Clinical characteristics and prognosis of acute myeloid leukemia associated with DNA-methylation regulatory gene mutations

Takeshi Ryotokuji,[1]* Hiroki Yamaguchi,[1]* Toshimitsu Ueki,[2] Kensuke Usuki,[3] Saiko Kurosawa,[4] Yutaka Kobayashi,[5] Eri Kawata,[5] Kenji Tajika,[6] Seiji Gomi,[6] Junya Kanda,[7] Anna Kobayashi,[1] Ikuko Omori,[1] Atsushi Marumo,[1] Yusuke Fujiwara,[1] Shunsuke Yui,[1] Kazuki Terada,[1] Keiko Fukunaga,[1] Tsuneaki Hirakawa,[1] Kunihito Arai,[1] Tomoaki Kitano,[1] Fumiko Kosaka,[1] Hayato Tamai,[1] Kazutaka Nakayama,[1] Satoshi Wakita,[1] Takahiro Fukuda,[4] and Koiti Inokuchi[1]

[1]Department of Hematology, Nippon Medical School, Tokyo; [2]Department of Hematology, Nagano Red Cross Hospital; [3]Department of Hematology, NTT Medical Center Tokyo; [4]Department of Hematopoietic Stem Cell Transplantation, National Cancer Center Hospital, Tokyo; [5]Department of Hematology, Japanese Red Cross Kyoto Daini Hospital; [6]Department of Hematology, Yokohama Minami Kyousai Hospital, Kanagawa; and [7]Division of Hematology, Saitama Medical Center Jichi Medical University, Japan

*TR and HY contributed equally to this work

Haematologica 2016
Volume 101(9):1074-1081

Correspondence:

y-hiroki@fd6.so-net.ne.jp

Received: January 23, 2016.

Accepted: May 30, 2016.

Pre-published: May 31, 2016.

doi:10.3324/haematol.2016.143073

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/101/9/1074

ABSTRACT

In recent years, it has been reported that the frequency of DNA-methylation regulatory gene mutations – mutations of the genes that regulate gene expression through DNA methylation – is high in acute myeloid leukemia. The objective of the present study was to elucidate the clinical characteristics and prognosis of acute myeloid leukemia with associated DNA-methylation regulatory gene mutation. We studied 308 patients with acute myeloid leukemia. DNA-methylation regulatory gene mutations were observed in 135 of the 308 cases (43.8%). Acute myeloid leukemia associated with a DNA-methylation regulatory gene mutation was more frequent in older patients (P<0.0001) and in patients with intermediate cytogenetic risk (P<0.0001) accompanied by a high white blood cell count (P=0.0032). DNA-methylation regulatory gene mutation was an unfavorable prognostic factor for overall survival in the whole cohort (P=0.0018), in patients aged ≤70 years, in patients with intermediate cytogenetic risk, and in *FLT3*-ITD-negative patients (P=0.0409). Among the patients with DNA-methylation regulatory gene mutations, 26.7% were found to have two or more such mutations and prognosis worsened with increasing number of mutations. In multivariate analysis DNA-methylation regulatory gene mutation was an independent unfavorable prognostic factor for overall survival (P=0.0424). However, patients with a DNA-methylation regulatory gene mutation who underwent allogeneic stem cell transplantation in first remission had a significantly better prognosis than those who did not undergo such transplantation (P=0.0254). Our study establishes that DNA-methylation regulatory gene mutation is an important unfavorable prognostic factor in acute myeloid leukemia.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease whose onset involves a variety of chromosomal abnormalities and gene mutations.[1,2] To improve the outcome of AML treatment, it is very important to establish a prognosis from cytogenetic analysis and provide accordingly differentiated treatment.[1,2] Standard

chemotherapy is given to patients with a favorable prognosis according to their cytogenetic profile, whereas allogeneic transplantation in first remission is actively promoted for patients with an unfavorable cytogenetic prognosis.[3]

Meanwhile, for the approximately 60% of cases that fall into the intermediate prognosis group, clinicians are seeking to provide a clear prognosis and differentiated therapy based on cytogenetic analysis, which have not been available for this group of patients. In the latter half of the 2000s, gene mutations in AML were successively discovered, and attempts have been made to use these to provide a differentiated prognosis. The most important of these gene mutations for differentiated prognosis are *FMS-like tyrosine kinase* 3 internal tandem duplications (*FLT3*-ITD), *nucleophosmin 1 (NPM1)* gene mutation, and *CCAAT/enhancer binding protein A* (*CEBPA*) gene mutation.[4-9] In 2008, Richard *et al.* reported that AML cases that were *FLT3*-ITD-negative and *NPM1*-mutation-positive, or that were accompanied by *CEBPA* biallelic mutation, were associated with a favorable prognosis and that allogeneic hematopoietic stem cell transplantation in first remission was not indicated.[9,10] It was also reported that *FLT3*-ITD-positive AML has a very unfavorable prognosis, which might be improved by allogeneic hematopoietic stem cell transplantation in first remission.[10] These findings have been integrated as prognostic factors in the European Leukemia Net (ELN) and National Comprehensive Cancer Network (NCCN) guidelines,[12,13] which are becoming widely applied in clinical practice.

