Characteristics of *DNMT3A* mutations in acute myeloid leukemia

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Background

DNMT3A mutations occur in approximately 20% of AML cases and are associated with changes in DNA methylation. *CDKN2B* plays an important role in the regulation of hematopoietic progenitor cells and *DNMT3A* mutation is associated with *CDKN2B* promoter methylation. We analyzed the characteristics of *DNMT3A* mutations including their clinical significance in AML and their influence on promoter methylation and *CDKN2B* expression.

Methods

A total of 142 adults, recently diagnosed with de novo AML, were enrolled in the study. Mutations in *DNMT3A*, *CEBPA*, and *NPM1* were analyzed by bidirectional Sanger sequencing. We evaluated *CDKN2B* promoter methylation and expression using pyrosequencing and RT-qPCR.

Results

We identified *DNMT3A* mutations in 19.7% (N=28) of enrolled patients with AML, which increased to 29.5% when analysis was restricted to cytogenetically normal-AML. Mutations were located on exons from 8–23, and the majority, including R882, were found to be present on exon 23. We also identified a novel frameshift mutation, c.1590delC, in AML with biallelic mutation of *CEBPA*. There was no significant difference in *CDKN2B* promoter methylation according to the presence or type of *DNMT3A* mutations. *CDKN2B* expression inversely correlated with *CDKN2B* promoter methylation and was significantly higher in AML with R882H mutation in *DNMT3A*. We demonstrated that *DNMT3A* mutation was associated with poor AML outcomes, especially in cytogenetically normal-AML. The *DNMT3A* mutation remained as the independent unfavorable prognostic factor after multivariate analysis.

Conclusion

We characterized *DNMT3A* mutations in AML and revealed the association between the *DNMT3A* mutation and *CDKN2B* expression and clinical outcome.

Key Words   *DNMT3A*, *CDKN2B*, Methylation, R882H

INTRODUCTION

In hematologic malignancies, recurrent chromosomal abnormalities play a significant role in leukemogenesis [1]. Gene fusions and mutations, including those in *CEBPA* and *NPM1*, are major recurrent genetic abnormalities in acute myeloid leukemia (AML) and have been established as major indicators for classification [2]. Further, genes involved in DNA methylation, including *DNMT3A*, *IDH1*, *IDH2*, and *TET2*, were found to be frequently mutated, especially in the cytogenetically normal karyotype (CN)-AML [3-6]. DNA methylation, involved in the silencing of tumor-suppressor genes, has been associated with not only leukemogenesis but also with the clonal evolution of the myelodysplastic syndrome to AML [7, 8]. Therefore, mutations in these genes

may alter DNA methylation and may play an important role in disease pathogenesis in CN-AML. Somatic mutations in *DNMT3A* have been reported in approximately 20% and ~30%–35% of total AML and CN-AML, respectively [4]. Most *DNMT3A* mutations in AML have been found to be heterozygous and have been associated with changes in DNA methylation [9, 10]. A missense mutation, R882H, located in the methyltransferase domain, has been found to be the most common mutation [11, 12]. R882H DNMT3A inhibits the expression of the wild-type DNMT3A and reduces the de novo methyltransferase activity, which results in the focal hypomethylation at specific CpGs throughout the genomes of AML cells [13].

CDKN2B, located in band 9p21 adjacent to *CDKN2A*, encodes a cyclin-dependent kinase inhibitor, which forms a complex with the cyclin-dependent kinase 4 (CDK4) or CDK6, and induces a G1-phase cell-cycle arrest by inhibiting CDK4/6. It also plays an important role in the regulation of cellular commitment of the hematopoietic progenitor cells and myeloid cell differentiation [14]. *DNMT3A* mutations in patients with AML have been reported to cause different degrees of the DNMT3A activity and have resulted in a heterogeneous DNA methylation (hyper-methylation or hypo-methylation) [15]. Various studies have shown conflicting data on the association of *DNMT3A* mutation and *CDKN2B* promoter methylation in AML. *CDKN2B* has been reported to be commonly silenced by deletion or hypermethylation in AML, but R882 DNMT3A has been reported to reduce the DNA methylation activity, causing a focal methylation loss [16]. A comprehensive understanding of the association of *DNMT3A* mutation with *CDKN2B* promoter methylation remains to be elucidated, along with the effect on its expression and clinical significance.

In this study, we analyzed the characteristics and clinical significance of *DNMT3A* mutations in AML and their influence on promoter methylation and expression of *CDKN2B*. In addition, we evaluated the changes of the R882H DNMT3A mutational burden after chemotherapy to understand its significance.

