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ORIGINAL ARTICLE

Prognostic significance of nucleophosmin mutations and *FLT3* internal tandem duplication in adult patients with cytogenetically normal acute myeloid leukemia

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Background

Nucleophosmin (NPM1) gene and fms-like tyrosine kinase 3 gene-internal tandem duplication (FLT3-ITD) mutations are the most frequent mutations in patients with cytogenetically normal (CN)-AML. We analyzed the prognostic impact of these mutations and their interactions in adults with CN-AML.

Methods

NPM1 mutation (*NPM1*mut) and *FLT3*-ITD mutation (*FLT3*-ITD+) were analyzed by GeneScan and PCR assays of bone marrow samples obtained from 121 adult patients with CN-AML (age≤60 years at diagnosis).

Results

The incidence of *FLT3*-ITD+ was higher in the *NPM1*mut group than in the wild-type *NPM1* gene (*NPM1*wt) group. The patients were divided according to their mutation status into the *NPM1*mut/*FLT3*-ITD (isolated *NPM1*mut), *NPM1*mut/*FLT3*-ITD+ or *NPM1*wt/*FLT3*-ITD-, and *NPM1*wt/*FLT3*-ITD+ (isolated *FLT3*-ITD+) groups. The isolated *NPM1*mut group showed significantly better clinical outcomes in terms of relapse rate, 5-year relapse-free survival (RFS), and overall survival (OS) than the other groups. In contrast, the isolated *FLT3*-ITD+ group had a higher relapse rate and shorter RFS and OS than the other groups. The 5-year RFS rate was much higher among the patients who underwent allogeneic stem cell transplantation (alloSCT) than among those treated with high-dose cytarabine chemotherapy (HDAC) only as consolidation therapy in the isolated *NPM1*mut group and the *NPM1*mut/*FLT3*-ITD+ or *NPM1*wt/*FLT3*-ITD- group.

Conclusion

Adult patients with CN-AML carrying isolated *NPM1*mut and isolated *FLT3*-ITD+ exhibit different clinical outcomes than those with *NPM1*mut/*FLT3*-ITD+ or *NPM1*wt/*FLT3*-ITD-. Although isolated *NPM1*mut leads to favorable clinical outcomes of CN-AML, the role of alloSCT in such patients remains to be considered.

Key Words *NPM1*, *FLT3*-ITD, Acute myeloid leukemia, Normal karyotype

INTRODUCTION

AML is a clinically and genetically heterogeneous disease. Three cytogenetically defined risk groups (favorable, intermediate, and adverse) are used to design risk-adapted treatment protocols for patients with AML. However, about 35-50% of the successfully karyotyped patients lack clonal chromosomal aberrations [1], and the prognostic implications have not been clearly established for cytogenetically normal (CN)-AML [2]. Therefore, it is important to develop a molec-

ular-level genetic approach for discriminating between prognostically different subsets of CN-AML.

In the recent years, CN-AML has been identified as a heterogeneous disease with mutations of the nucleophosmin (*NPM1*) and *fms*-like tyrosine kinase 3 (*FLT3*) genes. *NPM1*, encoded by the *NPM1* gene on chromosome 5q35, is a multifunctional nucleocytoplasmic shuttling protein localized primarily in the nucleolus but shuttles rapidly between the nucleus and the cytoplasm. The protein appears to be important for various cellular processes. NPM may assist in ribosomal protein assembly [3] and maintain genomic stability through its participation in DNA repair [4]. It also plays a crucial role in cell cycle regulation and apoptosis via its interactions with tumor suppressor p53 and alternate reading frame protein [5, 6].

The *NPM1* gene frequently acts as a target of chromosomal translocations and causes the cytoplasmic dislocation of proteins in various types of leukemia and lymphoma, indicating its role in malignant transformation. Recent studies have demonstrated that aberrant cytoplasmic localization of NPM (NPMc+) in leukemic blasts is associated with mutations at exon-12 of the *NPM1* gene [7-9]. *NPM1* exon-12 mutations can encode mutant proteins with a novel nuclear export signal (NES) motif inserted at the *C*-terminus and disruption of the nucleolar localization signal due to mutations of tryptophan residues 288 and 290 [7, 8]. Such mutations are classified according to the type of NES motif inserted into the mutant protein. In adult NPMc+ AML, mutation 'A' (tandem duplication of TCTG) accounts for approximately 80% of all the NPMc+ cases [9]. Mutations at *NPM1* exon-12 and the resultant shift of *NPM1* into the cytoplasm are found in approximately 35% of the adults with AML. One of the most frequent mutations seen in CN-AML is *NPM1* mutation (*NPM1*mut), found in 45.7-64% of the patients. Besides the characteristic biological features such as increased frequency of monocytic leukemia and distinctive gene expression profiles, patients with CN-AML carrying isolated *NPM1*mut show better clinical outcomes in terms of the responsiveness to chemotherapy or disease-free survival (DFS) than those with the wild-type *NPM1* (*NPM1*wt) gene [9-12].

FLT3 belongs to the class III receptor tyrosine kinase family. It is expressed in early hematopoietic progenitors and its dimerization by the *FLT3* ligand induces growth control signals in normal hematopoiesis. The *FLT3* gene maps to chromosome band 13q12 [13], and an internal tandem duplication (ITD) of the gene (*FLT3*-ITD) has been detected in 20-30% of the young adults with AML [14]. The duplication involves a segment of the juxtamembrane domain-coding sequence, which frequently involves exon-14 and rarely intron-14 or exon-15, and is always in-frame [14]. In support of the potential role of this mutation in leukemogenesis, *FLT3*-ITD from patients with AML has been shown to induce autonomous proliferation in cytokine-dependent cell lines. In contrast to *NPM1*mut, patients with isolated *FLT3*-ITD mutation (*FLT3*-ITD+) show poor clinical outcomes, similar to those with poor-risk cytogenetics [9, 12, 14].