However, these gene mutations are observed in only around 30% of cases with intermediate cytogenetic prognosis, meaning that differentiation of prognosis is still insufficient. In recent years, the use of next-generation sequencers has facilitated energetic exploration of gene mutations, leading to the discovery of other AML-related gene mutations,[14-17] and mutations in the genes that regulate *DNA methylation,* such as *DNA methyltransferase 3 alpha (DNMT3A), Tet methylcytosine dioxygenase 2 (TET2), isocitrate dehydrogenase1 (IDH1)*, and *isocitrate dehydrogenase 2 (IDH2)*.[18-23] It has been suggested that these gene mutations may also have prognostic relevance in some cases of AML, but this view has yet to become established. Moreover, a further series of gene mutations has recently been discovered, so that differentiated prognosis based on gene mutations has actually become more confused.[14,16,18,24]

The focus of the present study was DNA-methylation regulatory gene mutations (DMRGM), which are mutations of the genes that regulate gene expression through DNA methylation (*IDH1*, *IDH2*, *DNMT3A*, *TET2*). Studies have been carried out of the various DMRGM and other genes reported as indicating an unfavorable prognosis in AML.[18-20,22, 23,25-30] Our group has also reported that AML with DMRGM at onset is associated with a high frequency of *FLT3*-ITD at relapse and has an unfavorable prognosis.[31] However, since comprehensive gene mutation analysis using a next-generation sequencer is costly, there have so far been few reports on DMRGM-based prognostic analysis covering a large number of cases. In particular, there are very few reports on large cohorts in which there is a combined analysis of both *TET2* mutation, which is mutually exclusive with *IDH1* and *IDH2* mutations, and *DNMT3A* mutation.[20] Since *IDH1/2* mutations and *TET2* mutation are mutually complementary, DMRGM need to be subjected to integrated analysis as a group, but there have so far been no reports of such analysis having been performed. We therefore analyzed the clinical characteristics of a group of AML patients with DMRGM and the prognostic impact of these mutations.

Methods

Patients

We studied 308 patients with *de novo* AML (excluding M3) treated at Nippon Medical School Hospital or its affiliated institutions. A comprehensive genetic mutational analysis, as described below, was conducted among patients with ≥20% blasts in bone marrow or peripheral blood. The study was conducted in accordance with the Declaration of Helsinki; written informed consent was obtained from the participants, and the patients were analyzed and treated with respect for their welfare and free will. The study protocol was approved by our institutional review board.

Screening for cytogenetic mutations

G-band analysis was performed on bone marrow samples obtained from patients at initial presentation. When it was difficult to obtain bone marrow samples, peripheral blood was used instead. For patients suspected of having M2, M3, or M4e AML based on the French-American-British classification, fluorescence *in situ* hybridization analysis was used to search additionally for *RUNX1-RUNX1T1*, *PML-RARA*, and *CBFB-MYH11* mutations. The cytogenetic prognosis was then classified in accordance with the system recommended by the ELN.

Screening for molecular genetic abnormalities in all exons of 20 genes and hotspots of eight genes

An oligonucleotide library was generated by emulsion polymerase chain reaction using order-made probes designed against the exons of the following 20 genes: *TET2* (HGNC:25941), *DNMT3A* (HGNC:2978), *ASXL1* (HGNC:18318), *KMT2A* (HGNC:7132: the HGNC nomenclature of *MLL* has recently changed to *KMT2A*), *RUNX1* (HGNC:10471), *KIT* (HGNC:6342), *TP53* (HGNC:11998), *PTPN11* (HGNC:9644), *GATA2* (HGNC:4171), *WT1* (HGNC:12796), *STAG2* (HGNC:11355), *RAD21* (HGNC:9811), *SMC1A* (HGNC:11111), *SMC3* (HGNC:2468), *DAXX* (HGNC:2681), *BCOR* (HGNC:20893), *BCORL1* (HGNC:25657), *NF1* (HGNC:7765), *DDX41* (HGNC:18674), and *PHF6* (HGNC:18145). The library was sequenced with the next-generation sequencer Ion PGM™. With respect to detected mutations, the NCBI and COSMIC databases were used to search for polymorphisms and cancer-related mutations. For newly identified mutations, genetic polymorphisms were checked using Sanger sequencing with remission-stage samples.

Genes located in known hot spots (*FLT3*-ITD and *FLT3*-TKD, *NPM1*, *IDH1*, *IDH2*, *NRAS*, and *KRAS*), those for which probe design for emulsion sequencing was difficult (*CEBPA*), and those for which analysis with Ion PGM™ was difficult (*KMT2A*-PTD), were analyzed using previously reported methods.[31]

Statistical analysis

The primary endpoint was overall survival of DMRGM-positive AML patients in the study cohort. A hazard ratio of 0.707 and an overall survival rate of 50% were assumed for the sample size calculation. Recruitment of approximately 300 patients allowed for a power of 80% in detecting a difference of that size with a type I error of 5%. During the study period, there were 80 deaths among the 135 DMRGM-positive AML patients. This provided an 80%

statistical power to detect a hazard ratio of 0.542 with a significance level (alpha) of 0.05 (two-tailed) regarding overall survival, which was defined as the time interval from the date of diagnosis to the date of death. Relapse-free survival for patients who had achieved complete remission was calculated as the time interval from the date of complete remission to the date of relapse.