MATERIALS AND METHODS

Patients

This study was approved by the institutional review board of the Seoul St. Mary's Hospital, the Catholic University of Korea (KC17SESE0768). A total of 142 adult patients, recently diagnosed with de novo AML at the Seoul St. Mary's Hospital from June 2015–February 2017, were enrolled in the study, consecutively. Patients who had undergone chemotherapy previously or had an antecedent hematologic disease, acute promyelocytic leukemia with *PML-RARA*, pure erythroid leukemia, acute megakaryoblastic leukemia, and acute leukemias of an ambiguous lineage were excluded. Diagnosis and classification of AML were based on the guidelines by WHO (2016) [17].

Cytogenetic and molecular analyses

Bone marrow cells were aspirated from patients, cultured under unstimulated culture conditions for 1 d–2 d, and harvested. Karyotyping was carried out using the Giemsa banding techniques. At least 20 metaphase-cells were analyzed. Cytogenetic abnormalities were classified according to the 2016 guidelines of the International System for Human Cytogenetic Nomenclature [18]. Mutations in *DNMT3A*, *CEBPA*, and *NPM1* were analyzed by a bidirectional Sanger sequencing using primers designed with Primer3 (http://bioinfo.ut.ee/primer3/) and described in terms of GenBank sequences (*DNMT3A*, NM_022552.4; *CEBPA*, NM_004364.4; *NPM1*, NM_002520.6). Internal tandem duplications of *FLT3* (*FLT3*-ITD) were analyzed, as per a previously reported method [19]. Primer sequences have been listed in Supplementary Table 1.

Analysis of *CDKN2B* promoter methylation

CpG methylation in the promoter region of *CDKN2B* was quantified using pyrosequencing. The EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) was used for bisulfite conversion of genomic DNA; the PyroMark Gold Q96 Reagents (Qiagen) and the PyroMarkTM Q96 ID instrument (Biotage AB, Uppsala, Sweden) were used for pyrosequencing, according to the manufacturer's instructions. A total of seven CpG sites were analyzed per sample. The primers for the analysis of *CDKN2B*-specific CpG regions were the same as those used by our group in a previous study [20].

CDKN2B expression

CDKN2B mRNA expression was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using TaqMan (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. RNA was isolated from the bone marrow aspirates using the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany). Taqman probes for *CDKN2B* (HS00793225_m1) with transcript p15, and the endogenous control (glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*); Hs99999905_m1) were used. All reactions were performed using an ABI 7500 Real-Time PCR system (Applied Biosystems). Gene expression levels were calculated based on the $2^{-\Delta\Delta Ct} \times 1{,}000$ method, after normalization to the transcript levels of *GAPDH*.

Quantitative analysis of *DNMT3A* c.2645G>A (R882H)

Primers and probes were derived from RefSeq NM_022552.4 using the OLIGO ver. 7.51 software (Molecular Biology Insights, Inc., Cascade, CO, USA). The PCR reaction was carried out as follows: 2 min at 50°C, 10 min at 95°C, 50 cycles of 15 s at 95°C, and 1 min at 60°C on a Mx3000PTM Real-Time PCR system (Stratagene, San Diego, CA, USA). Data were analyzed using the MxPro version 4.10 (Stratagene). *DNMT3A* mutant allele burden was calculated as a ratio of the copy number of *DNMT3A* mutant to that of β-actin. The analytical performance of the quantitative PCR assay has been described in the Supplementary data.

Statistical analysis

The correlations between parameters compared in contingency tables were found using the Chi-squared (χ^2) test. The Kaplan-Meier survival analysis was used to estimate overall survival (OS) and to compare differences between survival curves. OS was measured from date of initial diagnosis to the date of death, or last follow-up, from any cause. The multivariate Cox proportional-hazards regression method was used to analyze independent prognostic factors for OS. The variables, including age; karyotype; presence of mutations in *DNMT3A* and *FLT3*-ITD; subgroup of AML, such as a biallelic *CEBPA* mutation with a mutated *NPM1*, and AML with myelodysplasia-related changes (AML-MRC), were used as covariates. All statistical analyses were done using MedCalc (MedCalc Software, Ltd., Ostend, Belgium) and the level of statistical significance was set at $P < 0.05$.

Table 1. Characteristics of patients with de novo acute myeloid leukemia with *DNMT3A* mutations.

	Total (N=142)	*DNMT3A*-mt (N=28)	*DNMT3A*-wild (N=114)	*P*
	N of patients (%)	N of patients (%)	N of patients (%)	
Median age (range)	53.5 (17–88)	55.5 (34–88)	52 (17–82)	0.0042
Sex (male), N	82 (57.7)	12 (42.9)	70 (61.4)	0.0895
Cytogenetic risk				
Favorable	24 (16.9)	1 (3.6)	23 (20.2)	0.0363
Intermediate	92 (64.8)	24 (85.7)	68 (59.6)	0.0099
Adverse	26 (18.3)	3 (10.7)	23 (20.2)	0.2478
Cytogenetically normal AML	61 (43.0)	18 (64.3)	43 (37.7)	0.0112
2016 WHO classification				
AML with recurrent genetic abnormalities				
AML with t(8;21)(q22;q22);*RUNX1-RUNX1T1*	19 (13.4)	1 (3.6)	18 (15.8)	0.0998
AML with inv(16) or t(16;16);*CBFB-MYH11*	5 (3.5)	0	5 (4.4)	0.2828
AML with t(9;11);*KMT2A-MLLT3*	4 (2.8)	0	4 (3.5)	0.3400
AML with t(6;9);*DEK-NUP214*	1 (0.7)	0	1 (0.9)	0.6400
AML with inv(3) or t(3;3);*GATA2, MECOM*	2 (1.4)	0	2 (1.8)	0.5056
AML with t(9;22);*BCR-ABL1*	1 (0.7)	0	1 (0.9)	0.6400
AML with mutated *NPM1*	29 (20.4)	12 (42.9)	17 (14.9)	<0.001
AML with biallelic mutation of *CEBPA*	17 (12.0)	1 (3.6)	16 (14.0)	0.1452
AML, not otherwise specified				
AML with minimal differentiation	4 (2.8)	2 (7.1)	2 (1.8)	0.1632
AML without maturation	17 (12.0)	5 (17.9)	12 (10.5)	0.3841
AML with maturation	20 (14.1)	5 (17.9)	15 (13.2)	0.6858
Acute myelomonocytic leukemia	8 (5.6)	1 (3.6)	7 (6.1)	0.4963
Acute monoblastic and monocytic leukemia	3 (2.1)	1 (3.6)	2 (1.8)	0.6256
AML with myelodysplasia-related changes	12 (8.5)	0	12 (10.5)	0.0436
FLT3-ITD mutation	33 (23.2)	12 (42.9)	21 (18.4)	0.0063

Abbreviations: AML, acute myeloid leukemia; *FLT3*, fms-like tyrosine kinase 3; ITD, internal tandem duplication.
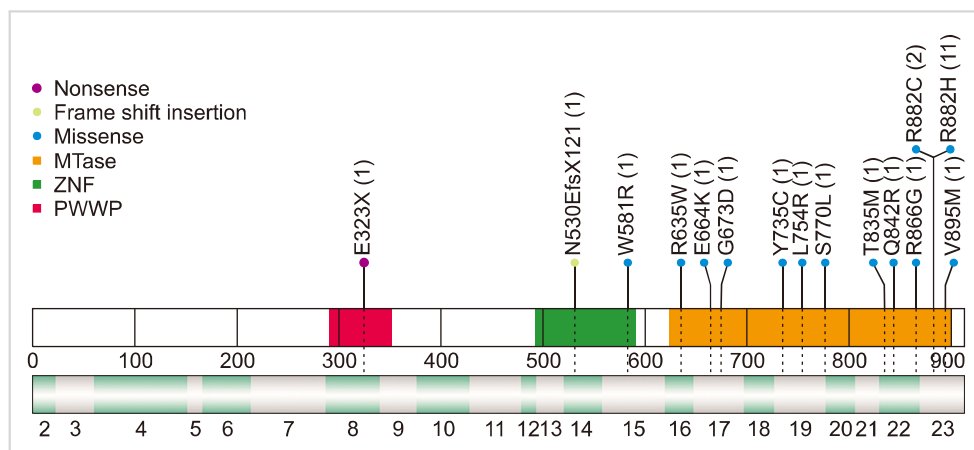

Fig. 1. Location of 15 different exonic mutations of *DNMT3A*.

RESULTS

Clinical, cytogenetic features and WHO 2016 based classification of *DNMT3A* mutations

Among the 142 patients enrolled with de novo AML, 82 were males and 60 were females. The median age was 53.5 years (range, 17–88 yr). *DNMT3A* mutations were identified in 28 patients (19.7%). Patients with *DNMT3A* mutations were older than those with wild type *DNMT3A* (median age, 55.5 vs. 52 yr; *P*=0.0042). For recurrent genetic abnormalities, *DNMT3A* mutations were not detected in patients with fusions, except for one with t(8;21) and Q842R DNMT3A. On the other hand, *DNMT3A* mutations were more commonly detected in patients with *NPM1* mutations (*P*<0.001). *DNMT3A* mutations were more frequently identified in patients with cytogenetically normal (CN)-AML (*P*=0.0112). In addition, the incidence of *DNMT3A* mutations was higher in patients with *FLT3-ITD* mutations than in those without *FLT3-ITD* mutations (*P*=0.0063) (Table 1).

