2	12.1	ns	0.002	0.003	0.025
Survival after relapse, median (months)	10.3	43.5	5.3	4.9	ns	0.017	0.024	0.028

Abbreviations: CR, complete remission; *FLT3* ITD, fms-like tyrosine kinase3 internal tandem duplication; NN, negative at diagnosis and relapse; PN, positive at diagnosis and negative at relapse; NP, negative at diagnosis and positive at relapse; PP, positive at diagnosis and relapse; ns, not significant.

between groups with different *FLT3* ITD mutation status at diagnosis and relapse. A comparison between the NN+NP group, which comprised patients with negative *FLT3* ITD mutation status at diagnosis, and the PN+PP group, which included patients with positive *FLT3* ITD mutation status at diagnosis, showed that the median WBC count at diagnosis was significantly higher in the PN+PP group (44.1×10^9/L) than in the NN+NP group (12.4×10^9/L; P=0.007). Moreover,

Table 3. Comparison of clinical variables and prognosis between groups with different *FLT3* ITD mutation status at diagnosis and relapse.

Clinical variables	*FLT3* ITD status (% with each status)				*P*	
	NN+NP (Negative at diagnosis)	PN+PP (Positive at diagnosis)	NN+PN (Negative at relapse)	NP+PP (Positive at relapse)	NN+NP vs. PN+PP	NN+PN vs. NP+PP
Sex (M : F)	29 : 20	13 : 7	30 : 19	12 : 8	ns	ns
Age, median (range)	56.5 (5.0-79.0)	41.0 (5.0-79.0)	53.0 (6.0-79.0)	55.0 (5.0-79.0)	ns	ns
SCT in 1st CR status						
No	36 (73.5)	12 (66.7)	35 (71.4)	13 (65.0)	ns	ns
Yes	13 (26.5)	8 (33.3)	14 (28.6)	7 (35.0)		
SCT after 1st relapse						
No	36 (73.5)	11 (55.0)	39 (79.6)	8 (33.3)	ns	<0.001
Yes	13 (26.5)	9 (45.0)	10 (20.4)	12 (66.7)		
Laboratory findings at diagnosis, median						
Hb (g/dL)	8.3	8.6	8.1	8.9	ns	ns
WBC ($\times 10^9$/L)	12.4	44.1	17.1	39.5	0.007	ns
PLT ($\times 10^9$/L)	58.0	41.0	51.0	69.0	ns	ns
PB blasts (%)	47.0	68.0	55.0	60.0	0.006	ns
BM blasts (%)	61.3	87.0	64.6	64.2	0.005	ns
Duration of CR median (months)	8.6	6.9	10.0	6.8	ns	0.034
Overall survival, median (months)	17.6	13.7	23.1	12.0	ns	<0.001
Survival after relapse, median (months)	6.9	6.4	11.8	5.5	ns	<0.001

Abbreviations: CR, complete remission; Hb, hemoglobin; WBC, white blood cell; PLT, platelet; PB, peripheral blood; BM, bone marrow; SCT, stem cell transplantation; *FLT3* ITD, fms-like tyrosine kinase3 internal tandem duplication; NN, negative at diagnosis and relapse; PN, positive at diagnosis and negative at relapse; NP, negative at diagnosis and positive at relapse; PP, positive at diagnosis and relapse; ns, not significant.

