pISSN 1598-2998, eISSN 2005-9256

https://doi.org/10.4143/crt.2022.1407 Cancer Res Treat. 2023;55(3):1011-1022

Original Article

Clinical Significance of bZIP In-Frame *CEBPA*-Mutated Normal Karyotype Acute Myeloid Leukemia

Seo-Yeon Ahn[1], **TaeHyung Kim**[2,3], **Mihee Kim**[1], **Ga-Young Song**[1], **Sung-Hoon Jung**[1], **Deok-Hwan Yang**[1], **Je-Jung Lee**[1], **Mi Yeon Kim**[4], **Chul Won Jung**[5], **Jun-Ho Jang**[5], **Hee Je Kim**[6], **Joon Ho Moon**[7], **Sang Kyun Sohn**[7], **Jong-Ho Won**[8], **Sung-Hyun Kim**[9], **Hyeoung-Joon Kim**[1,4], **Jae-Sook Ahn**[1,4], **Dennis Dong Hwan Kim**[10]

[1]*Department of Hematology-Oncology, Chonnam National University Hwasun Hospital, Hwasun, Korea,* [2]*The Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto,* [3]*Department of Computer Science, University of Toronto, Toronto, Canada,* [4]*Genomic Research Center for Hematopoietic Diseases, Chonnam National University Hwasun Hospital, Hwasun,* [5]*Division of Hematology-Oncology, Samsung Medical Center, Seoul,* [6]*Department of Hematology, Cancer Research Institute, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul,* [7]*Department of Hematology-Oncology, Kyungpook National University Hospital, Daegu,* [8]*Department of Hematology-Oncology, Soon Chun Hyang University Hospital, Seoul,* [9]*Department of Hematology-Oncology, Dong-A University College of Medicine, Busan, Korea,* [10]*Department of Medical Oncology and Hematology, Princess Margaret Cancer Centre, University of Toronto, Toronto, Canada*

Purpose We evaluated the characteristics of CCAAT/enhancer-binding protein α (*CEBPA*) mutations and the significance of a basic leucine zipper in-frame mutation (bZIP$^{in\text{-}f}$) of *CEBPA* in patients with acute myeloid leukemia with a normal karyotype.

Materials and Methods Based on updated knowledge of *CEBPA* mutations, we conducted next-generation sequencing analyses in a previously established real-world cohort.

Results Among 78 of a total of 395 patients (19.7%), 50 had bZIP$^{in\text{-}f}$ *CEBPA*, and 28 had non-bZIP$^{in\text{-}f}$ *CEBPA*. In the multivariate analysis, patients with *NPM1*^{mut}, those with bZIP$^{in\text{-}f}$ *CEBPA*, and those who underwent allogeneic hematopoietic cell transplantation (allo-HCT) had favorable overall survival (OS), but *FLT3*-ITD^{mut} was a poor prognostic indicator. For relapse-free survival (RFS) and cumulative incidence of relapse, bZIP$^{in\text{-}f}$ *CEBPA*, and allo-HCT were associated with favorable outcomes; *FLT3*-ITD^{pos} was associated with worse outcomes. In the *CEBPA* double-mutated group (*CEBPA*^{dm}), bZIP$^{in\text{-}f}$ *CEBPA* was associated with superior outcomes in terms of OS (p=0.007) and RFS (p=0.007) compared with non-bZIP$^{in\text{-}f}$ *CEBPA*. Of 50 patients with bZIP$^{in\text{-}f}$ *CEBPA*, 36 patients had at least one mutation. When grouped by the presence of mutations in chromatic/DNA modifiers (C), cohesion complex (C), and splicing genes (S) (CCS mutations), CCS-mutated bZIP$^{in\text{-}f}$ *CEBPA* was associated with poor OS (p=0.044; hazard ratio [HR], 2.419) and a trend in inferior RFS (p=0.186; HR, 1.838).

Conclusion Only bZIP$^{in\text{-}f}$ *CEBPA* was associated with favorable outcomes in patients with *CEBPA*^{dm}. However, some mutations accompanying bZIP$^{in\text{-}f}$ *CEBPA* showed inferior OS; thus, further studies with larger numbers of patients are required for clear conclusions of the significance of bZIP$^{in\text{-}f}$ *CEBPA*.

Key words Leukemia, Myeloid, Acute, *CEBPA*, Next-generation sequencing, Allogeneic transplantation

Introduction

CCAAT/enhancer-binding protein α (*CEBPA*) is a member of the family of basic leucine zipper (bZIP) transcription factors. It regulates tissue-specific gene expression and proliferation arrest and is critical for neutrophil development [1-3]. *CEBPA* mutations occur in 7.5%-11% of cases of de novo acute myeloid leukemia (AML) and predominantly in patients with a normal karyotype (NK-AML) with 15%-18% [4-6].

Two major types of *CEBPA* mutations have been identified in AML, and patients can have double or single mutations. *CEBPA* mutations affect either the N-terminus with nonsense mutations, leading to a dominant negative protein, or the C-terminus, resulting in decreased DNA binding of a leucine zipper region. Single mutations of *CEBPA* (*CEBPA*^{sm}) occur in either the N- or C-terminus of the gene, and double mutations of *CEBPA* (*CEBPA*^{dm}) dominantly involve both N-terminal frameshift mutations and C-terminal in-frame (bZIP) mutations, resulting in a lack of *CEBPA* wild-type allele expression [6,7].

The World Health Organization (WHO) Working Group classified AML with mutated *CEBPA* as a provisional entity [8,9]. NK-AML with a *CEBPA* mutation is associated with a favorable prognosis, similar to that of core binding factor AML [10]. Several studies have demonstrated that *CEBPA*^{dm}

Correspondence: Jae-Sook Ahn
Department of Hematology-Oncology, Chonnam National University Hwasun Hospital, Chonnam National University, 322 Seoyang-ro, Hwasun 58128, Korea
Tel: 82-61-379-7635 Fax: 82-61-379-7628 E-mail: f0115@jnu.ac.kr
Received October 25, 2022 **Accepted** January 24, 2023 **Published Online** January 26, 2023

Copyright © 2023 by the Korean Cancer Association

This is an Open-Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

is associated with a specific gene signature and a favorable outcome in patients with NK-AML, whereas *CEBPA*^{sm} is not significantly different from wild-type (*CEBPA*^{wild}) [11-13]. Due to advancements in understanding of *CEBPA* mutations and accumulation of clinical data, a favorable prognosis in patients with *CEBPA* mutations depends on the bZIP location of the *CEBPA* mutation but not on whether there is a single or double mutation [14-16]. The reason that *CEBPA*^{dm} has been reported to be a good prognostic factor because most cases of *CEBPA*^{dm} (94%) harbor bZIP mutations [15]. In particular, cases with an in-frame mutation of bZIP (bZIP$^{in\text{-}f}$) show favorable outcomes; this is accompanied by an in-frame mutation in approximately 81% of bZIP mutations [16]. Approximately 36% of patients with *CEBPA*^{sm} carry bZIP$^{in\text{-}f}$ and share a similar spectrum of co-mutations and clinical factors with patients with *CEBPA*^{dm}. Based on these results, the 2022 European LeukemiaNet (ELN) classified only bZIP$^{in\text{-}f}$ *CEBPA* as a favorable risk category rather than including existing biallelic *CEBPA* mutations [17].

In a previous study, we reported on the clinical significance of single or double *CEBPA* mutations in NK-AML, and *CEBPA*^{dm} was associated with a favorable prognosis with consolidation chemotherapy alone without allogeneic hematopoietic cell transplantation (allo-HCT) [13]. Based on the updated knowledge about *CEBPA* mutations, we conducted next-generation sequencing (NGS) analyses in a previously established real-world cohort to evaluate the characteristics of *CEBPA* mutations and the significance of bZIP$^{in\text{-}f}$ *CEBPA*. To clarify the prognostic significance of bZIP$^{in\text{-}f}$ *CEBPA* in AML, we directly compared the treatment results according to the consolidation type (consolidation chemotherapy alone or allo-HCT) in patients with bZIP$^{in\text{-}f}$ *CEBPA*. In addition, we analyzed the clinical significance of accompanying mutations in bZIP$^{in\text{-}f}$ *CEBPA* NK-AML.

Materials and Methods

1. Patients and treatment

The previous study included a total of 404 patients diagnosed with NK-AML from October 1998 to September 2012 at seven participating institutes [13]. A total of 395 samples of 404 were available for additional targeted NGS [18]. All patients met the following eligibility criteria: (1) age ≥ 15 years; (2) diagnosis of NK-AML confirmed by conventional cytogenetic analysis; and (3) treatment with induction chemotherapy using a standard protocol (a 3-day course of anthracyclines with a 7-day course of cytosine arabinoside). Patients who achieved complete remission (CR) received consolidation chemotherapy with or without allo-HCT depending on the availability of a matched related or unrelated donor. Genetic factors were not considered when choosing allo-HCT as a consolidation treatment.

2. *CEBPA* mutations and other genetic analyses

Cryopreserved bone marrow or peripheral blood samples taken at diagnosis were archived prior to genomic DNA extraction using QIAamp DNA blood mini-kits (Qiagen, Valencia, CA) according to the manufacturer's protocol. Mutation analysis was performed for *CEBPA* using Sanger sequencing employing polymerase chain reaction methodology [13]. For NGS analysis, Agilent custom probes were designed to cover the entire exon regions of targeted genes (92 genes) and sequenced according to the manufacturer's protocol using an Illumina HiSeq 2000 sequencer [18]. The variant calling was performed under identical conditions to a previously reported study with a detection threshold of 3% variant allele frequency [18].

3. Endpoints of response and survival

The CR criteria were defined as follows [19]: The incidence of relapse was defined as the time from attainment of remission to the date of relapse in all patients who achieved CR, considering the competing risk of death without relapse. Non-relapse mortality (NRM) was defined as death occurring in the absence of relapse. Relapse-free survival (RFS) was defined as the time from attainment of remission to the date of death from any cause or relapse, whichever occurred first. Overall survival (OS) was defined as the time from commencement of induction chemotherapy to the date of last follow-up or death from any cause.

4. Statistical analysis

The chi-squared test was used to compare differences in the distributions of categorical data, and Student's t test was used to evaluate the significance of differences in continuous variables. RFS and OS were estimated using Kaplan-Meier survival curves; differences among groups were compared using the log-rank test. The prognostic impacts of various risk factors on RFS and OS were evaluated using a Cox proportional hazard model. Time-dependent Cox proportional hazard models were used to examine time-dependent covariates by considering allo-HCT as a time-dependent covariate. To evaluate the risk of relapse and NRM, the cumulative incidence of relapse (CIR) and NRM were calculated using the Gray method and Fine–Gray proportional hazard regression model, considering competing events [20]. To clarify the immortal time bias, we performed a landmark analysis to compare the consolidation type. Covariates with parameters that were significant in the univariate analyses were included in the multivariate analyses. p-values of < 0.05 were considered to indicate statistical significance, and mutations

Table 1. Location and type of *CEBPA* mutation in 395 patients in normal karyotype acute myeloid leukemia

No. of *CEBPA* mutation	Location and type of mutation	CR achieved (%)	Consolidation type	
			Chemotherapy alone	Allo-HCT
Wild (n=317)	Wild (n=317)	252 (79.5)	135	90
Double (n=51)	bZIP^{in-f} (n=41)	40 (98.0)	15	24
	Non-bZIP^{in-f} (n=10)	10 (100)	3	5
Single (n=27)	bZIP^{in-f} (n=9)	7 (78.0)	3	2
	Non-bZIP^{in-f} (n=18)	16 (89.0)	10	5

Allo-HCT, allogeneic hematopoietic cell transplantation; bZIP^{in-f}, basic leucine zipper in-frame *CEBPA* mutation; *CEBPA*, CCAAT/enhancer-binding protein α; CR, complete remission; non-bZIP^{in-f}, non bZIP or non-inframe *CEBPA* mutation.

detected with a frequency of 5% or more were analyzed. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated using a predetermined reference risk value of unity. All statistical analyses were performed using EZR software using the 'R' language (available at http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html) [20].

Results

1. Reclassification according to the location of *CEBPA* mutations

CEBPA mutations were observed in 78 of 395 patients (19.7%). Fifty patients had bZIP^{in-f} *CEBPA*, and 28 patients had non-bZIP^{in-f} *CEBPA*. *CEBPA*^{dm} and *CEBPA*^{sm} were observed in 51 and 27 patients, respectively. Among patients with *CEBPA*^{dm}, bZIP^{in-f} *CEBPA* was detected in 41 patients (80%), and non-bZIP^{in-f} *CEBPA* was observed in 10 patients (20%). Of the 51 patients with *CEBPA*^{dm}, double C-terminal bZIP^{in-f} was observed in only one patient. In 27 patients with *CEBPA*^{sm}, nine (33%) instances of bZIP^{in-f} *CEBPA* and 18 (67%) instances of non-bZIP^{in-f} *CEBPA* were detected. In patients with *CEBPA* mutations (n=78), the risk stratification changed in 24% (from *CEBPA*^{dm} [favorable] to non-bZIP^{in-f} *CEBPA* [intermediate]:10 patients, from *CEBPA*^{sm} [intermediate] to bZIP^{in-f} *CEBPA* [favorable]: 9 patients) according to the location and type of mutation rather than the accompanying number of *CEBPA* mutations (Table 1).

2. Clinical characteristics and their associations with mutations

Younger patients were more likely to have bZIP^{in-f} *CEBPA* mutations than *CEBPA*^{wild} ($p < 0.001$) and non-bZIP^{in-f} (p=0.001) (Table 2). Non-bZIP^{in-f} *CEBPA* mutations were associated with high peripheral blast counts (p=0.031) in comparison with *CEBPA*^{wild}.

bZIP^{in-f} *CEBPA* mutations and their associations with other molecular mutations are described in Table 1 and Fig. 1. bZIP^{in-f} *CEBPA* mutations were associated with *GATA2* mutations (p=0.029) and less frequently associated with *FLT3*-ITD (p=0.003), *NPM1* mutations ($p < 0.001$), *DNMT3A* mutations (p=0.008), and *IDH1* mutations (p=0.013) than *CEBPA*^{wild}. bZIP^{in-f} *CEBPA* was also less frequently associated with *NPM1* mutations (p=0.001) and *FLT3*-ITD (p=0.028) than bZIP^{in-f} *CEBPA* and non-bZIP^{in-f} *CEBPA* (Table 2).

3. Favorable prognostic impact of bZIP^{in-f} *CEBPA* mutations

CR was achieved in 325 of 395 patients (82.3%). The CR rate was 94% in patients with bZIP^{in-f} *CEBPA*, 93% in patients with non-bZIP^{in-f} *CEBPA*, and 79.5% in patients with *CEBPA*^{wild}. The bZIP^{in-f} *CEBPA* mutation was associated with a higher CR rate than *CEBPA*^{wild} (p=0.011). Allo-HCT at first CR (CR1) was performed in 39.1% (127/325) of patients who achieved CR after induction chemotherapy. Allo-HCT in CR was performed in 26/50 (52%) patients with bZIP^{in-f} *CEBPA*, in 10/28 (36%) patients with non-bZIP^{in-f} *CEBPA*, and in 91/252 (36.1%) patients with *CEBPA*^{wild}.