Characteristics of *DNMT3A* mutations

We identified 19 different *DNMT3A* mutations in 28 patients with AML (Fig. 1, Table 2); 13 missense mutations, one nonsense mutation, one deletion mutation, and four splicing mutations. The most common mutation was c.2645 G>A (R882H, N=11), followed by c.2644C>T (R882C, N=2). Mutations were located in all exons, from exon 8 to exon 23, and the majority (N=14), including R882 mutations, occurred on exon 23. We identified a novel frameshift mutation, c.1590delC [p.(Asp530Glufs*121)], in AML with a biallelic mutation of *CEBPA*.

Association of *DNMT3A* mutation and *CDKN2B*

We measured the value of *CDKN2B* promoter methylation using pyrosequencing and evaluated the association of methylation with *DNMT3A* mutation (N=51). The average value of *CDKN2B* promotor methylation was 18.63±13.36%. It was lower in CN-AML and in patients with *NPM1* mutations (*P*=0.0472 and *P*=0.0096, respectively). There was no significant difference of *CDKN2B* promoter methylation, based on the presence or type of *DNMT3A* mutations.

Table 2. Identified *DNMT3A* mutations in 28 patients with acute myeloid leukemia.

No.	Sex/age	WHO 2016 classification	Location	Domain	Nucleotide change	AA change
1	F/51	AML with maturation	Exon 23	MTase	c.2645G>A	R882H
2	F/38	AML with mutated *NPM1*	Exon 23	MTase	c.2645G>A	R882H
3	F/37	AML with mutated *NPM1*	Exon 23	MTase	c.2645G>A	R882H
4	M/86	AML with minimal differentiation	Exon 23	MTase	c.2645G>A	R882H
5	F/79	AML with mutated *NPM1*	Exon 15	ZNF	c.1741T>A	W581R
6	F/55	AML with maturation	Intron 19	-	c.2323-2A>G	Splicing error
7	F/59	AML with maturation	Exon 16	MTase	c.1903C>T	R635W
8	M/56	AML without maturation	Exon 23	MTase	c.2644C>T	R882C
9	M/73	AML with mutated *NPM1*	Exon 23	MTase	c.2645G>A	R882H
10	F/80	AML with minimal differentiation	Intron 19,	-	c.2322+1G>A	Splicing error
			Exon 23	MTase	c.2683G>A	V895M
11	M/67	AML with mutated *NPM1*	Exon 17,	MTase	c.2018G>A	G673D
			Exon 22		c.2596A>G	R866G
12	F/62	AML with maturation	Exon 23	MTase	c.2644C>T	R882C
13	F/54	Acute monocytic leukemia	Exon 19	MTase	c.2204A>G	Y735C
14	F/81	AML with biallelic mutation of *CEBPA*	Exon 14	ZNF	c.1590delC	N530Efs
15	F/75	AML with mutated *NPM1*	Intron 14	-	c.1667+1G>A	Splicing error
16	M/52	AML with mutated *NPM1*	Exon 23	MTase	c.2645G>A	R882H
17	M/57	AML without maturation	Exon 19	MTase	c.2261T>G	L754R
18	F/81	AML without maturation	Exon 22	MTase	c.2504C>T	T835M
19	F/73	Acute myelomonocytic leukemia	Exon 23	MTase	c.2645G>A	R882H
20	F/52	AML with maturation	Exon 17	MTase	c.1990G>A	E664K
21	M/79	AML without maturation	Exon 23	MTase	c.2645G>A	R882H
22	M/40	AML with mutated *NPM1*	Exon 8	PWWP	c.967G>T	E323[a]
23	M/37	AML with mutated *NPM1*	Exon 23	MTase	c.2645G>A	R882H
24	M/51	AML without maturation	Intron 16	-	c.1937-12T>A	Splicing error
25	M/38	AML with *RUNX1-RUNX1T1*	Exon 22	MTase	c.2525A>G	Q842R
26	M/54	AML with mutated *NPM1*	Exon 23	MTase	c.2645G>A	R882H
27	F/36	AML with mutated *NPM1*	Exon 23	MTase	c.2645G>A	R882H
28	F/40	AML with mutated *NPM1*	Exon 19	MTase	c.2309C>T	S770L

[a]non-sense mutation.
Abbreviations: AML, acute myeloid leukemia; Fs, frame shift mutation; MTase, methyltransferase; PWWP, P (proline)-W (tryptophan)-W (tryptophan)-P(proline) motif; ZNF, zinc finger.

Table 3. The comparison of promoter methylation and expression of *CDKN2B* in acute myeloid leukemia.

