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ORIGINAL ARTICLE

Expression of myeloperoxidase and gene mutations in AML patients with normal karyotype: double *CEBPA* mutations are associated with high percentage of MPO positivity in leukemic blasts

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Abstract The percentage of myeloperoxidase (MPO)-positive blast cells is a simple and highly significant prognostic factor in AML patients. It has been reported that the high MPO group (MPO-H), in which >50% of blasts are MPO activity positive, is associated with favorable karyotypes, while the low MPO group (≤50% of blasts are MPO activity positive, MPO-L) is associated with adverse karyotypes. The MPO-H group shows better survival even when restricted to patients belonging to the intermediate chromosomal risk group or those with a normal karyotype. It has recently been shown that genotypes defined by the mutational status of *NPM1*, *FLT3*, and *CEBPA* are associated with treatment outcome in patients with cytogenetically normal AML. In this study, we aimed to evaluate the relationship between MPO positivity and gene mutations found in normal karyotypes. Sixty AML patients with normal karyotypes were included in this study. Blast cell MPO positivity was assessed in bone marrow smears stained for MPO. Associated genetic lesions (the *NPM1*, *FLT3*-ITD, and *CEBPA* mutations) were studied using nucleotide sequencing. Thirty-two patients were in the MPO-L group, and 28 patients in the MPO-H group. *FLT3*-ITD was found in 11 patients (18.3%), *NPM1* mutations were found in 19 patients (31.7%), and *CEBPA* mutations were found in 11 patients (18.3%). In patients with *CEBPA* mutations, the carrying two simultaneous mutations ($CEBPA^{double\text{-}mut}$) was associated with high MPO expression, while the mutant *NPM1* without *FLT3*-ITD genotype was not associated with MPO activity. Both higher MPO expression and the $CEBPA^{double\text{-}mut}$ genotype appeared to be associated with improved overall survival after intensive chemotherapy. Further studies are required to determine the importance of blast MPO activity as a prognostic factor, especially in *CEBPA* wild-type patients with a normal karyotype.

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1 Introduction

The AML87, -89, and -92 studies conducted by Japan Adult Leukemia Study Group (JALSG) revealed that patient age, ECOG performance status, leukocyte count, FAB subclass, the number of induction courses required to achieve complete remission (CR), the presence of good prognostic chromosomal abnormalities [t(8;21) or inv(16)], and percentage of myeloperoxidase (MPO)-stained positive blast cells at diagnosis were significant risk factors for overall survival (OS) of patients with acute myeloid leukemia (AML) [1]. In more recent AML201 study, it was shown that significant unfavorable prognostic features for OS were adverse cytogenetic risk group [2], age of more than 50 years, WBC more than 20×10^9/L, FAB classification of either M0, M6, or M7, and MPO-positive blasts less than 50% [3]. These observations imply that the percentage of MPO-positive blast cells is one of the important prognostic markers along with cytogenetics and molecular genetic information.

MPO, a microbicidal protein, is considered to be a golden marker for the diagnosis of AML in the French–American–British (FAB) and WHO classifications [4, 5]. In our previous reports [6–8], AML patients with a high percentage of MPO-positive blasts (>50% of blasts are MPO activity positive, MPO-H) had a significantly better complete remission (CR) rate, disease-free survival, and overall survival compared with the low MPO activity positive blast group (≤50% of blasts are MPO activity positive, MPO-L). Most patients with a favorable chromosomal risk profile were in the MPO-H group, and most of the patients with an adverse chromosomal risk profile were in the MPO-L group. The difference in OS between the low and high MPO groups was still observed in a cohort of patients with normal karyotypes, suggesting that MPO is highly expressed in the leukemic blasts of AML patients with a favorable prognosis. To fully understand this phenomenon, it would be important to analyze genetic factors associated with MPO expression, especially in patients with a normal karyotype.

In the WHO classification, mutations of *FLT3*, *NPM1* and *CEBPA* have been emphasized to have prognostic significance in AML patients with normal karyotype. The nucleophosmin 1 gene (*NPM1*) has been shown to be mutated in 45–64% of AML cases with a normal karyotype [9, 10], and *NPM1* mutations are associated with a favorable prognosis in the absence of the internal tandem duplication (ITD) type of fms-related tyrosine kinase-3 gene (*FLT3*) mutation, a known adverse prognostic factor [11]. The CCAAT/enhancer binding protein-alpha gene (*CEBPA*) is another gene that has been shown to be mutated in AML patients with a normal karyotype [12, 13]. Mutations in the *CEBPA* gene are found in 5–14% of all AML cases and are associated with a relatively favorable outcome, and hence, have gained interest as a prognostic marker [14]. Recently, it has been shown that most AML patients with *CEBPA* mutations carry 2 simultaneous mutations ($CEBPA^{\text{double-mut}}$), whereas single mutations ($CEBPA^{\text{single-mut}}$) are less common. In addition it was found that the $CEBPA^{\text{double-mut}}$ genotype is associated with a favorable overall and event-free survival [15, 16]. It is still unclear why $CEBPA^{\text{double-mut}}$ AML patients have better outcomes than those with a single heterozygous mutation.

In this study, we retrospectively examined 60 de novo adult AML patients with normal karyotypes in order to obtain a better insight into the relationships between MPO positivity and other prognostic factors (*NPM1*, *FLT3*, and *CEBPA* mutations). In line with previous reports, both high MPO positivity in AML blasts and the $CEBPA^{\text{double-mut}}$ genotype appeared to be associated with a favorable outcome, and it appeared that it was the $CEBPA^{\text{double-mut}}$ genotype that associated with high blast MPO activity.

2 Materials and methods

2.1 Patients and treatments

The study population included 60 patients with newly diagnosed de novo AML that had been treated at the Department of Internal Medicine, Nagasaki National Medical Center, between 1990 and 2010. All patients had normal karyotype AML. AML was diagnosed according to the FAB classification. Two members independently assessed the percentage of MPO-positive blast cells in MPO-stained bone marrow smears. The main biological and clinical features of the patients are shown in Table 1. Excluding the 25 patients who did not receive conventional induction chemotherapy, all patients were treated according to the Japan Adult Leukemia Study Group (JALSG) protocols (AML89, -92, -95, -97, and -201 studies) [3, 17–19]. CR was determined as when blasts accounted for less than 5% of the cells in normocellular bone marrow with normal peripheral neutrophil and platelet counts. This study was approved by the Ethical Committees of the participating hospitals.

2.2 Analysis of the *FLT3*, *NPM1*, and *CEBPA* genes

High molecular weight genomic DNA was extracted from bone marrow and peripheral blood samples after Ficoll

Table 1 Characteristics of de novo AML patients with a normal karyotype

	All patients ($n = 60$)	Patients who received intensive chemotherapy ($n = 36$)
Median age (range) (year)	59.5 (15–81)	49 (15–67)
Male/female	32/28	18/18
FAB type		
M0	5	3
M1	10	5
M2	21	14
M4	18	11
M5	3	1
M6	3	2
M7	0	0
WBC ($\times 10^9$/L), median (range)	14.9 (0.7–556)	13.0 (0.7–246)
Performance status		
0–2	55	34
3–4	5	2
LDH (IU/L), median (range)	296 (120–5,325)	291 (140–2,606)
MPO		
Low (≤50%)	32	20
High (>50%)	28	16

FAB French–American–British, *WBC* white blood cells, *LDH* lactate dehydrogenase, *MPO* myeloperoxidase

separation of mononucleated cells (35 and 4 patients, respectively) using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). In addition, we isolated genomic DNA from the BM smears of the AML patients (21 samples) using the QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany).

Mutations in the *FLT3*, *NPM1*, and *CEBPA* genes were detected by genomic DNA PCR and direct sequencing. Exons 14 and 15 and the intervening intron of the *FLT3* gene were amplified from DNA using the previously described primers FLT3-11F and FLT3-12R [20]. PCR for NPM1 exon 12 was performed with genomic DNA, the same reagent, and the published primer molecules NPM1-F and NPM1-R [21]. PCR for *CEBPA* was performed using 2 overlapping primer pairs: CEBPA-CT3F (5′-TGCCGGGTATAAAA-GCT GGG-3′) and CT3R (5′-CTCGTTGCTGTTCTTGTCCA -3′), CEBPA-PP2F (5′-TGCCGGGT-ATAAAAGCTGG G-3′) and PP2R (5′-CACGGTCTGGGCAAGCCTCG AGAT-3′). The PCR reactions were run in a final volume of 50 μL containing 10 ng DNA, 5× buffer, 0.2 mmol/L of each deoxynucleotide triphosphate, primers (0.3 μmol/L of each), nucleotides (0.2 mmol/L of each), and 1 U of KOD-Plus-Neo polymerase (TOYOBO, Osaka, Japan). The mixture was initially heated at 94°C for 2 min, before being subjected to 35 cycles of denaturation at 94°C for 10 s and annealing and extension at 68°C for 1 min. The amplified products were cut out from a 1.2% agarose gel and purified with the MinElute Gel extraction kit (QIAGEN, Germany). To screen for mutations, the PCR products were sequenced in both directions with the following primers: FLT3-11F, FLT3-12R, NPM1-F, NPM-R, CEBPA-CT1F, CEBPA-1R, CEBPA-PP2F, CEBPA-PP2R, CEBPA-2F (5′-GCTG GGCGGCATCTGCG-A-3′), and CEBPA-1R (5′-TGT-GC TGGAACAGGTCGGCCA-3′) using a BigDye Terminator v3.1 Cycle Sequencing Kit and the ABI Prism 3100 ×1 Genetic Analyzer (Applied Biosystems, CA, USA). In the case of *NPM1* and *CEBPA* genes, when heterozygous data were identified by sequence screening, mutations were confirmed by cloning with the StrataClone Blunt PCR Cloning Kit (Stratagene, CA, USA) according to the manufacturer's recommendations. Four to ten recombinant colonies were chosen and cultured in LB medium. Plasmid DNA was prepared using a QIAprep spin plasmid miniprep kit (Qiagen, Hilden, Germany), and both strands were sequenced using the T3 and T7 primers and the CEBPA-2F and CEBPA-1R primers.

2.3 Statistical methods

To evaluate the relationship between the frequency of mutations status and clinical characteristics, the following variables were included in the analysis: age, FAB classification, peripheral WBC count, MPO-positivity rate, JALSG score [1], and CR achievement. A comparison of frequencies was performed using Fisher's exact test. Differences in percentage of MPO-positive blasts among patients with different mutational status of genes were compared using the non-parametric Kruskal–Wallis test and followed by Dunn's multiple comparison post-test. Overall survival (OS) was calculated using the Kaplan–Meier method [22], and the group differences were compared using the log-rank test. Thirteen patients who underwent allogeneic or autologous hematopoietic stem cell transplantation were not censored at the time of transplantation. For all analyses, statistical significance was considered at the level of two-tailed 0.05.

3 Results

3.1 Patients' characteristics

As shown in Table 1, the series included 60 patients. Their median age was 59.5 (15–81 years), and there were 32 males (53.3%) and 28 females (46.7%). All patients had normal cytogenetics. Using the percentage of MPO-positive leukemic blasts, as judged from bone marrow slides, the cases

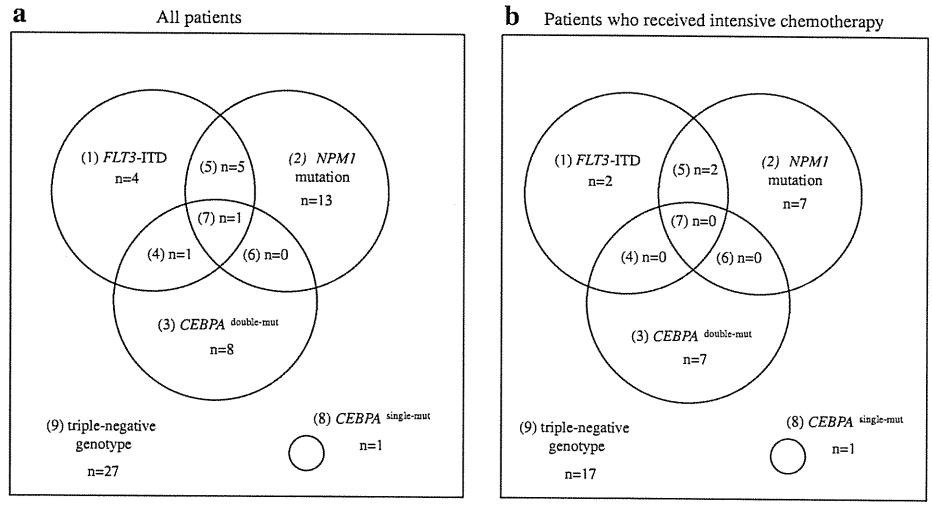

Fig. 1 Frequency and overlapping patterns of AML patients with a normal karyotype. Data are shown for all patients (**a**) and for patients who received intensive chemotherapy (**b**). **a** (*1*) *FLT3*-ITD + wt *NPM1* + wt *CEBPA* ($n = 4$, 6.7%), (*2*) wt *FLT3* + *NPM1* mutation + wt *CEBPA* ($n = 13$, 21.7%), (*3*) wt *FLT3* + wt *NPM1* + $CEBPA^{\text{double-mut}}$ ($n = 8$, 13.3%), (*4*) *FLT3*-ITD + wt *NPM1* + $CEBPA^{\text{double-mut}}$ ($n = 1$, 1.7%), (*5*) *FLT3*-ITD + *NPM1* mutation + wt *CEBPA* ($n = 5$, 8.3%), (*6*) wt *FLT3* + *NPM1* mutation + $CEBPA^{\text{double-mut}}$ ($n = 0$, 0%), (*7*) *FLT3*-ITD + *NPM1* mutation + $CEBPA^{\text{double-mut}}$ ($n = 1$, 1.7%), (*8*) wt *FLT3* + wt *NPM1* + $CEBPA^{\text{single-mut}}$ ($n = 1$, 1.7%), (*9*) triple-negative genotype ($n = 27$, 45%). **b** (*1*) *FLT3*-ITD + wt *NPM1* + wt *CEBPA* ($n = 2$, 5.6%), (*2*) wt *FLT3* + *NPM1* mutation + wt *CEBPA* ($n = 7$, 19.4%), (*3*) wt *FLT3* + wt *NPM1* + $CEBPA^{\text{double-mut}}$ ($n = 7$, 19.4%), (*4*) *FLT3*-ITD + wt *NPM1* + $CEBPA^{\text{double-mut}}$ ($n = 0$, 0%), (*5*) *FLT3*-ITD + *NPM1* mutation + wt *CEBPA* ($n = 2$, 5.6%), (*6*) wt *FLT3* + *NPM1* mutation + $CEBPA^{\text{double-mut}}$ ($n = 0$, 0%), (*7*) *FLT3*-ITD + *NPM1* mutation + $CEBPA^{\text{double-mut}}$ ($n = 0$, 0%), (*8*) wt *FLT3* + wt *NPM1* + $CEBPA^{\text{single-mut}}$ ($n = 0$, 0%), (*9*) triple-negative genotype ($n = 17$, 47.2%). *wt* wild-type

were divided into the High group (MPO-positive blasts > 50%) and Low group (MPO-positive blasts ≤ 50%). Thirty-two patients were classified into the Low group, and 28 patients were classified into the High group.

3.2 Mutational analysis

FLT3-ITD was found in 11 patients (18.3%), *NPM1* mutations were found in 19 patients (31.7%), and *CEBPA* mutations were found in 11 patients (18.3%). Frequency and an overlapping pattern of mutations are shown in Fig. 1. Among the patients with *CEBPA* mutations, approximately 90% (10 of 11 patients) of the patients had two *CEBPA* mutations ($CEBPA^{\text{double-mut}}$), whereas 10% (1 of 11 patients) had a single mutation. As previously reported, the mutations in the $CEBPA^{\text{double-mut}}$ patients were clustered in the N- and C-terminal hotspots (Table 2; Fig. 2). *FLT3*-ITD mutation was associated with a higher WBC at the time of diagnosis, as reported previously. Neither *NPM1* nor *CEBPA* mutation status displayed a significant association with age, PS, WBC, FAB subtype, JALSG score, or CR achievement (Table 3).