The median follow-up was 79 months (range, 1.5 to 219 months) among survivors. The 5-year RFS and OS rates were 37.0% (95% CI, 31.5 to 42.5) and 33.9% (95% CI, 29.1 to 38.9), respectively. Among 325 patients who achieved CR, the 5-year CIR was 43.6% (95% CI, 38.0 to 49.1), and the 5-year NRM was 18.1% (95% CI, 14.4 to 23.1). The long-term outcomes were subsequently analyzed by *CEBPA* mutational status. The 5-year OS rates were 60.8% (95% CI, 45.5 to 73.0) in patients with bZIP^{in-f} *CEBPA* (n=50), 50.0% (95% CI, 30.6 to 66.6) in patients with non-bZIP^{in-f} *CEBPA* (n=28), and 26.9% (95% CI, 24.4 to 35.0) in patients with *CEBPA*^{wild} group (n=317) (Fig. 2A). bZIP^{in-f} *CEBPA* was associated with a statistically favorable OS compared with *CEBPA*^{wild} ($p < 0.001$). However, there was no statistical difference between patients with bZIP^{in-f} and those with non-bZIP^{in-f} *CEBPA* mutations (p=0.106) or between patients with non-bZIP^{in-f} *CEBPA* and those with *CEBPA*^{wild} (p=0.336) (Fig. 2A). The 5-year RFS rates were 60.7% (95% CI, 44.9 to 73.2) in patients with bZIP^{in-f} *CEBPA* (n=47), 45.5% (95% CI, 25.8 to 63.2) those

Table 2. Clinical characteristics and mutation status in patients with normal karyotype acute myeloid leukemia

Parameter	Median or frequency (n=395)	*CEBPA*^{wild} (n=317)	bZIP^{in-f} *CEBPA* mutations (n=50)	Non-bZIP^{in-f} *CEBPA* mutations (n=28)	p-value[a] (wild vs. bZIP^{in-f})	p-value[b] (wild vs. non-bZIP^{in-f})	p-value[c] (bZIP^{in-f} vs. non-bZIP^{in-f})
Age (yr)	52 (15-84)	54 (15-84)	38 (15-67)	57 (17-75)	< 0.001[d]	0.793	0.001[d]
Male sex	201 (50.9)	161 (50.3)	23 (63.0)	17 (43.1)	0.547	0.332	0.244
WBC ($\times 10^9$/L)	28.2 (0.3-397.2)	27.1 (0.3-384.0)	31.6 (2.0-333.2)	49.8 (1.7-397.2)	0.142	0.031[d]	0.327
Bone marrow blasts (%)	63 (3-100)	70 (3-100)	82 (21-100)	70 (30-100)	0.135	0.231	0.964
NPM1^{mut}	128 (32.4)	117 (36.9)	2 (4.0)	9 (32.1)	< 0.001[d]	0.686	0.001[d]
FLT3-ITD^{mut}	107 (27.1)	93 (29.3)	5 (10.0)	9 (32.1)	0.003[d]	0.829	0.028[d]
DNMT3A^{mut}	95 (24.1)	87 (27.4)	5 (10.0)	3 (10.7)	0.008[d]	0.070	> 0.99
NRAS^{mut}	68 (17.2)	57 (18.0)	8 (16.0)	3 (10.7)	0.844	0.440	0.737
IDH2^{mut}	55 (13.9)	48 (15.1)	3 (6.0)	4 (14.3)	0.120	> 0.99	0.243
PTPN11^{mut}	36 (9.1)	32 (10.1)	2 (4.0)	2 (7.1)	0.289	> 0.99	0.615
FLT3-TKD^{mut}	33 (8.4)	32 (10.1)	1 (2.0)	0	0.065	0.092	> 0.99
IDH1^{mut}	32 (8.1)	31 (9.8)	0	1 (3.6)	0.013[d]	0.494	0.359
TET2^{mut}	27 (6.8)	20 (6.3)	3 (6.0)	4 (14.3)	> 0.99	0.118	0.243
KRAS^{mut}	22 (5.6)	19 (6.0)	2 (4.0)	1 (3.6)	0.752	> 0.99	> 0.99
SRSF2^{mut}	21 (5.3)	19 (6.0)	1 (2.0)	1 (3.6)	0.497	> 0.99	> 0.99
GATA2^{mut}	16 (4.1)	9 (2.8)	5 (10.0)	2 (7.1)	0.029[d]	0.222	> 0.99
Achieved CR	325 (82.3)	252 (79.5)	47 (94.0)	26 (92.9)	0.011[d]	0.131	> 0.99
Received allo-HCT in CR achieved patients (n=325)	127 (39.1)	91 (36.1)	26 (52.0)	10 (35.7)	0.015[d]	> 0.99	0.223

Values are presented as median (range) or number (%). Allo-HCT, allogeneic hematopoietic cell transplantation; bZIP^{in-f} *CEBPA*, bZIP in-frame *CEBPA* mutation; *CEBPA*, CCAAT/enhancer-binding protein α; *CEBPA*^{wild}, *CEBPA* wild-type; CR, complete remission; non-bZIP^{in-f} *CEBPA*, non bZIP or non-in-frame *CEBPA* mutation; WBC, white blood cell. [a]The p-values refer to comparisons among groups with *CEBPA*^{wild} and bZIP^{in-f} *CEBPA*, [b]The p-values refer to comparisons among groups with *CEBPA*^{wild} and non-bZIP^{in-f} *CEBPA*, [c]The p-values refer to comparisons between those with bZIP^{in-f} *CEBPA* and non-bZIP^{in-f} *CEBPA*, [d]Significant variables are shown.

Table 3. Multivariate analysis of clinical and molecular parameters in patients with normal karyotype acute myeloid leukemia

Parameter	Variable	Multivariate		
		OR/HR	95% CI	p-value
CR achieve	Age < 60 yr	2.93	1.69-5.09	0.001
	NPM1^{mut}	2.48	1.31-4.67	0.005
	bZIP^{in-f} *CEBPA*	3.97	1.16-13.50	0.028
	PTPN11^{mut}	0.37	0.17-0.81	0.013
OS	Allo-HCT	0.61	0.44-0.85	0.003
	bZIP^{in-f} *CEBPA*	0.49	0.30-0.81	0.006
	NPM1^{mut}	0.57	0.41-0.80	< 0.001
	FLT3-ITD^{mut}	1.97	1.43-2.71	< 0.001
RFS	Allo-HCT	0.63	0.45-0.87	0.006
	bZIP^{in-f} *CEBPA*	0.56	0.35-0.91	0.019
	FLT3-ITD^{mut}	1.64	1.21-2.23	0.001
CIR	Allo-HCT	0.41	0.28-0.60	< 0.001
	bZIP^{in-f} *CEBPA*	0.49	0.25-0.96	0.036
	FLT3-ITD^{mut}	1.78	1.23-2.59	0.002

Allo-HCT, allogeneic hematopoietic cell transplantation; bZIP^{in-f} *CEBPA*, bZIP in-frame *CEBPA* mutation; *CEBPA*, CCAAT/enhancer-binding protein α; CI, confidence internal; CIR, cumulate incidence of relapse; CR, complete remission; HR, hazard ratio; OR, odd ratio; OS, overall survival; RFS, relapse-free survival.

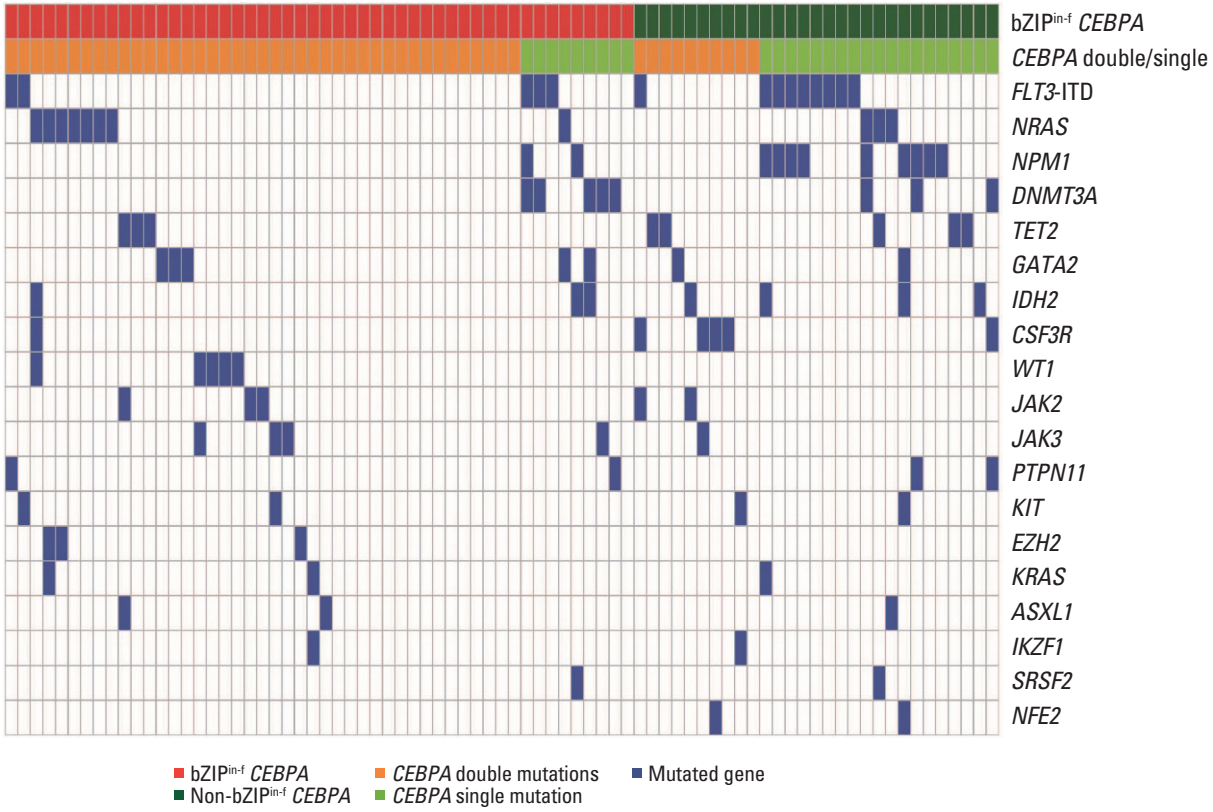

Fig. 1. Prevalence of genetic alterations in the samples obtained at diagnosis from patients with *CEBPA*-mutated normal karyotype acute myeloid leukemia (n=78). bZIP^{in-f} *CEBPA*, bZIP in-frame *CEBPA* mutation; *CEBPA*, CCAAT/enhancer-binding protein α; non-bZIP^{in-f} *CEBPA*, non bZIP or non-in-frame *CEBPA* mutation.

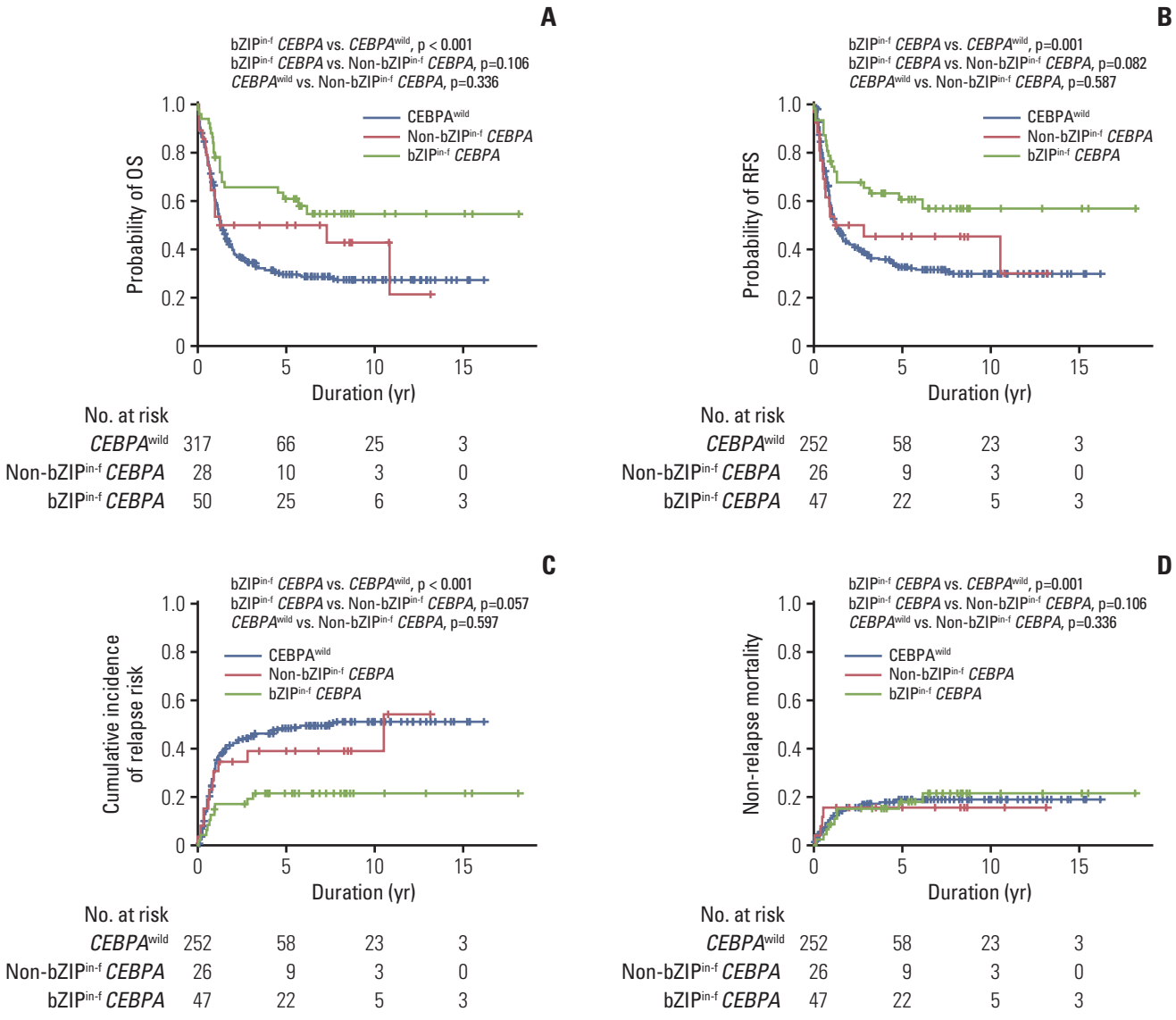

Fig. 2. Prognostic significance according to the *CEBPA* mutation status for all 395 patients with normal karyotype acute myeloid leukemia. OS (A), RFS (B), cumulative incidence of relapse (C), and non-relapse mortality (D). bZIP$^{in\text{-}f}$ *CEBPA*, bZIP in-frame *CEBPA* mutation; CEBPA, CCAAT/enhancer-binding protein α; *CEBPA*^{wild}, *CEBPA* wild-type; non-bZIP$^{in\text{-}f}$ *CEBPA*, non bZIP or non-in-frame *CEBPA* mutation; OS, overall survival; RFS, relapse-free survival.

with non-bZIP$^{in\text{-}f}$ *CEBPA* (n=26), and 32.1% (95% CI, 26.0 to 38.2) in those with *CEBPA*^{wild} (n=252). Patients with bZIP$^{in\text{-}f}$ *CEBPA* also showed a statistically favorable RFS compared with those with *CEBPA*^{wild} (p=0.001), and there was a trend of a favorable outcome compared with those with non-bZIP$^{in\text{-}f}$ *CEBPA* (p=0.082). However, there was no statistical difference between patients with non-bZIP$^{in\text{-}f}$ *CEBPA* and those with *CEBPA*^{wild} (p=0.587) (Fig. 2B). The 5-year CIRs among patients with bZIP$^{in\text{-}f}$ *CEBPA*, non-bZIP$^{in\text{-}f}$ *CEBPA*, and *CEBPA*^{wild} were 21.6%, 39.2%, and 48.4%, respectively. Patients with bZIP$^{in\text{-}f}$ *CEBPA* showed a lower relapse rate than those with *CEBPA*^{wild} (p < 0.001); they also showed a trend of a lower CIR than those with non-bZIP$^{in\text{-}f}$ *CEBPA* (p=0.057). However, there was no statistical difference between patients with non-bZIP$^{in\text{-}f}$ *CEBPA* and those with *CEBPA*^{wild} (p=0.597) (Fig. 2C). There was no difference in the 5-year NRM in all three groups (Fig. 2D).

4. Multivariate analyses on achievement of CR, OS, RFS, and CIR

Multivariate analyses were conducted using the p-value–based stepwise selection method for achievement of CR, OS,

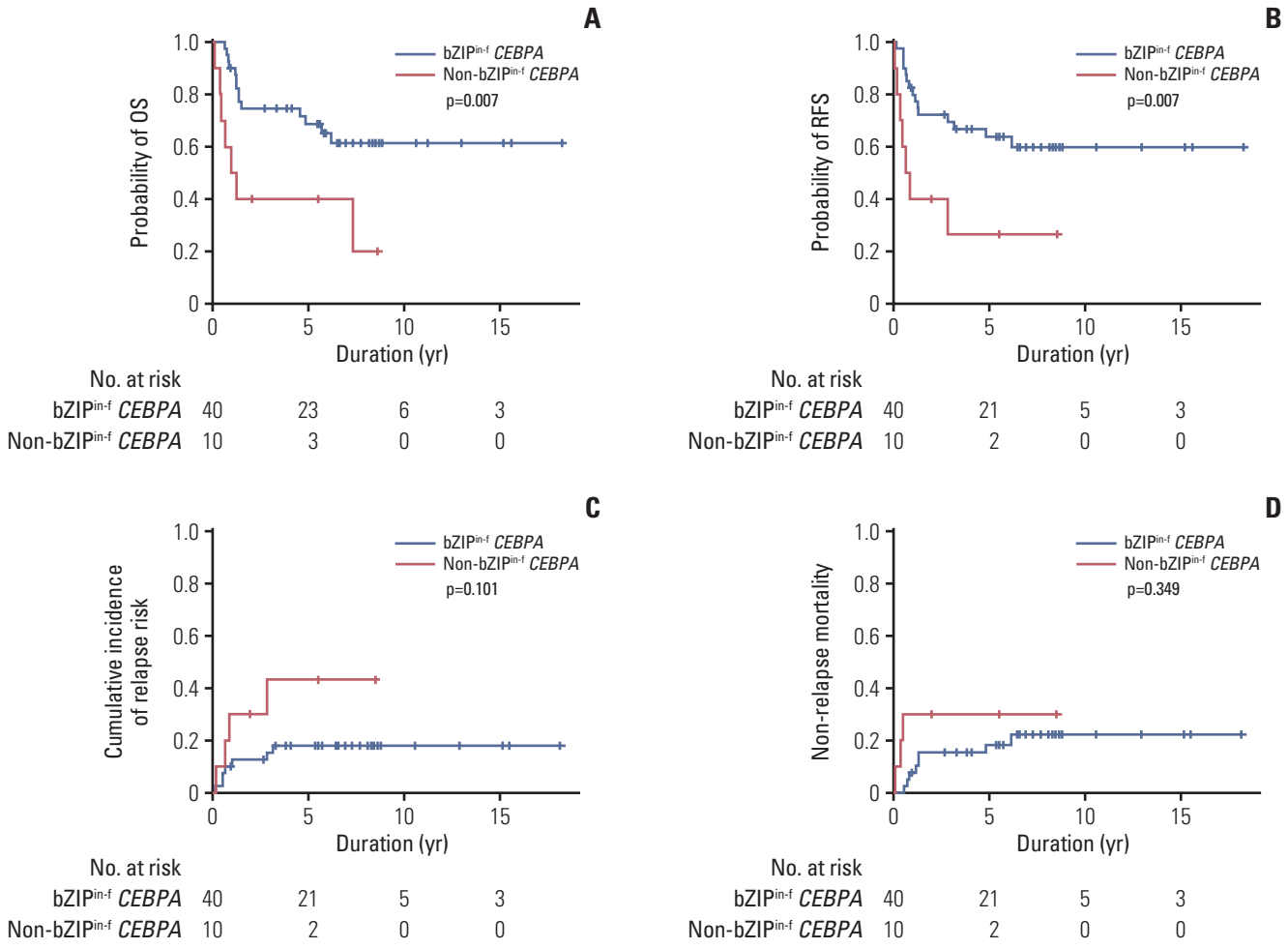

Fig. 3. Prognostic significance according to bZIP^{in-f} or non-bZIP^{in-f} *CEBPA* in patients with *CEBPA* double mutations. OS (A), RFS (B), cumulative incidence of relapse (C), and non-relapse mortality (D). bZIP^{in-f} *CEBPA*, bZIP in-frame *CEBPA* mutation; *CEBPA*, CCAAT/enhancer-binding protein α; non-bZIP^{in-f} *CEBPA*, non bZIP or non-in-frame *CEBPA* mutation; OS, overall survival; RFS, relapse-free survival.

RFS, and CIR. Allo-HCT was included as a time-dependent covariate for OS and RFS. In the multivariate analysis, a high rate of achieving CR was observed in younger patients (age < 60 years), those with *NPM1*^{mut}, and those with bZIP^{in-f} *CEBPA*, while patients with *PTPN11*^{mut} showed low achievement of CR. In terms of OS, *NPM1*^{mut}, bZIP^{in-f} *CEBPA*, and allo-HCT were favorable factors, but *FLT3-ITD*^{pos} was a poor prognostic factor. For RFS and CIR, bZIP^{in-f} *CEBPA*, and allo-HCT were associated with favorable outcomes; *FLT3-ITD*^{pos} was associated with worse outcomes (Table 3).

5. Comparison of outcomes according to bZIP^{in-f} mutations in patients with *CEBPA*^{dm}

Among patients with *CEBPA*^{dm} (n=51), bZIP^{in-f} *CEBPA* was detected in 41 patients (80%), and non-bZIP^{in-f} *CEBPA* was observed in 10 patients (20%). All patients with *CEBPA*^{dm} except one patient with a bZIP^{in-f} *CEBPA* mutation achieved CR. Among them, patients with bZIP^{in-f} *CEBPA* mutations showed superior outcomes in terms of OS (p=0.007) and RFS (p=0.007) compared with patients with non-bZIP^{in-f} *CEBPA* mutations (Fig. 3). Patients with bZIP^{in-f} *CEBPA* also showed a trend of a lower CIR (p=0.101) and a lower NRM (p=0.349); however, these findings were not statistically significant.

Direct comparison of the long-term outcomes by *CEBPA* mutational status had limitations in our cohort because the proportion of patients who received allo-HCT as a consolidation treatment was higher in patients with bZIP^{in-f} *CEBPA* mutations. Therefore, we compared the outcomes to identify the role of either consolidation chemotherapy or allo-HCT in patients with bZIP^{in-f} *CEBPA* mutations alone.

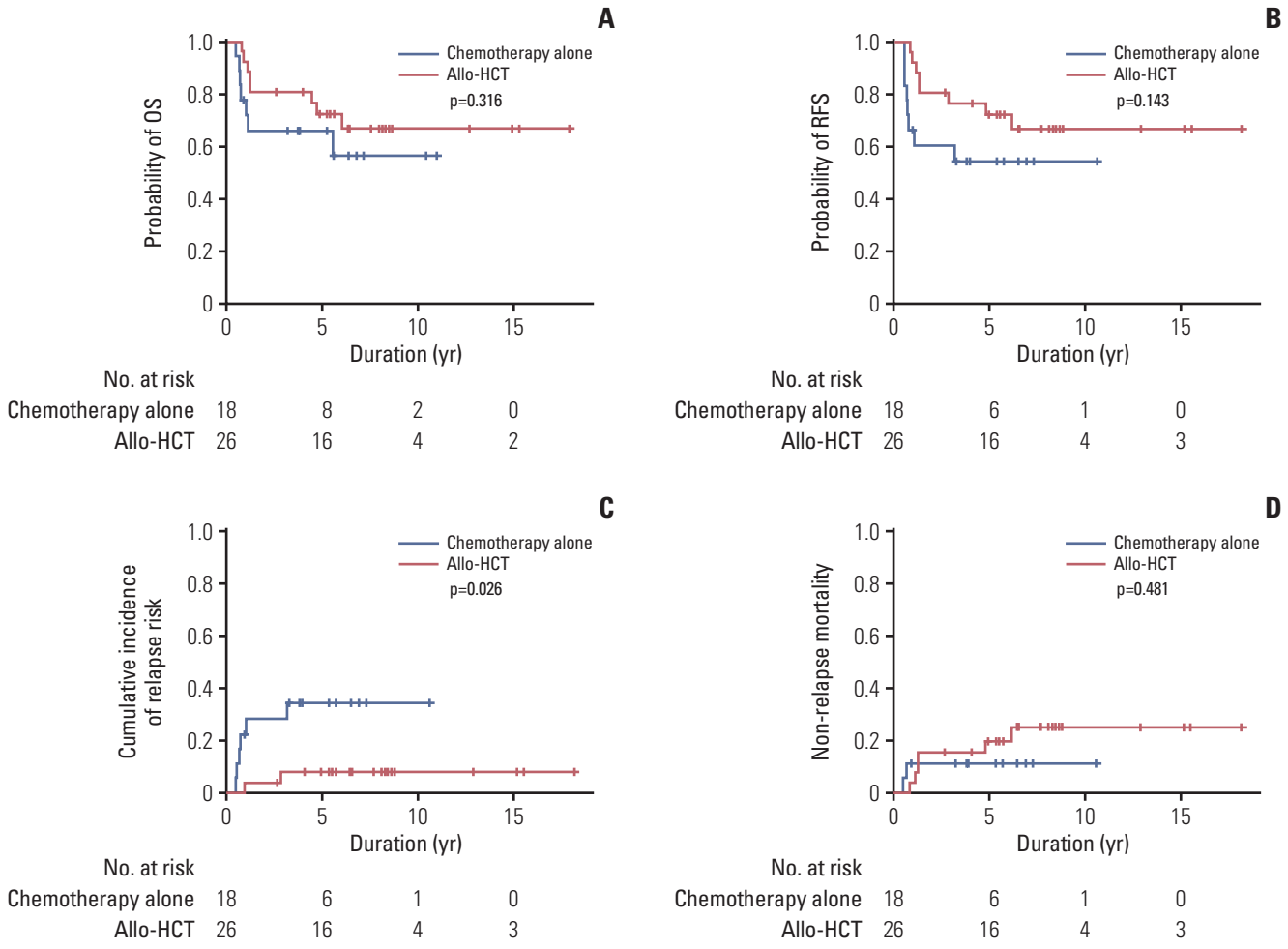

Fig. 4. OS (A), RFS (B), cumulative incidence of relapse (C), and non-relapse mortality (D) by landmark analysis according to the type of consolidation therapy in patients with bZIP$^{in\text{-}f}$ *CEBPA* with normal karyotype acute myeloid leukemia. Allo-HCT, allogeneic hematopoietic cell transplantation; bZIP$^{in\text{-}f}$ *CEBPA*, bZIP in-frame *CEBPA* mutation; OS, overall survival; RFS, relapse-free survival.

6. Favorable outcomes of consolidation chemotherapy alone compared with allo-HCT in patients with bZIP$^{in\text{-}f}$ *CEBPA* mutations

Of 50 patients with bZIP$^{in\text{-}f}$ *CEBPA* mutations, 47 patients achieved CR. We analyzed the treatment outcome by consolidation modalities in patients with bZIP$^{in\text{-}f}$ *CEBPA* mutations who achieved CR. The median duration from allo-HCT to the achievement of CR was 4.0 months. Therefore, we excluded three patients in the chemotherapy group who relapsed (n=2) or died (n=1) within four months. Twenty-six patients underwent allo-HCT, and 18 patients received consolidation chemotherapy alone. Of these 18 patients, six patients relapsed. Five of six patients received intensive reinduction chemotherapy, two patients achieved 2nd CR, and one of them underwent allo-HCT. Three patients died of complications during consolidation chemotherapy. Of 26 patients who underwent allo-HCT, only two patients relapsed and died of leukemia. The other six patients died of complications of allo-HCT including chronic graft versus host disease. The 5-year RFS was 54.5% (95% CI, 29.2 to 74.2) vs. 72.5% (95% CI, 50.6 to 85.8) in those who received chemotherapy alone vs. those who received allo-HCT (p=0.143) (Fig. 4B). The CIR was significantly different in favor of patients who underwent allo-HCT (p=0.026) (Fig. 4C). However, both groups of patients showed a similar OS, RFS, and NRM (all p > 0.05) (Fig. 4A and D).

7. Inferior outcomes of co-occurrence of some mutations in bZIP$^{in\text{-}f}$ *CEBPA*

Of 50 patients with bZIP$^{in\text{-}f}$ *CEBPA* mutations, 36 patients had at least one mutation. However, the number of patients in each co-mutated group was too small to compare them

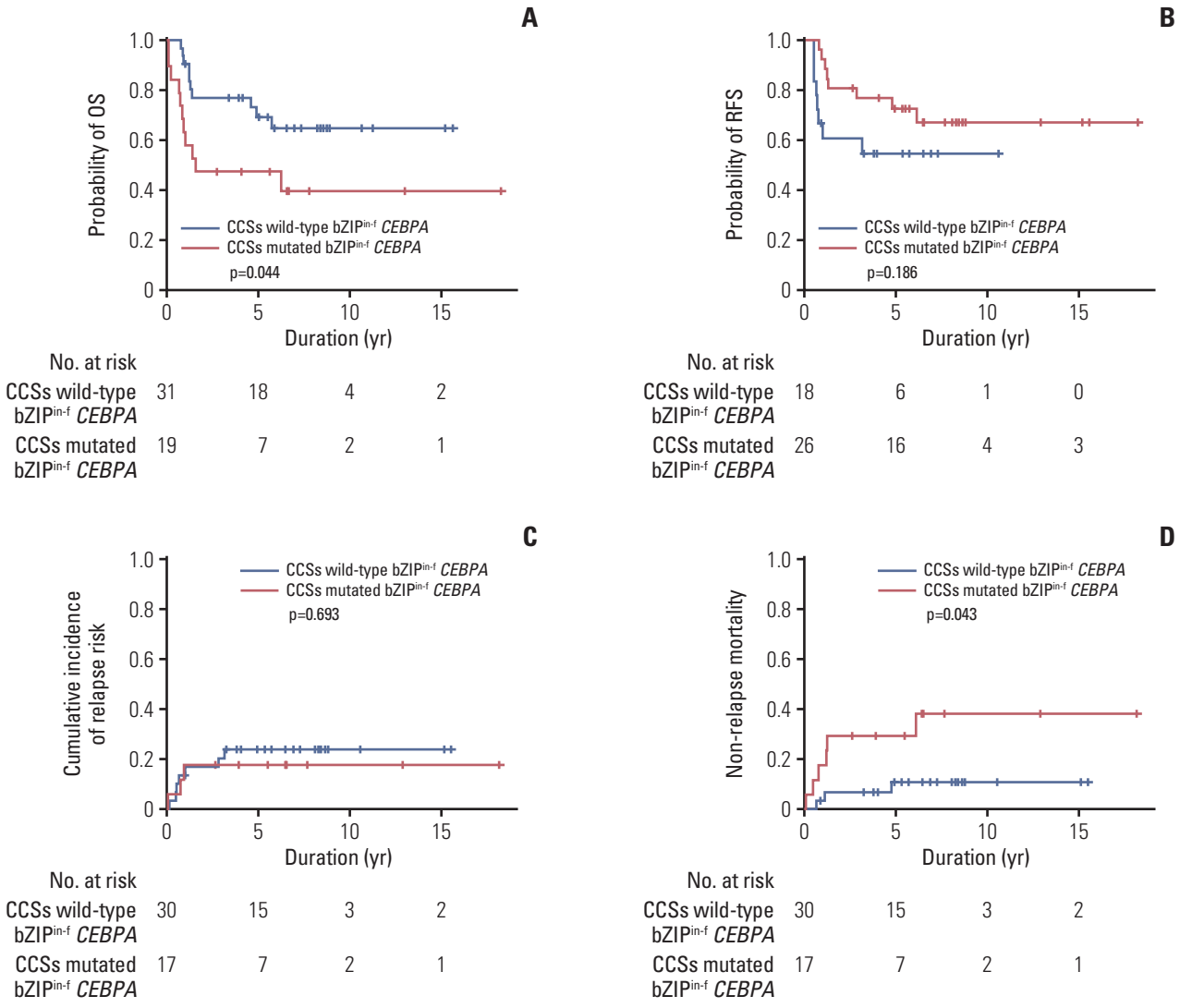

Fig. 5. Prognostic significance by co-occurrence of mutations in patients with bZIP^{in-f} *CEBPA*–mutated normal karyotype acute myeloid leukemia. OS (A), RFS (B), cumulative incidence of relapse (C), and non-relapse mortality (D). bZIP^{in-f} *CEBPA*, bZIP in-frame *CEBPA* mutation; *CEBPA*, CCAAT/enhancer-binding protein α; CCSs mutations, mutations in chromatic/DNA modifiers (C), cohesion complex (C), and splicing genes (S).

directly. By grouping the presence of mutations in chromatic/DNA modifiers (C), cohesion complex (C), and splicing genes (S) (CCS mutations), CCS-mutated bZIP^{in-f} *CEBPA* showed poor outcomes in terms of OS (p=0.044; HR, 2.419; 95% CI, 1.025 to 5.709) and NRM (p=0.043; HR, 3.984; 95% CI, 1.042 to 15.23), and a trend of inferior outcomes without statistical significance in terms of RFS (p=0.186; HR, 1.838; 95% CI, 0.746 to 4.531) (Fig. 5).

Discussion

We evaluated the clinical impact of *CEBPA* mutations in 395 patients with NK-AML. In patients with *CEBPA* mutations (n=78), the risk stratification changed in 24% (n=19) according to the location and type of mutation rather than the number of *CEBPA* mutations. In patients with *CEBPA*^{dm}, only those with bZIP^{in-f} *CEBPA* mutations showed favorable outcomes. The results showed that consolidation chemotherapy alone showed similar results to allo-HCT in patients with bZIP^{in-f} *CEBPA* mutations. However, some accompanying mutations in patients with bZIP^{in-f} *CEBPA* were associ-

ated with inferior OS.

The frequency of *CEBPA* mutations in AML is 7%-20%, and these mutations are mostly associated with a normal karyotype, and more patients present with *CEBPA*^{dm} in Asian populations [21,22]. Since the first report of *CEBPA* mutations in AML was published in 2001, understanding of *CEBPA* mutations has improved due to advancements in biological aspects and the accumulation of clinical data [3]. Recently, gene expression profile studies have suggested that bZIP$^{in\text{-}f}$ *CEBPA* is associated with a unique gene expression profile and can be distinguished from non-bZIP$^{in\text{-}f}$ *CEBPA* or *CEBPA*^{wild}; in contrast, *CEBPA*^{sm} with bZIP$^{in\text{-}f}$ does not express a discriminating signature from bZIP$^{in\text{-}f}$ *CEBPA*^{dm} [16]. The favorable outcomes associated with *CEBPA* mutations in patients with AML are known to be restricted in those with bZIP$^{in\text{-}f}$ *CEBPA*. In previous studies, favorable outcomes associated with *CEBPA*^{dm} were reported in patients with AML, which can be interpreted as showing a good prognosis because approximately 90% of cases of *CEBPA*^{dm} are accompanied by bZIP$^{in\text{-}f}$ [16]. In our cohort, bZIP$^{in\text{-}f}$ *CEBPA* was detected in 80% and 33% of patients with *CEBPA*^{dm} and *CEBPA*^{sm}, respectively. The locations of bZIP and in-frame *CEBPA* mutations were prominently favorable in patients with *CEBPA*^{dm}.

Allo-HCT is not recommended at first CR in patients with AML with bZIP$^{in\text{-}f}$ *CEBPA* according to the 2022 ELN recommendations [17]. In our study, the relapse incidence was significantly reduced in the allo-HCT group compared with chemotherapy alone group among patients with bZIP$^{in\text{-}f}$ *CEBPA*; however, there were no statistically significant differences in OS. The benefit of reducing the relapse rates in the allo-HCT group was likely neutralized by the higher transplant-related mortality (26%). On the other hand, the consolidation chemotherapy alone group may have overcome the higher relapse rates by reduced treatment mortality (11%).

Although patients with AML with bZIP$^{in\text{-}f}$ *CEBPA* showed favorable outcomes, relapse after treatment was inevitable in some patients. The co-occurrence of other genetic mutations may be a clue to predicting relapse in patients with bZIP$^{in\text{-}f}$ *CEBPA*. Approximately 70%-87% of patients with *CEBPA* mutations had other mutations detected by NGS [14-16]. Our results were similar to those of a previous report that bZIP$^{in\text{-}f}$ *CEBPA*^{dm} was accompanied by rare concurrent mutations such as *FLT3-ITD*^{mut}, *NPM1*^{mut}, and *DNMT3A*^{mut} compared with *CEBPA*^{wild} or non-bZIP$^{in\text{-}f}$ *CEBPA* [14-16]. Some studies have reported that the associated mutations in patients with *CEBPA*-mutated AML can be used to predict prognosis [14,15,23]. In *CEBPA*-mutated AML, *GATA2*^{mut} is relatively common, which is reported to be associated with a favorable outcome, whereas *CSF3R*^{mut} or *TET2*^{mut} is associated with a poor prognosis [14,15,23]. In our study, 78 patients had *CEBPA* mutations, which is an insufficient sample size to demonstrate the significance of associated mutations. An effective way to solve the issue of a small cohort is to combine patients with mutations according to genetic pathways. As reported by Konstandin et al. [23], our study also showed poor OS in cases of CCS mutations in patients with bZIP$^{in\text{-}f}$ *CEBPA*. This result may provide evidence for considering alternative treatment in patients with poor survival through precise risk classification by NGS analysis performed at diagnosis.

The present study has several limitations in interpreting the clinical significance of bZIP$^{in\text{-}f}$ *CEBPA* mutations. This was a retrospective study that included patients treated at several centers where consolidation therapies were not uniform. Interestingly, the current cohort showed that a higher proportion of patients with bZIP$^{in\text{-}f}$ *CEBPA* who underwent allo-HCT than those in other populations. Patients with bZIP$^{in\text{-}f}$ *CEBPA* may have had more opportunities to undergo an allo-HCT because they were younger and had higher CR achievement after intensive induction chemotherapy. In addition, the number of patients with *CEBPA* mutations was small, limiting the power to demonstrate the role of consolidation therapy. In our cohort, bZIP$^{in\text{-}f}$ mutations in *CEBPA*^{sm} did not significantly improve survival, which is a limitation to demonstrate the significance of bZIP$^{in\text{-}f}$ mutations in patients with *CEBPA*^{sm} because only a small number of patients were included in this analysis. However, this work has significant clinical relevance in that the selection of consolidation therapy was relatively randomized since genetic factors were not considered when choosing allo-HCT as a consolidation treatment. Furthermore, the association with other mutations according to *CEBPA* mutational status and the prognostic significance of bZIP$^{in\text{-}f}$ *CEBPA* mutations were clarified.

Our results are considered good evidence in support of the 2022 ELN new risk stratification. The present study demonstrated that consolidation therapy alone was associated with similar OS to allo-HCT in patients with bZIP$^{in\text{-}f}$ *CEBPA* with NK-AML. However, precaution is needed in patients with accompanying mutations in bZIP$^{in\text{-}f}$ *CEBPA* because some mutations may be associated with poor outcomes. Further studies with larger numbers of patients are required for clear conclusions of the significance of bZIP$^{in\text{-}f}$ in the *CEBPA*^{sm} subgroup.

Ethical Statement

The study was approved by the Institutional Review Board (IRB) of Chonnam National University Hwasun Hospital, Korea (IRB reference number: CNUHH-2014-153). All study participants provided written informed consent.

Author Contributions

Conceived and designed the analysis: Ahn SY, Kim HJ (Hyeoung-Joon Kim), Ahn JS, Kim DDH.
Collected the data: Ahn SY, Kim M, Song GY, Jung SH, Yang DH, Lee JJ, Kim MY, Jung CW, Jang JH, Kim HJ (Hee Je Kim), Moon JH, Sohn SK, Won JH, Kim SH, Ahn JS, Kim DDH.
Contributed data or analysis tools: Ahn SY, Kim T, Ahn JS, Kim DDH.
Performed the analysis: Ahn SY, Kim T, Ahn JS.
Wrote the paper: Ahn SY, Ahn JS.

ORCID iDs

Seo-Yeon Ahn : https://orcid.org/0000-0002-6061-1490
Jae-Sook Ahn : https://orcid.org/0000-0003-3078-5024

Conflicts of Interest

Conflict of interest relevant to this article was not reported.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Science, ICT, and Future Planning (NRF-2015R1A2A1A10054579) and the National R&D Program for Cancer Control, Ministry of Health & Welfare, Republic of Korea (1720160). This study was supported by a grant from (BCRI22008) Chonnam National University Hospital and (HCRI21006) Chonnam National University Hwasun Hospital Institute for Biomedical Science. This work was also supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (No. 2018R1A2A1A05078480). This research was supported by a Basic Research Program through the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (No. 2022R1F1A1063836). The biospecimens used in this study were provided by the Biobank of Chonnam National University Hwasun Hospital, a member of the Korea Biobank.

References

1. Koschmieder S, Halmos B, Levantini E, Tenen DG. Dysregulation of the C/EBPalpha differentiation pathway in human cancer. J Clin Oncol. 2009;27:619-28.
2. Martelli MP, Sportoletti P, Tiacci E, Martelli MF, Falini B. Mutational landscape of AML with normal cytogenetics: biological and clinical implications. Blood Rev. 2013;27:13-22.
3. Pabst T, Mueller BU, Zhang P, Radomska HS, Narravula S, Schnittger S, et al. Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. Nat Genet. 2001;27:263-70.
4. Green CL, Koo KK, Hills RK, Burnett AK, Linch DC, Gale RE. Prognostic significance of CEBPA mutations in a large cohort of younger adult patients with acute myeloid leukemia: impact of double CEBPA mutations and the interaction with FLT3 and NPM1 mutations. J Clin Oncol. 2010;28:2739-47.
5. Schlenk RF, Taskesen E, van Norden Y, Krauter J, Ganser A, Bullinger L, et al. The value of allogeneic and autologous hematopoietic stem cell transplantation in prognostically favorable acute myeloid leukemia with double mutant CEBPA. Blood. 2013;122:1576-82.
6. Fasan A, Haferlach C, Alpermann T, Jeromin S, Grossmann V, Eder C, et al. The role of different genetic subtypes of CEBPA mutated AML. Leukemia. 2014;28:794-803.
7. Park SH, Chi HS, Cho YU, Jang S, Park CJ. CEBPA single mutation can be a possible favorable prognostic indicator in NPM1 and FLT3-ITD wild-type acute myeloid leukemia patients with intermediate cytogenetic risk. Leuk Res. 2013;37:1488-94.
8. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H. WHO classification of tumours of haematopoietic and lymphoid tissues. 4th ed. Lyon: International Agency for Research on Cancer; 2008.
9. Hwang SM. Classification of acute myeloid leukemia. Blood Res. 2020;55:S1-4.
10. Schlenk RF, Dohner K, Krauter J, Frohling S, Corbacioglu A, Bullinger L, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. N Engl J Med. 2008;358:1909-18.
11. Wouters BJ, Lowenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R. Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. Blood. 2009;113:3088-91.
12. Li HY, Deng DH, Huang Y, Ye FH, Huang LL, Xiao Q, et al. Favorable prognosis of biallelic CEBPA gene mutations in acute myeloid leukemia patients: a meta-analysis. Eur J Haematol. 2015;94:439-48.
13. Ahn JS, Kim JY, Kim HJ, Kim YK, Lee SS, Jung SH, et al. Normal karyotype acute myeloid leukemia patients with CEBPA double mutation have a favorable prognosis but no survival benefit from allogeneic stem cell transplant. Ann Hematol. 2016;95:301-10.
14. Tarlock K, Lamble AJ, Wang YC, Gerbing RB, Ries RE, Loken MR, et al. CEBPA-bZip mutations are associated with favorable prognosis in de novo AML: a report from the Children's Oncology Group. Blood. 2021;138:1137-47.
15. Wakita S, Sakaguchi M, Oh I, Kako S, Toya T, Najima Y, et al. Prognostic impact of CEBPA bZIP domain mutation in acute myeloid leukemia. Blood Adv. 2022;6:238-47.
16. Taube F, Georgi JA, Kramer M, Stasik S, Middeke JM, Rollig C, et al. CEBPA mutations in 4708 patients with acute myeloid leukemia: differential impact of bZIP and TAD mutations on outcome. Blood. 2022;139:87-103.
17. Dohner H, Wei AH, Appelbaum FR, Craddock C, DiNardo

CD, Dombret H, et al. Diagnosis and management of AML in adults: 2022 recommendations from an international expert panel on behalf of the ELN. Blood. 2022;140:1345-77.
18. Ahn JS, Kim HJ, Kim YK, Lee SS, Ahn SY, Jung SH, et al. Assessment of a new genomic classification system in acute myeloid leukemia with a normal karyotype. Oncotarget. 2018; 9:4961-8.
19. Dohner H, Estey E, Grimwade D, Amadori S, Appelbaum FR, Buchner T, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 2017;129:424-47.
20. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 2013;48:452-8.
21. Su L, Shi YY, Liu ZY, Gao SJ. Acute myeloid leukemia with CEBPA mutations: current progress and future directions. Front Oncol. 2022;12:806137.
22. Su L, Tan Y, Lin H, Liu X, Yu L, Yang Y, et al. Mutational spectrum of acute myeloid leukemia patients with double CEBPA mutations based on next-generation sequencing and its prognostic significance. Oncotarget. 2018;9:24970-9.
23. Konstandin NP, Pastore F, Herold T, Dufour A, Rothenberg-Thurley M, Hinrichsen T, et al. Genetic heterogeneity of cytogenetically normal AML with mutations of CEBPA. Blood Adv. 2018;2:2724-31.