	N of patients (%)	Promoter methylation (mean±SD)	P	Expression (mean±SD)	P
DNMT3A					
All mutations	19 (37.3)	19.82±16.33	0.6302[a]	$1.64×10^{-3}±2.17×10^{-3}$	0.1732[a]
R882H	8 (15.7)	15.79±10.29	0.6327[a]	$3.07×10^{-3}±2.38×10^{-3}$	0.0358[a]
Other mutations	11 (21.6)	22.75±19.58	0.4546[a]	$0.59×10^{-3}±1.28×10^{-3}$	0.5375[a]
Wild-type	32 (62.7)	17.93±11.48		$0.87×10^{-3}±1.27×10^{-3}$	
Cytogenetically normal					
Yes	36 (70.6)	15.52± 9.42	0.0472	$1.39×10^{-3}±1.87×10^{-3}$	0.0537
No	15 (29.4)	26.11±18.19		$0.60×10^{-3}±0.95×10^{-3}$	
Recurrent genetic abnormality					
NPM1 mutations	17 (33.3)	12.79±8.68	0.0096	$1.70×10^{-3}±2.29×10^{-3}$	0.1875
No *NPM1* mutation	34 (66.7)	21.56±14.41		$0.89×10^{-3}±1.24×10^{-3}$	
FLT3-ITD	14 (27.5)	20.99±18.24	0.5414	$0.89×10^{-3}±1.09×10^{-3}$	0.3847
No *FLT3*-ITD	37 (72.5)	17.74±11.17		$1.26×10^{-3}±1.86×10^{-3}$	
NPM1-mutant AML	17				
DNMT3A mutations	9 (52.9)	11.77±10.39	0.6226	$2.95×10^{-3}±2.57×10^{-3}$	0.0147
No *DNMT3A* mutation	8 (47.1)	13.94±6.79		$0.28×10^{-3}±0.31×10^{-3}$	

[a]Comparison with wild-type.
Abbreviations: AML, acute myeloid leukemia; *FLT3*, fms-like tyrosine kinase 3; ITD, internal tandem duplication; *NPM1*, nucleophosmin 1.
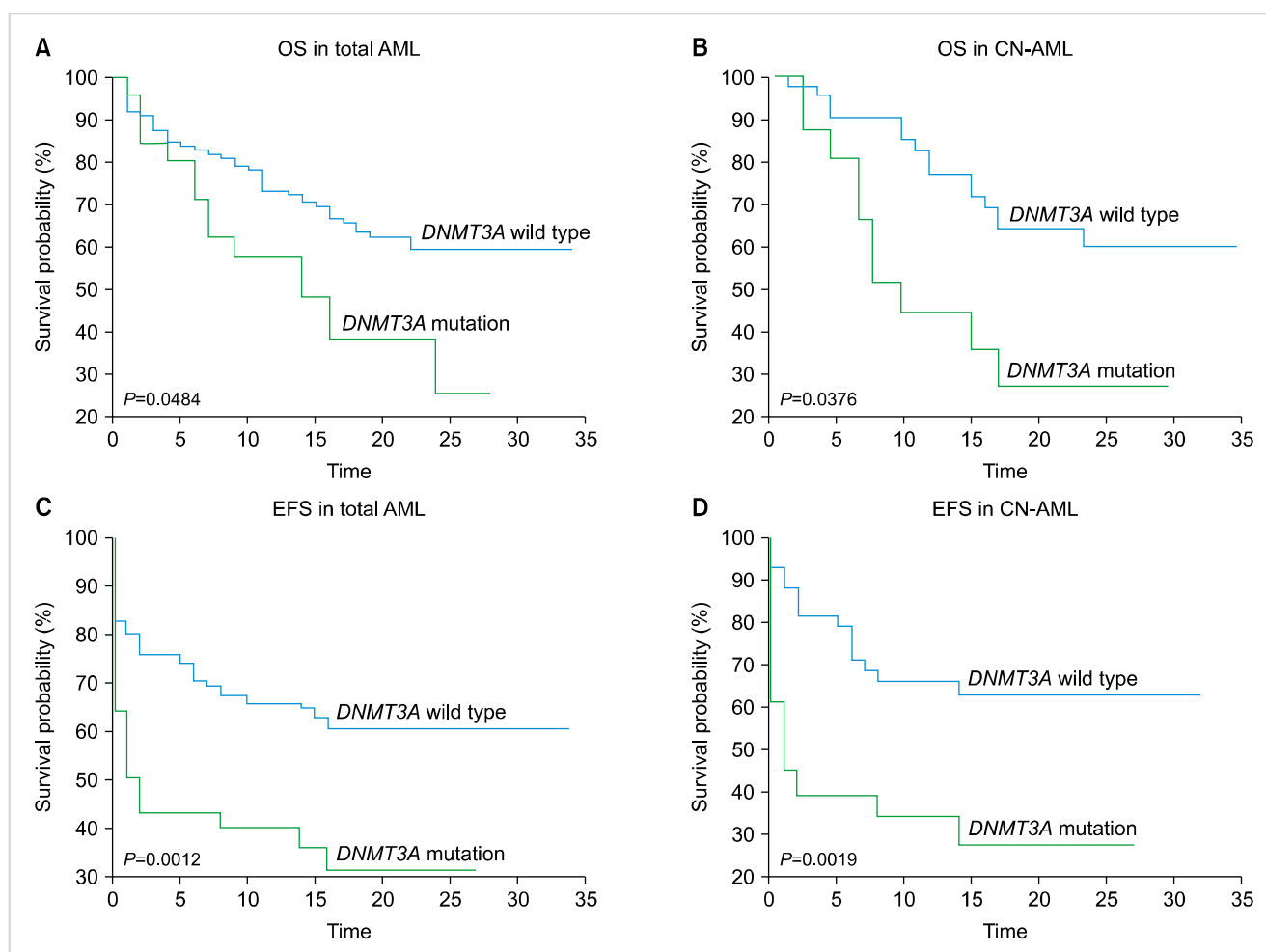

Fig. 2. Prognostic factors for patients with acute myeloid leukemia. **(A)** OS and **(C)** EFS in total patients with AML. **(B)** OS and **(D)** EFS in patients with CN-AML. **(A)** Kaplan-Meier survival curves for OS in 142 total patients with AML (P=0.0484) and **(B)** 61 patients with CN-AML (P=0.0376). **(C)** EFS curve in total 142 patients with AML (P=0.0012) and **(D)** EFS curve in 61 patients with CN-AML (P=0.0019).

We also measured *CDKN2B* expression using RT-qPCR and analyzed its association with *CDKN2B* promoter methylation. *CDKN2B* expression was inversely correlated with *CDKN2B* promoter methylation (*R*=-0.50, *P*<0.001). *CDKN2B* expression was significantly higher in R882H DNMT3A AML than in *DNMT3A* wild-type AML (*P*= 0.0358) (Table 3). In addition, patients with R882H DNMT3A showed higher *CDKN2B* expression compared to patients with AML and other *DNMT3A* mutations (*P*=0.0094). Among patients with the *NPM1* mutations (N=17), *CDKN2B* promoter methylation was not different between the *DNMT3A* mutated and the wild-type AML. *CDKN2B* expression was significantly different in accordance with the *NPM1* mutation with the *DNMT3A* status (Table 3).

Prognostic effect of *DNMT3A* mutation in AML and CN-AML

DNMT3A mutated AML showed poor OS and event-free survival (EFS), compared to that from the *DNMT3A* wild-type AML (*P*=0.0484 and *P*=0.0012, respectively) (Fig. 2A, B). In addition, we analyzed the prognostic effect of *DNMT3A* mutations in CN-AML and found that the *DNMT3A*-mutated CN-AML showed poorer OS and EFS

compared to that shown by the *DNMT3A* wild-type CN-AML (*P*=0.0376 and *P*=0.0019) (Fig. 2C, D).

Clinical outcomes according to *DNMT3A* mutation, age, and cytogenetics as independent prognostic factors

We performed multivariate analysis with variables that included the *DNMT3A* mutation, age, favorable cytogenetics, the *NPM1* mutation without *FLT3*-ITD, the *NPM1* mutation with *FLT3*-ITD, a biallelic *CEBPA* mutation, and AML-MRC. Among these, old age (>60 yr) remained an independent unfavorable prognostic factor. For OS, *DNMT3A* mutations remained an independent unfavorable prognostic factor. However, *DNMTA* mutations did not show significant difference in the prognosis of EFS. Favorable cytogenetics and presence of the *NPM1* mutation in the absence of *FLT3*-ITD were independent favorable prognostic factors (Table 4).

Quantitative analysis of *DNMT3A* c.2645G>A (R882H)

We performed quantitative analysis of the R882H mutation in *DNMT3A* in 17 samples from eight patients. We used *DNMT3A*/β-actin ratio as quantitative R882H mutation index. The average value of the mutation (DNMT3A/β-actin

Table 4. Multivariate analysis (Cox regression) for overall survival (OS) and event free survival (EFS) for total patients with acute myeloid leukemia.

Variable	OS				EFS			
	HR	Lower	Upper	*P*	HR	Lower	Upper	*P*
DNMT3A	1.952	1.022	3.728	0.0428	1.704	0.901	3.221	0.1009
Age	4.593	2.614	8.070	<0.0001	3.305	1.912	5.715	<0.0001
Favorable cytogenetics	0.278	0.096	0.803	0.0180	0.266	0.092	0.772	0.0148
NPM1 without *FLT3*-ITD	0.190	0.057	0.631	0.0067	0.234	0.071	0.772	0.0170
NPM1 with *FLT3*-ITD	0.890	0.388	2.041	0.7832	0.936	0.409	2.143	0.8759
AML-MRC	1.423	0.605	3.349	0.4185	1.408	0.599	3.311	0.4330
Biallelic *CEBPA*	0.541	0.219	1.332	0.1813	0.542	0.220	1.331	0.1814

Abbreviations: AML-MRC, acute myeloid leukemia with myelodysplasia-related changes; EFS, event free survival; ITD, internal tandem duplication; OS, overall survival.
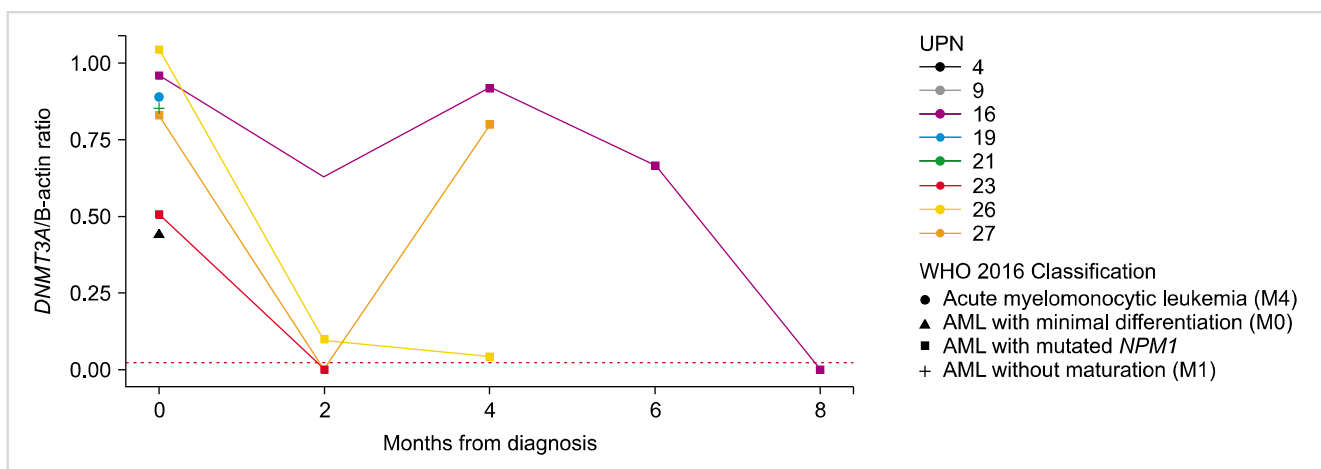

Fig. 3. The results of quantitative analysis of DNMT3A R882H in eight patients with acute myeloid leukemia with regard to the WHO 2016 classification.

ratio) was 0.81±0.22 at the time of diagnosis. Sequential follow-up was done for four patients (UPN 16, 23, 26, and 27) of AML with *NPM1* mutation. The *DNMT3A* mutant burden decreased after induction chemotherapy; however, the reduced value was variable. *DNMT3A* mutations disappeared after induction chemotherapy in the patient, UPN23. Patient UPN27 showed a marked decrement of the mutant burden in complete remission status (0.39% of the initial value), but it increased up to the initial value at relapse (96.57%). Patient UPN16 showed persistent *DNMT3A* mutations after achieving morphological CR (65.16%, 94.46%, and 69.34%). The patient received an allogeneic stem-cell transplantation, after which the *DNMT3A* mutation disappeared. In patient UPN26, the *DNMT3A* mutant burden decreased to 8.70% and 4.16% in the CR status (Fig. 3).

DISCUSSION

DNMT3A is a de novo DNA methyltransferase that catalyzes the addition of a methyl group to the cytosine residue of CpG dinucleotides, forming 5-methylcytosine. DNA methyltransferase plays a significant role in epigenetically regulated gene expression and repression. In this study, we identified *DNMT3A* mutations in 19.7% of the enrolled patients with AML, which increased to 29.5% when restricted to the CN-AMLs. For the WHO 2016-based classification, *DNMT3A* mutations were frequently accompanied in AML with *NPM1* mutations and were less frequently observed in AML-MRC. Noteworthy, we found a Q842R mutation in AML with t(8;21). To our knowledge, AML with t(8;21), inv(16), and inv(3)/t(3;3) rarely harbored the *DNMT3A* mutation, and this is the first reported case of *DNMT3A* mutation in AML with t(8;21) [5, 21, 22]. R882H DNMT3A was the most commonly found mutation, consistent with previous reports [23, 24]. We also detected a novel frameshift mutation located on ZNF.

