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ORIGINAL RESEARCH

A novel non-invasive monitoring assay of 5-azacitidine efficacy using global DNA methylation of peripheral blood in myelodysplastic syndrome

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Purpose: Monitoring response and resistance to 5-azacitidine (AZA) is essential when treating patients with myelodysplastic syndrome (MDS). To quantify methylated DNA not only in the promoter region but also in the gene body, we established a single-molecule methylation assay (SMMA).

Patients and methods: We first investigated the methylation extent (expressed as methylation index [MI]) by SMMA among 28 MDS and 6 post-MDS acute myeloid leukemia patients. We then analyzed the MI in 13 AZA-treated patients.

Results: Whole-blood DNA from all 34 patients had low MI values compared with healthy volunteers ($P<0.0001$). DNA hypomethylation in MDS patients was more evident in neutrophils ($P=0.0008$) than in peripheral mononuclear cells ($P=0.0713$). No consistent pattern of genome-wide DNA hypomethylation was found among MDS subtypes or revised International Prognostic Scoring System (IPSS-R) categories; however, we found that the MI was significantly increased for patients at very high risk who were separated by the new cytogenetic scoring system for IPSS-R ($P=0.0398$). There was no significant difference in MI before AZA, regardless of the response to AZA ($P=0.8689$); however, sequential measurement of MI in peripheral blood demonstrated that AZA non-responders did not have normalized MI at the time of next course of AZA ($P=0.0352$).

Conclusion: Our results suggest that sequential SMMA of peripheral blood after AZA may represent a non-invasive monitoring marker for AZA efficacy in MDS patients.

Keywords: myelodysplastic syndrome, azacytidine, peripheral blood, methylation index

Introduction

Myelodysplastic syndrome (MDS) is a clonal hematopoietic disorder characterized by peripheral blood cytopenia and dysplastic features of hematopoietic cells. The prognosis of MDS patients has been defined by cytopenia, percentage of bone marrow (BM) blasts, and cytogenetic risk factors, which comprise the so-called International Prognostic Scoring System (IPSS) established in 1997.[1] Low and intermediate-1 scores have been defined as low-risk MDS according to the IPSS, while intermediate-2 and high scores have been defined as high-risk MDS. The goal of therapy for low-risk MDS patients is to deviate from transfusion dependency; for high-risk MDS patients, the goal is to prevent leukemic transformation.[2] The European LeukemiaNet has provided clinical recommendations for diagnosis and

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appropriate therapeutic interventions based on evidence- and consensus-based guidelines for adult patients with primary MDS in 2013.[3] In addition, the US National Comprehensive Cancer Network (NCCN) guidelines for the management of MDS have been updated. Both of these guidelines commonly recommend hypomethylating agents (HMA) as a key medication for high-risk MDS patients.[3] Moreover, NCCN guidelines have proposed HMA for low-risk MDS patients who do not respond to or are intolerant to other therapeutic options (https:www.nccn.org/professionals/physician_gls/pdf/mds/pdf).

5-azacytidine (azacytidine; AZA) is the most widely used HMA, and approximately 50% of AZA-treated MDS patients experience hematological improvement.[4,5] Because AZA incorporates both RNA and DNA, it exerts bipolar effects on hematological improvement. AZA induces apoptosis via RNA incorporation and demethylation via DNA integration; therefore, this double effect reflects the reduction in blast numbers and recovery from cytopenia. The remaining 50% of AZA-treated MDS patients do not show any response, even after treatment with at least six courses of 75 mg/m^2 AZA.[6] For this primary AZA treatment failure, it remains unclear which MDS patients respond to AZA treatment, although several genes are considered linked to AZA sensitivity.[7–9] Moreover, some AZA responders do not maintain consistent hematologic improvement and subsequently develop acute leukemia, termed secondary AZA failure. Evidence suggests that hypomethylation of the promoter region in tumor suppressor genes is not always related to a clinical response attributable to AZA.[9–11] Recent reports have also demonstrated that somatic mutations, such as TP53 and TET2 mutation,[7,8,12–15] has been identified as a poor prognostic factor for MDS patients, and AZA response among TP53 mutated patients is better compared with non-mutated patients.[12] However, this response is temporary, and overall survival is not improved. TET2 is one of the most frequently identified mutation,[13] although its effect on HMA response is controversial. Some studies have shown that TET2 mutation is a favorable indicator of AZA efficacy,[7,8] while others do not support such a -relationship.[14,15] To date, no predictive biomarker for secondary AZA failure has been described. BM specimens are typically used in the initial diagnosis or relapsed phase, but are less appropriate during the follow-up period. We therefore applied a single-molecule methylation assay (SMMA), whereby methylated DNA in samples could be quantified using the methylation index (MI), to establish a non-invasive monitoring method for AZA efficacy using peripheral blood cells.[16] We first analyzed MI in 34 untreated patients with MDS, including 6 post-MDS acute myeloid leukemia (AML) patients, and then measured the MI sequentially in 13 AZA-treated patients.