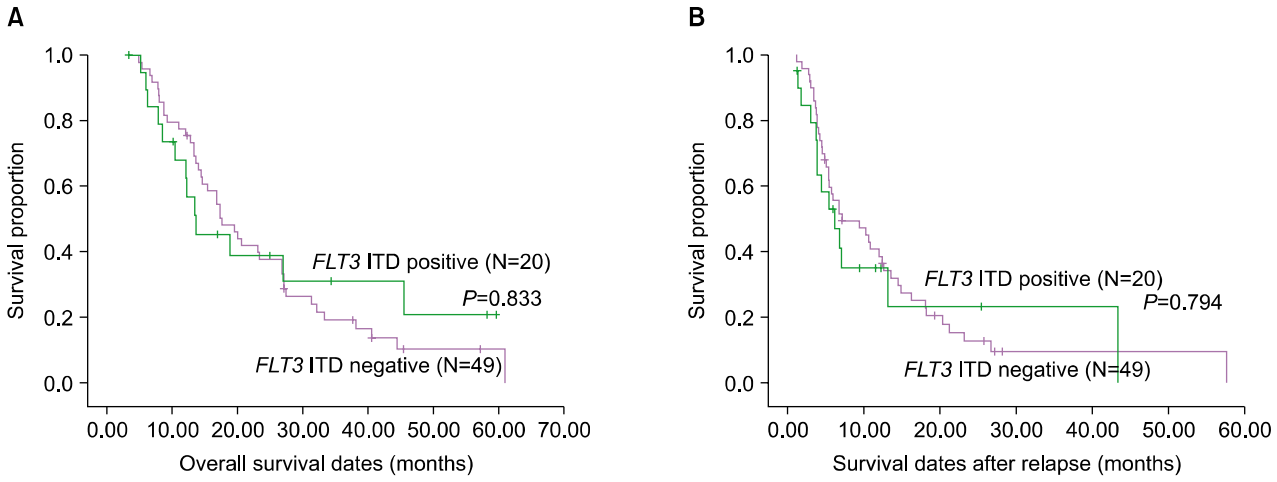

Fig. 1. Comparison of overall survival **(A)** and survival after relapse **(B)** between patients positive and negative for *FLT3* ITD mutation at diagnosis.

the PN+PP group showed significantly higher blast percentage in PB (68.0% vs. 47.0%; *P*=0.006) and in BM (87.0% vs. 61.3%, *P*=0.005) than the NN+NP group did. However, there was no difference in the duration of CR, there was no difference in OS and survival after relapse between these groups (Fig. 1). Another comparison was performed between the NN+PN group, which included patients with negative *FLT3* ITD mutation status at relapse, and the NP+PP group, which included patients with positive *FLT3* ITD mutation status at relapse, revealed that the median duration of CR was significantly shorter in the NP+PP group (6.8 months) than in the NN+PN group (10.0 months; *P*=0.034). In particular, the median OS was significantly shorter in the NP+PP group (12.0 months) than in the NN+PN group (23.1 months; *P*<0.001), as was survival after relapse (5.5 vs. 11.8 months; *P*<0.001), despite the fact that SCT was performed significantly more frequently in the NP+PP group than in the NN+PN group after the first relapse (66.7% vs. 20.4%; *P*<0.001) (Fig. 2).

4. Comparison of prognosis in patients without *FLT3* ITD mutation stratified by *NPM1* genotype status at diagnosis and relapse