3.3 Clinical outcome

OS was analyzed only in patients who received intensive chemotherapy ($n = 36$). They received chemotherapy based on the treatment protocol described in the JALSG AML89, -92, -95, -97, and -201 studies. As reported previously [6], we observed an association between the percentage of MPO-positive blasts and the survival rate in the normal karyotype patients treated with intensive chemotherapy, although the significance in this cohort was rather low ($P = 0.10$) (Fig. 3). Figure 4 shows Kaplan–Meier curves according to genotype. 'Other genotypes' included the *FLT3*-ITD genotype, the $CEBPA^{\text{single-mut}}$ genotype, and the triple-negative genotype consisting of the wild-type *NPM1* and *CEBPA* genotypes without *FLT3*-ITD. In line with previous reports [14], the patients with the $CEBPA^{\text{double-mut}}$ genotype tended to show higher survival rate compared with patients displaying other genotypes ($P = 0.07$). In this study, the mutant *NPM1* without *FLT3*-ITD genotype was not significantly associated with treatment outcome, possibly due to the small number of patients.

3.4 Difference of MPO-positivity rate by gene mutation status

Figure 5 shows the level of the percentage of MPO-positive blasts by gene mutational status of the *CEBPA*, *FLT3*-ITD, and *NPM1*. The MPO-positivity rate was very high, over 50% (median 96, range 71–100), in all $CEBPA^{\text{double-mut}}$ cases, but it was 20% in one case displaying the

Table 2 Genetic findings of the patients with *CEBPA* mutations

Patient	Category	Nucleotide changes	Amino acid changes	Comments
4	Double	218_219insC	P23fsX107	Produces N-terminal stop codon
		1129_1130insATGTGGAGACGCAGCAGAAGGTGCTGGAGCTG ACCAGTGACAATGACCGCCTGCGCAAGC	K326_327insHVETQQKVLELTSDNDRLRKR	In-frame insertion in bZIP
6	Double	200_218delinsCT	S16fsX101	Produces N-terminal stop codon
		1087_1089dup	K313dup	In-frame duplication in bZIP
7	Double	368_369insA	A72fsX107	Produces N-terminal stop codon
		1080_1082del	T310_Q311del	In-frame deletion in bZIP
13	Double	303_316del	P50fsX102	Produces N-terminal stop codon
		1062_1063insTTG	K304_Q305insV	In-frame insertion in bZIP
19	Double	215_225del	P21fsX103	Produces N-terminal stop codon
		1101_1102insCAGCGCAACGTGGAGACGCAGCAGA AGGTGCTGGAGCTG	L317_T318insQRNVETQQKVLEL	In-frame insertion in bZIP
22	Double	213del	P22fsX159	Produces N-terminal stop codon
		1064_1129dup	K304_Q305insQRNVETQQKVLELTSDNDRLRKR	In-frame insertion in bZIP
27	Double	324_328dup	E59fsX161	Produces N-terminal stop codon
		1062_1063insTTG	K304_Q305insV	In-frame insertion in bZIP
39	Double	213del	P22fsX159	Produces N-terminal stop codon
		1081_1086dup	Q311_Q312dup	In-frame duplication in bZIP
47	Double	397del	F82fsX159	Produces N-terminal stop codon
		1101_1102insCAGCGCAACGTGGAGACGCAGCA GAAGGTGCTGGAGCTG	L317_T318insQRNVETQQKVLEL	In-frame insertion in bZIP
49	Double	297_304del	A48fsX104	Produces N-terminal stop codon
		758del	A202fsX317	Frameshift between TAD2 and bZIP; produces stop codon in bZIP
35	Single	1087_1089dup	K313dup	In-frame duplication in bZIP

Nucleotide numbering was performed according to NCBI Entrez accession no. XM_009180.3, in which the major translational start codon starts at nucleotide position 151. The locations of functional domains are derived from Mueller and Pabst.1

bZIP basic leucine zipper region, *TAD2* second transactivation domain

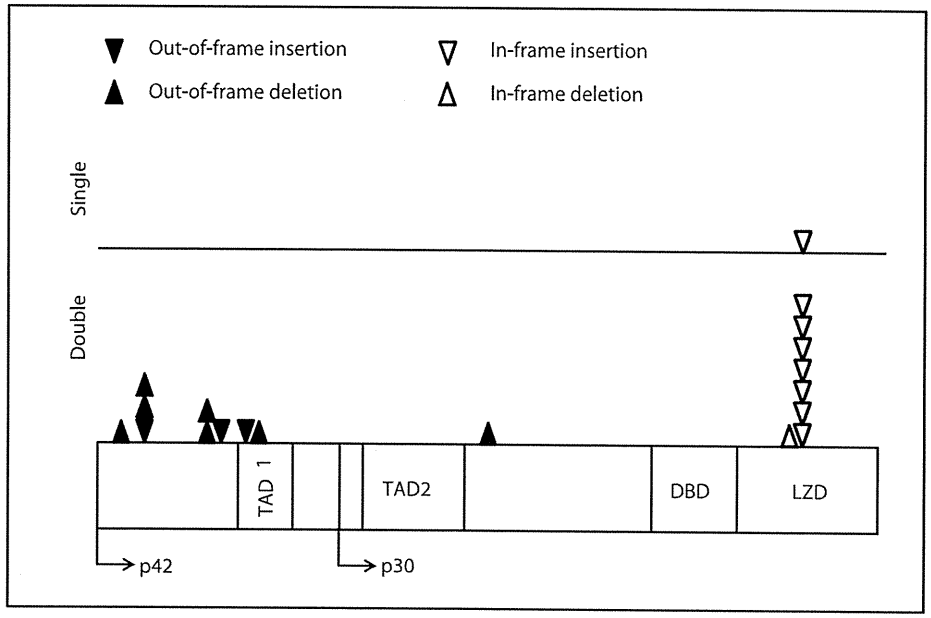

Fig. 2 Location of mutations detected in the *CEBPA*$^{single\text{-}mut}$ and *CEBPA*$^{double\text{-}mut}$ patients. Transactivation domain (TAD) 1, amino acids (AA) 70–97; p30 ATG, AA120; TAD2, AA 126–200; DNA-binding domain (DBD), AA 278–306; leucine zipper domain (LZD), AA 307–358

CEBPA$^{single\text{-}mut}$ genotype (data not shown). The MPO-positivity rate was widely distributed in patients who had mutant *NPM1* without *FLT3*-ITD genotype (median 26, range 0–100) and other genotypes (median 31, range 0–100). Kruskal–Wallis test showed that a significant difference of the MPO-positivity rate among three groups ($P = 0.005$). When comparing the individual groups by Dunn's Multiple Comparisons post hoc test for each group, there was a significant difference only for patients with *CEBPA*$^{double\text{-}mut}$ versus patients with other genotypes.

4 Discussion

While cytogenetic group is considered to be the primary prognostic indicator in AML, the percentage of MPO-positive blast cells could be used to predict the prognosis of patients with normal karyotypes [6]. In this study, we found that *CEBPA* gene mutational status has impact on the frequency of MPO expression: the patients with the *CEBPA* mutation genotype displayed a significantly higher percentage of cells expressing MPO than those with other genotypes ($P < 0.01$). The association was even more significant when analyzed without the *CEBPA*$^{single\text{-}mut}$ carrying patient, suggesting that high blast MPO activity is related to double *CEBPA* mutations. Although the mutant *NPM1* without *FLT3*-ITD genotype has been reported to be associated with a favorable prognosis in AML patients, there was no relationship between this type of mutation and the percentage of blasts showing MPO expression.

It is not clear how the *CEBPA*$^{double\text{-}mut}$ genotype enhances MPO activity in AML blasts. It has been shown that the MPO enhancer contains a *CEBPA* site contributing to its functional activity [23, 24], suggesting that the MPO gene is a major target of C/EBPα. Since it has been shown that both N-terminal frame-shift mutant and C-terminal mutant do not show transcriptional activity [25], we first speculated that mutations of the *CEBPA* gene might lead to decreased MPO activity, which turned out to be wrong. AML1 is another gene that has been reported to participate in up-regulation of MPO gene [26]. An AML1 site was identified in upstream enhancer of the human MPO gene, which appears to be necessary for maximal stimulation of MPO promoter activity. In patients with AML with t(8;21), the translocation results in an in-frame fusion between 5 exons of the AM1 gene and essentially all of the ETO gene producing a chimeric protein [27]. This protein, AML1-ETO, acts as a negative dominant inhibitor of wild-type AML1 [28], which theoretically could lead to down-regulation of AML1 target genes, such as MPO gene. However, blasts with t(8;21) have been shown to display higher levels of MPO expression both in clinical samples and in vitro experiments [29, 30], suggesting that the transcriptional alterations caused by these mutations are complex. The upregulation of blast MPO activity seen in CEBP/$\alpha^{double\text{-}mut}$ cases may be due to alterations in the gene expression profile, rather than a simple dominant negative effect of mutated CEBP/α. Further experiments including investigation of transactivation potential of CEBP/α mutants on MPO promoter is necessary to clarify this mechanism.

CEBPA mutations are associated with a relatively favorable outcome, and it was recently shown in a multivariable analysis including cytogenetic risk and the

Table 3 Frequency *of FLT3*-ITD, *NPM1*, and *CEBPA* mutations by clinical characteristics in de novo AML cases with a normal karyotype

	FLT3		*P*	*NPM1*		*P*	*CEBPA*		*P*
	ITD ($n = 11$)	Other type ($n = 49$)		Mutation without *FLT3*-ITD ($n = 13$)	Other type ($n = 47$)		Double mutation without *FLT3*-ITD ($n = 8$)	Other type ($n = 52$)	
Age			0.08			0.74			0.10
≤50	1	19		5	15		5	15	
>50	10	30		8	32		3	37	
PS			1.00			0.20			0.52
0–2	10	45		11	45		7	48	
3–4	1	4		2	2		1	4	
WBC			0.02			1.00			1.00
≤20,000	2	30		7	25		4	28	
>20,000	9	19		6	22		4	24	
FAB subtype			0.33			0.18			0.58
M1, M2, M4, M5	11	41		13	39		8	44	
M0, M6, M7	0	8		0	8		0	8	
JALSG score[a]			0.79			0.72			0.09
Favorable	0	5		0	5		2	3	
Intermediate	2	18		5	15		5	15	
Adverse	2	9		2	9		0	11	
CR[a]			1.00			0.56			0.56
Achievement	4	27		7	24		7	24	
Failure	0	5		0	5		0	5	

[a] Analysis was carried in 36 patients with intensive chemotherapy

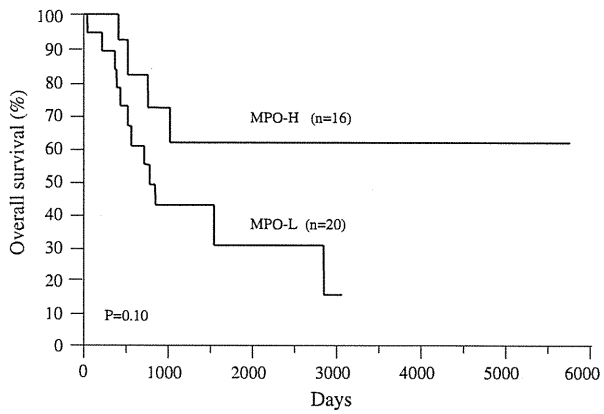

Fig. 3 Kaplan–Meier estimates of the probability of overall survival in 36 patients who received intensive chemotherapy, according to the percentage of myeloperoxidase-positive blasts. MPO-H (MPO-positive blasts: >50%) tended to have a positive effect on overall survival compared with MPO-L (MPO-positive blasts: ≤50%), although the difference was not statistically significant. The statistical significance of differences was evaluated with the log-rank test

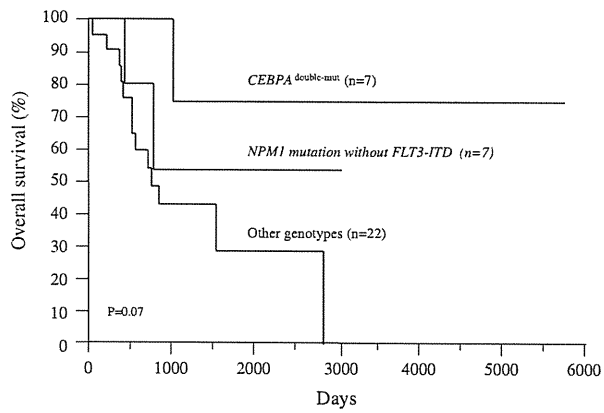

Fig. 4 Overall survival according to genotype in patients administered intensive chemotherapy. 'Other genotypes' was defined as the *FLT3*-ITD genotype, the *CEBPA*$^{\text{single-mut}}$ genotype, and the triple-negative genotype consisting of the wild-type *NPM1* and *CEBPA* genotypes without *FLT3*-ITD. The patients with the *CEBPA*$^{\text{double-mut}}$ genotype tended to show higher overall survival compared with the patients with 'other genotypes' ($P = 0.07$)

FLT3-ITD and *NPM1* mutations that the *CEBPA*$^{\text{double-mut}}$ genotype is associated with favorable overall and event-free survival [15, 16]. In a cohort of 60 cases of adult de novo AML, we identified 1 *CEBPA*$^{\text{single-mut}}$ case and 10 *CEBPA*$^{\text{double-mut}}$ cases, and in line with previous reports, our study tended to show better overall survival in *CEBPA*$^{\text{double-mut}}$ cases compared to cases with wild-type *CEBPA* in patients treated with intensive chemotherapy. We failed to find a prognostic effect in relation to the *CEBPA*$^{\text{double-mut}}$ in patients treated with low dose

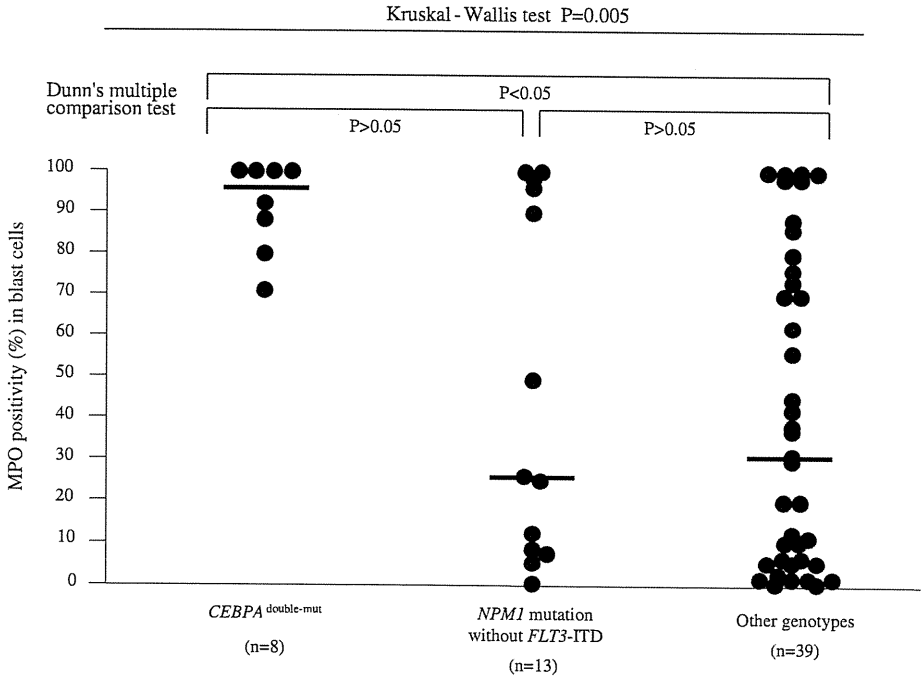

Fig. 5 MPO-positivity rate in blast according to genetic abnormalities in de novo AML patients with a normal karyotype. 'Other genotypes' was defined as the *FLT3*-ITD genotype, the *CEBPA*$^{\text{single-mut}}$ genotype, and the triple-negative genotype consisting of the wild-type *NPM1* and *CEBPA* genotypes without *FLT3*-ITD. The median MPO-positivity rate (*horizontal line*) was significantly different between the *CEBPA*$^{\text{double-mut}}$ genotype and 'other genotypes' (Kruskal–Wallis test followed by Dunn's multiple comparisons test: $P < 0.05$)

chemotherapy (data not shown), suggesting that the standard chemotherapy dose is necessary to improve the outcome of *CEBPA*$^{\text{double-mut}}$ cases.

It is unclear why *CEBPA*$^{\text{double-mut}}$ AML patients have a better outcome than those with *CEBPA* wild-type AML. One explanation is that high MPO expression leads to increased sensitivity to chemotherapeutic agents, such as to Ara-C [8]. To test this hypothesis, we also examined the association between blast MPO positivity and overall survival in *CEBPA* wild-type cases. Unexpectedly, when the patients were treated with intensive chemotherapy, the percentage of MPO-positive blasts was not significantly associated with overall survival in this group (data not shown), suggesting that the level of MPO expression itself is not responsible for the improvement in overall survival. However, as this analysis only involved 28 cases, we need to increase the number of cases in order to draw a definitive conclusion.

In summary, the data presented here suggested that the *CEBPA*$^{\text{double-mut}}$ genotype was associated with high MPO blast activity in patients with a normal karyotype. Although the results were obtained from a single institution, the presence of *CEBPA*$^{\text{double-mut}}$ genotype in high MPO group could explain, at least in part, why high MPO blast activity is associated with better overall survival. Further studies in a larger cohort of patients are necessary to assess blast MPO activity as a prognostic factor, especially in *CEBPA* wild-type patients with a normal karyotype.

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Conflict of interest All authors have no conflict of interest to report.

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ORIGINAL ARTICLE

Clinical significance of subcategory and severity of chronic graft-versus-host disease evaluated by National Institutes of Health consensus criteria

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Abstract To evaluate the clinical significance of subcategory and severity of chronic graft-versus-host disease (GVHD) as defined by the National Institutes of Health (NIH) consensus criteria, we retrospectively studied 211 patients with hematologic neoplasms who survived beyond 100 days after allogeneic hematopoietic cell transplantation. Endpoints included chronic GVHD-specific survival (cGSS), duration of immunosuppressive treatment, and non-relapse mortality (NRM). A total of 96 patients fulfilled the NIH diagnostic criteria for cGVHD. In univariable analysis, patients with NIH overlap syndrome tended to exhibit lower cGSS compared to those with NIH classic cGVHD [hazard ratio (HR) = 2.76, $P = 0.060$], while patients with severe cGVHD at onset had a significantly lower cGSS compared to those with mild-to-moderate cGVHD (HR = 3.10, $P = 0.034$). The duration of immunosuppressive treatment was not significantly affected by either subcategory or severity of NIH cGVHD. In multivariable analysis treating cGVHD as a time-dependent covariate, development of overlap syndrome (HR = 3.90, $P = 0.014$) or severe cGVHD at peak worsening (HR = 6.21, $P < 0.001$) was significantly associated with higher risk of NRM compared to the absence of cGVHD. Our results suggest that both the subcategory and severity of NIH cGVHD are partly correlated with cGSS and may play a useful role in distinguishing patients at high risk for NRM, warranting validation of this approach through future prospective studies.

Keywords Hematopoietic cell transplantation · Chronic graft-versus-host disease · NIH consensus criteria

1 Introduction

Chronic graft-versus-host disease (cGVHD) remains a serious complication associated with substantial late morbidity and mortality after allogeneic hematopoietic cell transplantation (allo-HCT). In contrast to acute GVHD (aGVHD), which preferentially affects specific organs such as the skin, liver, and gastrointestinal tract, cGVHD presents with protean organ dysfunctions and various degrees of immunodeficiency that is further worsened by immunosuppressive medications used for relieving symptoms associated with GVHD [1]. Previous studies have identified a variety of factors that increase the risk of the development of cGVHD, including a prior history of aGVHD, older patient age, use of alloimmune female donors for male recipients, transplants from unrelated or human leukocyte antigen (HLA)-mismatched donors, and use of peripheral blood grafts [2–10]. In this context, clinical management of cGVHD has increasingly become more important, because recent trends in allo-HCT such as expanding applications of peripheral blood stem cell

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transplantation after reduced-intensity conditioning in older patients may increase the incidence of cGVHD [11].

Historically, aGVHD and cGVHD were distinguished based on whether immune-mediated organ dysfunction occurred within 100 days or more than 100 days after transplantation. However, accumulating experience has indicated that clinical manifestations similar to aGVHD can develop even several months after allo-HCT, while GVHD with typical features of the "chronic" form can occur as early as 2 months post-transplantation [12, 13]. Therefore, an arbitrary classification using the timing of GVHD onset is no longer considered appropriate. Another drawback in the management of cGVHD is that the grading criteria for its severity has not been standardized: it is difficult to predict the risk of GVHD-associated mortality by using historic classification that categorizes cGVHD into limited and extensive subtypes [14], because clinical severity as well as organ involvement of patients classified as having extensive cGVHD varies considerably [15–17].

To resolve these issues, the National Institutes of Health (NIH) consensus criteria were recently proposed to standardize the diagnosis and global assessment of cGVHD with a new severity scoring system based on organ-specific manifestations taking functional impact into account [18]. The NIH criteria distinguished two subcategories of cGVHD, "classic cGVHD" without features of aGVHD and "an overlap syndrome" in which characteristic features of both cGVHD and aGVHD are simultaneously present. In particular, features of aGVHD occurring beyond day 100 without manifestations of classic cGVHD are classified as "persistent", "recurrent", or "late-onset" aGVHD. Based on the number of involved organs and the severity within affected organs, each subcategory of cGVHD was graded into mild, moderate, or severe subtype. However, clinical significance of NIH cGVHD subcategory as well as their severity is not fully established, although several studies have shown their impact on overall survival, cGVHD-specific survival (cGSS), and non-relapse mortality (NRM) [19–23].

In the present study, we retrospectively evaluated patients who received allo-HCT for intractable hematologic disorders with special focus on the influences of subcategory and severity of NIH cGVHD on clinical outcomes. Since probabilities of GVHD-specific survival and discontinued immunosuppressive treatment (IST) have been most commonly used as surrogate endpoints representing the clinical resolution of cGVHD [24–26], we analyzed factors associated with these outcomes in patients who developed NIH cGVHD. We also evaluated the impact of the presence or absence of each subtype of NIH cGVHD on NRM.

2 Patients and methods

2.1 Patients

We retrospectively reviewed the medical records of 259 consecutive patients with hematologic disorders who underwent allo-HCT between January 2000 and December 2008 in our department and survived at least 100 days after transplantation. Patients were excluded if they had a history of previous allo-HCT ($n = 24$), rejected graft ($n = 4$), or relapsed before day 100 ($n = 20$); thus, a total of 211 patients were included in the present analysis. No patients received donor lymphocyte infusions before day 100. Patients with malignant hematologic neoplasms were defined as having standard-risk disease if they underwent transplantation in first complete remission or without prior chemotherapy, while those who underwent transplantation in any other status were classified as having high-risk disease. Patients with aplastic anemia were considered to have standard-risk disease. This study was approved by the Ethics Committee of Kyoto University Graduate School of Medicine. Written informed consent for the transplantation protocol was obtained from all patients.

2.2 Transplantation procedure

Patients with malignant hematologic neoplasms received myeloablative or fludarabine-based reduced-intensity conditioning regimens with or without total-body irradiation (TBI) as described elsewhere [27, 28]. Patients with aplastic anemia received conditioning regimens consisting of high-dose cyclophosphamide and horse or rabbit antilymphocyte globulin with or without 2–4 Gy TBI. None of these patients received T-cell-depleted grafts. All patients received GVHD prophylaxis by the use of cyclosporine or tacrolimus combined with or without short-term methotrexate. A proportion of patients given transplants from HLA-mismatched family members or unrelated marrow donors received mycophenolate mofetil in addition to tacrolimus plus methotrexate as GVHD prophylaxis [28]. All patients received supportive care including blood product transfusion and prophylaxis against opportunistic infections according to our institutional protocols [29].

2.3 Evaluation and management of acute and chronic GVHD

All patients were graded for aGVHD using conventional criteria, and the maximum grade until day 100 after transplantation was assigned [30]. Patients who developed grade II–IV aGVHD were initially treated with methylprednisolone or prednisolone usually at a dose of 1–2 mg/kg. Treatment of steroid-refractory aGVHD was variable.

The incidence of cGVHD was retrospectively evaluated by using the NIH consensus criteria [18]. Patients who had at least one "diagnostic" clinical sign or at least one "distinctive" manifestation, confirmed by relevant laboratory tests or histologic examination, were defined as having cGVHD if other possible diagnoses were excluded. Subclassification of cGVHD into "classic cGVHD" and "overlap syndrome" was strictly according to the NIH criteria. If patients had any features of aGVHD along with classic cGVHD, they were classified as having an overlap syndrome. The severity of cGVHD was assessed at its onset and at maximal clinical worsening and graded into "mild", "moderate", and "severe" categories according to the global scoring system defined by the NIH criteria. Treatment of cGVHD was variable, but followed some general principles; patients with isolated mouth, ocular, or localized skin cGVHD were treated only with topical therapy, while patients with more symptomatic cGVHD were treated with systemic immunosuppressive agents such as prednisolone at a dose of 0.5–1.0 mg/kg per day combined with calcineurin inhibitors. Although the duration and dosing of those agents were not standardized, patients typically received treatment until all symptoms of cGVHD were resolved or stabilized. Patients with less severe symptoms were often treated with peroral low-dose prednisolone at a dose of less than 0.5 mg/kg per day.

2.4 Statistical analysis

Descriptive statistics were used to summarize variables related to patient and transplant characteristics. Comparisons among the groups were performed by use of extended Fisher exact test for categorical variables and Wilcoxon–Mann–Whitney test for continuous variables. The primary endpoint of the study was cGSS, which is defined as the time from the day of diagnosis of cGVHD to the day of death in the absence of relapse or secondary malignancy, among patients who developed NIH cGVHD stratified by its subcategory or severity at onset. The probabilities of cGSS were estimated according to the Kaplan–Meier method, and univariable comparison between groups was made using the log-rank test. Patients who were alive without recurrent or secondary malignancy were censored at their last follow-up visit and those who experienced recurrent or secondary malignancy were censored at the time of its diagnosis. The time to discontinuation of IST was defined among patients who received systemic IST for the treatment of NIH cGVHD as the time from the day of diagnosis of cGVHD to the day of withdrawal of systemic IST. NRM was defined among all patients included in the study as rates of death without evidence of primary disease recurrence. The incidence rates of IST withdrawal and those of NRM were estimated with the use of the cumulative incidence method to accommodate the following competing events [31]: the onset of recurrent or secondary malignancy and death from any cause for IST withdrawal, and the recurrent primary disease for NRM. Cox proportional-hazards regression models were used to evaluate variables potentially associated with cGSS, while competing risks regression models were used to evaluate variables potentially associated with IST withdrawal and NRM [32]. The variables included in the analysis were as follows: patient age, donor–recipient sex combination, disease status at the time of transplantation, donor–recipient HLA compatibility, stem cell sources, type of conditioning regimens, grades of prior aGVHD (grades 0–1 vs. grades 2–4), subcategory of NIH cGVHD (classic cGVHD vs. overlap syndrome), global severity of NIH cGVHD at onset (mild to moderate vs. severe), platelet counts, eosinophil counts, and administration of systemic corticosteroids at the onset of cGVHD. In the analysis to evaluate the impact of the presence of each NIH cGVHD subtype on NRM for the entire cohort of patients in the study, development of each subtype of cGVHD was treated as a time-dependent covariate under the assumption that a patient who developed moderate or severe cGVHD could not revert to less severe cGVHD and that classic cGVHD and overlap syndrome could not switch to each other [33]. Factors having two-sided P values less than 0.1 for association with outcome were included in multivariable model using a forward and backward stepwise method with a predetermined risk of 0.1. Two-sided P values <0.05 were considered to be statistically significant. All analyses were performed using STATA version 11 (College Station, TX, USA) according to patient information available as of 1 July 2009.

3 Results

3.1 Patient characteristics

Table 1 shows the characteristics of the 211 patients included in the study; they had a median age of 46 years, included 113 males and 98 females, and underwent transplantation for malignant hematologic neoplasms in most cases. The number of patients who received bone marrow, peripheral blood, and cord blood unit was 152 (72%), 44 (21%), and 15 (7%), respectively. After a median follow-up of 37.2 months (range 3.3–111.6), a total of 96 patients (45%) developed manifestations of cGVHD that met the NIH consensus criteria. There was no statistically significant difference in background characteristics between patients who developed NIH cGVHD and those who did not, except that the former group included higher proportion of patients with a history of antecedent grade II–IV aGVHD.

Table 1 Patient and transplantation characteristics

Characteristic	All patients ($n = 211$)	NIH cGVHD		
		Absent ($n = 115$)	Present ($n = 96$)	P value
Median patient age, years (range)	46 (17–69)	46 (19–69)	47 (17–67)	0.90
Donor/recipient sex combination, n (%)				0.17
Male/male	66 (31)	41 (35)	25 (26)	
Male/female	42 (20)	21 (18)	21 (22)	
Female/female	56 (27)	33 (29)	23 (24)	
Female/male	47 (22)	20 (17)	27 (28)	
Diagnosis, n (%)				0.59
Myeloid neoplasms	113 (54)	65 (57)	48 (50)	
Precursor lymphoid neoplasms	31 (15)	17 (15)	14 (15)	
Mature lymphoid neoplasms	61 (29)	29 (25)	32 (33)	
Aplastic anemia	6 (3)	4 (3)	2 (2)	
Disease status at transplant, n (%)				0.41
Standard risk	105 (50)	54 (47)	51 (53)	
High risk	106 (50)	61 (53)	45 (47)	
Donor type[a], n (%)				0.71
HLA-matched related	83 (39)	45 (39)	38 (40)	
HLA-mismatched related	23 (11)	12 (10)	11 (11)	
HLA-matched unrelated	89 (42)	47 (41)	42 (44)	
HLA-mismatched unrelated	16 (8)	11 (10)	5 (5)	
Donor/recipient HLA compatibility[a], n (%)				0.59
Matched	172 (82)	92 (80)	80 (83)	
Mismatched	39 (18)	23 (20)	16 (17)	
Stem cell source, n (%)				0.30
Bone marrow	152 (72)	85 (74)	67 (70)	
Peripheral blood	44 (21)	20 (17)	24 (25)	
Cord blood	15 (7)	10 (9)	5 (5)	
Conditioning regimen, n (%)				0.55
Myeloablative with TBI	113 (54)	64 (56)	49 (51)	
Myeloablative without TBI	15 (7)	10 (9)	5 (5)	
Reduced intensity with TBI	65 (31)	33 (29)	32 (33)	
Reduced intensity without TBI	18 (9)	8 (7)	10 (10)	
GVHD prophylaxis, n (%)				0.73
Tacrolimus based	169 (80)	91 (79)	78 (81)	
Cyclosporine based	42 (20)	24 (21)	18 (19)	
Prior aGVHD, n (%)				0.048
Grade 0–1	117 (55)	70 (61)	47 (49)	
Grade 2	72 (34)	38 (33)	34 (35)	
Grade 3–4	22 (10)	7 (6)	15 (16)	
Median months (range) after transplantation[b]	37.2 (3.3–111.6)	35.6 (3.3–111.6)	40.6 (4.0–105.3)	0.14

cGVHD chronic graft-versus-host disease, *aGVHD* acute graft-versus-host disease, *TBI* total-body irradiation

[a] HLA matching was defined by 2-digit compatibility at HLA-A, -B, and -DRB1 loci

[b] Median follow-up months among patients who were alive at the time of last follow-up

Table 2 summarizes the characteristics of 96 patients who developed NIH cGVHD according to its subcategory; 77 (80%) developed "classic cGVHD" and 19 (20%) developed "overlap syndrome". A total of 31 (40%) patients with classic GVHD and 18 (95%) with overlap syndrome had a prior history of grade II–IV aGVHD. The

median time from transplantation to the onset of cGVHD in patients with overlap syndrome was shorter compared to patients with classic cGVHD (4.1 vs. 7.1 months, $P < 0.001$). All patients with overlap syndrome were graded as having moderate or severe cGVHD, whereas the proportion of patients who developed severe cGVHD was similar between patients with classic cGVHD and those with overlap syndrome. Proportions of patients with platelet counts less than $100 \times 10^3/\mu L$, eosinophil counts less than $500/\mu L$, and ongoing systemic corticosteroid treatment at the onset of cGVHD were higher among patients who developed overlap syndrome compared with those who developed classic cGVHD.

3.2 Chronic GVHD-specific survival

Of the 96 patients who developed NIH cGVHD, recurrent or secondary malignant neoplasm occurred in 27 patients and death due to any cause occurred in 31 patients. The respective 3-year probabilities of cGSS among patients who developed classic cGVHD and overlap syndrome were 88 and 70% ($P = 0.060$) (Fig. 1a), while those among subgroups of patients graded to have mild, moderate, and severe cGVHD at onset were 100, 86, and 69% (mild to moderate vs. severe, $P = 0.034$) (Fig. 1b). Table 3 shows the results of univariable and multivariable analyses for factors potentially associated with cGSS among the patients who developed NIH cGVHD. In univariable analysis, the presence of severe cGVHD and thrombocytopenia at cGVHD onset were significantly associated with lower cGSS, whereas the presence of an overlap syndrome and high-risk malignant disease tended to be associated with lower cGSS. In multivariable analysis, the presence of thrombocytopenia at cGVHD onset was the only significant factor that adversely affected cGSS [hazard ratio (HR) for mortality = 4.05, 95% confidence interval (CI) = 1.35–12.1, $P = 0.013$], although patients with severe cGVHD (HR = 2.58, 95% CI = 0.90–7.39, $P = 0.077$) or those with high-risk underlying disease (HR = 2.75, 95% CI = 0.86–8.80, $P = 0.088$) also had a trend toward lower cGSS.

3.3 Duration of systemic immunosuppressive treatment

A total of 81 patients received systemic immunosuppressive agents for the treatment of NIH cGVHD. In this group of patients, the cumulative incidence of withdrawal of systemic IST was 40% (95% CI = 29–51%) at 3 years after the onset of cGVHD, while the cumulative incidence of the competing risks of death or recurrent/secondary malignancy during systemic IST was 42% (95% CI = 32–55%) (Fig. 2). In univariable analysis, no significant association was found between discontinuation of IST and subcategory or global severity of NIH cGVHD (overlap syndrome vs. classic cGVHD, HR for IST withdrawal = 0.51, 95% CI = 0.20–1.31, $P = 0.16$; severe vs. mild to moderate, HR = 0.90, 95% CI = 0.42–1.96, $P = 0.80$). Multivariable analysis revealed two factors significantly associated with prolonged administration of systemic IST; high-risk primary disease (HR = 0.39, 95% CI = 0.19–0.77, $P = 0.007$) and the ongoing use of systemic corticosteroids at the onset of cGVHD (HR = 0.40, 95% CI = 0.19–0.84, $P = 0.015$).

3.4 Non-relapse mortality

Death from non-relapse causes occurred in 16 (17%) of 96 patients who developed NIH cGVHD and in 10 (9%) of 115 patients who did not. In a multivariable analysis of the entire series of 211 patients, treating the subcategory or peak severity of NIH cGVHD as a time-dependent covariate, development of the overlap syndrome or severe cGVHD was significantly associated with higher risk of NRM compared to the absence of cGVHD (overlap syndrome vs. no cGVHD, HR = 3.90, 95% CI = 1.32–11.6, $P = 0.014$; severe cGVHD vs. no cGVHD, HR = 6.21, 95% CI = 2.25–17.1, $P < 0.001$). Development of classic cGVHD or mild-to-moderate cGVHD was not significantly associated with higher risk of NRM when compared with the absence of NIH cGVHD (classic cGVHD vs. no cGVHD, HR for mortality = 1.39, 95% CI = 0.55–3.53, $P = 0.49$; mild-to-moderate cGVHD vs. no cGVHD, HR = 2.25, 95% CI = 0.62–8.18, $P = 0.22$).

4 Discussion

In the present study, we evaluated the clinical significance of subcategory and severity of NIH cGVHD in terms of their influences on cGSS, discontinuation of IST, and NRM using a retrospective cohort of patients who underwent allo-HCT for hematologic disorders. In univariable analysis, patients with overlap syndrome tended to have a lower probability of cGSS than those with classic cGVHD, while patients who developed severe cGVHD had significantly worse cGSS compared with those who developed mild-to-moderate cGVHD. Although such differences in cGSS according to NIH cGVHD subtypes did not reach statistical significance by multivariable analysis, patients who developed overlap syndrome or severe NIH cGVHD had a significantly higher NRM than those who did not develop any manifestation of NIH cGVHD. These results suggest that both subcategory and global severity of NIH cGVHD might be useful for evaluating the risk of GVHD-associated mortality in patients diagnosed to have cGVHD by the NIH criteria. In

Table 2 Characteristics of chronic GVHD according to subcategory defined by the National Institutes of Health criteria

Characteristics	Total ($n = 96$)	NIH cGVHD subcategory		
		Classic cGVHD ($n = 77$)	Overlap syndrome ($n = 19$)	P value
Median months (range) to onset of cGVHD	6.7 (2.1–29.9)	7.1 (2.7–29.9)	4.1 (2.1–20.7)	<0.001
Involved organs or sites[a], n (%)[b]				0.92
Skin	55 (57)	40 (52)	15 (79)	
Mouth	69 (72)	56 (73)	13 (68)	
Eyes	29 (30)	23 (30)	6 (32)	
Gastrointestinal tract	34 (35)	25 (32)	9 (47)	
Liver	76 (79)	61 (79)	15 (79)	
Lungs	12 (12)	9 (12)	3 (16)	
Joints and fascia	4 (4)	3 (4)	1 (5)	
Genital tract	2 (2)	2 (3)	0 (0)	
Number of involved organs or sites[a], n (%)				0.14
1	7 (7)	7 (9)	0 (0)	
2	27 (28)	24 (31)	3 (16)	
3 or more	62 (65)	46 (60)	16 (84)	
Maximum score of involved organs[a], n (%)				0.18
Score 1	22 (23)	20 (26)	2 (11)	
Score 2 (other than lungs)	26 (27)	18 (62)	8 (42)	
Score 2 (lungs)	6 (6)	4 (5)	2 (11)	
Score 3	42 (44)	35 (45)	7 (37)	
Severity at onset, n (%)				0.023
Mild	20 (21)	20 (26)	0 (0)	
Moderate	53 (55)	39 (51)	14 (74)	
Severe	23 (24)	18 (23)	5 (26)	
Severity at peak, n (%)				0.17
Mild	12 (13)	12 (16)	0 (0)	
Moderate	39 (41)	29 (38)	10 (53)	
Severe	45 (47)	36 (47)	9 (47)	
Platelet count at cGVHD onset, n (%)				0.002
$100 \times 10^3/\mu L$ or more	65 (68)	58 (75)	7 (37)	
Less than $100 \times 10^3/\mu L$	31 (32)	19 (25)	12 (63)	
Eosinophil count at cGVHD onset, n (%)				0.010
Less than 500/μL	68 (71)	50 (65)	18 (95)	
500/μL or more	28 (29)	27 (35)	1 (5)	
Systemic corticosteroids at cGVHD onset, n (%)				<0.001
Not received	63 (66)	61 (79)	2 (11)	
Received	33 (34)	16 (21)	17 (89)	

cGVHD chronic graft-versus-host disease

[a] Data evaluated at peak clinical worsening are shown

[b] The sum of the number per involved site is not equal to the number of evaluable patients, because the involvement of more than one organ can occur in a single patient. Accordingly, the sum of percentage among the total number of patients does not equal to one hundred

contrast, duration of IST was neither affected by NIH cGVHD subcategory nor by its severity.

While cGSS has been frequently used as a study endpoint to describe the mortality attributable to cGVHD-associated organ dysfunction, there have been no established early surrogates that help to guide the clinical management of patients with evidence of ongoing cGVHD. Given that the historic limited/extensive grading system is not a useful predictor for the severity of organ involvement in terms of mortality risk, several studies have attempted to develop

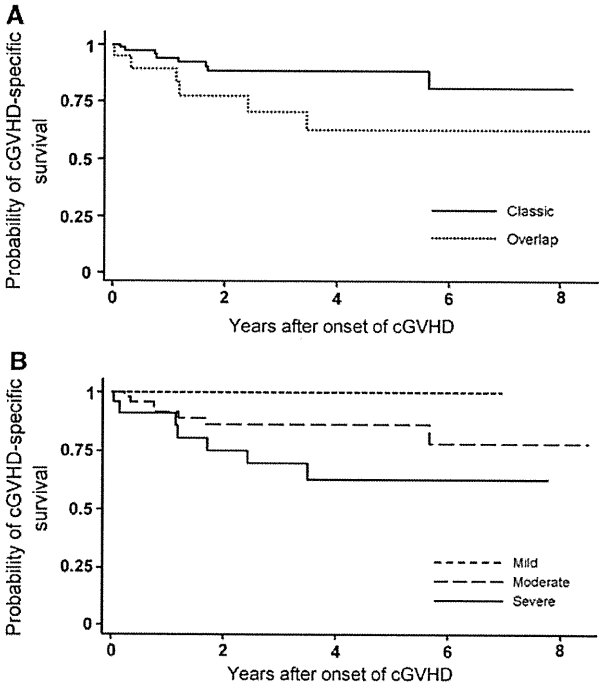

Fig. 1 Chronic GVHD-specific survival in patients with chronic GVHD diagnosed by the NIH consensus criteria. **a** Probability of chronic GVHD-specific survival (cGSS) among patients who developed classic chronic GVHD (*solid line*) and overlap syndrome (*dotted line*). **b** Probability of cGSS among patients who developed mild (*short dashed line*), moderate (*long dashed line*), and severe chronic GVHD (*solid line*)

improved grading scales for established cGVHD. A retrospective analysis of data on HLA-identical sibling transplantation reported to the International Bone Marrow Transplant Registry identified five variables independently associated with worse survival of those who developed historic cGVHD: low Karnofsky performance status at cGVHD diagnosis (<80), chronic diarrhea, weight loss, presence of cutaneous manifestation, and lack of oral involvement [15]. The Seattle group also proposed a revised classification for distinguishing limited and extensive cGVHD by the use of 16 clinical criteria [16]. Although these new classifications do not clearly discriminate between cGVHD and delayed onset GVHD with features resembling aGVHD, they have been shown to be at least useful for identifying patients at higher risk of NRM. Future studies are strongly warranted to compare the prognostic values of NIH cGVHD subcategories with those determined by other cGVHD grading system [21].

So far, several groups have reported the prognostic relevance of cGVHD severity graded by the NIH criteria and consistently found the inferior survival of patients with severe cGVHD [20–23], although such association was not observed in one earlier study [19]. While only a few of these studies focused on the significance of distinction between "overlap syndrome" and "classic cGVHD", our study revealed a trend toward worse survival in patients with overlap syndrome compared to those with classic GVHD, as was recently reported by Kim et al. [23]. In the present study, patients with overlap syndrome had a significantly shorter median time to the development of cGVHD than patients with classic cGVHD and were more likely to receive corticosteroid treatment for prior aGVHD at the onset of cGVHD. Intriguingly, these observations were very similar to the findings by Arora et al. [22], who reported that most of patients with overlap syndrome had a history of prior aGVHD and a progressive cGVHD onset, although they did not observe worse survival of this subgroup of patients compared to those with classic cGVHD. Given that nearly all patients who developed overlap syndrome had a prior history of aGVHD in our study cohort, NIH overlap syndrome in most instances could be considered as a flare of preexisting aGVHD, concomitant with development of classic cGVHD. In this context, it is important to note that early flare of cGVHD or early treatment change for exacerbation of cGVHD has been reported to be associated with increased NRM and inferior cGSS [34, 35]. It is also of note that a significantly higher proportion of patients with overlap syndrome had thrombocytopenia less than $100 \times 10^3/\mu L$ at cGVHD onset in our study. Since the progressive cGVHD onset and the presence of thrombocytopenia were consistently associated with an increased NRM across various studies [16, 36], more effective management of patients with overlap syndrome and thrombocytopenia might be needed.

Duration of systemic immunosuppressive therapy is suggested to be a useful surrogate endpoint to evaluate the response to specific treatment for cGVHD [26]. Although we could not find significant association of NIH cGVHD subtypes with duration of systemic IST, patients who had been given ongoing systemic corticosteroids at the onset of cGVHD were found to receive significantly prolonged systemic IST in multivariable analysis, consistent with the findings of Vigorito et al. [37]. In our study, the duration of systemic IST was also prolonged in patients who had high-risk underlying disease compared with those who had standard-risk disease. If the activity of cGVHD were likely to worsen in the high-risk subgroup of patients, one possible explanation might be the preference of physicians to taper systemic IST faster for patients at higher risk of relapse.

The present study, however, has several limitations; the retrospective study design, small cohort size, recording bias, and heterogeneity of underlying diseases and transplantation procedures might substantially influence the results. In addition, diagnostic cGVHD manifestations of affected organs or sites might have originated from other causes, including drug reactions, infection, and

Table 3 Univariable and multivariable analysis of factors potentially associated with chronic GVHD-specific survival among patients who developed chronic GVHD defined by the National Institutes of Health criteria

Variable	*n* (%)	Univariable analysis		Multivariable analysis	
		HR (95% CI)	*P* value	HR (95% CI)	*P* value
Patient age					
Less than 50 years	51 (53)	1.00		–	
50 years or more	45 (47)	1.40 (0.49–4.05)	0.53	–	
Donor/recipient sex combination					
Other than female/male	69 (72)	1.00		–	
Female/male	27 (28)	1.03 (0.32–3.28)	0.97	–	
Disease status at transplant					
Standard risk	51 (53)	1.00		1.00	
High risk	45 (47)	3.03 (0.95–9.68)	0.061	2.75 (0.86–8.80)	0.088
Donor/recipient HLA compatibility					
Matched	80 (83)	1.00		–	
Mismatched	16 (17)	0.33 (0.04–2.53)	0.29	–	
Conditioning regimen					
Myeloablative intensity	54 (56)	1.00		–	
Reduced intensity	42 (44)	1.04 (0.36–3.00)	0.95	–	
Stem cell source					
Bone marrow	67 (70)	1.00		–	
Peripheral blood	24 (25)	2.07 (0.69–6.19)	0.19	–	
Cord blood	5 (5)	1.63 (0.57–4.68)	0.37	–	
Prior aGVHD					
Grade 0–1	47 (49)	1.00		–	
Grade 2–4	49 (51)	1.16 (0.40–3.37)	0.78	–	
Subcategory of cGVHD					
Classic cGVHD	77 (80)	1.00		–	
Overlap syndrome	19 (20)	2.76 (0.96–7.97)	0.060	–	
Severity of cGVHD at onset					
Mild to moderate	73 (76)	1.00		1.00	
Severe	23 (24)	3.10 (1.09–8.86)	0.034	2.58 (0.90–7.39)	0.077
Platelet count at cGVHD onset					
$100 \times 10^3/\mu L$ or more	65 (68)	1.00		1.00	
Less than $100 \times 10^3/\mu L$	31 (32)	4.19 (1.40–12.5)	0.010	4.05 (1.35–12.1)	0.013
Eosinophil count at cGVHD onset					
Less than 500/μL	68 (71)	1.00		–	
500/μL or more	28 (29)	0.90 (0.28–2.88)	0.86	–	
Systemic corticosteroids at cGVHD onset					
Not received	63 (66)	1.00		–	
Received	33 (34)	1.74 (0.61–4.97)	0.30	–	

CI confidence interval, *aGVHD* acute graft-versus-host disease, *cGVHD* chronic graft-versus-host disease

comorbidity before transplantation. Furthermore, genital tract involvement might be underestimated because female patients do not always report about their genital symptoms to physicians.

In conclusion, our present study suggests that both the subcategory and global severity of cGVHD proposed by NIH consensus criteria have effects on cGSS and the risk of NRM among patients who develop NIH cGVHD. Future prospective studies are warranted to more precisely characterize the clinical significance of the subcategory and severity of cGVHD evaluated by the NIH consensus criteria.

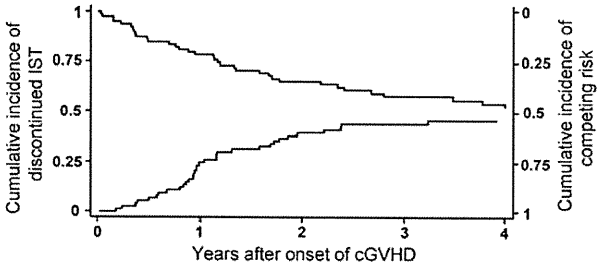

Fig. 2 Cumulative incidence of discontinued systemic immunosuppressive treatment. The *lower curve* shows the cumulative incidence of discontinued systemic immunosuppressive treatment (IST) in the absence of death, recurrent primary disease, or secondary malignancy among 81 patients who developed NIH cGVHD and received systemic IST (*left-hand scale*). The *upper curve* shows the competing risks of death or recurrent/secondary malignancy during systemic IST (*right-hand scale*). At the onset of cGVHD, 69 patients had been already given ongoing systemic IST consisting of calcineurin inhibitors alone ($n = 36$), calcineurin inhibitors plus corticosteroids ($n = 27$), corticosteroids alone ($n = 4$), or corticosteroids plus mycophenolate mofetil ($n = 2$)

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