The χ^2 test was used to test the association between categorical variables and the presence and absence of mutations. The Fisher exact test was used if the expected frequency of an event was less than five in any cell of a 2 × 2 table. The non-parametric Mann–Whitney U test was used to determine the statistical significance of differences in median values. All statistical tests were two-sided. The Kaplan-Meier method and log-rank test were applied to analyze overall survival and relapse-free survival. With respect to prognostic factors, multivariate analysis was conducted with the Cox proportional hazards model. A stepwise backward procedure selection model was used to extract independent events. Events at a significance level of $P \leq 0.20$ were analyzed. Statistical analyses were performed using GraphPad Prism (version 6.00 for Windows, GraphPad Software, La Jolla, CA, USA) and IBM SPSS Statistics (version 21.0 for Windows, IBM Corp., Armonk, NY, USA), while the power calculation was performed using GraphPad StatMate (version 2.00 for Windows).

Results

Patients' background

The average age of the 308 patients studied was 54.1 years (range, 17-86 years). There were 181 males (58.8%) and 127 females (41.2%). Based on cytogenetic analysis, 60 cases (19.5%) were assigned to the favorable risk group, 51 (16.6%) to the unfavorable risk group, and 184 (59.7%) to the intermediate risk group. Overall, 148 (48.1%) had a normal karyotype (Table 1, *Online Supplementary Table S1*). Gene mutations were observed in 265 cases (86.0%), with the most frequent being *NPM1* mutation (88 cases, 28.6%), *DNMT3A* mutation (71, 23.1%), and *FLT3*-ITD (65, 21.1%) (*Online Supplementary Figure S1*, *Online Supplementary Table S2*). Gene mutations with an incidence of ≤3% were excluded from the analysis as they were too infrequent to be studied as prognostic factors (*Online Supplementary Figure S1*).

Clinical characteristics of patients with DNA-methylation regulatory gene mutations

A *DNMT3A* mutation was found in 71 cases (23.1%), *TET2* mutation in 57 (18.5%), *IDH2* mutation in 28

Table 1. Clinical background of the AML patients studied.

	All		DMRGM-positive		DMRGM-negative		
	n=308	%	n=135	%	n=173	%	*P* value
Age, years							
Mean	54.1		59.0		50.2		<0.0001
Range	17-86		18-86		17-82		
Sex							
Male	181	58.8	72	53.3	109	63.0	0.1025
Female	127	41.2	63	46.7	64	37.0	
White cell count, x10^9/L							
Mean	57.2		75.2		43.1		0.0032
Range	0.3-677		0.3-677		0.6-483		
Cytogenetic risk group							
Favorable	60	19.5	4	3.0	56	32.4	<0.0001
Intermediate	184	59.7	109	80.7	75	43.2	
Adverse	51	16.6	16	11.9	35	20.2	
Unknown	13	4.2	6	4.4	7	4.0	
FAB classification							
M0	17	5.5	5	3.7	12	6.9	0.3150
M1	75	24.4	42	31.1	33	19.1	0.0163
M2	115	37.3	39	28.9	76	43.9	0.0089
M4	40	13.0	22	16.3	18	10.4	0.1712
M4e	12	3.9	0	0.0	12	6.9	0.0015
M5	33	10.7	21	15.6	12	6.9	0.0246
M6	7	2.3	2	1.5	5	2.9	0.4729
M7	1	0.3	1	0.7	0	0.0	0.4383
Not determined	8	2.6	3	2.2	5	2.9	1.0000
Induction therapy							
(IDA/DNR/ACR)+AraC	292	94.8	125	82.6	167	96.5	0.2989
Others (AVV, VP16+AraC)	3	1.0	2	1.5	1	0.6	
Unknown	13	4.2	8	5.9	5	2.9	
Stem cell transplantation							
First CR	37	12.0	14	10.4	23	13.3	0.4831
Second CR	13	4.2	4	3.0	9	5.2	0.4015
≥Third CR/disease	36	11.7	12	8.9	24	13.9	0.2122

CR: complete remission. IDA: idarubicin; DNR: daunorubicin; ACR: aclarubicin; AraC: cytarabine; AVV: cytarabine+etoposide+vincristine+vinblastine; VP16: etoposide.

(9.1%), and *IDH1* mutation in 17 (5.5%). *TET2* mutation was more frequent in older patients (average 63.5 years, *P*<0.0001) (*Online Supplementary Table S3*). High white blood cell counts were found in patients with mutations of *DNMT3A* (average 86.6x10^9/L, *P*=0.0028) and *TET2* (average 80.7x10^9/L, *P*=0.0389). Mutations in *DNMT3A* (*P*<0.0001), *TET2* (*P*=0.0087), and *IDH2* (*P*=0.0199) were more frequent in the intermediate cytogenetic risk group (*Online Supplementary Table S3*).

A DMRGM was found in 135 cases (43.8%), indicating that this group of gene mutations occurs with very high frequency in AML. There were 99 cases (73.3%) with one DMRGM (DMRGM1), 34 (25.2%) with two (DMRGM2), and two (1.5%) with three (DMRGM3). No cases had four or more DMRGM (Figure 1). Compared to AML without DMRGM (non-DMRGM), AML with DMRGM was more frequent in older patients (DMRGM: average 59.0 years; non-DMRGM: average 50.2 years; *P*<0.0001), in patients with high white blood cell count (DMRGM: average 75.2 x10^9/L; non-DMRGM: average 43.1x10^9/L; *P*=0.0032), and in the intermediate cytogenetic risk group (DMRGM: 80.7%; non-DMRGM: 43.4%; *P*<0.0001) (Table 1).

Overlapping gene mutations

DNMT3A mutation was frequently present together with *FLT3*-ITD (*P*=0.0005), *FLT3*-TKD (*P*=0.0200), and mutations of *PTPN11* (*P*=0.0254), *NPM1* (*P*<0.0001), and *IDH2* (*P*=0.0002), but was mutually exclusive with *NRAS* mutation (*P*=0.0364) and *CEBPA* double mutation (*CEBPA* dm, *P*=0.0265). *TET2* mutation was frequently present together with *NPM1* mutation (*P*=0.0031) and *CEBPA* monoallelic mutation (*P*=0.0441), but was mutually exclusive with *NRAS* mutation (*P*=0.0229), *IDH1* mutation (*P*=0.0497), and *IDH2* mutation (*P*=0.0380) (*Online Supplementary Table S4*). DMRGM was frequently present together with *FLT3*-ITD (*P*=0.0007) and mutations of *PTPN11* (*P*=0.0190) and *NPM1* (*P*<0.0001), but was mutually exclusive with mutations of *NRAS* (*P*=0.0035) and *WT1* (*P*=0.0241) (*Online Supplementary Table S4*).

Prognostic analysis in all subjects

With regards to overall survival, the factors associated with an unfavorable prognosis were age >70 years (*P*<0.0001), adverse cytogenetic risk (*P*<0.0001), *FLT3*-ITD (*P*<0.0001), *KMT2A*-PTD (*P*=0.0152), *DNMT3A* mutation (*P*=0.0017), *TET2* mutation (*P*=0.0043), and *TP53* mutation (*P*<0.0001). In recent years, the development of a non-myeloablative regimen for hematopoietic stem cell transplantation has made it possible also for patients aged 65 to 69 years to undergo allogeneic hematopoietic stem cell transplantation. In the present study, analysis of prognosis was therefore stratified between patients aged ≤70 and >70 years. Favorable cytogenetic risk (*P*<0.0001), allogeneic hematopoietic stem cell transplantation (in first or second remission) (*P*<0.0001), and *CEBPA* dm (*P*=0.0282) were associated with a favorable prognosis (*Online Supplementary Table S5*).

As regards relapse-free survival, the factors associated with an unfavorable prognosis were adverse cytogenetic risk (*P*=0.0210), *FLT3*-ITD (*P*<0.0001), *TET2* mutation (*P*=0.0219), and *TP53* mutation (*P*=0.0011), whereas allogeneic hematopoietic stem cell transplantation (in first remission) (*P*<0.0001) and *NRAS* mutation (*P*=0.0142) were associated with a favorable prognosis (*Online Supplementary Table S5*).

Stratified analysis of FLT3-ITD-negative patients aged below 70 years with intermediate cytogenetic prognosis

Age >70 years, adverse cytogenetic risk, and *FLT3*-ITD were powerful poor prognostic factors. Rates of overall survival and relapse-free survival were therefore analyzed following stratification based on age ≤70 years, intermediate cytogenetic prognosis, and *FLT3*-ITD negativity. For overall survival, *DNMT3A* mutation was associated with an unfavorable prognosis (*P*=0.0429). On the other hand, allogeneic hematopoietic stem cell transplantation (in first or second remission) was associated with a favorable prognosis (*P*=0.0283) (*Online Supplementary Table S5*). For relapse-free survival, *TP53* mutation was associated with an unfavorable prognosis (*P*=0.0068), while, allogeneic hematopoietic stem cell transplantation (in first remission) was associated with a favorable prognosis (*P*=0.0008) (*Online Supplementary Table S5*).

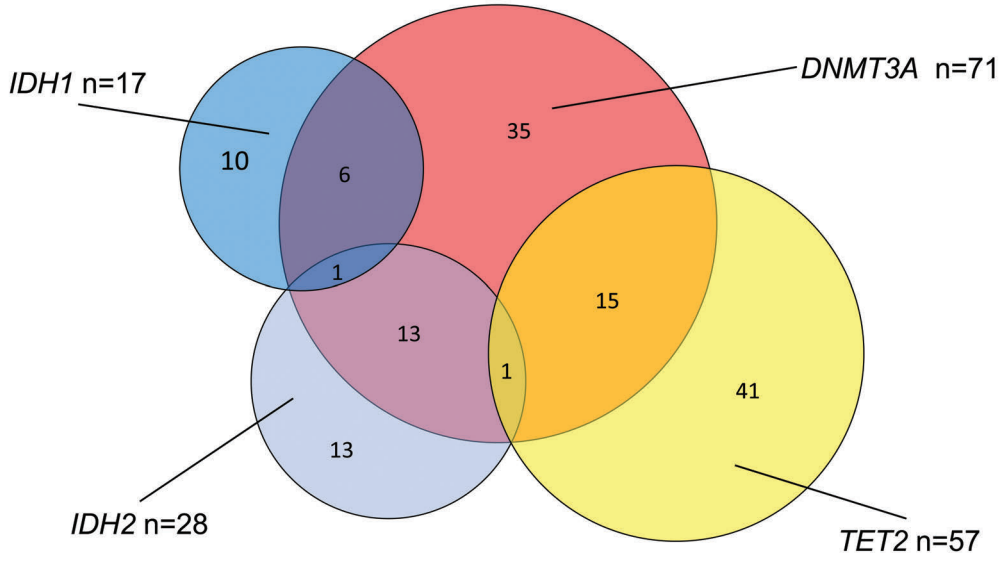

Figure 1. Frequency and overlap of DMRGM. There were 173 cases of DMRGM-negative AML (DMRGM0), 99 cases with one DMRGM (DMRGM1: 10 cases with *IDH1*, 13 with *IDH2*, 35 with *DNMT3A*, and 41 with *TET2*), 34 cases with two DMRGM (DMRGM2: 6 with *IDH1+DNMT3A*, 13 with *DH2+DNMT3A*, and 15 with *DNMT3A+TET2*), and two cases with three DMRGM (DMRGM3: 1 with *IDH1+IDH2+DNMT3A* and 1 with *IDH2+DNMT3A+TET2*).