Several studies have attempted to identify the interactions between these frequent mutations and their prognostic implications for patients with CN-AML. On the basis of these reports, the recently updated National Comprehensive Cancer Network (NCCN) Guidelines [15] recommend that isolated *NPM1*mut and *FLT3*-ITD+ CN-AML should be regarded as better-risk and poor-risk cytogenetics, respectively; hence, transplantation as a postremission therapy should be reserved until relapse in patients with CN-AML carrying isolated *NPM1*mut. In contrast, patients with *FLT3*-ITD+ CN-AML should be enrolled in clinical trials as the standard therapy on account of their poor prognosis. Nonetheless, controversy exists as to the prognostic significance of these two mutations in patients with CN-AML [9, 16, 17]. Therefore, we examined the incidence and interactions of *NPM1*mut and *FLT3*-ITD+ in such patients and assessed the validity of the newly proposed risk-adapted treatment strategies based on these mutations.

MATERIALS AND METHODS

1. Patients and treatment protocol

Diagnostic bone marrow (BM) samples from 121 adult patients with CN-AML (age≤60 years) who had received at least one cycle of intensive induction chemotherapy were analyzed retrospectively for the presence of *NPM1*mut and *FLT3*-ITD+. To establish CN-AML, 20 or more metaphase cells from the samples had to test positive for normal karyotypes [18]. Approval was obtained from the institutional review board for this procedure. All patients gave written informed consent for both the treatment and the cryopreservation of BM.

All enrolled patients received intensive remission induction therapy consisting of 3 days of idarubicin (IDA) at 12 mg/m^2/day and 7 days of cytarabine at 100 mg/m^2/day or N^4-behenoyl-1-D-arabinofuranosylcytosine (BH-AC; 300 mg/m^2/day for patients younger than 40 years, 200 mg/m^2/day for patients older than 40 years). If the patients failed to achieve complete remission (CR) after the first round of induction chemotherapy, they received reinduction chemotherapy using the same regimen. Patients achieving CR received three courses of high-dose cytarabine chemotherapy (HDAC; 3 g/m^2 every 12 h/day on days 1, 3, and 5) for consolidation. These patients were allowed to proceed to allogeneic (alloSCT) or autologous stem cell transplantation (autoSCT) after one or two courses of HDAC consolidation therapy.

2. Mutational analysis

Genomic DNA was extracted from the diagnostic BM samples, such as cryopreserved mononuclear cells, by using a DNA blood minikit (QIAamp; Qiagen, Valencia, CA, USA) according to the manufacturer's protocol.

For *NPM1*mut analysis, *NPM1* exon-12 was amplified by genomic PCR using primers 5'-TCTGAGTATAAATTTTCT TGGAGTCA-3' (sense) and 5'-ACCAAGCAAAGGGTGGAG TT-3' (antisense). The reaction mixture contained 1.25 pmol

of each primer, 50 ng of genomic DNA, 250 μM dNTPs, and 0.5 U f-taq polymerase (Solgent, Daejeon, Korea) in the buffer provided by the manufacturer. Amplification was performed in a thermal cycler (PTC 200; MJ Research, Inc., Waltham, MA, USA), and the PCR fragments were purified (GENEALL PCR Purification Kit; General Biosystem, Seoul, Korea). The sequencing reactions were analyzed by using a sequencer (ABI 3100) and cycle sequencing kit (BigDye Terminator; Applied Biosystems, Foster City, CA, USA).

For *FLT3*-ITD+ analysis, wild-type *FLT3* exon-11 and exon-12 were amplified by genomic PCR using primers 5'-CAATTTAGGTATGAAAGCC-3' (sense) and 5'-CTTTCA GCATTTTGACGGCAACC-3' (antisense). The reaction mixture contained 2.5 mM dNTPs, 2.5 mM $MgCl_2$, 0.5 μM of each primer, and 0.5 U f-taq polymerase in a total volume of 20 μL. The samples were amplified by initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 53°C for 1 min, and 72°C for 2 min, and final extension at 72°C for 10 min. The PCR products (10 μL) were resolved on 6% polyacrylamide gels, stained with ethidium bromide, and photographed under ultraviolet light.

3. Statistical analysis

The response to initial therapy was evaluated after induction or after salvage chemotherapy. The definition of CR followed the recommended criteria [19]. Relapse was defined as the reappearance of blasts post-CR in the peripheral blood or BM. Relapse-free survival (RFS) endpoints, measured from the date of documented CR, included relapse, patient death from any cause, and alive in CR at last follow-up (censored). The overall survival (OS) endpoints, measured from the date of diagnosis, were death from any cause and alive at last follow-up (censored) [19]. RFS before transplantation and OS before transplantation were also assessed to eliminate confounding bias and were defined as the time without relapse, death, or transplantation from the date of CR and the time from diagnosis to death or transplantation, respectively.

For between-group comparisons, Fisher's exact test (categorical data) and the Mann-hitney U test (continuous data) were used. Categorical data were compared among three groups defined by the *NPM1* and *FLT3*-ITD mutation status using one-way analysis of variance (ANOVA) and the *post hoc* Tukey's honestly significant difference (HSD) test. Continuous variables were compared among the three groups by using the Kruskal-Wallis test. RFS and OS were analyzed by means of Kaplan-Meier survival curve estimates and log-rank tests to compare differences in the distribution of survival for the three groups. Multivariate analysis using forward conditional selection of variables was performed with the Cox's proportional-hazards model to analyze the influence of high WBC count ($>50\times10^9$/μL versus $\leq50\times10^9$/μL), secondary AML (versus *de novo* AML), alloSCT, autoSCT, and the *NPM1* and *FLT3*-ITD mutation status or the interaction of these mutations. A *P*-value of less than 0.05 was considered statistically significant. All statistical computations were performed by using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

1. Patient characteristics

The median age of the 57 male and 64 female patients was 44 years (range, 15-60 years). All patients received intensive remission induction chemotherapy. Fifty-three (43.8%) and five (4.1%) of the patients underwent alloSCT and autoSCT, respectively. The median follow-up time was 11.8 months (range, 0.6-86.1 months).

Among the patients, 65 (53.7%) had *NPM1*mut at exon-12. All patients carrying *NPM1*mut had the type A mutation. Details of the presenting features by the *NPM1*mut status are listed in Table 1. The median peripheral WBC count at diagnosis was significantly higher in the *NPM1*mut group than in the *NPM1*wt group (*P*=0.03). Furthermore, the *NPM1*mut group showed a higher incidence of *FLT3*-ITD+ than the unmutated group (*P*=0.04).

To follow the current NCCN guidelines, we categorized the patients into three groups: *NPM1*mut/*FLT3*-ITD-, *NPM1*mut/

Table 1. Patient characteristics according to the *NPM1*mut status.

Parameter	*NPM1*wt	*NPM1*mut	Total AML	*P*
No. of patients	56 (46.3)	65 (53.7)	121	
Age (yr)	43 (15-60)	47 (16-60)	44 (15-60)	0.65
Sex				0.86
Male	27 (48.2)	30 (46.2)	57 (47.1)	
Female	29 (51.8)	35 (53.8)	64 (52.9)	
FAB subtype				NA
M0	3 (5.4)	2 (3.1)	5 (4.1)	
M1	12 (21.4)	12 (18.5)	24 (19.8)	
M2	18 (32.1)	20 (30.8)	38 (31.4)	
M4	9 (16.1)	9 (13.8)	18 (14.9)	
M5	4 (7.1)	10 (15.4)	14 (11.6)	
M6	2 (3.6)	4 (6.2)	6 (5.0)	
M7	3 (5.4)	0	3 (2.5)	
Secondary leukemia	4 (7.1)	8 (12.3)	12 (9.9)	
Unknown	1 (1.8)	0	1 (0.8)	
WBC ($\times10^9$/L)	11.8 (0.5-333.2)	29.7 (1.3-190.4)	25.0 (0.5-333.2)	0.03
Hb (g/dL)	7.2 (3.1-10.6)	8.4 (4.9-12.9)	7.6 (3.1-12.9)	0.23
Platelets ($\times10^9$/L)	46 (19-87)	62 (5-150)	61 (5-150)	0.22
BM blasts (%)	78 (20-99)	80 (20-100)	80 (20-100)	0.50
AlloSCT	24 (42.9)	29 (44.6)	53 (43.8)	0.86
AutoSCT	4 (7.1)	1 (1.5)	5 (4.1)	0.18
FLT3-ITD+	16 (28.6)	30 (46.2)	46 (38.0)	0.04

The values represent either the number (percentage) or the median (range).
Abbreviations: *NPM1*wt, wild-type nucleophosmin gene; *NPM1*mut, nucleophosmin gene mutation NA, not applicable; BM, bone marrow; alloSCT, allogeneic stem cell transplantation autoSCT; autologous stem cell transplantation FLT3-ITD, *fms*-like tyrosine kinase 3 gene-internal tandem duplication.

FLT3-ITD+ or *NPM1*wt/*FLT3*-ITD-, *NPM1*wt/*FLT3*-ITD+. Table 2 shows the baseline characteristics of these groups. There was no difference among the groups with regard to age and gender. The *NPM1*wt/*FLT3*-ITD+ group showed the highest initial WBC count; however, there was no statistical significance among the groups.

2. Prognostic impact of *NPM1*

Of the 121 patients, 93 (76.9%) achieved CR. Among these, 19 patients failed to achieve CR after the first round of induction chemotherapy and therefore received reinduction chemotherapy (Table 3). There was no difference in the remission rate to induction chemotherapy between the patients with and those without *NPM1*mut (76.9% versus 76.8%, *P*=0.99). Among the patients who achieved CR, the *NPM1*mut group showed a lower relapse rate before SCT (18.0% versus 34.9%, *P*=0.06) and lower overall relapse rate (36.0% versus 47. 6%, *P*=0.26), although the differences were not statistically significant. Before undergoing SCT, the *NPM1*mut group showed a significantly higher rate of 4-year RFS (45.4% versus 0%, *P*=0.00) and OS (59.8% versus 15.6%, *P*=0.04) than the *NPM1*wt group (Fig. 1A). Overall RFS and OS also showed a trend toward better prognosis in the *NPM1*mut group (Fig. 1B).

In the subgroup analysis, the overall RFS was significantly longer among the patients who underwent alloSCT than among the nontransplant patients (*P*=0.01) in the *NPM1*wt group (Fig. 2A). On the other hand, in the *NPM1*mut group, a trend for longer RFS was observed among the alloSCT patients than the nontransplant patients, but the difference was not statistically significant (*P*=0.34; Fig. 2B). Although few patients underwent autoSCT (N=5), this procedure did not influence the overall relapse rate, RFS, and OS (data not shown).

According to the multivariate analysis for the overall RFS, *NPM1*mut (versus *NPM1*wt, OR=0.49, 95% CI=0.26-0.92, *P*=0.03), *FLT3*-ITD- (versus *FLT3*-ITD+, OR=0.32, 95% CI=0.17-0.60, *P*=0.00), and alloSCT (versus chemotherapy only, OR=0.41, 95% CI=0.22-0.77, *P*=0.01) were independently associated with longer overall RFS, after adjusting for high WBC counts ($\geq 50\times10^9$/μL versus $<50\times10^9$/μL) and secondary AML (versus *de novo* AML) (Table 4). In the multivariate analysis for the overall OS, *NPM1*mut failed to be an independent prognostic factor, whereas *FLT3*-ITD- and alloSCT were independent, favorable prognostic factors (Table 4).

3. Prognostic impact of the interactions between *NPM1*mut and *FLT3*-ITD+

In agreement with the previous reports, our study showed that the *FLT3*-ITD+ status was significantly associated with *NPM1*mut in terms of the incidence and clinical outcomes;

Table 2. Patient characteristics according to the *NPM1*mut and *FLT3*-ITD+ status.