Previously, a strong correlation between decreased DNA methylation and *DNMT3A* mutations in the methylation of 12 select tumor suppressor genes, including *CDKN2B*, has been reported [9]. However, we did not find a significant difference in *CDKN2B* promoter methylation, in patients with *DNMT3A* mutations, although promoter methylation was low in AML with R882H DNMT3A. Moreover, methylation levels were significantly lower in CN-AML and in AML with the *NPM1* mutations, which frequently accompanied the *DNMT3A* mutations. These differences may be due to the presence of heterogeneous *DNMT3A* mutations, a limited number of patients with mutations, and the technique used to determine the level of methylation, in the present study. Previously, various techniques, including Southern blotting, methylation specific PCR, and pyrosequencing, have been used to determine *CDKN2B* methylation. However, no standardized method has been identified; therefore, the methylation was heterogeneous with inter-individual differences [14].

Moreover, the presence of *CDKN2B* methylation is not always associated with reduced expression of *CDKN2B* mRNA; therefore, it will be important to investigate the expression of *CDKN2B* in AML with various *DNMT3A* mutations. We found that *CDKN2B* expression was inversely correlated with *CDKN2B* promoter methylation. Notably, patients with R882H DNMT3A showed higher *CDKN2B* expression compared to those with the wild-type DNMT3A or other *DNMT3A* mutations.

In addition, we observed that *CDKN2B* expression showed significant difference in accordance with the *NPM1* mutation with the *DNMT3A* mutation status. However, in this study, the number of cases analyzed were limited and further studies will be necessary to determine whether the statistical difference arises from DNMT3A R882H (Table 3).

We demonstrated that the *DNMT3A* mutations were associated with poor outcomes in AML, especially in CN-AML. Multivariate analysis results showed that *DNMT3A* mutations remained a factor affecting poor OS. Previously, *DNMT3A* mutations have been shown to be independently associated with poor outcomes [4, 22]; however, there is no consensus on the effect of *DNMT3A* mutations [25]. It seems worthwhile to evaluate their effect on prognosis in the presence of other known prognostic markers and therapeutic strategies, such as the hematopoietic stem-cell transplantation [26, 27]. Studies that have investigated the suitability of *DNMT3A* mutations for MRD monitoring, have shown the persistence of *DNMT3A* mutations during CR [28, 29]. However, pre-leukemic hematopoietic clones with *DNMT3A* mutations may be resistant to leukemic therapy and may lead to further clonal expansion during remission, and may eventually cause recurrent disease [30]. We have developed a qPCR method to measure the R882H DNMT3A allele burden accurately. The mutant allele burden was variable at the time of diagnosis and changed differently during CR. A recent study has shown that the R882 DNMT3A mutant allele burden determined AML prognosis [31]. Although no clear association between the persistence of *DNMT3A* mutations and clinical outcomes has been reported, a large prospective study, using a suitable method to measure *DNMT3A* mutations, will clarify the significance of the initial mutant allele burden and its change after treatment.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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Supplementary data

Validation of *DNMT3A* R882H quantitative PCR (qPCR)

We prepared serial concentrations (1×10^2 copies/mL to 1×10^6 copies/mL) of *DNMT3A* R882H mutation by serial dilutions of plasmid DNA. A linear regression analysis of *DNMT3A* qPCR showed a valid linearity, with R^2=0.9954, slope=-3.4327, and the y-axis intercept points=41.042. We also performed a sensitivity test using 40 replicates at different concentrations (5,000 copies/mL, 2,500 copies/mL, 1,000 copies/mL, 500 copies/mL, 250 copies/mL, 100 copies/mL, and 50 copies/mL). The detection limit of this qPCR method was estimated to be 189 copies/mL for *DNMT3A* mutations. We analyzed 95% probity using the IBM SPSS Statistics 20. The number of copies for *DNMT3A* mutations confirmed in patient samples ranged from 189 copies/mL to 11,192,661 copies/mL. The median number of *DNMT3A* mutant copies was 4,736,001 (60–11192661).

Supplementary Table 1. Oligonucleotide primer sequences.

Gene name	Sequence
NPM1	F: ACC ACA TTT CTT TTT TTT TTT TTC CAG GCT
	R: CCT GGA CAA CAT TTA TCA AAC ACG GTA
DNMT3A R882	F: GTG TGG TTA GAC GGC TTC CG
	R: CCC ATG TCC CTT ACA CAC ACG
CEBPA	F1: TCG CCA TGC CGG GAG AAC TCT AAC
	R1: CTG GTA AGG GAA GAG GCC GGC CAG
	F2: CCG CTG GTG ATC AAG CAG GA
	R2: CAC GGT CTG GGC AAG CCT CGA GAT
FLT3 ITD[a]	F: FAM-TTT AGG TAT GAA AGC CAG CTA CA
	R: AGC ATT TTG ACG GCA ACC T

[a]Primers for fragment analysis.
Abbreviation: ITD, internal tandem duplication.