Our study indicates that whole-blood DNA from all 34 patients had low MI values compared with healthy volunteers, regardless of the percentage of blasts, indicating that the sequential analysis of MI in each patient may be more reliable for determining AZA efficacy. Indeed, appropriate MI recovery of peripheral blood after AZA treatment was found in AZA responders. The sequential analysis of MI using peripheral blood may therefore represent a non-invasive monitoring marker for use in the treatment of patients with MDS.

Materials and methods

Patients

Thirty-four patients with MDS and post-MDS AML (aged 23−83 years; mean, 66.2 years) were enrolled in this study. For the methylation assay, we subdivided patients according to percentage of BM blasts. Fifteen patients had <5% BM blasts (Table 1): four patients witefractory anemia (RA), one with RA with ringed sideroblasts, eight with refractory cytopenia with multilineage dysplasia, and two with unclassifiable MDS (MDS-U). Twenty-eight patients had ≥5% BM blasts but <20% BM blasts (Table 1): two patients with RA with excess blasts-1 and 11 with RA with excess blasts-2. Six patients with post-MDS AML were also enrolled in this study. Samples from all MDS or post-MDS AML patients were obtained before treatment. Among these, 13 MDS patient samples were analyzed sequentially before and after AZA treatment. Blood samples were collected weekly, and the response to AZA was evaluated using the International Working Group (IWG) response criteria for myelodysplasia.[17] For NGS analysis, BM mononuclear cells were also used. Thirteen healthy volunteers (aged 35−58 years; mean, 48.9 years) served as the control group. This study was approved by the institutional review board of Tokyo Medical University (no. 1979). Written informed consent was obtained from all patients and volunteers prior to participation in accordance with the Declaration of Helsinki.

Isolation of DNA from whole blood, neutrophils, and mononuclear cells

For the methylation assay, DNA from either whole blood or the separated fraction was extracted using a robotic

Table 1 Methylation index of patients with myelodysplastic syndrome and post-MDS AML

UPN	Sex	Age (y)	FAB	IPSS	IPSS-R	PB blasts (%)	BM blasts (%)	Karyotype	New cytogenetic scoring	Methylation index (%)*					
										Whole blood		N		MNC	
I. Bone marrow blasts <5% (n=15) (mean ± SD)										64.8 ±9.0	(63.0: 49.3~82.7)	61.6 ±14.8	(63.7: 28.9~78.3)	59.3 ±13.7	(60.2: 32.8~79.1)
10[a]	M	60	RA	Int-1	Int	0	3.6	del(20q)	Good	60.6		78.3		73	
101	F	83	RA	Int-1	L	0	0	NC	Good	63.0		28.9		57	
103	F	77	RA	Int-1	Int	0	4	NC	Good	66.0		55.1		77.6	
104	M	81	RA	Int-1	Int	0	0	NC	Good	57.2		40.6		32.8	
107	M	68	RARS	Int-1	Int	0	0.8	−9,+mar	Int	59.8		75.4		66.4	
18[a]	M	75	RCMD	Int-2	H	0	0	complex:-Y, del(5q), del (17p)	Very poor	67.9		62.8		60.6	
20[a]	M	62	RCMD	Int-1	Int	1	1.2	NC	Good	61.1		66.1		52.1	
25[a]	M	72	RCMD	Int-2	Int	0.5	2	del 7, +marker	Int	64.6		ND		ND	
31[a]	M	66	RCMD	Int-2	Int	0	4.4	add(21q)	Good	82.0		ND		ND	
106	M	41	RCMD	Int-1	Int	0	0	NC	Good	57.7		75.2		60.2	
108	M	44	RCMD	Int-1	Int	0	0	del(9q)/N	Int	72.0		47.7		55.5	
119	F	74	RCMD	L	L	0	0	NC	Good	58.9		63.5		38.5	
120	M	77	RCMD	Int-1	Int	0	0	der(1;7)	Int	49.3		72.4		79.1	
105	M	82	MDS-U	Int-1	Int	0	0	del(20q),I (17q)	Int	68.8		63.7		52.4	
130	M	72	MDS-U	Int-1	H	0	0	del(3p)	Int	82.7		70.7		65.9	
II. Bone marrow blasts ