Table 1 (continued)

Clinical features	CD56-positive		CD56-negtive		*P*-value
	No. of patients (%)	Median (range)	No. of patients (%)	Median (range)	
0–2	0 (0)		3 (4)		0.02
3–9	7 (64)		75 (88)		
≥10	4 (36)		7 (8)		
FAB subtype					
Typical	11 (100)		74 (85)		0.17
Variant	0 (0)		13 (15)		
ACAs	2 (25)		22 (30)		0.76

†Fibrinogen degradation product (FDP) ratio calculated by dividing the FDP value by its upper normal limit. ‡Disseminated intravascular coagulation (DIC) score:[18] 0–2 indicates improbable DIC; score 3, suspected DIC; score 4–9, definitive DIC; ≥10, severe DIC. ACAs, additional chromosomal abnormalities; APL, Acute promyelocytic leukemia; ECOG, Eastern Cooperative Oncology Group; FAB, French–American–British; FDP, fibrin degradation product; WBC, white blood cell.

institutional review boards of each participating institution, and registered with the UMIN Clinical Trials Registry (http://www.umin.ac.jp/ctrj/) under trial number C000000206. Informed consent was obtained from each patient before registration to the study in accordance with the Declaration of Helsinki.

Study design and treatments. The detail of treatment schedule was as described previously.[15] Remission induction therapy consisted of ATRA and chemotherapy with idarubicin and Ara-C, with dose and duration determined by initial WBC counts. After obtaining CR and receiving three courses of intensive consolidation chemotherapy including anthracyclines, Ara-C, and etoposide, patients negative for the *PML-RARA* fusion transcript were randomly allocated either to receive six courses of intensified maintenance chemotherapy or to observation. Patients who were positive for the *PML-RARA* fusion transcript received late ATRA therapy followed by maintenance therapy, and were scheduled to receive allogeneic hematopoietic stem cell transplantation, if they had a human leukocyte antigen-identical donor. Risk stratification according to initial WBC counts ($<3.0 \times 10^9$/L; 3.0×10^9/L to less than 10.0×10^9/L; $\geq 10.0 \times 10^9$/L) used in the current JALSG APL study are based on the results of the JALSG APL92 study.[3] In consideration of this background and the number of cases in each group, we adopted the value and divided the patients into two groups (i.e., initial WBC counts $<3.0 \times 10^9$ and $\geq 3.0 \times 10^9$) to analyze the prognostic impact of CD56 expression.

Immunophenotypic analysis. Immunophenotypic analysis was carried out using bone marrow samples taken at diagnosis and analyzed in the reference laboratory by standard immunofluorescence methods. Cells were stained with anti-CD45 (mAb), gated by CD45 expression and analyzed by flow cytometer. Cells were additionally stained with fluorescein-conjugated mAb against CD2, CD5, CD7, CD4, CD8, CD19, CD20, CD11b, CD13, CD14, CD15, CD33, CD34, CD56, and HLA-DR surface antigens. According to the criteria defined by the European Group for the Immunological Characterization of Leukemias,[16] surface markers were defined as positive if more than 20% of APL cells expressed a specific antigen.

Definition and evaluation of patients. Hematological response was evaluated by standard criteria.[17] Molecular relapse detected by RT-PCR analysis of *PML-RARA* was also considered as a relapse. Overall survival was calculated from the first day of therapy to death or last visit. Event-free survival was determined from the first day of therapy to relapse, death from any cause, or last visit. Cumulative incidence of relapse (extramedullary relapse) was measured from the date of CR to the first relapse, whereas non-relapse mortality was censored as a competing risk event.

Statistical analysis. Categorical data were compared using the χ^2-test or Fisher's exact test. Continuous data were compared using Wilcoxon's rank-sum test. The OS and EFS were estimated by Kaplan–Meier methods and compared by the log–rank test. The CIR was analyzed according to Kalbfleisch and Prentice, and differences were compared using Gray statistics. Cox's proportional hazards model was used for multivariate analysis of EFS. Factors significant at the 0.2 level in the univariate analysis were included in the multivariate analysis model. Statistical analyses were carried out using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA) and R 2.12.1 (R Foundation for Statistical Computing, Vienna, Austria; available at http://www.r-project.org/). All hypothesis testing was two-tailed with a significance level of 0.05.

Results

Patient characteristics. Among 283 evaluable patients of 302 registered to the JALSG APL97 study,[15] 239 (85%) (median age, 48 years; range, 15–70 years) had satisfactory data for CD56 surface antigen expression, and were evaluated in this study. The median follow-up period was 8.5 years (0–12.2 years).

Of 239 patients, 23 (9.6%) were positive for CD56. The clinical and biological characteristics according to CD56 expression are shown in Tables 1 and 2. Expression of CD56 was significantly associated with lower platelet count ($<10 \times 10^9$/L) and severe DIC ($P = 0.04$ and $P = 0.04$, respectively); $CD56^+$ APL significantly coexpressed CD2, CD7, CD34, and/or HLA-DR antigen. ($P = 0.03$, $P = 0.04$, $P < 0.001$, and $P < 0.001$, respectively).

Treatment outcome. The CR rate and incidence of early death during induction therapy were not different between $CD56^+$ and $CD56^-$ APL (91% *vs* 95%, $P = 0.4$, and 9% *vs* 5%, $P = 0.54$, respectively; Table 3). Primary resistance to induction therapy was not observed in either group. The incidence of differentiation syndrome was not different between the two groups (22% *vs* 21%, $P = 0.9$; Table 3).

Overall survival was not different between the two groups (73.9% *vs* 79.2%, $P = 0.52$, at 9 years; Fig. 1a), whereas EFS

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Table 2. Immunophenotypic features of acute promyelocytic leukemia patients (n = 239) according to CD56 expression

Parameters	CD56-positive No. of patients (%)	CD56-negative No. of patients (%)	P-value
CD2			
Positive	5 (22)	16 (8)	0.03
Negative	18 (78)	191 (92)	
CD5			
Positive	1 (5)	3 (2)	0.25
Negative	18 (95)	195 (98)	
CD7			
Positive	2 (9)	4 (2)	0.04
Negative	20 (91)	208 (98)	
CD19			
Positive	1 (4)	5 (2)	0.56
Negative	22 (96)	210 (98)	
CD20			
Positive	0 (0)	1 (0.5)	0.75
Negative	19 (100)	191 (99.5)	
CD11b			
Positive	3 (19)	11 (7)	0.08
Negative	13 (81)	157 (93)	
CD15			
Positive	7 (54)	50 (33)	0.12
Negative	6 (46)	103 (67)	
CD41a			
Positive	1 (5)	19 (10)	0.46
Negative	20 (95)	177 (90)	
CD34			
Positive	9 (41)	27 (13)	$P < 0.01$
Negative	13 (59)	185 (87)	
HLA-DR			
Positive	7 (30)	16 (8)	$P < 0.01$
Negative	16 (70)	197 (92)	

HLA, human leukocyte antigen.

Table 3. Clinical outcomes of acute promyelocytic leukemia patients according to CD56 expression (n = 239)

Clinical features	CD56-positive No. of patients (%)	CD56-negative No. of patients (%)	P-value
No. of patients	23	216	
Induction outcome			
CR rate	21 (91)	206 (95)	0.40
Differentiation syndrome	5 (22)	44 (21)	0.90
Induction death	2 (9)	10 (5)	0.54
Hemorrhage	2 (100)	6 (60)	0.13
Infection	0 (0)	1 (10)	0.74
Differentiation syndrome	0 (0)	2 (20)	0.64
Others	0 (0)	1 (10)	0.74
Postremission outcome			
No. of patients	21	206	
Relapse			
All patients	9 (43)	49 (24)	0.06
Intial WBC counts <3.0	3 (14)	27 (13)	0.88
Initial WBC counts ≥3.0	6 (29)	22 (11)	0.02
Extramedullary relapse			
All patients	1 (5)	3 (1.5)	0.27
Intial WBC counts <3.0	0 (0)	2 (1.0)	0.65
Initial WBC counts ≥3.0	1 (5)	1 (0.5)	0.05
CIR (%)			
All patients	39.1	24.3	0.08
Intial WBC counts <3.0	20.0	20.1	0.98
Initial WBC counts ≥3.0	53.8	28.9	0.03
CIR (extramedullary relapse) (%)			
All patients	5.0	1.5	0.27
Intial WBC counts <3.0	0.0	1.8	0.69
Initial WBC counts ≥3.0	9.3	1.1	0.07

CIR, cumulative incidence of relapse; CR, complete remission; WBC, white blood cell.

and CIR tended to be inferior in $CD56^+$ APL (47.8% *vs* 64.8%, P = 0.08, and 39.1% *vs* 24.3%, P = 0.08, at 9 years, respectively; Figs 2a,3a). In patients with initial WBC counts ≥3.0 × 10^9/L, EFS and CIR for 11 $CD56^+$APL patients were significantly inferior to those for 87 $CD56^-$ APL patients (30.8% *vs* 63.6%, P = 0.008, and 53.8% *vs* 28.9%, P = 0.03, at 9 years, respectively; Figs 2b,3b). In patients with initial WBC counts <3.0 × 10^9/L, EFS and CIR were not different between the two groups (P = 0.99 and P = 0.98, at 9 years, respectively). The OS in patients with initial WBC counts ≥3.0 × 10^9/L was similar between the two groups (61.5% *vs* 78.8%, P = 0.13, at 9 years; Fig. 1b). Although the number was small, EFS and CIR for five $CD56^+$ APL patients among those with initial WBC counts of ≥10 × 10^9/L were inferior to those for 41 $CD56^-$ APL patients (20.0% *vs* 60.9%, P = 0.03, and 60.0% *vs* 30.7%, P = 0.09, at 9 years, respectively). Cumulative incidence of extramedullary relapse tended to be more frequent in patients with $CD56^+$ APL whose initial WBC counts were ≥3.0 × 10^9/L (9.3% *vs* 1.1%, at 9 years, P = 0.07). We also analyzed the influence of CD56 expression on clinical outcomes according to Sanz's relapse risk score.[7] Both CIR and EFS in patients with $CD56^+$ APL were inferior in the high risk group (60.0% *vs* 31.4%, P = 0.09 and 20.0% *vs* 62.5%, P = 0.02, respectively), but not in low and intermediate risk groups (P = 0.17 and P = 0.55, respectively).

In the multivariate analysis, CD56 expression was an independent adverse prognostic factor for EFS in patients whose initial WBC counts were ≥3.0 × 10^9/L (hazard ratio = 2.54; 95% confidence interval, 1.07–6.06, P = 0.04) (Table 4).

Discussion

Expression of CD56 has been reported as one of the adverse prognostic factors in AML with t(8;21), associated with a short remission duration and survival as well as higher incidence of extramedullary relapse.[19,20] Recently, several investigators have suggested that CD56 expression is also associated with short remission duration in APL, higher CIR, and extramedullary relapse (Table 5).[9–12] However, large-scale studies with long-term follow-up are limited,[12] and the prognostic significance of CD56 expression has not been fully elucidated.