Parameter	*NPM1*mut/ *FLT3*-ITD-	*NPM1*wt/ *FLT3*-ITD- *NPM1*mut/ *FLT3*-ITD+	*NPM1*wt/ *FLT3*-ITD+	*P*
No. of patients	35 (28.9)	70 (57.9)	16 (13.2)	
Age (yr)	50 (23-60)	43 (16-60)	44 (15-54)	0.49
Sex				0.51
Male	19 (54.3)	32 (45.7)	6 (37.5)	
Female	16 (45.7)	38 (54.3)	10 (62.5)	
FAB subtypes				NA
M0	2 (5.7)	3 (4.3)	0	
M1	5 (14.3)	15 (21.4)	4 (25.0)	
M2	15 (42.9)	20 (28.6)	3 (18.8)	
M4	3 (8.6)	9 (12.9)	6 (37.5)	
M5	5 (14.3)	8 (11.4)	1 (6.3)	
M6	2 (5.7)	4 (5.7)	0	
M7	0	2 (2.9)	1 (6.3)	
Secondary leukemia	3 (8.6)	8 (11.4)	1 (6.3)	
Unknown	0	1 (1.4)	0	
WBC (10^9/L)	29.7 (1.3-178.8)	20.7 (0.5-333.2)	37.9 (6.4-74.9)	0.38
Hb (g/dL)	9.2 (4.9-12.9)	7.5 (3.1-10.8)	8.7 (7.1-9.2)	0.42
Platelets (10^9/L)	66.6 (5-150)	45 (19-116)	68 (25-87)	0.50
BM blasts (%)	74 (20-90)	80 (20-100)	79 (20-98)	0.27
AlloSCT	13 (37.1)	34 (48.6)	6 (37.5)	0.57
AutoSCT	1 (2.9)	3 (4.3)	1 (6.3)	0.82

The values represent either the number (percentage) or the median (range).
Abbreviations: *NPM1*wt, wild-type nucleophosmin gene; *NPM1*mut, nucleophosmin gene mutation *FLT3*-ITD, *fms*-like tyrosine kinase 3-internal tandem duplication; NA, not applicable; BM, bone marrow; alloSCT, allogeneic stem cell transplantation; autoSCT; autologous stem cell transplantation.

Table 3. Treatment outcomes according to the *NPM1*mut status.

Parameter	*NPM1*wt	*NPM1*mut	Total AML	*P*
No. of patients	56 (46.3)	65 (53.7)	121	
CR1[a]	34 (60.7)	40 (61.5)	74 (61.2)	0.94
CR total[b]	43 (76.8)	50 (76.9)	93 (76.9)	0.99
Resistant	12 (21.4)	14 (21.5)	26 (21.5)	0.99
Early death	1 (1.8)	1 (1.5)	2 (1.7)	NA
Relapse before SCT	15 (34.9)	9 (18.0)	24 (25.8)	0.06
Overall relapse	20 (47.6)[c]	18 (36.0)	38 (41.3)[c]	0.26
4-yr RFS before SCT	0	45.4±1.4	24.7±10.3	0.00
4-yr OS before SCT	15.6±12.5	59.8±11.3	41.8±9.0	0.04
5-yr RFS	20.2±15.1	49.1±8.7	39.5±7.8	0.18
5-yr OS	44.2±9.1	52.5±7.7	48.8±5.8	0.29

The values represent either the number (percentage) or the mean ±SD.
[a]Patients achieved CR after one course of remission induction chemotherapy, [b]Patients achieved CR regardless of induction chemotherapy, [c]One patient was missing due to follow-up loss.
Abbreviations: *NPM1*wt, wild-type nucleophosmin gene; *NPM1*mut, nucleophosmin gene mutation CR, complete remission; NA, not applicable; SCT, stem cell transplantation; RFS, relapse-free survival; OS, overall survival.

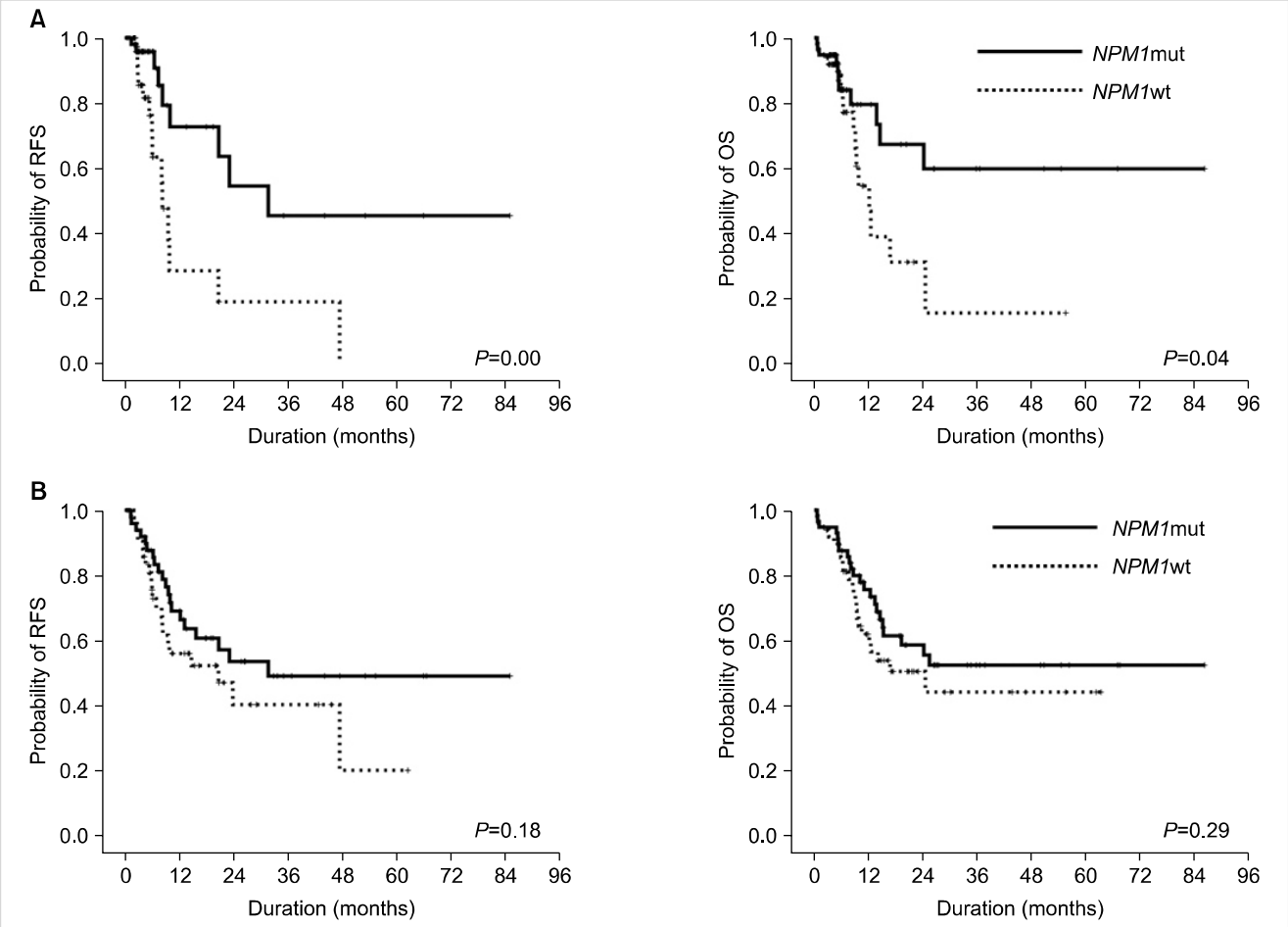

Fig. 1. Kaplan-Meier analysis of CN-AML according to the *NPM1*mut status. **(A)** RFS and OS before SCT. **(B)** Overall RFS and OS. Abbreviations: CN, cytogenetically normal; *NPM1*mut, nucleophosmin gene mutation; *NPM1*wt, wild-type nucleophosmin gene; RFS, relapse-free survival; OS, overall survival; SCT, stem cell transplantation.

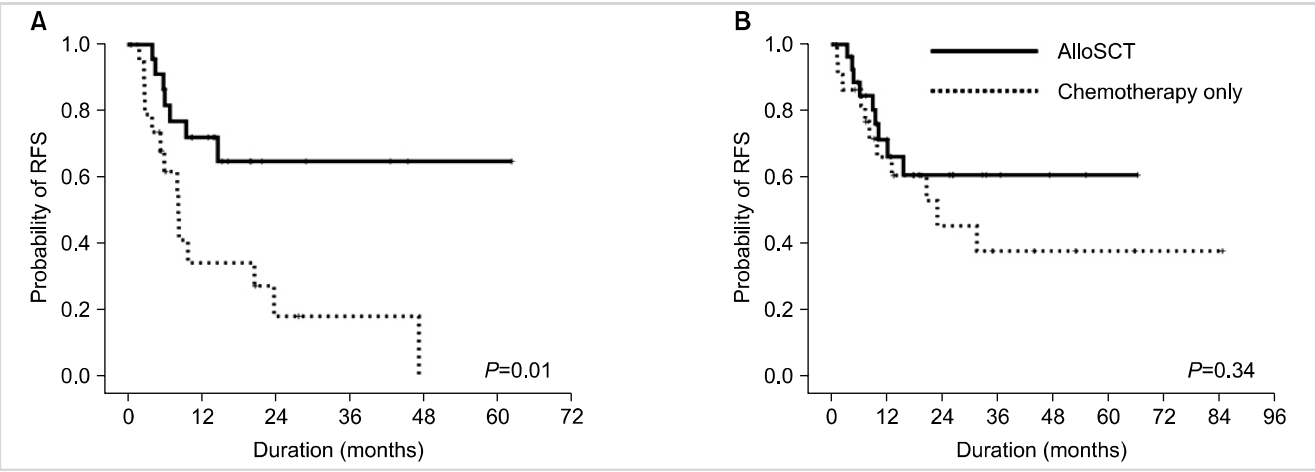

Fig. 2. RFS analysis of the alloSCT patients versus the chemotherapy-alone patients according to the *NPM1*mut status. **(A)** Overall RFS in the *NPM1*wt group. The overall RFS was significantly longer among the alloSCT patients than among the chemotherapy-alone patients ($P=0.01$). **(B)** Overall RFS in the *NPM1*mut group. A trend of longer survival was observed among the alloSCT patients than among the chemotherapy-alone patients, but the difference was not statistically significant ($P=0.34$). Abbreviations: *NPM1*mut, nucleophosmin gene mutation; *NPM1*wt, wild-type nucleophosmin gene; RFS, relapse-free survival; alloSCT, allogeneic stem cell transplantation.

hence, we assessed the prognostic impact of the three groups according to the *NPM1*mut and *FLT3*-ITD+ status (Table 5). The highest remission rate was found in the *NPM1*mut/*FLT3*-ITD- group (80.0%); however, the differences among the groups were not statistically significant. The relapse rate before SCT was higher in the *NPM1*wt/*FLT3*-ITD+ group

Table 4. Multivariable analysis for outcome according to the *NPM1*mut and *FLT3*-ITD+ status.

Variable	RFS		OS	
	OR (95% CI)	*P*	OR (95% CI)	*P*
*NPM1*wt				
*NPM1*mut	0.49 (0.26-0.92)	0.03	NA	NA
FLT3-ITD+				
FLT3-ITD−	0.32 (0.17-0.60)	0.00	0.42 (0.23-0.77)	0.01
Interaction of *NPM1* and *FLT3*-ITD				
*NPM1*mut/*FLT3*-ITD−				
*NPM1*wt/*FLT3*-ITD−	0.30 (0.14-0.64)	0.00	0.32 (0.15-0.69)	0.00
*NPM1*mut/*FLT3*-ITD−				
*NPM1*wt/*FLT3*-ITD+	0.16 (0.06-0.40)	0.00	0.20 (0.08-0.50)	0.00
AlloSCT				
No				
Yes	0.43 (0.23-0.81)	0.01	0.35 (0.18-0.66)	0.00

Abbreviations: *NPM1*wt, wild-type nucleophosmin gene; *NPM1*mut, nucleophosmin gene mutation *FLT3*-ITD, *fms*-like tyrosine kinase 3 gene-internal tandem duplication; RFS, relapse-free survival; OS, overall survival; OR, odds ratio; 95% CI, 95% confidence interval; NA, not applicable; alloSCT, allogeneic stem cell transplantation.

Table 5. Treatment outcomes according to the *NPM1*mut and *FLT3*-ITD+ status.

Parameter	*NPM1*mut/ *FLT3*-ITD-	*NPM1*wt/ FLT3-ITD- *NPM1*mut/ FLT3-ITD+	*NPM1*wt/ *FLT3*-ITD+	*P*
No. of patients	35 (28.9)	70 (57.9)	16 (13.2)	
CR1[a]	21 (60.0)	43 (61.4)	10 (62.5)	1.00
CR total[b]	28 (80.0)	53 (75.7)	12 (75.0)	0.78
Resistant	6 (17.1)	16 (22.9)	4 (25.0)	0.78
Early death	1 (2.9)	1 (1.4)	0	0.75
Relapse before SCT	6 (21.4)	13 (24.5)	5 (41.7)	0.40
Overall relapse	8 (28.6)	20 (38.5)[c]	10 (83.3)	0.00
5-yr RFS before SCT	52.4±15.7	11.9±10.7	NA	0.00
5-yr OS before SCT	61.4±12.0	34.8±14.8	0	0.02
5-yr RFS	60.5±10.8	35.4±12.3	0	0.00
5-yr OS	62.6±9.1	47.1±8.7	NA	0.00

The values represent either the number (percentage) or the mean ±SD.
[a]Patients achieved CR after one course of remission induction chemotherapy, [b]Patients achieved CR regardless of induction chemotherapy, [c]One patient was missing due to follow-up loss.
Abbreviations: *NPM1*wt, wild-type nucleophosmin gene; *NPM1*mut, nucleophosmin gene mutation *FLT3*-ITD, *fms*-like tyrosine kinase 3 gene-internal tandem duplication; CR, complete remission; SCT, stem cell transplantation; RFS, relapse-free survival; NA, not applicable; OS, overall survival.

(41.7%) than in the *NPM1*mut/*FLT3*-ITD- group (21.4%) and the other groups (*NPM1*wt/*FLT3*-ITD- or *NPM1*mut/*FLT3*-ITD+, 24.5%). Furthermore, the overall relapse rate was significantly higher in the isolated *FLT3*-ITD+ group (83.3%) than in the isolated *NPM1*mut (28.6%) and the other groups (38.5%). The post hoc test also revealed that the *NPM1*wt/*FLT3*-ITD+ group had a significantly higher overall relapse rate than the *NPM1*mut/*FLT3*-ITD- (*P*= 0.00) or the other groups (*P*=0.01). The highest 5-year RFS and OS were observed in the isolated *NPM1*mut group regardless of SCT, followed by the *NPM1*wt/*FLT3*-ITD- or *NPM1*mut/*FLT3*-ITD+ group and the isolated *FLT3*-ITD+ groups (Fig. 3).

We also conducted further analysis for the overall RFS following alloSCT compared with chemotherapy alone as a consolidation therapy. In the isolated *NPM1*mut group, 13 patients (37.1%) underwent alloSCT whereas 20 (57.1%) received chemotherapy alone. The CR rate in the alloSCT group and chemotherapy-alone group was 69.2% and 55%, respectively (*P*=0.49). Twelve patients (92.3%) achieved CR at transplantation. One (7.7%) was refractory to all three courses of previous remission induction chemotherapy; in spite of alloSCT, the patient could not achieve CR and died of pneumonia 3 months after SCT. Peripheral blood stem cells collected from sibling donors (s-PBSCs, N=11), unrelated bone marrow stem cells (u-BMs, N=1), and unrelated PBSCs (u-PBSCs, N=1) were used as the hematopoietic stem cell sources. Conditioning regimens consisted of Flu/Bu (fludarabine at 30 mg/m^2/day for 6 days and busulfan at 3.2 mg/kg/day for 4 days, N=9), TBI/Cy (total body irradiation at 13.2 Gy in 4 days with cyclophosphamide at 60 mg/day in 2 days, N=3), and Bu/Cy (busufan with cyclophosphamide, N=1). The median number of infused CD34+ cells was 5.3×10^6/kg (range=0.7-13.2). As depicted in Fig. 4, alloSCT significantly influenced the longer (5-year) RFS in the isolated *NPM1*mut group (alloSCT versus chemotherapy alone, 87.5%. versus 42.7%, *P*=0.03; Fig. 4A). In addition, we conducted a subgroup analysis of 21 patients who achieved CR after one cycle of induction chemotherapy (9 in alloSCT group, 11 in chemotherapy-alone group, and 1 in autoSCT group). The alloSCT group showed a significantly lower rate of overall relapse (0% versus 36.4%, *P*=0.04) and higher 5-year overall RFS (100% versus 52.5%, *P*=0.04) than the chemotherapy-alone group. Although statistically insignificant, the patients with *NPM1*wt/*FLT3*- ITD- or *NPM1*mut/*FLT3*-ITD+ who underwent alloSCT (5-year RFS=63.6%) showed better overall RFS than the chemotherapy-alone patients (15.1%, *P*=0.12; Fig. 4B). Among the patients with isolated *FLT3*-ITD+, the overall RFS in the alloSCT group (N=6) was not significantly different from that in the chemotherapy-alone group (N=9; Fig. 4C). s-PBSCs (N=4), u-PBSCs (N=1), and u-BMs (N=1) were used as the stem cell sources and all patients underwent alloSCT at CR status. The con-