Patients with *NPM1* mutation at diagnosis showed a trend

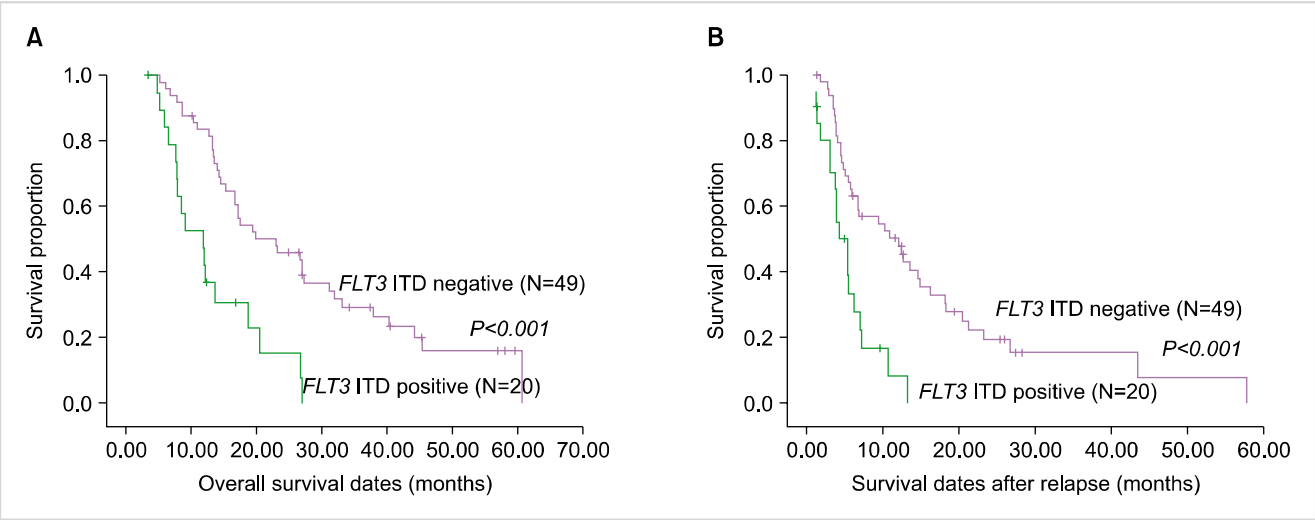

Fig. 2. Comparison of overall survival **(A)** and survival after relapse **(B)** between patients positive and negative for *FLT3* ITD mutation at relapse.

Table 4. Multivariate analysis of OS and survival after relapse with respect to *FLT3* ITD mutation status at diagnosis and relapse.

Clinical variables	Overall survival		Survival after relapse	
	HR (95% CI)	*P*	HR (95% CI)	*P*
FLT3 ITD mutation at relapse (compared with negative at relapse)	2.486 (1.203-5.138)	0.014[a]	2.042 (1.062-4.045)	0.039[a]
FLT3 ITD mutation at diagnosis (compared with negative at diagnosis)	1.084 (0.528-2.144)	ns[b]	1.147 (0.534-2.447)	ns[b]

[a]*P*-value was adjusted for WBC count, duration of CR, blast percentage in PB and BM at diagnosis, and SCT performance rates after 1st relapse, [b]*P*-value was adjusted for WBC count and blast percentage in PB and BM at diagnosis.
Abbreviations: HR, hazard ratio; *FLT3* ITD, fms-like tyrosine kinase3 internal tandem duplication; ns, not significant; CI, confidence interval.

towards a better outcome, such as longer OS (24.6 vs. 17.3 months; *P*=0.173) and duration of CR (10.0 vs. 7.5 months; *P*=0.558) than those without *NPM1* mutation at diagnosis. However, these differences were not statistically significant. Patients with *NPM1* mutation at relapse also showed no differences in OS (15.4 vs. 23.1 months; *P*=0.609) and duration of CR (8.6 vs. 10.3 months; *P*=0.657) compared with those without *NPM1* mutation at relapse.

5. Comparison of prognosis in patients with *FLT3* ITD mutation at diagnosis or relapse according to the amount of *FLT3* ITD mutants

The high/low cut-off ratio was set at 0.66, as described in a previous study [23]. Six patients with a high mutant:wild ratio (≥0.66) at diagnosis showed trends towards shorter OS (9.3 vs. 22.5 months; *P*=0.370) and duration of CR (4.1 vs. 6.6 months; *P*=0.682) than 14 patients with a low ratio (<0.66) at diagnosis, suggesting possible poor prognosis, but the results were not statistically significant. Eight patients with high mutant:wild ratios (≥0.66) at relapse also showed similar OS (9.2 vs. 10.1 months; *P*=0.734) and duration of CR (4.1 vs. 5.4 months; *P*=0.701) as shown by 12 patients with a low ratio (<0.66) at relapse.

6. Multivariate analysis of OS and survival after relapse related to *FLT3* ITD mutation status at diagnosis and relapse

Table 4 shows the results of multivariate analysis of OS and survival after relapse related to *FLT3* ITD mutation status at diagnosis and relapse. *FLT3* ITD mutation at relapse, irrespective of the initial mutation status, showed a statistically significant association with a poor prognosis on OS (HR, 2.486; *P*=0.014) and survival after relapse (HR, 2.042; *P*=0.039). *FLT3* ITD mutation at diagnosis did not show statistically significant prognostic impact on OS or survival after relapse.

DISCUSSION

In this study, the *FLT3* ITD mutation was present in 20 (29.0%) patients each at diagnosis and relapse, similar to the findings of a recent study [22]. Between diagnosis and relapse, the *FLT3* ITD mutation status changed in 18 patients (26.1%). This figure is quite high relative to the recently reported value of 17.5%, and reflects the heterogeneity of *FLT3* ITD mutation populations [24].