Prognostic impact of DNA-methylation regulatory gene mutations

The significance of DMRGM as a prognostic factor was investigated. With regards to overall survival, cases with DMRGM had a significantly poorer prognosis than cases without DMRGM (P=0.0018) (Figure 2A). Furthermore, patients with DMRGM tended to have a higher rate of relapse than patients without DMRGM, but the difference was not statistically significant (Figure 2B). Additionally, overall and relapse-free survival rates were analyzed following stratification based on age ≤70 years, intermediate cytogenetic risk, and *FLT3*-ITD negativity. With regards to overall survival, cases with DMRGM had a significantly poorer prognosis than cases without DMRGM (P=0.0409) (Figure 2C). However, there was no significant difference in relapse-free survival between patients with and without DMRGM (Figure 2D).

Prognostic impact of number of DNA-methylation regulatory gene mutations

Overall and relapse-free survival rates were studied relative to the number of DMRGM mutations. The greater the number of DMRGM, the poorer the prognosis (DMRGM0 *versus* DMRGM2+3: P<0.0001; DMRGM1 *versus* DMRGM2+3: P=0.0244; DMRGM0 *versus* DMRGM1: P=0.0651) (Figure 3A). As far as concerns relapse-free survival, DMRGM2+3 was associated with a poorer prognosis than DMRGM0 (P=0.0244) and DMRGM1 (P=0.0824) (Figure 3B). Additionally, overall and relapse-free survival rates were analyzed following stratification based on age ≤70 years, intermediate cytogenetic risk, and *FLT3*-ITD negativity. The overall survival rate of patients with DMRGM2+3 was significantly lower than that of patients with DMRGM0 (P=0.0189) (Figure 3C). The relapse-free survival rate of patients with DMRGM2+3 tended to be worse than that of patients with DMRGM1, but not significantly so (P=0.0600) (Figure 3D).

Prognostic significance of DNA-methylation regulatory gene mutation combinations

In the present analysis, prognosis was poor for patients with DMRGM, but was found to be poorer still for those with DMRGM2+3. We, therefore, investigated whether the combinations of DMRGM influence prognosis in patients with DMRGM2+3. The 15 patients with *DNMT3A* mutation accompanied by *TET2* mutation (*DNMT3A* mutation$^+$/*TET2* mutation$^+$) were compared with the 20 patients with *DNMT3A* mutation accompanied by *IDH* mutation (*DNMT3A* mutation$^+$/*IDH1* or *IDH2* mutation$^+$). No clear significant difference was

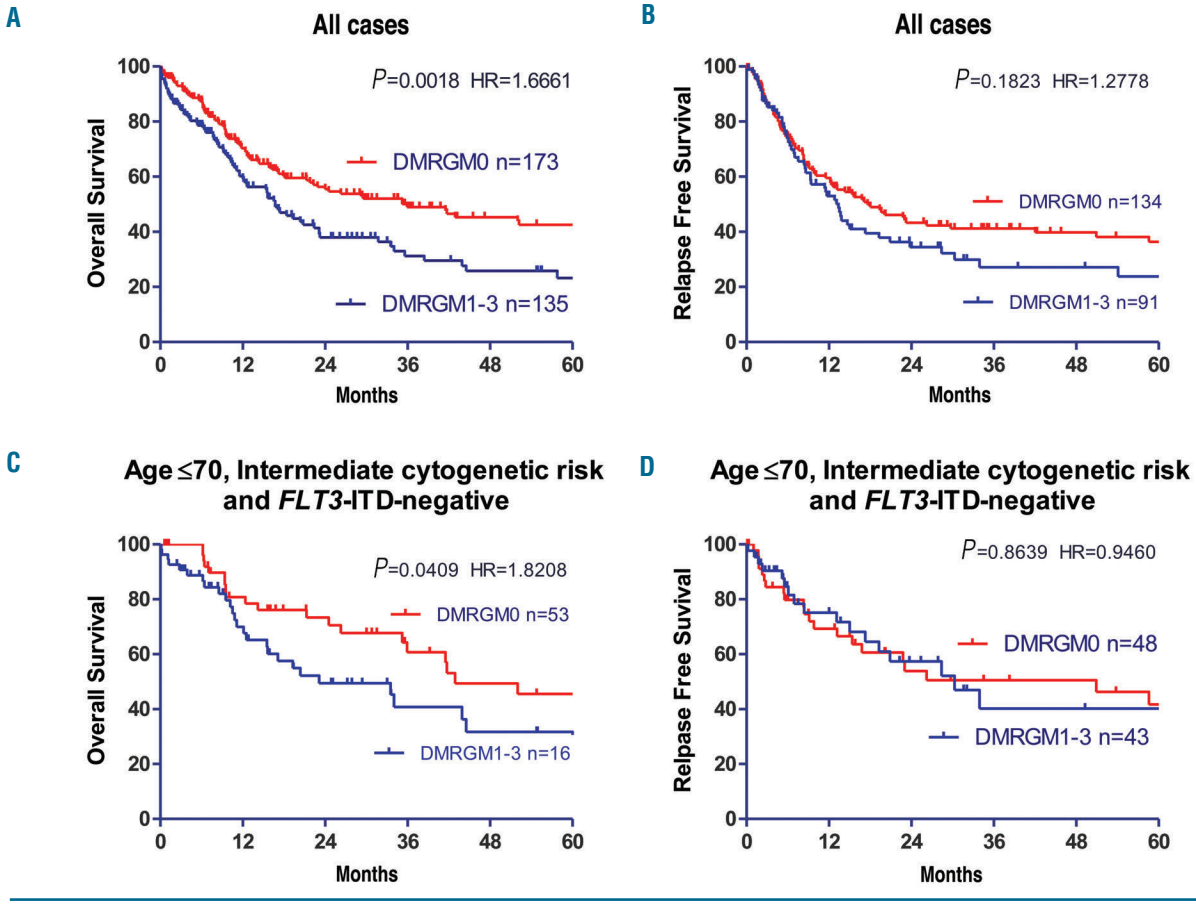

Figure 2. Overall and relapse-free survival rates in AML cases with and without DMRGM. (A) Overall survival rate for all cases. (B) Relapse-free survival rate for all cases. (C) Overall survival rate in *FLT3*-ITD-negative cases aged ≤ 70 years with intermediate cytogenetic prognosis. (D) Relapse-free survival rate in *FLT3*-ITD-negative cases aged ≤ 70 years with intermediate cytogenetic prognosis. HR: Hazard ratio.

established in overall survival rate (*Online Supplementary Figure S2A*), but *DNMT3A* mutation$^+$/*TET2* mutation$^+$ was associated with a poorer relapse-free survival rate (P=0.0349) (*Online Supplementary Figure S2B*).

Multivariate analysis

Multivariate analysis (Cox proportional hazard model) *via* the step-wise method was carried out using the following variables: age >70 years, poor cytogenetic risk, transplantation time (in first or second complete remission), DMRGM, and gene mutation associated with poor prognosis. The following were identified as independent unfavorable prognostic factors for overall survival: age >70 years (P=0.0001); adverse cytogenetic risk (P<0.0001); DMRGM (P=0.0424); *FLT3*-ITD (P<0.0001); and *TP53* mutation (P=0.0003) (Table 2), whereas the independent unfavorable prognostic factors for relapse-free survival were: unfavorable cytogenetic risk (P=0.0005); *FLT3*-ITD (P<0.0001); *TP53* mutation (P=0.0089); and *KMT2A*-PTD (P=0.0236) (Table 2).

Efficacy of allogeneic transplantation in first remission for patients with DNA-methylation regulatory gene mutation-positive acute myeloid leukemia

The efficacy of allogeneic transplantation in first remission for patients with DMRGM aged ≤70 years was examined. In terms of overall survival, patients with DMRGM who underwent allogeneic stem cell transplantation in first remission [SCT (1st CR)$^+$/DMRGM$^+$] had a significantly more favorable prognosis than those who did not undergo such transplantation [SCT(1st CR)$^-$/DMRGM$^+$] (P=0.0254) (Figure 4A). Likewise, in terms of relapse-free survival, SCT (1st CR)$^+$/DMRGM$^+$ cases had a significantly more favorable prognosis than SCT (1st CR)$^-$/DMRGM$^+$ cases (P=0.0049) (Figure 4B). A similar analysis was undertaken in cases aged ≤70 years, cases with intermediate cytogenetic risk, and *FLT3*-ITD-negative cases, but as the number of SCT(1st CR)$^+$/DMRGM$^+$ cases was low, at just seven, the efficacy of allogeneic stem cell transplantation in first remission is not shown here.

Discussion

It was confirmed that DMRGM are very frequently present in AML, being observed in 135 of the 308 cases (43.8%) studied. A DMRGM was an unfavorable prognostic factor for overall survival in the whole group and in patients aged ≤70 years, in patients with an intermediate cytogenetic risk group, and in *FLT3*-ITD-negative patients. Allogeneic stem cell transplantation in first remission may improve the prognosis of cases with DMRGM mutation.

Until now, the significance of individual DMRGM as prognostic factors in AML has not been clear. Regarding *DNMT3A*, the most frequent of the DMRGM, Ley *et al.* reported that *DNMT3A* R882 mutation and non-R882