Our study, analyzing 239 APL patients, showed a significant correlation between CD56 expression with lower platelet counts and severe DIC. In contrast to previous reports,[9,10,12] CD56 expression was not associated with higher WBC counts, lower albumin levels, or higher frequency of M3 variant. Severity of DIC was related to platelet counts in $CD56^+$ APL,

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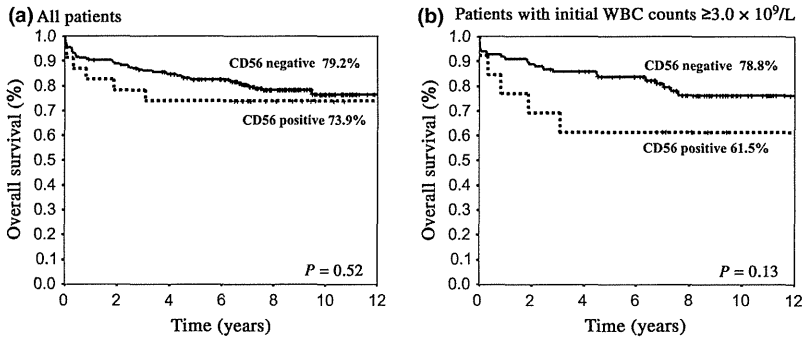

Fig. 1. Overall survival (OS) of patients with acute promylocytic leukemia according to CD56 expression. (a) OS was not different between the two groups for all patients (73.9% *vs* 79.2% at 9 years, $P = 0.52$). (b) In patients whose white blood cell (WBC) count was $\geq 3.0 \times 10^9$/L, OS did not differ between the two groups (61.5% *vs* 78.8%, $P = 0.13$).

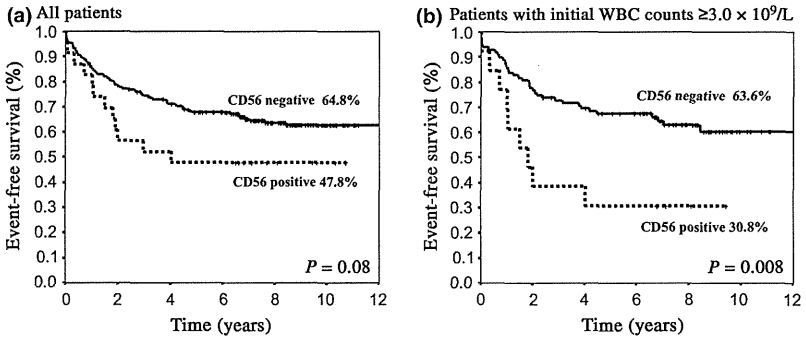

Fig. 2. Event-free survival (EFS) of patients with acute promylocytic leukemia (APL) according to CD56 expression. (a) EFS for all patients showed an inferior trend in $CD56^+$ APL (47.8% *vs* 64.8% at 9 years, $P = 0.08$). (b) In patients whose white blood cell (WBC) count was $\geq 3.0 \times 10^9$/L, EFS for $CD56^+$ APL was significantly inferior to that for $CD56^-$ APL (30.8% *vs* 63.8%, $P = 0.008$).

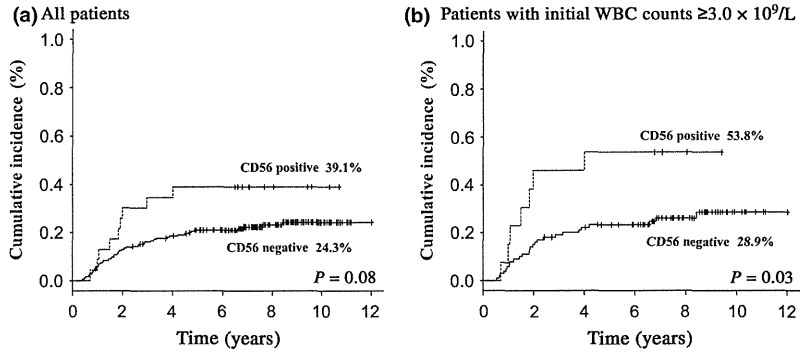

Fig. 3. Cumulative incidence of relapse (CIR) of patients with acute promylocytic leukemia (APL) according to CD56 expression. (a) CIR for all patients showed an inferior trend in the $CD56^+$ APL group (39.1% *vs* 24.3% at 9 years, $P = 0.08$). (b) In patients whose white blood cell (WBC) count was $\geq 3.0 \times 10^9$/L, CIR for the $CD56^+$ group was significantly higher compared to that for the $CD56^-$ APL group (53.8% *vs* 28.9%, $P = 0.03$).

although fibrinogen levels and fibrinogen degradation product ratios (fibrinogen degradation product value/its upper limit of normal value) were not different (Table 1). The relationship between CD56 expression and DIC in AML, including APL, has not been elucidated. As statistically significant findings associated with $CD56^+$ APL in previous reports were not the same as our present study, further studies with sufficient numbers of patients will be needed to clarify the characteristic features of $CD56^+$ APL.

Consistent with the report from the PETHEMA/HOVON group,[12] $CD56^+$ APL cells frequently coexpressed CD2, CD7, CD34, and/or HLA-DR antigen in our study. Although the mechanism leading to aberrant expression of lymphoid markers, such as CD2 and CD7 in $CD56^+$ APL cells, remains unclear, the expression of these antigens, as well as CD34 and HLA-DR, may indicate that $CD56^+$ APL cells arise in more immature, undifferentiated, and progenitor cells, as previously suggested in acute leukemia.[21]

The PETHEMA/HOVON group have reported lower CR rates in their patients with $CD56^+$ APL.[12] However, our study showed no difference in CR and induction mortality rates. Their patients with $CD56^+$ APL showed poorer ECOG PS scores and lower albumin levels compared with our patients. Higher ECOG PS scores and lower albumin levels were reportedly associated with induction mortality.[22] Therefore, the difference may be explained by the characteristics of patients enrolled in both studies.

Our study indicated that CD56 expression was correlated with higher CIR and inferior EFS, and was an independent adverse prognostic factor for EFS by multivariate analysis among APL patients whose initial WBC counts were $\geq 3.0 \times 10^9$/L. These results verified that CD56 expression was one of the adverse prognostic factors in APL patients. However, the direct molecular mechanism why CD56 expression in APL is associated with poor prognosis still remains unclear. CD56 expression is reportedly associated with higher

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expression of P-glycoprotein in AML,[23,24] but their adverse prognostic roles seem independent.[24] Unfortunately, neither ours nor other studies focusing on $CD56^+$ APL have tested the association between CD56 and P-glycoprotein. However, APL expressing CD34 was reportedly less sensitive to ATRA therapy.[25,26] Therefore, coexpression of CD34 antigen might explain the higher CIR in $CD56^+$ APL, although the RT-PCR negativity after the consolidation chemotherapy was not different between $CD56^+$ and $CD56^-$ APL.

In this study, CD56 expression was not determined as one of the prognostic factors in APL patients whose initial WBC counts were $<3.0 \times 10^9$/L. One explanation might be that it has become difficult to determine significant risk factors in patients with APL, whose prognosis has considerably improved.[1–5] In particular, in patients with lower initial WBC counts, the outcome has been dramatically improved in the ATRA era.[3,27] Another considerable reason is that there might be synergistic action between CD56 expression and some undetermined proliferation molecular factors. Additionally, extramedullary relapse, observed frequently in patients with $CD56^+$ APL whose initial WBC counts are $\geq 3.0 \times 10^9$/L, might also be a reason. The molecular mechanism behind why $CD56^+$ APL patients with higher initial WBC counts show poor prognosis should be clarified in a future study.

Recently, arsenic trioxide, gemtuzumab ozogamicin, and tamibarotene have been shown to be effective for APL,[28–33] and, in fact, most of our relapsed patients received these drugs as well as stem cell transplantation. This may be a plausible reason why EFS and CIR tended to be worse in $CD56^+$ APL, but not OS, because these drugs and transplantation salvaged the relapsed patients.

Although our study confirmed CD56 expression as an independent adverse prognostic factor in APL patients with higher initial WBC counts who were treated with ATRA and chemotherapy (Table 4), the clinical significance of CD56 expression might change with the introduction of more potent agents as front-line therapy. Expression of CD56 has not been included so far in standard treatments recommended by the European LeukemiaNet.[14] However, some recent

Table 4. Prognostic factors affecting event-free survival of acute promyelocytic leukemia patients (initial white blood cell counts $\geq 3.0 \times 10^9$/L) (n = 239)

Factors for event-free survival	Univariate analysis		Multivariate analysis	
	P-value	Hazard ratio	95% CI	*P*-value
DIC score† >10 (*vs* DIC score ≤10)	0.17	1.06	0.90–1.24	0.48
Age >60 years (*vs* age ≤60 years)	0.04	2.00	0.86–4.65	0.11
HLA-DR antigen positive (*vs* negative)	0.02	1.46	0.49–4.33	0.49
CD56 antigen positive (*vs* negative)	0.008	2.54	1.07–6.06	0.04

†Disseminated intravascular coagulation (DIC) score:[18] 0–2 indicates improbable DIC; score 3, suspected DIC; score 4–9, definitive DIC; ≥10, severe DIC. Factors with *P*-value <0.20 in univariate analysis were included in the multivariate analysis. CI, confidence interval; HLA, human leukocyte antigen; HR, hazard ratio.

Table 5. Clinical features and outcomes in acute promyelocytic leukemia (APL) patients with CD56 expression, as reported in published works

Authors	No. of patients	Treatment	$CD56^+$ APL (%)	Clinical features in patients with $CD56^+$ APL*	CR rate		CIR		CIR (extramedullary)		DFS†		OS	
					$CD56^+$	$CD56^-$	$CD56^+$	$CD56^-$	$CD56^+$	$CD56^-$	$CD56^+$	$CD56^-$	$CD56^+$	$CD56^-$
Murray *et al.*[9]	50	CT alone / ATRA alone / ATRA + CT	24%	S-isoform↑, Fibrinogen↓	50%*	84%	NA	NA	NA	NA	NA	NA	5 weeks*	232 weeks
Ferrara *et al.*[10]	100	ATRA + CT	15%	No effect	87%	94%	NA	NA	13%	8%	22 months	NR	62%*	86%
Ito *et al.*[11]	28	ATRA + CT	14%	Coexpression of CD34	100%	87%	NA	NA	75%*	0%	4 months*	NR	26 months	NR
Montesinos *et al.*[12]	651	CT alone / ATRA + CT	11%	Initial WBC counts↑, Albumin↓, S-isoform↑, Coexpression of CD2, CD7, CD15, CD34, CD117, and HLA-DR	85%	92%	22%*	10%	7%*	1%	73%*	85%	78%	84%
Present study (all patients)	225	ATRA + CT	10%	Initial platelet counts↓, Severe DIC†, Coexpression of CD2, CD7, CD34, and HLA-DR	91%	95%	39%	24%	5%	1.5%	48%	65%	74%	79%
Present study (initial WBC counts ≥3.0 × 10^9/L)	112	ATRA + CT	12%		92%	94%	54%*	29%	9.3%	1.1%	31%*	64%	62%	79%

*Significant difference. †Event-free survival in present study. APL, acute promyelocytic leukemia; ATRA, all-*trans* retinoic acid; CIR, cumulative incidence of relapse; CR, complete remission; CT, chemotherapy; DFS, disease-free survival; DIC, disseminated intravascular coagulation; HLA, human leukocyte antigen; NA, not available; NR, not reached; OS, overall survival; WBC, white blood cell.