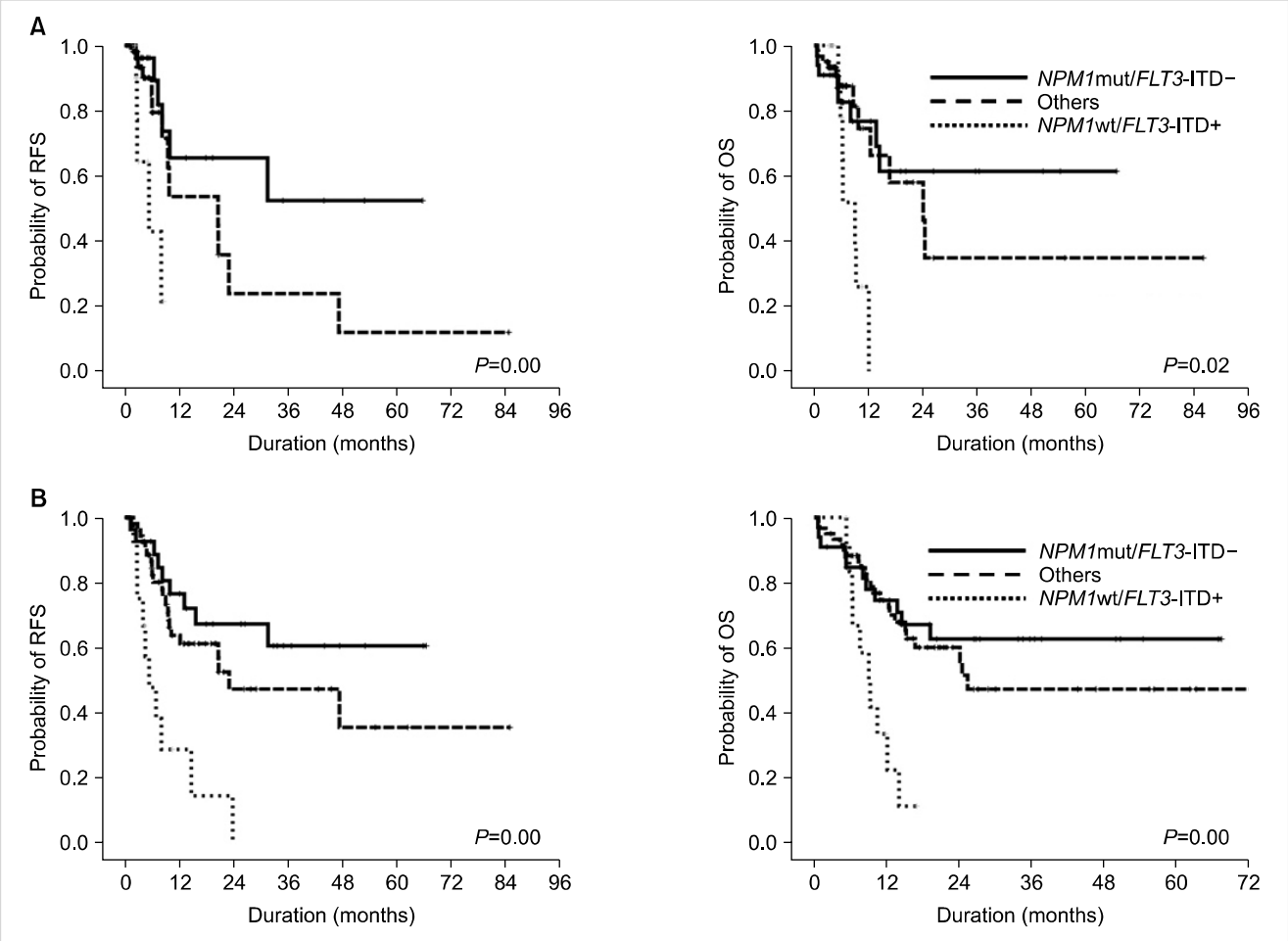

Fig. 3. Kaplan-Meier analysis of CN-AML for RFS and OS according to the combined *NPM1*mut and *FLT3*-ITD+ status. **(A)** RFS and OS before transplantation. **(B)** Overall RFS and OS. Abbreviations: CN, cytogenetically normal; *NPM1*mut, nucleophosmin gene mutation; *NPM1*wt, wild-type nucleophosmin gene; *FLT3*-ITD, *fms*-like tyrosine kinase 3 gene-internal tandem duplication; RFS, relapse-free survival; OS, overall survival.

ditioning regimens consisted of Flu/Bu (N=4) and TBI/Cy (N=2). The median number of infused CD34+ cells was 4.5×10^6/kg (range=2.6-7.8). Four patients in the alloSCT group died of leukemic relapse, and there was no significant correlation between the relapse rate or RFS and the conditioning regimen or the stem cell source.

The multivariate analysis for the overall RFS and OS also identified the combination of *NPM1* and *FLT3*-ITD mutations and alloSCT as independent prognostic factors after adjusting for high WBC count ($\geq 50\times10^9$/μL versus $<50\times10^9$/μL) and secondary AML (versus *de novo* AML) (Table 4).

DISCUSSION

We evaluated the prevalence and prognostic impact of *NPM1*mut and *FLT3*-ITD+ and their interactions in adult patients with CN-AML. The incidence of *NPM1*mut and *FLT3*-ITD+ was 53.7% and 38%, respectively, suggesting that they are frequent molecular events in such patients. The incidence of *FLT3*-ITD+ was higher in the *NPM1*mut group than in the *NPM1*wt group. Previous studies have reported a considerable correlation between these two mutations, suggesting that they are secondary events from a primary process that predisposes myeloid stem and progenitor cell errors in DNA replication [10, 11]. Others have demonstrated that the *NPM1*mut/allele ratio is higher than the *FLT3*-ITD+/wild-type ratio, suggesting that *NPM1*mut occurs prior to *FLT3*-ITD+ in cases with both mutations [9].

According to previous studies, patients with CN-AML carrying *NPM1*mut show a higher rate of CR, suggesting that these patients are more sensitive to chemotherapeutic agents [11, 12, 20]. These studies assumed that NPMc+ may interact with and sequester nuclear factor kappaB (NF-κB), contributing to the maintenance and survival of malignant clones and an impaired response to chemotherapy in the cytoplasm, leading to its inactivation and reduced DNA binding [20]. However, in other studies, patients younger than 60 years and pediatric patients did not show a significantly different CR rate between *NPM1*wt and *NPM1*mut [9, 16, 17]. In the present study, there were no significant differences in the CR rate between *NPM1*mut and *NPM1*wt. Although the patients with *NPM1*mut/*FLT3*-ITD- CN-AML showed a slightly higher CR rate than the other groups, the differ-