Patients with persistent *FLT3* ITD mutation showed higher initial WBC counts than those with persistent wild-type

status. Patients with loss of the *FLT3* ITD mutation at relapse showed higher initial WBC count, blast percentage in PB and BM than those with persistent wild-type status, consistent with the previous findings [11-16]. Patients who acquired the *FLT3* ITD mutation at relapse showed significantly shorter OS and survival after relapse than those with persistent wild-type status, and similar differences were observed between patients who acquired the *FLT3* ITD mutation at relapse and patients with persistent *FLT3* ITD mutation. Additionally, patients with persistent *FLT3* ITD mutation showed a significantly shorter duration of CR than those with persistent wild-type status. These findings were most pronounced in patients with *FLT3* ITD mutation at relapse, compared to those with mutation-negative status at relapse, despite SCT being significantly more frequently performed after the first relapse in patients with *FLT3* ITD mutation. Collectively, the results suggest that *FLT3* ITD mutation at relapse is a poor prognostic indicator, and are consistent with recent data that reported a shorter time-to-relapse in patients with *FLT3* ITD mutation at relapse compared to those without the mutation [24]. On the other hand, *FLT3* ITD mutation status at diagnosis influenced WBC count and blast percentage in PB and BM at diagnosis, but prognostic differences according to mutation status at diagnosis were not statistically significant. These differences between the prognostic potential of mutation status at diagnosis and relapse may indicate that the prognostic impact of *FLT3* ITD mutation status at relapse is more pronounced than the mutation status at diagnosis in normal-karyotype AML patients with relapse.

Multivariate analysis revealed the *FLT3* ITD mutation at relapse to be an independent indicator of poor prognosis for both OS and survival after relapse. Otherwise, the presence of *FLT3* ITD mutation at diagnosis did not show any prognostic effect. These results support our speculation that the impact of *FLT3* ITD mutation status at relapse is of greater prognostic value than the mutation status at diagnosis in normal-karyotype AML patients with relapse. Thus far, *FLT3* ITD mutation status at diagnosis has been firmly established as a prognostic indicator for normal-karyotype AML [25, 26]. Moreover, the ratio of mutant:wild-type cells has been suggested to be a possible prognostic indicator by a previous study, which showed significantly lower 5-year relapse-free survival rates (57% vs. 79%, P=0.048) in patients with a high mutant:wild-type ratio ($\geq$0.66) than in those with a low ratio. However, in our study, not only did the *FLT3* ITD mutation status at diagnosis not predict outcome but there was also no evidence of a prognostic impact of the relative amount of the mutant cells. One possible explanation for the discordant results is the different nature of the patient group included in the analysis. Previous studies have focused on *FLT3* ITD mutation status at initial diagnosis; therefore, all patients could be included into analysis regardless of their status at relapse. However, in our study, only patients who had undergone a relapse could be enrolled. Because the clinical outcome between relapsed and non-relapsed patients is quite different, this heterogeneity might influence the clinical outcome.

Our study had some limitations. First, several molecular aberrations which have potential prognostic impacts in normal karyotype AML, such as CCAAT-enhancer binding protein-α (*CEBPA*) and Wilm's tumor 1 (*WT1*), were not analyzed. These factors may have influenced our results. Second, the number of patients was relatively small, which may lead to false positives or negatives and thus would have affected our conclusions. Third, although we found that a prognostic impact of *NPM1* mutation was not evident at either diagnosis or relapse in patients in the absence of *FLT3* ITD mutation, this analysis suffered from low statistical power due to the limited number of patients. For this reason, we could not perform more categorized analysis based on the different mutation status combination of *FLT3* ITD and *NPM1*, which has been widely used in recent studies. Therefore, further studies with larger patient populations will be required for more accurate analysis.

In conclusion, patients with acquired or persistent *FLT3* ITD mutation at relapse showed significantly shorter OS and survival after relapse than those with persistent wild-type status. These statistical differences were most pronounced in patients with *FLT3* ITD mutation at relapse, when compared to those with mutation-negative status at this time point. However, the mutation status at diagnosis did not affect the outcome. Collectively, these results suggest that the impact of *FLT3* ITD mutation status at relapse is expected to be of greater prognostic value than the mutation status at diagnosis in normal karyotype-AML patients with relapse.

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