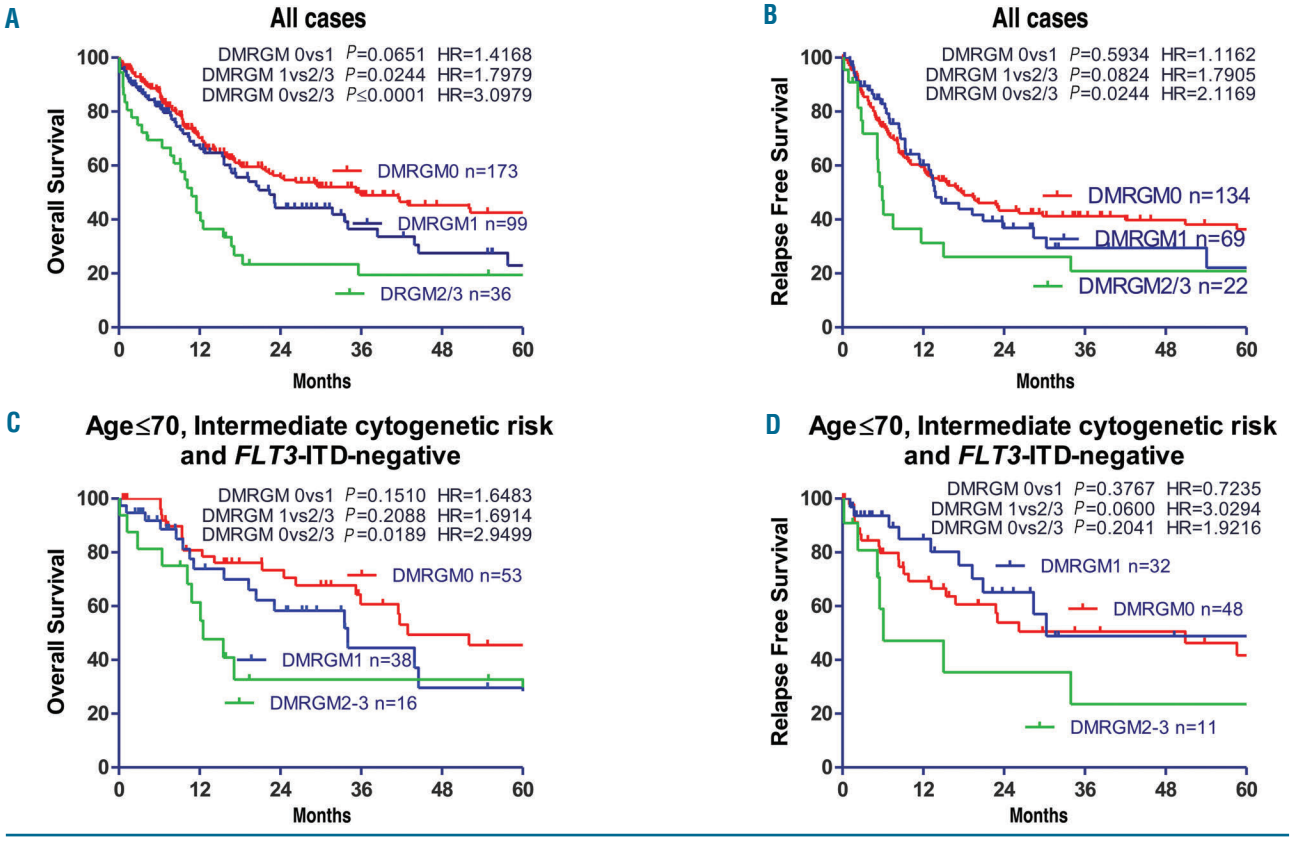

Figure 3. Overall and relapse-free survival rates in AML patients with no DMRGM, one DMRGM, and two or more DMRGM (A) Overall survival rate for all cases. (B) Relapse-free survival rate for all cases. (C) Overall survival rate in *FLT3*-ITD-negative cases aged ≤ 70 years with intermediate cytogenetic prognosis. (D) Relapse-free survival rate in *FLT3*-ITD-negative cases aged ≤ 70 years with intermediate cytogenetic prognosis. HR: Hazard ratio.

mutation are both associated with unfavorable prognosis, independently of whether *FLT3*-ITD is present.[18] Meanwhile, Patel *et al.* reported that patients with intermediate cytogenetic risk who have a *DNMT3A* mutation do not have an unfavorable prognosis even if *FLT3*-ITD is not present.[24]

Because of the expense associated with comprehensive gene mutation analysis using a next-generation sequencer, there have so far been few reports on DMRGM-based prognostic analysis involving a large number of patients. Such studies consist of the one by Patel *et al.*, based on 398 cases, and one by Hou *et al.*, based on 500 cases.[24,25] Our analysis covered four DMRGM – *IDH1*, *IDH2*, *DNMT3A*, and *TET2* – in 308 cases of AML, a large cohort suggesting reliable results. Moreover, ours is the first study in which AML prognosis was explored with division into groups based on DMRGM.

Our study revealed that the greater the number of DMRGM, the poorer the prognosis is. It has been reported that increasing numbers of DMRGM are associated with unfavorable outcome in a number of hematologic malignancies. For example, Papaemmanuil *et al.* described that leukemia-free survival in patients with myelodysplastic syndromes becomes shorter with increasing number of gene mutations.[32] Guglielmelli *et al.* also reported that, in primary myelofibrosis, the greater the number of mutations of *ASXL1*, *EZH2*, *SRSF2*, and *IDH1/2*, the poorer the patients' prognosis is.[33] We have also reported recently that three or more gene mutations is an unfavorable prognostic factor in *de novo* AML.[34] According to whole-exon mutation analysis using a next-generation sequencer, there are on average 2.5-5.0 gene mutations per case in AML,[14,18,34] and it has been established that the onset of AML requires the combination of a number of gene mutations.[16,17] These findings suggest that cases with a large number of gene mutations have a correspondingly high level of genomic instability and point to the strong possibility of mutations in genes not yet examined.[34] This is thought to result in a poorer prognosis. Our finding that prognosis in AML worsens with increasing number of DMRGM suggests that to improve prognostic analysis in AML it will be important not only to focus on each individual gene mutation, but also to seek to identify overall genomic instability in individual cases.