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published research, including ours (summarized in Table 5), will promote the modification of treatment for CD56$^+$ APL. In fact, it is proposed in some recently published studies. We should not only continue to monitor CD56 expression in APL patients, but use more effective therapeutic strategies for patients with CD56$^+$ APL, especially those with higher initial WBC counts.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
Ara-C	cytosine arabinoside
ATRA	all-*trans* retinoic acid
CIR	cumulative incidence of relapse
CR	complete remission
DIC	disseminated intravascular coagulation
ECOG	Eastern Cooperative Oncology Group
EFS	event-free survival
HLA	human leukocyte antigen
HOVON	Hemato-Oncologie voor Volwassenen Nederland
JALSG	Japan Adult Leukemia Study Group
OS	overall survival
PETHEMA	Programa de Estudio y Tratamiento de las Hemopatı'as Malignas
PS	performance status
WBC	white blood cell

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ORIGINAL ARTICLE

Comprehensive analysis of genetic alterations and their prognostic impacts in adult acute myeloid leukemia patients

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To clarify the cooperative roles of recurrently identified mutations and to establish a more precise risk classification system in acute myeloid leukemia (AML), we comprehensively analyzed mutations in 51 genes, as well as cytogenetics and 11 chimeric transcripts, in 197 adult patients with *de novo* AML who were registered in the Japan Adult Leukemia Study Group AML201 study. We identified a total of 505 mutations in 44 genes, while only five genes, *FLT3, NPM1, CEBPA, DNMT3A* and *KIT*, were mutated in more than 10% of the patients. Although several cooperative and exclusive mutation patterns were observed, the accumulated mutation number was higher in cytogenetically normal AML and lower in AML with *RUNX1-RUNX1T1* and *CBFB-MYH11*, indicating a strong potential of these translocations for the initiation of AML. Furthermore, we evaluated the prognostic impacts of each sole mutation and the combinations of mutations and/or cytogenetics, and demonstrated that AML patients could be clearly stratified into five risk groups for overall survival by including the mutation status of *DNMT3A*, *MLL*-PTD and *TP53* genes in the risk classification system of the European LeukemiaNet. These results indicate that the prognosis of AML could be stratified by the major mutation status in combination with cytogenetics.

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Keywords: acute myeloid leukemia; gene mutations; prognosis; risk factor

INTRODUCTION

Acute myeloid leukemia (AML) is a clinically and genetically heterogeneous disease.[1,2] Although about 80% of younger adults with AML achieve complete remission (CR) with induction chemotherapy, more than half of the CR patients relapse, even if they receive intensive consolidation therapies. Allogeneic hematopoietic stem cell transplantation (allo-SCT) is applied to the patients who have risk factors for relapse, and it has been demonstrated by meta-analysis that allo-SCT at the first CR improves the long-term prognosis of the cytogenetically intermediate- and adverse-risk groups.[3] Cytogenetic-risk classification for AML is well established and commonly used as criteria for the application of allo-SCT at the first CR, whereas there is clinical heterogeneity in the intermediate-risk group, particularly cytogenetically normal (CN)-AML.[4] Recent advances and the accumulation of information on the prognostic relevance of recurrent genetic alterations have made more detailed risk stratification possible in AML patients.[5–19] The European LeukemiaNet (ELN) has recommended a novel risk classification system on the basis of the cytogenetic and genetic status.[2] In this system, CN-AML is stratified into two risk groups according to the mutation status of *FLT3*, *NPM1* and *CEBPA*: patients with *NPM1* mutation but not *FLT3*-ITD and those with *CEBPA* mutation are included in the favorable-risk (FR) group, and patients with *FLT3*-ITD and those with neither *NPM1* mutation nor *FLT3*-ITD are categorized into the intermediate-I-risk (IR-I) group. Long-term prognosis according to the ELN classification system was retrospectively evaluated in well-established cohorts, and it has

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been demonstrated that the ELN system is useful for further risk stratification of younger adult patients with CN-AML.[20,21] However, it has been reported that another genetic status, such as mutations in epigenetic modifier-encoding genes, could more precisely distinguish the prognosis in each ELN-risk group.[5,22] In addition, the prognostic impacts of recently identified mutations in spliceosome and cohesin complex genes on AML remain unclear.

The Japan Adult Leukemia Study Group (JALSG) conducted six phase III trials for adult *de novo* AML from 1987 (AML87, AML89, AML92, AML95, AML97 and AML201).[23] In the JALSG AML201 study, we prospectively compared a standard dose of idarubicin (IDR) with a higher dose of daunorubicin (HiDNR) in combination with cytarabine (Ara-C) as induction therapy, and three courses of high-dose Ara-C (HiDAC) with four courses of conventional standard-dose multiagents as consolidation therapy in CR patients.[24,25] We demonstrated that HiDNR was equivalent to IDR, as induction therapy and HiDAC was of benefit only to patients with core-binding factor (CBF)-AML as consolidation therapy. Although the CR rate remained at 75–80% during the six JALSG studies, 7-year overall survival (OS) was improved to 48% in the AML201 study from 29% in the AML87 study. Allo-SCT was conducted in only 7.1% of registered patients in the AML87 study, whereas 45.8% of registered patients received allo-SCT not only at the first CR but also after relapse or primary induction failure in the AML201 study, indicating that active application of allo-SCT even after relapse or primary induction failure might contribute to the improvement of OS. These results collectively suggested that a novel risk stratification system for decision making of allo-SCT at the first CR is required.

In this study, we comprehensively analyzed mutations in 51 genes that have been recurrently identified in myeloid neoplasm as well as cytogenetics, and evaluated the association of genetic status with prognostic and clinical features in patients who were registered in the AML201 study.

PATIENTS AND METHODS

Patients and samples

The study population included 197 newly diagnosed *de novo* AML patients, except for those with acute promyelocytic leukemia, who were registered in the JALSG AML201 study (UMIN Clinical Trials Registry C000000157, http://www.umin.ac.jp/ctrj/). The diagnosis of AML was on the basis of the French–American–British (FAB) classification.[26] Median follow-up time was 32.5 months. The age distribution is presented in Table 1. In the AML201 study, patients were randomly assigned to receive either IDR or HiDNR for induction therapy, and those who achieved CR were again randomized to receive either four courses of conventional consolidation therapy or three courses of HiDAC therapy.[24,25] Of the 197 patients, 98 and 99 patients were assigned to IDR and HiDNR arms for induction therapy, respectively. CR was achieved in 161 of 197 (81.7%) patients, and 80 and 77 patients were assigned to HiDAC and conventional consolidation therapies, respectively (Table 1).

High molecular weight DNA and total RNA were extracted from bone marrow samples using standard methods.[27–29]

Cytogenetic G-banding analysis was performed by standard methods. We also examined 11 chimeric gene transcripts (Major: *BCR-ABL1*, Minor: *BCR-ABL1*, *PML-RARA*, *RUNX1-RUNX1T1*, *CBFB-MYH11*, *DEK-NUP214*, *NUP98-HOXA9*, *MLL-MLLT1*, *MLL-MLLT2*, *MLL-MLLT3*and *MLL-MLLT4*) by reverse transcriptase-mediated quantitative PCR as previously reported.[30]

Morphological diagnosis, the FAB classification and karyotypes were reviewed and confirmed by the central review committees of the JALSG using the BM samples obtained at diagnosis.

We obtained informed consent from all patients to use their samples for banking and molecular analysis, and approval was obtained from the ethics committees of the participating institutes.

Screening for mutations in 51 genes

A custom-made oligonucleotide probe library was designed to capture the exons of 51 genes that have been recurrently identified in myeloid neoplasm (Supplementary Table 1). Captured and enriched exons were subjected to sequencing on an Illumina HiSeq (Illumina, San Diego, CA, USA).[31–33] Sequence variation annotation was performed using known polymorphism databases, followed by mutation characterization. Each predicted variant sequence was confirmed by Sanger sequencing. Internal tandem duplication of the *FLT3* gene (*FLT3*-ITD) and partial tandem duplication of the *MLL* gene (*MLL*-PTD) were examined as previously reported.[28,34]

Table 1. Characteristics of the 197 patients

Characteristics	*Number*	*(%)*
Age (year)		
15–19	6	3.0
20–29	32	16.2
30–39	35	17.8
40–49	33	16.8
50–59	69	35.0
60–64	22	11.2
FAB subtype		
M0	7	3.6
M1	36	18.3
M2	89	45.2
M4	34	17.3
M4Eo	9	4.6
M5	21	10.7
M6	1	0.51
Cytogenetic-risk group		
Favorable	55	27.9
RUNX1-RUNX1T1	41	20.8
CBFB-MYH11	14	7.1
Intermediate	100	50.8
Normal cytogenetics	72	36.5
Unfavorable	23	11.7
Complex karyotype	16	8.1
t(11q23) excluding t(9;11) and t(11;19)	3	1.5
t(9;22)	2	1.0
−7	1	0.5
Not determined	19	9.6
Induction therapy		
IDR + Ara-C	98	49.7
DNR + Ara-C	99	50.3
Achieving CR	161	81.7
Consolidation therapy		
High-dose Ara-C	80	51.0
Multiagent CT	77	49.0

Abbreviation: IDR, idarubicin. The study population included 197 newly diagnosed *de novo* AML patients except for acute promyelocytic leukemia, and equally assigned to induction and consolidation arms. Nine patients showed the M4Eo FAB type, and all of them harbored the *CBFB-MYH11* transcript.

Statistical analysis

Differences in continuous variables were analyzed by the unpaired *t*-test or the Mann–Whitney *U*-test for distribution between two groups. Analysis of frequencies was performed using Fisher's exact test for 2×2 tables or Pearson's χ^2 test for larger tables. A multivariate analysis to identify risk factors for achieving CR was performed by the logistic regression model. Survival probabilities were estimated by the Kaplan–Meier method, and differences in the survival distributions were evaluated using the log-rank test. OS was defined as the time from the date of entry into the AML201 study to death due to any cause or last follow-up. Disease-free survival (DFS) was defined as the time from the day of achieving CR to relapse, death due to any cause or last follow-up. Patients undergoing SCT were not censored at the time of transplantation. The prognostic significance of the clinical variables was assessed using the Cox proportional hazards model. These statistical analyses were performed with Prism 5 (GraphPad Software, La Jolla, CA, USA) and JMP Pro10 (SAS Institute Japan,

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Tokyo, Japan). For all analyses, the *P*-values were two-tailed, and a *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Cytogenetic analysis

Cytogenetic analysis revealed a normal karyotype in 72 (36.5%) patients and an abnormal karyotype in 106 (53.8%) patients, including 41 AML with t(8;21) (q22;q22); *RUNX1-RUNX1T1* and 14 AML with inv(16) (p13q22); *CBFB-MYH11*. However, karyotypes could not be determined in 19 (9.6%) patients because we could not obtain sufficient mitotic cells. On the basis of the G-banding karyotype and chimeric transcript analyses, patients were assigned to favorable- ($n=55$, 27.9%), intermediate- ($n=100$, 50.8%) and adverse-risk ($n=23$, 11.7%) groups according to the refined MRC criteria (Table 1).[4]

Frequencies of mutations

We identified mutations in 44 of 51 genes analyzed in the 197 AML patients. However, there were only five genes (*FLT3, NPM1, CEBPA, DNMT3A* and *KIT*) that were mutated in more than 10% of the patients (Figure 1a and Supplementary Table 1). Each position and type of mutation is presented in Supplementary Figure 1. As germ-line controls were available in a limited number of patients, we could not completely confirm that all identified mutations were somatic mutations. Therefore, there is a possibility that a part of identified mutations might be rare SNPs. *FLT3* mutation was the most frequently identified (50 patients, 25.4%), followed by *NPM1* (38 patients, 19.2%), *DNMT3A* (32 patients, 16.2%), *CEBPA* (31 patients, 15.7%) and *KIT* mutations (28 patients, 14.2%). Of the 50 patients with *FLT3* mutations, 36 (18.3%) and 17 (8.6%) patients harbored *FLT3*-ITD and *FLT3*-KDM, respectively, and three patients harbored both mutations. Of the 31 patients with *CEBPA* mutations, 19 (9.6%) and 12 (6.1%) patients harbored double *CEBPA* (*CEBPA*-D) mutations and a single *CEBPA* (*CEBPA*-S) mutation, respectively. Of the 28 patients with *KIT* mutations, 4, 2 and 23 patients harbored mutations in exon 8, exons 10–11 and exon 17 of the *KIT* gene, respectively, and one patient harbored mutations in both exons 10–11 and exon 17.

Although mutations in the 51 analyzed genes were not identified in 14 (7.1%) patients, 183 (92.9%) patients harbored one or more mutations; one mutation in 36, two mutations in 56, three mutations in 40, four mutations in 27, five mutations in 17, six mutations in five and seven mutations in two patients. The mean mutation number per patient was 2.56 ± 0.11 in all patients, whereas it was significantly higher in patients with a normal karyotype (3.18 ± 0.16) than in those with an aberrant karyotype (2.10 ± 0.15) ($P<0.0001$). Furthermore, mean mutation numbers per patient in AML with *RUNX1-RUNX1T1* (1.68 ± 0.17) and *CBFB-MYH11* (1.57 ± 0.20) were significantly lower than that in all samples ($P=0.0008$ and 0.0123, respectively) (Figure 1b).

The mean mutation number per patient aged 60–64 years (3.18 ± 0.41) tended to be higher, although there was no significant difference between the mean mutation number and age (Supplementary Figure 2).

Genetic alterations found in AML have been conceptually grouped into class I mutation, which causes constitutive activation of intracellular signals that contribute to the growth and survival, and class II mutation that blocks differentiation and/or enhance self-renewal by altered transcription factors.[35–37] Recently, it has been suggested that mutations that modify the epigenetic status generate a new class because of their overlap mutations both with class I and class II mutations.[13,38] In this study, Class II mutations (*NPM1, CEBPA, RUNX1* and *GATA2* mutations, and *RUNX1-RUNX1T1* and *CBFB-MYH11*) were the most frequently identified (138/197; 70.1%), followed by Class I mutations (*FLT3, KIT, N/KRAS, PTPN11, JAK1/3* and *TP53* mutations) (116/197; 58.9%) and mutations that modify the epigenetic status (*ASXL1, ATRX, EZH2, TET2, PBRM1, DNMT3A, IDH1/2, KDM6A, MLL* and *DOT1L* mutations) (91/197; 46.2%). Furthermore, mutations of NOTCH family genes (*NOTCH1 and NOTCH2*), cohesin complex genes (*STAG2, SMC1A, SMC3* and *RAD21*), BCOR family genes (*BCOR* and *BCORL1*), NCOR family genes (*NCOR1, NCOR2* and *DIS3*) and spliceosome genes (*SF3B1, U2AF1, SRSF2* and *ZRSR2*) were identified in 19 (9.6%), 22 (11.2%), 17 (8.6%), 22 (11.2%) and 9 patients (4.6%), respectively (Figure 1c).

Association between gene mutations and cytogenetics

The prevalence of each gene mutation differed among the cytogenetic-risk groups. *KIT* mutations were preferentially identified in the favorable cytogenetic-risk group. *FLT3*-ITD, *NPM1, CEBPA* and *DNMT3A* mutations were preferentially identified in the intermediate-risk group, particularly in patients with a normal karyotype. *BCORL1* and *TP53* mutations were preferentially identified in the poor-risk group; in particular, *TP53* mutations were frequent in patients with a complex karyotype. In addition, *PHF6* mutations were also frequently identified in patients with a complex karyotype (Figure 2 and Supplementary Table 2).

Overlap mutations

Several patterns of overlap mutations were identified in this comprehensive mutation analysis (Supplementary Figures 3 and 4). Significantly overlapped mutations were observed between *FLT3* mutations and *NPM1, DNMT3A* and *MLL*-PTD mutations; *NPM1* mutations and *DNMT3A, IDH1* and *IDH2* mutations; *CEBPA* mutations and *TET2* mutations; *ASXL1* mutations and spliceosome gene mutations; *DIS3* mutations and *MLL* mutations; *DNMT3A* mutations and *PTPN11* mutations; *GATA2* mutations and *CEBPA*-D mutations; *K/NRAS* mutations and *WT1* mutations and *BCOR/BCORL1* mutations; *RUNX1* mutations and *U2AF1, MLL-PTD, BCOR/BCORL1* and *PHF6* mutations; *SF3B1* mutations and *NRAS* mutations; and *TET2* mutations and *STAG2* mutations. In contrast, mutually exclusive mutations were observed between *FLT3* mutations and *KIT, K/NRAS* and *CEBPA*-D mutations; *NPM1* mutations and *CEBPA*-D and *RUNX1* mutations; and *CEBPA* mutations and *IDH1/2* mutations.

According to the conceptual classification of the mutated genes, overlap mutations between Class I, Class II and epigenetic modifying gene mutations were frequently observed. However, these major mutations widely coexisted with other family gene mutations, such as the cohesin complex, BCOR family and spliceosome gene mutations (Figure 1d). Although biological functions of mutated genes have not been fully clarified, we also present frequencies and associations of mutated genes according to the provisional gene function in the Supplementary Figure 5.

Association of gene mutations with clinical characteristics

Several associations between mutations and clinical characteristics were observed. *DNMT3A* mutations and *MLL*-PTD were more frequently identified in patients over 50 years old than in those less than 50 years old ($P=0.0064$ and $P=0.0121$, respectively), whereas the other mutations were not significantly associated with age (Supplementary Table 3).

Several mutations were associated with the white blood cell count at diagnosis. *FLT3-ITD, NPM1, DNMT3A* and *NOTCH1* mutations were significantly associated with the high white blood cell count. In contrast, *ASXL1* and *IDH1* mutations were associated with a lower white blood cell count (Supplementary Table 4).

Association of gene mutations with the CR rate

We analyzed the association of mutations with the CR rate. By Fisher's exact test, *RUNX1-RUNX1T1* or *CBFB-MYH11, KIT, NPM1* and *CEBPA*-D mutations were identified as favorable factors for

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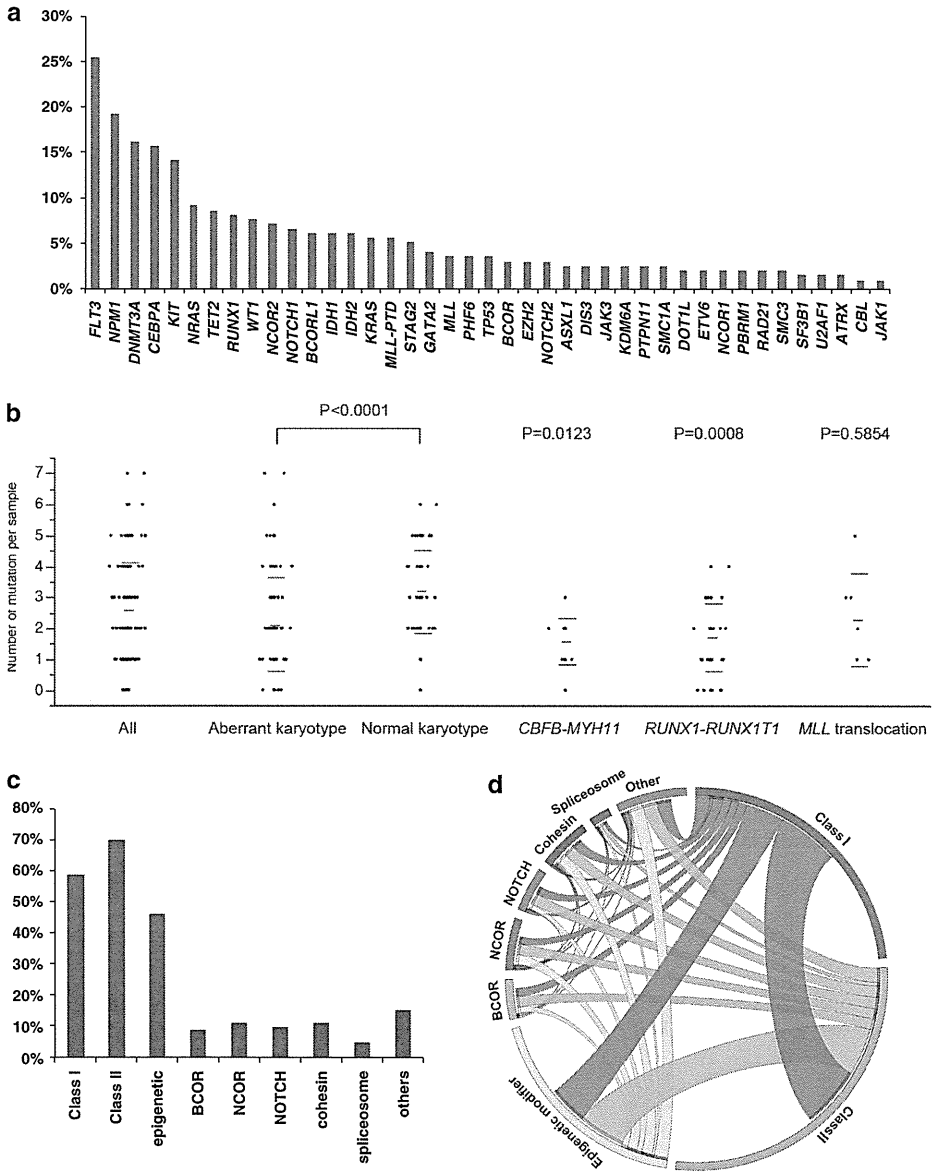

Figure 1. Frequencies and associations of mutated genes. (**a**) Frequencies of analyzed gene mutations. Frequency of each mutated gene is shown. Mutations were identified in 44 genes of 51 genes analyzed in 197 AML patients. Only five genes (*FLT3, NPM1, CEBPA, DNMT3A* and *KIT*) were mutated in more than 10% of the patients. (**b**) Mutated gene numbers according to the cytogenetics. Mean mutation number ± s.d. is indicated by horizontal lines. Mean mutation number per one patient in patients with normal karyotype (3.18 ± 0.16) was significantly higher than in that with aberrant karyotype (2.10 ± 0.15) ($P<0.0001$). Those in AML with *RUNX1-RUNX1T1* (1.68 ± 0.17) and *CBFB-MYH11* (1.57 ± 0.20) were significantly lower than that in all samples ($P=0.0008$ and $P=0.0123$, respectively). That in AML with *MLL*-translocation tended to be lower but not statistically significant (2.28 ± 0.57, $P=0.5854$). (**c**) Frequencies of mutations according to the conceptual classification. Mutations in Class I, Class II and epigenetic modifying genes were frequently identified. (**d**) Association of mutated genes according to the conceptual classification. Circos plot of mutated genes according to the function is shown.[45] Overlap mutations between Class I, Class II and epigenetic modifying genes mutations were frequently observed. These major mutations were widely coexistent with another family genes, such as cohesin complex, BCOR family and spliceosome genes.

achieving CR, and *TP53* mutation was an unfavorable factor; however, multivariate logistic regression analysis including all analyzed mutations showed that only *NPM1* (Hazard ratio (HR): 96.206, 95% Confidence interval (CI): 2.247–411.9, $P=0.0172$) and *TP53* (HR: 22.222, 95% CI: 1.597–333.3, $P=0.0172$) mutations were identified as favorable and unfavorable factors for achieving CR, respectively (Table 2 and Supplementary Table 5).

Importantly, *KIT* mutations were closely associated with *RUNX1-RUNX1T1* or *CBFB-MYH11*, whereas the other mutations that confer the achievement of CR were mutually exclusive (Supplementary Figures 3 and 6). In the patients with *RUNX1-RUNX1T1* or *CBFB-MYH11*, *NPM1* and *CEBPA*-D mutations, the CR rate (106/112; 94.6%) was significantly higher than for those with the other genotypes (55/85; 64.7%) ($P<0.0001$).