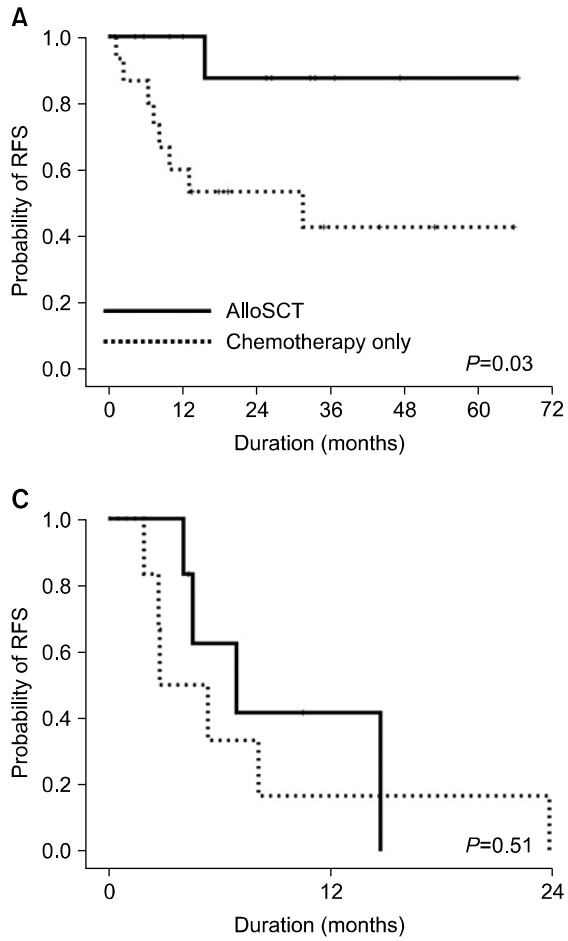

Fig. 4. RFS analysis of the alloSCT patients versus the chemotherapy-alone patients according to the combined *NPM1*mut and *FLT3*-ITD+ status. **(A)** *NPM1*mut/*FLT3*-ITD- (isolated *NPM1*mut) group. **(B)** *NPM1*mut/*FLT3*-ITD+ or *NPM1*wt/*FLT3*-ITD- group. **(C)** *NPM1*wt/*FLT3*-ITD+ (isolated *FLT3*-ITD+) group. In the *NPM1*mut/*FLT3*-ITD- group, the alloSCT patients had significantly longer RFS than the chemotherapy- alone patients **(A)**. The *NPM1*mut/*FLT3*-ITD+ or *NPM1*wt/*FLT3*-ITD- group also displayed a longer trend of RFS with alloSCT **(B)**. In contrast, the patients with *NPM1*wt/*FLT3*-ITD+ showed significantly shorter RFS and alloSCT did not improve the outcome of this subset **(C)**. Abbreviations: *NPM1*mut, nucleophosmin gene mutation; *NPM1*wt, wild-type nucleophosmin gene; *FLT3*-ITD, *fms*-like tyrosine kinase 3 gene-internal tandem duplication; RFS, relapse-free survival; alloSCT, allogeneic stem cell transplantation.

ences were not statistically significant.

On the other hand, we demonstrated that the *NPM1*mut group had better RFS and OS before SCT than the *NPM1*wt group, although the differences were not statistically significant. A recent study has demonstrated that non-A type *NPM1*mut individuals show different clinical outcomes compared with those having the type A mutation [21]. In the present study, however, all the *NPM1*mut individuals possessed type A mutations. In the multivariate analysis to evaluate other factors influencing the overall RFS and OS, *FLT3*-ITD+ and alloSCT treatment were identified as independent prognostic factors (besides the *NPM1*mut status).

For CN-AML, the proper risk-stratified postremission therapy has not yet been determined. Previous studies on young adult patients with CN-AML have reported a 4-year DFS of 48.5% with alloSCT [22] and 5-year DFS of 28-41% with repeated courses of HDAC consolidation [23, 24]. Hence, transplant-based options generally afford a lesser risk of relapse and higher DFS as consolidation for the patients. However, this benefit is accompanied by a treatment-related mortality of 15-25% [25]. To balance the treatment-related toxicity and risk of relapse, it is crucial to define distinct clinical and molecular subtypes that influence the prognosis of CN-AML.

It was previously reported that event-free survival (EFS) and DFS are better in patients with NPMc+/*FLT3*-ITD- than in those with other CN-AML subtypes [9, 12]. Some studies have also shown that the 5-year EFS and OS of patients with NPMc+/*FLT3*-ITD- are comparable to those with favorable cytogenetics [16, 17], suggesting that this group of patients could be considered a favorable group. Others have reported that the RFS and OS are significantly better in the NPMc+/*FLT3*-ITD- group than the other groups, and suggested that alloSCT has no effect on the RFS and OS in this subset of CN-AML [11]. Therefore, the NCCN guidelines for AML consider patients with isolated *NPM1*mut as a favorable-risk group and recommended multiple cycles of HDAC as a reasonable option. Further, patients carrying isolated *FLT3*-ITD+ constitute a poor-risk cytogenetic group that should be considered for clinical trials or early alloSCT [15]. However, other studies have reported variable results about the clinical outcomes according to the presence of these mutations; the *FLT3*-ITD+ status was prognostically significant only in the patients with *NPM1*wt [18, 19].

In our three-group analysis, the isolated *NPM1*mut group had a significantly better overall relapse rate and the highest rate of 5-year RFS and OS, regardless of SCT. Further, in this group, alloSCT significantly increased the 5-year RFS rate. For patients carrying favorable cytogenetics such as t(8;21) or inv (16), multiple courses of HDAC are known

to lead to 50-60% of 4-year DFS [24, 26]. In the present study, the 5-year RFS was 42.7% for the patients treated only with HDAC consolidation in the isolated *NPM1*mut group, lower than that among patients with favorable cytogenetics. The patients who underwent alloSCT, however, showed a significantly better 5-year RFS rate (87.5%) than the chemotherapy-alone patients in the isolated *NPM1*mut group. Therefore, the role of alloSCT in patients with *NPM1*mut/*FLT3*-ITD- CN-AML should be reconsidered.

In contrast, the isolated *FLT3*-ITD+ group showed poor clinical outcomes in terms of the overall relapse rate, RFS, and OS, regardless of SCT. All patients relapsed or died within 2 years after diagnosis, and alloSCT did not change their RFS.

In the *NPM1*wt/*FLT3*-ITD- or *NPM1*mut/*FLT3*-ITD+ group, the 5-year RFS increased remarkably (but not significantly) with alloSCT compared with the chemotherapy-alone patients, who showed a remarkably lower rate of 5-year RFS (15.1%) than the patients who underwent alloSCT (63.6%). Therefore, alloSCT seems to be a reasonable option for this subset of patients.

In summary, adult patients (≤60 years) with CN-AML carrying isolated *NPM1*mut and *FLT3*-ITD+ show different clinical outcomes than those bearing both mutated or wild-type *NPM1* and *FLT3*-ITD. Furthermore, isolated *NPM1*mut is associated with favorable clinical outcomes in patients with CN-AML; however, the efficacy of alloSCT as a treatment option for this group of patients remains to be determined.

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