In a whole-exon analysis of 12,380 healthy subjects, Genovese *et al.* found that healthy subjects in whom DMRGM mutations were observed had a high rate of myeloid malignancies in hematopoietic organs a number of years later.[35] Shlush *et al.* demonstrated that, in AML with *DNMT3A* mutation, the mutation also occurred in hematopoietic stem cells with normal differentiation potential, so that even in cases in which chemotherapy induced remission, the *DNMT3A* mutation remaining in the stem cells subsequently led the resulting blood cells to proliferate and cause relapse of the leukemia.[36] If this finding applies to all DMRGM-positive AML cases, then chemotherapy alone cannot be expected to be effective in DMRGM-positive AML. As indicated by Patel *et al.*, for young patients with DMRGM-positive AML, allogeneic hematopoietic stem cell transplantation in first remission may be the only curative therapy available. The findings above indicate that the prognosis of patients with DMRGM-positive AML is unfavorable,

Table 2. Multivariate analysis of prognostic factors.

	Hazard ratio	*P* value	95% Confidence interval
Overall survival			
Age>70 years	2.2901	0.0001	1.5049 – 3.4848
Adverse cytogenetic risk	2.7025	<0.0001	1.7472 – 4.1802
SCT (first or second CR)	0.2917	<0.0001	0.1658 – 0.5133
DMRGM	1.5782	0.0424	1.0158 – 2.4521
FLT3-ITD	2.6250	<0.0001	1.7393 – 3.38945
RUNX1	0.6524	0.0918	0.3971 – 1.0719
TP53	2.5915	0.0003	1.5488 – 4.3362
KMT2A-PTD	1.5755	0.0983	0.9191 – 2.7010
GATA2	0.5523	0.1717	0.2358 – 1.2939
Relapse free survival			
Age>70 years	1.4937	0.1443	0.8716 – 2.5599
Adverse cytogenetic risk	2.6814	0.0005	1.5347 – 4.6848
SCT(first remission)	0.1466	<0.0001	0.0670 – 0.3211
FLT3-ITD	3.1407	<0.0001	2.0494 – 4.8131
NRAS	0.5634	0.0888	0.2910 – 1.0909
RUNX1	0.6602	0.1802	0.3597 – 1.2117
TP53	2.3102	0.0089	1.2341 – 4.3244
KMT2A-PTD	2.2479	0.0236	1.1147 – 4.5330

SCT: stem cell transplantation, CR: complete remission.

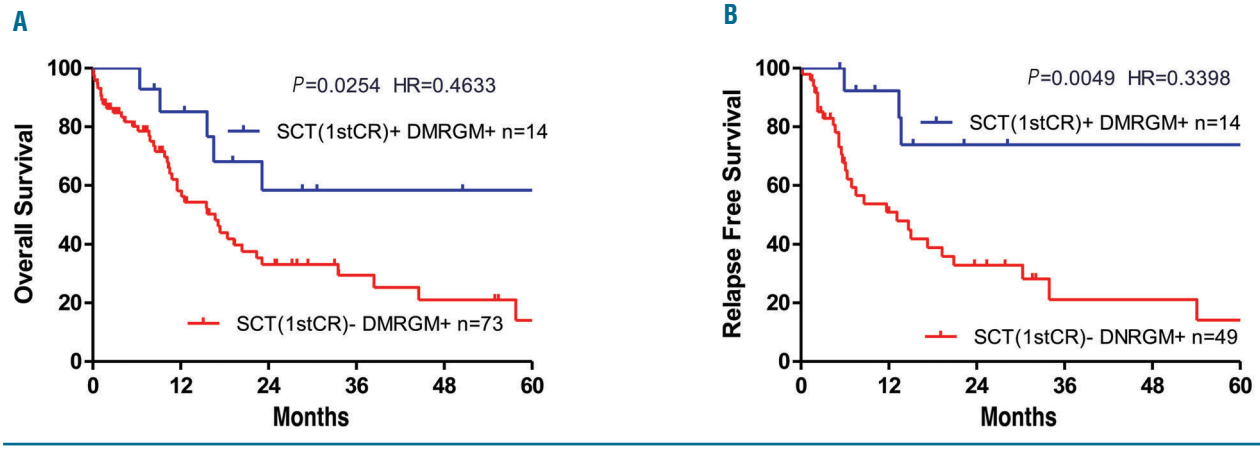

Figure 4. Efficacy of allogeneic stem cell transplantation in first remission for DMRGM-positive AML cases aged 70 years or below. (A) Overall survival rate for all cases. (B) Relapse-free survival rate for all cases. HR: hazard ratio; SCT: stem cell transplantation; 1stCR: first complete remission.

that the outcome worsens with increasing numbers of such mutations and that these patients should be treated with allogeneic hematopoietic stem cell transplantation in first remission.

One of the issues that needs to be considered when going forward with this research is that the subjects in this study were of Japanese ethnicity. So far. there have been no reports on the relation of ethnicity to frequency of gene mutations and prognosis in AML. Whether the present research findings would be replicated in cohorts of patients of other ethnicities is an area that requires investigation in the future. Moreover, the present study did not include patients treated with azacitidine or other demethylating agents. The efficacy of demethylating agents in DMRGM-positive AML is therefore another area requiring investigation.

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