Prognostic impacts of mutations

We next analyzed the prognostic impact of each mutation. By univariate analysis, *FLT3*-ITD (HR: 1.805, 95% CI: 1.130–2.885, $P=0.0135$), *DNMT3A* (HR: 1.696, 95% CI: 1.055–2.725, $P=0.0291$),

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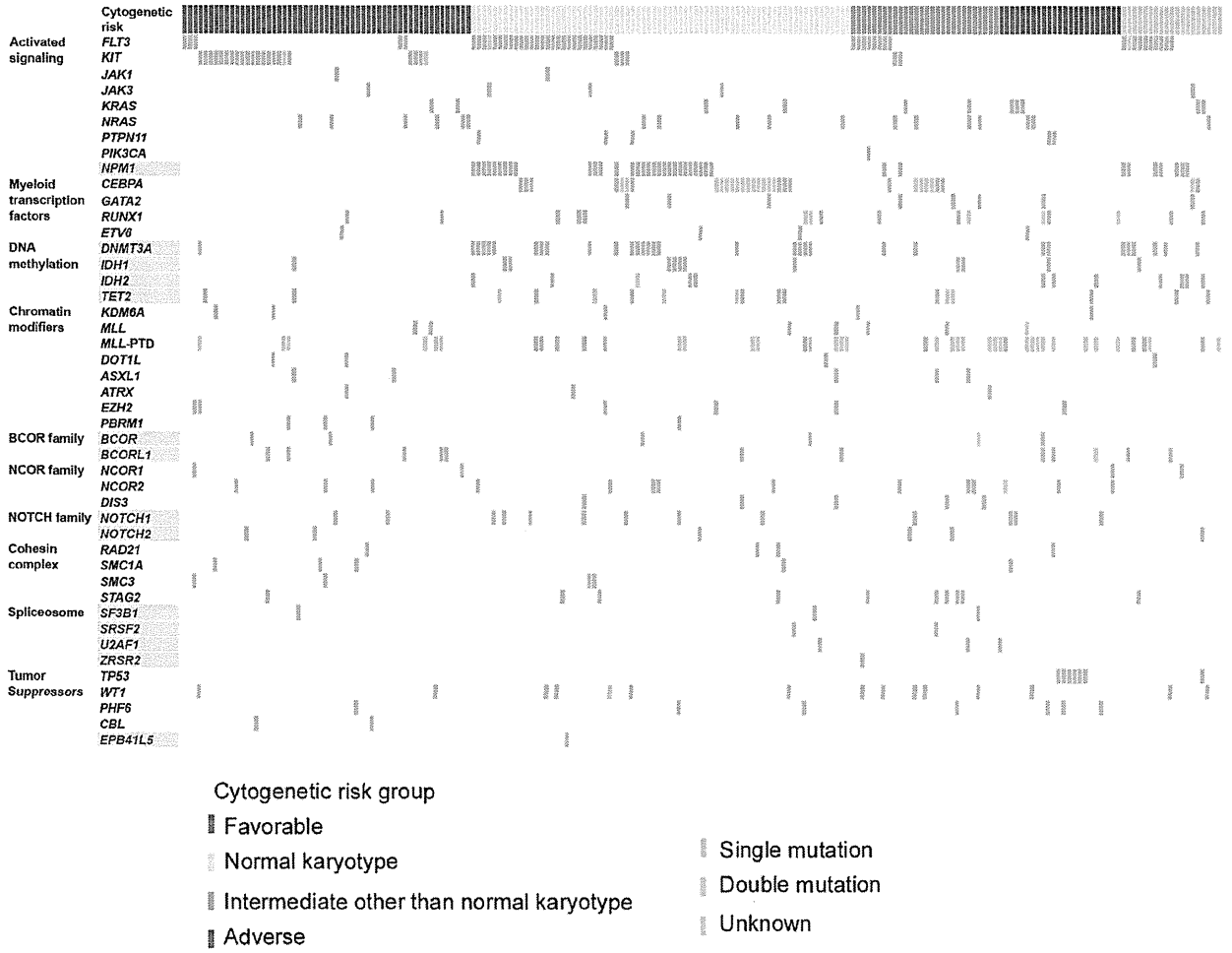

Figure 2. Mutation status according to the cytogetics-risk groups. Identified mutations in analyzed AML patients are shown according to the cytogenetic-risk groups. Pink boxes indicate single mutations and orange boxes indicate double mutations.

Table 2. Gene mutations affecting the CR achievement

Mutations	*CR rate (%)*		P-*value*
	Positive	*Negative*	
Fisher's exact test			
NPM1	97	78	0.0041
CEBPA-D-Mt.	100	80	0.0273
KIT	96	79	0.0326
RUNX1-RUNX1T1 or *CBFB-MYH11*	91	78	0.0409
TP53	14	84	0.0002

Mutations	*HR (95% CI)*	P-*value*
Multivariate analysis		
Wild-*NPM1*	96.206 (2.247–411.9)	<0.0001
TP53 mutation	22.222 (1.597–333.3)	0.0172

Abbreviations: CI, confidence interval; CR, complete remission; HR, hazard ratio. By the Fisher's exact test, *RUNX1-RUNX1T1* or *CBFB-MYH11*, *KIT*, *NPM1* and *CEBPA*-D mutations were identified as the favorable factor for achieving CR, and *TP53* mutation was for the unfavorable factor. The multivariate logistic regression analysis including all analyzed mutations showed that only wild-*NPM1* and *TP53* mutation were identified as unfavorable factors for achieving CR.

TP53 (HR: 15.167, 95% CI: 6.555–35.094, $P<0.001$), *MLL*-PTD (HR: 3.782, 95% CI: 1.948–7.346, $P<0.001$) and *RUNX1* (HR: 2.301, 95% CI: 1.278–4.146, $P=0.0055$) mutations and the karyotypes other than *RUNX1-RUNX1T1* or *CBFB-MYH11* (HR: 2.786, 95% CI: 1.608–4.831, $P=0.0003$) were identified as unfavorable prognostic factors for OS (Table 3 and Supplementary Figure 7). Multivariate Cox regression analysis with stepwise selection showed that *TP53* (HR: 14.803, 95% CI: 6.259–35.009, $P<0.001$), *MLL*-PTD (HR: 2.853, 95% CI: 1.401–5.810, $P=0.0039$) and *RUNX1* (HR: 1.965, 95% CI: 1.054–3.663, $P=0.0336$) mutations and the karyotypes other than *RUNX1-RUNX1T1* or *CBFB-MYH11* (HR: 2.353, 95% CI: 1.342–4.132, $P=0.0028$) were independent poor prognostic factors for OS (Table 3).

In this cohort, mutations of NOTCH family, the cohesin complex, BCOR family and spliceosome genes were frequently identified. NOTCH family and BCOR family genes were not associated with the CR rate, OS and DFS. Although mutations of cohesin complex genes were not associated with the CR rate and DFS, the patients harboring those mutations revealed better OS than those without mutations ($P=0.0274$) (Figure 3). The CR rate and DFS of patients with spliceosome gene mutations tended to be lower than for those without mutations, although both differences were not statistically significant: the CR was achieved in five of the nine (55.6%) and 156 of the 188 (83.0%) patients ($P=0.0601$), and 3-year DFS were 0% and 38.9% ($P=0.1117$) in those with and

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without mutations, respectively (Supplementary Table 3 and Supplementary Figure 8).

When the patients were stratified into the risk groups recommended by ELN, that is, FR, IR-I, IR-II and AR groups included 92, 35, 42 and 28 patients, respectively. The ELN system well stratified the long-term prognosis of adult AML patients, whereas the OSs of IR-I and AR groups were the same in the present cohort (Supplementary Figure 9). Therefore, we analyzed whether another mutations could further stratify the prognosis in each ELN-risk group. *MLL*-PTD, *GATA2* and *TP53* mutations were identified as further poor prognostic factors in IR-I, IR-II and AR groups, respectively. Furthermore, we identified that the *DNMT3A* mutation was a poor prognostic factor in the FR group except for the AML with t(8;21) (q22;q22); *RUNX1-RUNX1T1* or inv(16) (p13q22); *CBFB-MYH11* (CBF-AML) (Figure 4).

Table 3. Unfavorable prognostic factors for overall survival (OS)

Mutations	*HR (95% CI)*	P-*value*
Univariate analysis		
TP53	15.167 (6.555–35.094)	<0.0001
MLL-PTD	3.782 (1948–7.346)	<0.0001
Non CBF	2.786 (1.608–4.831)	0.0003
RUNX1	2.301 (1.278–4.146)	0.0055
FLT3-ITD	1.805 (2.247–4119)	0.0135
DNMT3A	1.696 (1.055–2.725)	0.0291
Multivariate analysis		
TP53	14.803 (6.259–35.009)	<0.0001
MLL-PTD	2.853 (1.4017–5.810)	0.0039
Non CBF	2.353 (1.342–4.132)	0.0028
RUNX1	1.965 (1.054–3.663)	0.0336

Abbreviations: CI, confidence interval; HR, hazard ratio. By the univariate analysis, *FLT3*-ITD, *DNMT3A*, *TP53*, *MLL*-PTD and *RUNX1* mutations and the karyotypes other than *RUNX1-RUNX1T1* or *CBFB-MYH11* were identified as adverse prognostic factors for OS. Multivariate Cox regression analysis with stepwise selection showed that *TP53*, *MLL*-PTD and *RUNX1* mutations and the karyotypes other than *RUNX1-RUNX1T1* or *CBFB-MYH11* were independent poor prognostic factors for OS.

In addition, we also evaluated two recently reported risk stratification systems on the basis of genetic status in our cohort.[5,39] Patel *et al.*[5] reported a risk stratification system on the basis of cytogenetics and genetic status According to their system, our patients were clearly stratified into three risk groups, although the patients in the intermediate cytogenetic risk with a favorable mutational risk profile and those in the favorable cytogenetic-risk profile showed the same probability of OS (Supplementary Figure 10a). Grossmann *et al.*[39] reported a prognostic model solely on the basis of molecular mutations. Although our cohort did not include AML with *PML-RARA*, our patients were clearly stratified into four risk groups. However, the patients in the very favorable group and those in the favorable group showed the same probability of OS (Supplementary Figure 10b).

On the other hand, *KIT* mutations were frequently identified in CBF-AML, while they were not a poor prognostic factor for either OS or DFS (Supplementary Figure 11a). According to the types of *KIT* mutations, CBF-AML patients harboring mutations in exon 17 of the *KIT* gene showed worse prognosis than those harboring the other types of *KIT* mutation, although this was not statistically significant (Supplementary Figure 11b). Notably, in the CBF-AML patients harboring *KIT* mutations, OS and DFS of those treated with three courses of HiDAC consolidation therapy tended to be better than those treated with four courses of conventional standard-dose multiagent therapy (Supplementary Figure 11c).

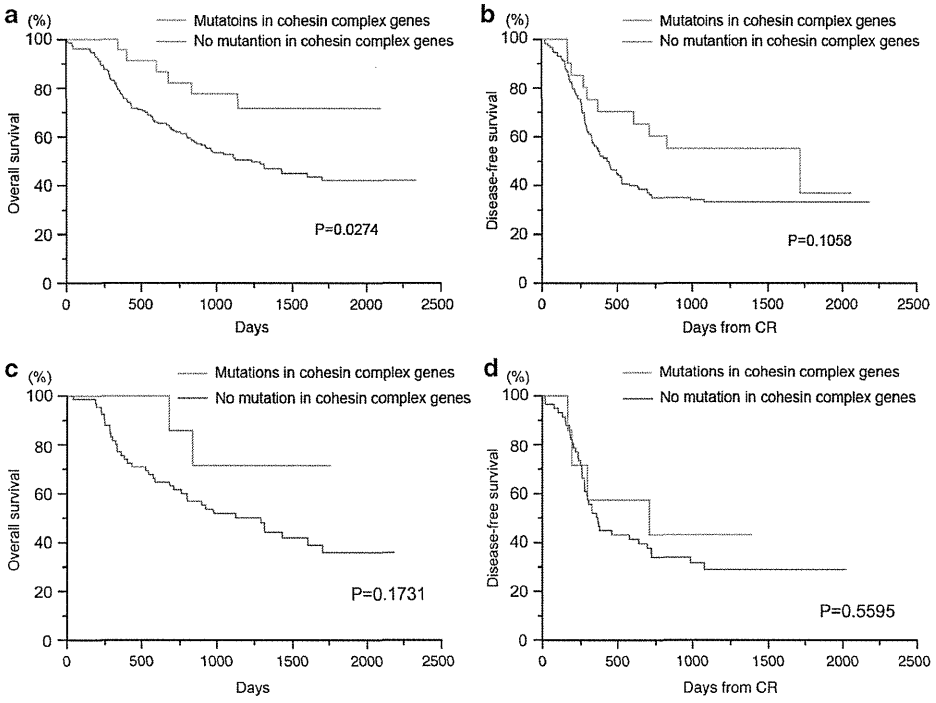

Figure 3. Prognostic impact of mutations in cohesin complex genes. Kaplan–Meier curves for OS and DFS according to the mutations in cohesin complex genes are shown. (**a**) OS in the total patients, (**b**) DFS in the total patient, (**c**) OS in the patients with normal karyotype, (**d**) DFS in the patients with normal karyotype. Although mutations of cohesin complex genes were not associated with the CR rate and DFS, the patients harboring those mutations revealed better OS than those without mutations ($P = 0.0274$). In the patients with normal karyotype, OS of the mutated patients tended to be better that that of unmutated patients, though statistical significance was not observed ($P = 0.1731$).

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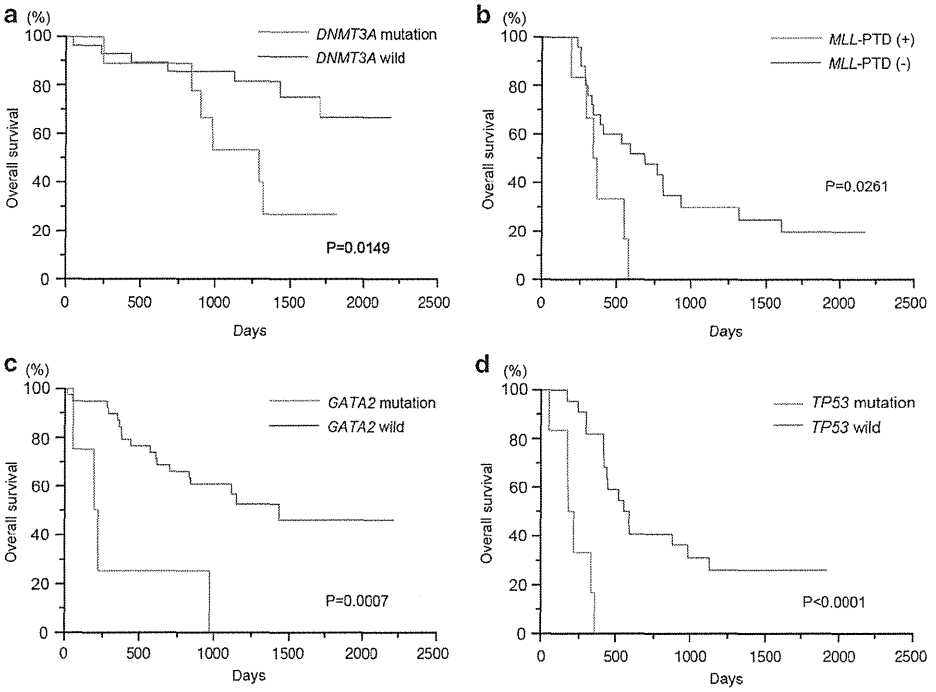

Figure 4. Mutations that could further stratify the ELN-risk groups into two risk groups. (**a**) *DNMT3A* mutation was a poor prognostic factor in the FR group except for the AML with t(8;21) (q22;q22); *RUNX1-RUNX1T1* or inv(16) (p13q22); *CBFB-MYH11* (CBF-AML). (**b**) *MLL*-PTD was a poor prognostic factor for the OS in the ELN IR-I group. (**c**) *GATA2* mutation was a poor prognostic factor for the OS in the ELN IR-II group. (**d**) *TP53* mutation was a poor prognostic factor for the OS in the ELN AR group.

In CBF-AML, the accumulated mutation number was lower than in the other types of AML, although many kinds of mutation were identified (Supplementary Figure 12). However, we could not identify a gene that affects the prognosis of CBF-AML.

Taking these results together, we tried to modify the genetic criteria for the ELN stratification system. When the CN-AML patients with *DNMT3A* mutations of the FR group and the patients with *MLL*-PTD of the IR-I group were included in the IR-I and the AR group, respectively, we could more clearly stratified the patients into four risk groups for OS than the original ELN system (Figures 5a and b). Furthermore, as the prognosis of the patients with *TP53* mutations were very unfavorable as previously reported,[39] we could more clearly stratify the patients into five risk groups for OS by classifying the *TP53*-mutated patients as the very adverse-risk group (Figures 5c and d).

DISCUSSION

In this study, we comprehensively analyzed mutations of 51 genes by the targeting sequence, and identified a total of 505 mutations in 44 genes in 197 adult *de novo* AML patients except for APL. The whole-genome and -exome analysis demonstrated recurrent mutations in a total of 260 genes in 200 AML patients, suggesting that another mutations might be accumulated in the presently analyzed AML cells.[40] However, frequencies of most mutated genes were reportedly less than 10%. In consistence, only five genes (*FLT3, NPM1, CEBPA, DNMT3A* and *KIT*) were mutated in more than 10% patients in our study, and each mutation frequency was almost the same as previous reports.[5,17,19,39] The frequency of *KIT* mutation was relatively higher in our study than previous reports,[5,40] while this is caused by the higher frequency of CBF-AML (28.0%) in the Japanese patients, in which *KIT* mutations are frequently identified. Our study, therefore, essentially includes major genetic regions, which may affect the pathogenesis and prognosis of AML. However, mutation analyses were not thoroughly performed in all subtypes of AML, such as acute erythroid leukemia and acute megakaryoblastic leukemia because of their lower frequencies. Further analyses are required to fully clarify the genetic alterations in AML.

The whole-genome and -exome analysis demonstrated that an average of mutated genes in coding regions per sample was 5.24.[40] Of note was that there were significant differences in mutated gene numbers among the types of cytogenetics and mutations: the mean mutation numbers were higher in AML with *RUNX1-RUX1T1*, and are lower in that with *PML-RARA* and *MLL* translocations than that of all samples. As analyzed gene numbers were limited, mean mutated gene number per sample (2.56 ± 0.11) was low in our study; however, there were different features from the previous report. In our study, higher mutation number was observed in CN-AML (3.18 ± 0.16), and lower was in CBF-AML. Furthermore, the mutation number in AML with *MLL*-translocation tended to be lower (2.28 ± 0.57, $P = 0.5854$). These results collectively suggested that recurrent cytogenetic abnormalities, such as *RUNX1-RUNX1T1, CBFB-MYH11* and *MLL*-translocation, have a strong potential for the initiation of AML, and that most of accumulated mutations in AML with these cytogenetics might be passenger mutations.

It has been reported that common mutations in AML, such as *DNMT3A, NPM1, CEBPA, IDH1/2* and *RUNX1*, were mutually exclusive of the transcription-factor fusions, indicating the high potential for leukemia initiation.[10,11,13,40,41] Consistently, *DNMT3A, NPM1* and *CEBPA* mutations were not identified in CBF-AML, but frequent in CN-AML. In addition, we identified that *MLL*-PTD mutation was also exclusive of CBF-AML. In CBF-AML, *KIT* mutations were preferentially identified, whereas several types of mutations were also accumulated, suggesting that many mutations could act as a driver mutation for the clonal expansion of the initiating clone with *RUNX1-RUNX1T1* and

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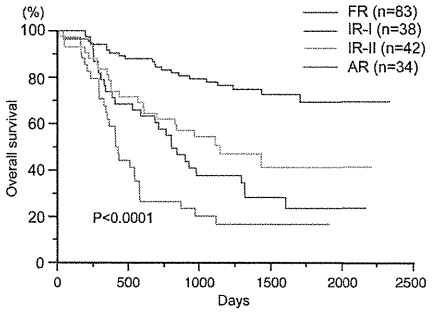
a

Genetic group	Subsets
Favorable	• CBF-AMLs • CN-AMLs with mutated *NPM1* without *FLT3*-ITD and mutated *DNMT3A* (normal karyotype) • CN-AMLs with mutated *CEBPA* without mutated *DNMT3A* (normal karyotype)
Intermediate-I	• CN-AMLs with mutated *DNMT3A* • CN-AMLs except for those included in the FR and AR
Intermediate-II	• AMLs included in the IR-II of the ELN stratification
Adverse	• CN-AMLs with *MLL*-PTD • AMLs included in the AR of the ELN stratification

b



c

Genetic group	Subsets
Favorable	• CBF-AMLs • CN-AMLs with mutated *NPM1* without *FLT3*-ITD and mutated *DNMT3A* (normal karyotype) • CN-AMLs with mutated *CEBPA* without mutated *DNMT3A* (normal karyotype)
Intermediate-I	• CN-AMLs with mutated *DNMT3A* • CN-AMLs except for those included in the FR and AR
Intermediate-II	• AMLs included in the IR-II of the ELN stratification except for those with mutated *TP53*
Adverse	• CN-AMLs with *MLL*-PTD • AMLs included in the AR of the ELN stratification except for those with mutated *TP53*
Very Adverse	• AMLs with mutated *TP53*

d

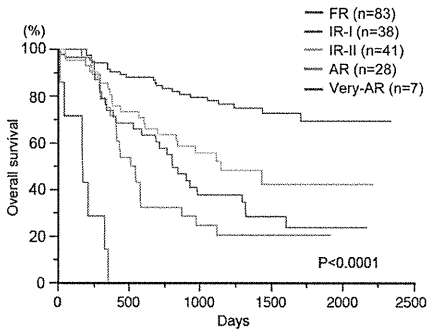


Figure 5. Risk stratification by modifying the ELN stratification system. When the CN-AML patients with *DNMT3A* mutations of the FR group and the patients with *MLL*-PTD of the IR-I group were included in the IR-I and the AR group, respectively (**a**), we could more clearly stratify the patients into four risk groups for OS than the original ELN system (**b**). When the patients with *TP53* mutations were classified as the very adverse-risk group (**c**), we could more clearly stratify the patients into five risk groups for OS (**d**).

CBFB-MYH11. Further study is required to clarify which combination is necessary for the clonal expansion, and whether different combinations cause clinical and phenotypical varieties.

After the completion of genetic alterations in AML, the most important issue is to clarify the prognostic impact of each mutation and/or co-occurring mutations.[7,42] The recently recommended ELN classification system is the first system that includes both cytogenetics and mutation status. Several groups reported that the ELN system clearly stratified the long-term prognosis of AML patients. However, the prognosis of FR groups except for the CBF-AML is still controversial. Paschka *et al.*[43] reported that the *IDH1/2* mutation was a poor prognostic factor in CN-AML with mutated *NPM1* without *FLT3*-ITD. In contrast, Patel *et al.*[5] reported that the *IDH1/2* mutation was a favorable prognostic factor in AML with mutated *NPM1* without *FLT3*-ITD. Furthermore, it has been reported that the *TET2* mutation was an adverse prognostic factor in AML with mutated *NPM1* or *CEBPA* without *FLT3*-ITD.[22] In the present cohort, we could not observe the statistically significant effects of *IDH1/2* and *TET2* mutations on the prognosis of CN-AML with mutated *NPM1* or *CEBPA* without *FLT3*-ITD, while we identified that *DNMT3A* mutation is an adverse prognostic factor in CN-AML with mutated *NPM1* or *CEBPA* without *FLT3*-ITD. In addition, we could not find the better prognostic impact of the *CEBPA* double mutations on the FR group recommended by the ELN in contrast to previous reports.[39,44]

Although different mutations might further stratify the prognosis of AML with mutated *NPM1* or *CEBPA* without *FLT3*-ITD, it was noteworthy that all mutations belonged to the class modifying methylation status.[13,38] These results collectively suggested that the epigenetic deregulation might contribute the pathogenesis of AML with mutated *NPM1* or *CEBPA* without *FLT3*-ITD. Prospective and large-scale study is necessary to clarify what genetic alterations influence the prognosis of AML with these genotypes.

In this study, we demonstrated that the prognosis of adult AML patients could be more clearly stratified by including the *DNMT3A* and *MLL*-PTD mutation status than the original ELN system, and that *TP53* mutations have a very adverse effect on the prognosis of AML patients. However, as most recurrently identified mutations were observed less than 5% of AML, it is highly expected to refine the genetic-based risk stratification system by much larger-scale studies. In addition, it is also important to evaluate the prognostic effects according to the functions of mutated genes rather than each sole mutation.

In the JALSG AML201 study, patients were randomized to the standard dose of IDR + Ara-C or HiDNR + Ara-C induction therapy, and the CR patients were again randomized to three courses of HiDAC or four courses of conventional standard-dose multiagent consolidation therapy. Therefore, we analyzed whether therapeutic regimens affect the CR rate and long-term survivals according to the mutation status and risk groups on the basis of the genetic status, while we could not observe any significant differences between therapeutic regimens and genetic status. Furthermore, we could not demonstrate that allo-SCT could improve the prognosis of the patients falling in the intermediate- and adverse-risk groups because of the small number of patients who underwent allo-SCT in the first CR in this cohort. It is, therefore, required to evaluate whether therapeutic regimens and allo-SCT affect the prognosis according to the genetic status.

In conclusion, we comprehensively analyzed 51 genes mutations in 197 *de novo* adult AML patients who were registered to a single prospective clinical study, and demonstrated that cooperative and exclusive mutation patterns and their prognostic impacts. Furthermore, we demonstrated that the prognosis of adult AML patients could be more clearly stratified by including the *DNMT3A, MLL*-PTD and *TP53* mutation status than the original ELN system. However, prognostic impacts of some mutation status are different from the previous reports. We must refine the risk

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stratification system by considering all known-risk factors in a large-scale and well-established cohort, although molecular genetic status has a strong impact on the prognosis of AML patients. We are now conducting a prospective large-scale study to confirm the present results.

CONFLICT OF INTEREST

H Kiyoi: Research funding from Bristol-Myers Squibb, Novartis Pharma, Chugai Pharmaceutical Co., Ltd. and Kyowa Hakko Kirin Co., Ltd. YM: Honoraria from Bristol-Myers Squibb, Novartis Pharma, Chugai Pharmaceutical Co., Ltd., Kyowa Hakko Kirin Co., Ltd. and Celgene Japan; Research funding from Bristol-Myers Squibb, Novartis Pharma, Chugai Pharmaceutical Co., Ltd. and Kyowa Hakko Kirin Co., Ltd. NU: Consultant for Kyowa Hakko Kirin Co., Ltd.; Honoraria from Bristol-Myers Squibb, Novartis Pharma and Chugai Pharmaceutical Co., Ltd.; Research funding from Bristol-Myers Squibb, Novartis Pharma, Chugai Pharmaceutical Co., Ltd. and Kyowa Hakko Kirin Co., Ltd. TN: Research funding from Bristol-Myers Squibb, Novartis Pharma, Chugai Pharmaceutical Co., Ltd., Kyowa Hakko Kirin Co., Ltd., Dainippon Sumitomo Pharma and Zenyaku Kogyo. The other authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

H Kiyoi, S Ogawa and TN designed the study, interpreted the data and wrote the manuscript; RK, YN, T Kato, EY, KS and FC performed molecular analysis and interpreted the data; YN, YS, KC, HT, SM and S Ogawa performed bioinformatics; NA, S Ohtake, SM, YM, TS, YO, N Usui, H Kanamori, T Kiguchi, KI, N Uike, FK, KK, CN, MO, AT, FI, HS, YK and HM collected samples and clinical data, contributed to the interpretation of the data and critically reviewed the draft; and all authors approved the final version submitted for the publication.

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Leukemic evolution of donor-derived cells harboring *IDH2* and *DNMT3A* mutations after allogeneic stem cell transplantation

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Although allogeneic stem cell transplantation is effective for the treatment of leukemia with poor prognosis, some such treated individuals experience disease relapse at various times after transplantation. Chimerism analysis of the relapsed disease has revealed infrequent cases in which the malignant cells originate from the donor and not from the initial leukemic clones.[1,2] Such donor cell leukemia (DCL) is often refractory to further treatment, with a mean overall survival for the affected patients of only 32.8 months.[2]

We recently described a 47-year-old Japanese man with acute myeloid leukemia (AML) who underwent a transplantation of peripheral blood stem cells (PBSCs) from his HLA-matched brother.[3] Although the allogeneic transplantation was successful, AML again became apparent in the patient 27 months later and chimerism analysis revealed that the leukemia was DCL. Genomic DNA was isolated and subjected to whole-exome sequencing from specimens of the initial AML (containing 70% myeloblasts, referred to as sample P1), the first complete remission after chemotherapy (sample P2), the first relapse (containing 24% myeloblasts; sample P3), donor PBSCs (sample D1), DCL at 27 months after allogeneic transplantation (containing 6% myeloblasts, sample D2) and DCL at 36 months after transplantation (containing 71% myeloblasts, sample D3).

Exome sequencing yielded a total of ~84.7 million, ~31.6 million, ~73.5 million, ~44.3 million and ~53.2 million unique, high-quality, paired-end reads for samples P1, P2, P3, D1 and D3, respectively (Supplementary Information). Although chimerism analysis for short tandem repeats had indicated that D3 was derived from D1 clones,[3] we further examined this possibility in a genome-wide manner. As demonstrated in Supplementary Figure 1a, the allele frequency of single-nucleotide polymorphisms (SNPs) detected in our data sets was highly concordant between P1 and P2 (Pearson's correlation coefficient (r) of 0.978) as well as between P1 and P3 ($r=0.986$), suggesting that these three samples originate from a single individual. However, as expected, the concordance dropped substantially for the P1 and D3 pair ($r=0.628$). In contrast, the concordance between D1 and D3 was high ($r=0.983$), suggesting that the relapsed leukemia after transplantation was indeed derived from the donor cell. Of note, the allele frequency of SNPs showed only a low level of concordance ($r=0.285$) between P1 and a cell line (KCL22)[4] derived from an unrelated Japanese patient with chronic myeloid leukemia (Supplementary Figure 1b). The correlation coefficient of 0.628 for P1 and D3 thus indicated that the patient and donor siblings share a substantial number of SNPs.

We next searched for somatic nonsynonymous mutations among the leukemic samples. For P1 and P3, we used P2 as a paired normal control. Given that D3 was shown to be derived from D1, we used the latter as the germline control for the former. Through our computational pipeline (Supplementary Information), nine missense mutations and two out-of-frame insertions/deletions (indels) were detected for P1, two missense mutations for P3 and nine missense mutations and one out-of-frame indel for D3 (Table 1). As described previously,[3] a 4-bp deletion of *CEBPA* was present in the initial AML but absent from the DCL. Similarly,

Table 1. Confirmed somatic mutations in the specimens analyzed

Specimen	*Gene symbol*	*GenBank accession no.*	*Nucleotide change*	*Amino-acid change*	*Mutation ratio (%)*				
					P1	*P2*	*P3*	*D1*	*D3*
P1	*ACSL5*	NM_016234	c.280G>A	p.V94I	40.6	0.0	30.6	0.0	0.0
	ANO4	NM_178826	c.2441C>T	p.S814L	42.3	0.0	16.7	0.0	0.0
	APOB	NM_000384	c.9175C>T	p.R3059C	32.8	0.0	7.4	0.0	0.0
	BANK1	NM_017935	c.222C>G	p.N74K	36.4	0.0	9.2	0.0	0.0
	CCDC88C	NM_001080414	c.3748G>A	p.E1250K	36.4	0.0	0.0	0.0	0.0
	FAM178B	NM_001122646	c.81G>A	p.M27I	41.2	0.0	25.0	0.0	0.0
	GABRB2	NM_021911	c.1009C>T	p.R337C	44.8	0.0	14.5	0.0	0.0
	JAK3	NM_000215	c.2570T>C	p.L857P	40.8	0.0	0.0	0.0	0.0
	SPATA31D1	NM_001001670	c.3793C>T	p.R1265C	36.6	0.0	6.7	0.0	0.0
	CEBPA	NM_004364	c.319_322delGACT	p.D107Tfs	63.6	0.0	10.0	0.0	0.0
	STAG2	NM_001042750	c.219_220insCG	p.H73Rfs	100.0	0.0	27.6	0.0	0.0
P3	*ACSL5*	NM_016234	c.280G>A	p.V94I	40.6	0.0	30.6	0.0	0.0
	NTNG2	NM_032536	c.1348G>T	p.G450C	0.0	0.0	37.5	0.0	0.0
D3	*CCDC168*	NM_001146197	c.11761G>C	p.D3921H	0.0	0.0	0.0	0.0	55.6
	GAL3ST1	NM_004861	c.1086G>T	p.M362I	0.0	0.0	0.0	0.0	32.6
	IDH2	NM_002168	c.419G>A	p.R140Q	0.0	0.0	0.0	7.1	50.0
	MYO7B	NM_001080527	c.635G>A	p.R212H	0.0	0.0	0.0	0.0	45.8
	NFATC1	NM_172390	c.736G>A	p.V246I	0.0	0.0	0.0	0.0	48.6
	PSMB8	NM_004159	c.637C>T	p.P213S	0.0	0.0	0.0	0.0	40.9
	TCAIM	NM_173826	c.668C>G	p.S223C	0.0	0.0	0.0	0.0	70.0
	TMEM132D	NM_133448	c.481G>A	p.A161T	0.0	0.0	0.0	0.0	35.3
	UBA2	NM_005499	c.419G>A	p.G140E	0.0	0.0	0.0	0.0	47.4
	DNMT3A	NM_153759	c.449delT	p.V150Gfs	0.0	0.0	0.0	8.7	61.1
	NRAS[a]	NM_002524	c.38G>A	p.G13D	0.0	0.0	0.0	0.0	18.4

[a]Below the threshold in the initial screening.

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none of the identified somatic mutations were shared between the initial AML and DCL, providing further support for the distinct nature of the two leukemias.

Given that P3 contains only 24% myeloblasts, our computational pipeline could not accurately detect all of the associated somatic mutations. Indeed, most of the somatic mutations found in P1 (such as those in *ANO4*, *APOB*, *BANK1*, *STAG2* and *CEBPA*) were still present in P3 at lower frequencies (Table 1) but were not isolated in our pipeline analysis for P3. Lowering the threshold for somatic calls, however, increased the number of pseudopositive mutations in all specimens. We therefore applied the 30% threshold for mutation calls to all analyses. Of note, our data still indicate that P3 is not completely identical to P1. Nonsynonymous mutations of *CCDC88C* and *JAK3* detected in P1 were thus absent in P3, whereas a mutation of *NTNG2* was newly apparent in P3, suggestive of a clonal evolution in P3 divergent from the original P1 clones.

Surprisingly, whereas most somatic mutations detected in D3 were not present in D1, our results suggested that IDH2(R140Q) and DNMT3A(V150Gfs) were already present in the healthy donor at a low frequency (Table 1). Polymerase chain reaction (PCR)-based cloning of the genomic fragments and Sanger sequencing for *IDH2* and *DNMT3A* from D1 indeed confirmed the presence of the corresponding mutations in 2 (2.3%) out of 87 DNA clones and 1 (1.1%) out of 93 clones, respectively (Supplementary Figure 2). Furthermore, although the mutation rate (18.4%) was below the threshold of the present study, the oncogenic mutation NRAS(G13D)[5] in D3 (Table 1) was confirmed by Sanger sequencing of the corresponding genomic DNA (Supplementary Figure 2).

We then verified these infrequent mutations by sequencing the corresponding DNA fragments at extra-high coverage (hundreds of thousand times) with the use of a next-generation sequencer. The D2 sample, which contains only 6% myeloblasts, was also examined in this analysis. We confirmed that 1.6% (5.96×10^3 mutant reads out of 3.67×10^5 total reads at the corresponding nucleotide position) and 2.1% (1.24×10^4 out of 6.01×10^5 reads) of D1 cells already harbored the IDH2(R140Q) and DNMT3A(V150Gfs) mutations, respectively (Figure 1a). These mutations were not detected in the primary AML (P1 to P3). Whereas the *NRAS* mutation was not detected in D1, it became apparent in D2 and D3 at a frequency similar to that of the *IDH2* mutation. In addition, the *JAK3* mutation present in P1 was no longer evident at the relapsed stage P3.

On the basis of the genetic mutation profiles identified in the present case, we propose the following scheme for disease progression (Figure 1b). Given the high frequency of *STAG2* and *CEBPA* mutations in the primary AML, the 2-bp insertion in *STAG2* on the X chromosome (with there being only one copy of *STAG2* per cell in the male patient) as well as the heterozygous 4-bp deletion in *CEBPA* may characterize the founding clone of the original leukemia, with subsets of this clone subsequently acquiring additional oncogenic hits such as JAK3(L857P). The disappearance of *JAK3* and *CCDC88C* mutations in P3 suggests that the leukemic subclones harboring these mutations were sensitive to the initial chemotherapy.

The molecular pathogenesis of DCL has been unclear and may differ among cases. For instance, germline predisposition to cancer, such as the Li-Fraumeni syndrome or Bloom syndrome, may be shared between recipients and related donors.[6] However, in the present case, mutations in *IDH2* and *DNMT3A* were detected only in the donor, not in the primary AML, rendering this scenario unlikely. Alternatively, occult leukemia may already be present in the donor blood system and is inadvertently transmitted to the recipient.[7] In such cases, however, leukemia usually emerges in the donor soon after transplantation. Our donor, in contrast, has not developed any hematologic malignancy at 10 years after the donation of his PBSCs.

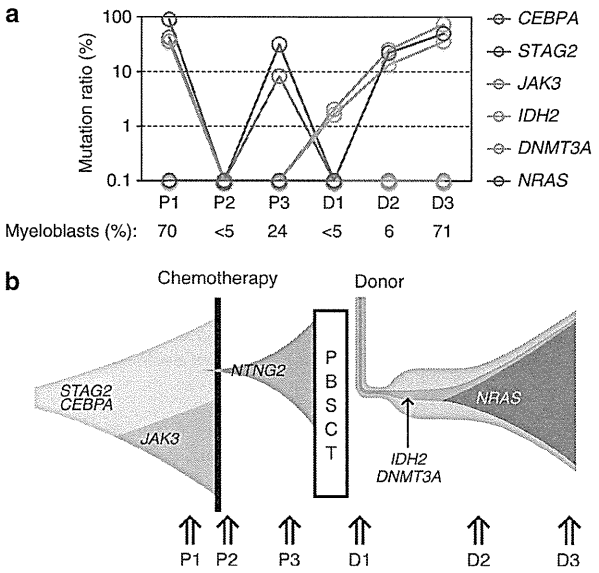

Figure 1. Genomic analysis of AML samples and donor PBSCs. (**a**) Genomic mutations corresponding to CEBPA(D107Tfs), STAG2(H73Rfs), JAK3(L857P), IDH2(R140Q), DNMT3A(V150Gfs) and NRAS(G13D) were examined by targeted deep sequencing in genomic DNA prepared from samples P1, P2, P3, D1, D2 and D3. The ratio of mutant reads to all reads at the corresponding position is shown as a percentage, with mutation frequencies of $<0.1\%$ being considered as 0.1% in the graph. The percentage of myeloblasts in each sample is indicated below the graph. (**b**) Founding clones of the primary AML harbored nonsynonymous mutations of *STAG2* and *CEBPA* and gave rise to subclones harboring a *JAK3* mutation. Whereas the latter cell population was sensitive to the initial chemotherapy, a subclone positive for an *NTNG2* mutation emerged from the former population and gave rise to relapse. All of these leukemic clones were successfully eradicated by peripheral blood stem cell transplantation (PBSCT). PBSCs of the donor, however, contained a small clonal population of cells positive for *IDH2* and *DNMT3A* mutations that eventually gave rise to AML on acquisition of additional mutations including NRAS(G13D).

Our present data therefore strongly suggest that apparently healthy individuals may harbor preleukemic subclones in their blood system (Figure 1b). Indeed, somatic mutations of *TET2* and *DNMT3A* were recently identified in clonal blood cells from one healthy elderly individual.[8] Furthermore, the *IDH2* and *DNMT3A* mutations identified in the present study may have had a specific role in the initiation of leukemia, given that mutations in the epigenetic modifiers including TET1/2, IDH1/2 and DNMT3A have been identified as early genetic events in AML progression.[9,10] Such mutations are indeed among the most frequently detected somatic alterations in AML.[11] These observations raise an important concern as to how 'appropriate' donors should be chosen, especially given that the incidence of DCL is increasing with the prevalence of molecular analysis for donor/recipient chimerism.[2] Prospective studies of whether and how examination of preleukemic subclones should be incorporated into the donor selection process for stem cell transplantation are thus warranted.

Furthermore, in our case, the oncogenic mutation NRAS(G13D) was likely a driver for leukemia progression, given that the frequency of this mutation was almost identical to that of the *IDH2* mutation in the D2 and D3 specimens. In contrast to the absence of leukemia in the donor, DCL rapidly developed in the recipient after transplantation in association with the accumulation of additional genetic hits, possibly as a result of a growth-promoting condition of the bone marrow after transplantation and due to a

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defective immune surveillance resulting from the immunosuppressive treatment to control graft-versus-host disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Cytogenetics and outcome of infants with acute lymphoblastic leukemia and absence of *MLL* rearrangements

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Acute lymphoblastic leukemia (ALL) in infants less than 1 year of age is rare and the biological features are different from ALL in older children.[1] Infant ALL is characterized by a high frequency of rearrangements of the *MLL* gene (*MLL*-R) and heterogeneous outcome. However overall, their event-free survival (EFS) is much worse than older children with ALL.[1–5] A large collaborative trial, Interfant-99, demonstrated improved outcome, while characterizing definitively the independent prognostic variables in infant ALL.[6] While cytogenetic data are reported within individual infant ALL clinical trials, the numbers are typically small and many reports are less detailed for those patients without *MLL* gene rearrangements (*MLL*-G). However, it was previously suggested that *MLL*-G had an important predictive influence on outcome.[7,8] These observations were later confirmed in Interfant-99,[6] in which *MLL*-G patients showed a threefold reduced risk of an event compared with *MLL*-R patients, although all *MLL*-G patients were grouped together into a single category. To better understand the association of different chromosomal abnormalities and outcome among *MLL*-G infants, here we have carried out detailed cytogenetic investigation of two infant ALL trials: Interfant-99 and Children's Oncology Group (COG)-P9407.

Patients were 365 days old or less with newly diagnosed ALL without a rearrangement of the *MLL* gene enrolled to Interfant-99 (May 1999–December 2005; $n=110$) and COG-P9407 (June 1996–October 2006; $n=52$).[6,9] Individual study groups obtained ethical approval, and treating physicians obtained informed consent from parents or guardians. The presence of *MLL* gene rearrangements was excluded using fluorescence *in situ* hybridization (FISH), reverse transcription (RT)-PCR and/or Southern blotting, as previously reported.[6] Each national study group provided patient data, including cytogenetics, FISH and molecular results. EFS and overall survival (OS) were calculated from the date of trial enrolement to the date of the first event (induction failure, relapse, second malignancy or death) or last follow-up. Median follow-up time was 7 years.

Among 162 *MLL*-G patients, no cytogenetic data were available for 34 (21%), resulting in a success rate of 79%. An abnormal karyotype was detected in 90/128 (70%) patients with a successful cytogenetic result (Supplementary Table 1) with the remainder classified as normal based on the presence of at least 10 (but usually 20) normal metaphases. They were categorized according to cytogenetic risk group as previously defined for childhood ALL.[10] Compared with childhood ALL (1–18 years) using data from the UKALL97/99 treatment trial,[10] the frequency of good risk cytogenetic abnormalities among *MLL*-G infants was significantly lower (12 vs 60%, $P<0.01$), whereas the frequency of poor risk abnormalities (excluding *MLL* translocations) was similar (8 vs 10%). Although *ETV6–RUNX1* fusion is present in 25% of childhood ALL, we found no *ETV6–RUNX1* cases among the 75 patients tested by FISH or RT-PCR. High hyperdiploidy (HeH) was the most

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nature genetics

Recurrent mutations in multiple components of the cohesin complex in myeloid neoplasms

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Cohesin is a multimeric protein complex that is involved in the cohesion of sister chromatids, post-replicative DNA repair and transcriptional regulation. Here we report recurrent mutations and deletions involving multiple components of the cohesin complex, including *STAG2*, *RAD21*, *SMC1A* and *SMC3*, in different myeloid neoplasms. These mutations and deletions were mostly mutually exclusive and occurred in 12.1% (19/157) of acute myeloid leukemia, 8.0% (18/224) of myelodysplastic syndromes, 10.2% (9/88) of chronic myelomonocytic leukemia, 6.3% (4/64) of chronic myelogenous leukemia and 1.3% (1/77) of classical myeloproliferative neoplasms. Cohesin-mutated leukemic cells showed reduced amounts of chromatin-bound cohesin components, suggesting a substantial loss of cohesin binding sites on chromatin. The growth of leukemic cell lines harboring a mutation in *RAD21* (Kasumi-1 cells) or having severely reduced expression of RAD21 and STAG2 (MOLM-13 cells) was suppressed by forced expression of wild-type RAD21 and wild-type RAD21 and STAG2, respectively. These findings suggest a role for compromised cohesin functions in myeloid leukemogenesis.

Recent genetic studies have led to the discovery of a number of new mutational targets in myeloid malignancies, unmasking unexpected roles for deregulated histone modification and DNA methylation in both acute and chronic myeloid neoplasms[1,2]. However, knowledge of the spectrum of gene mutations in myeloid neoplasms remains incomplete. We previously reported a whole-exome sequencing study of 29 paired tumor and normal samples of myeloid neoplasms with myelodysplastic features[3]. Although our major discovery was that frequent spliceosome mutations are uniquely associated with myelodysplasia phenotypes, we also identified hundreds of previously unreported gene mutations[3]. Most of those mutations affected single individuals only and are probably passenger changes. Therefore, their importance in leukemogenesis remains undetermined. However, through closer inspection of an updated list of mutations, including newly validated single-nucleotide variants, we identified additional recurrent mutations involving *STAG2*, a core component of the cohesin complex (Online Methods and **Supplementary Table 1**). In addition, we found that two other functionally related cohesin components, *STAG1* and *PDS5B*, were mutated in single specimens (**Supplementary Fig. 1**).

Cohesin is a multimeric protein complex that is conserved across species and is composed of four core subunits, SMC1, SMC3, RAD21

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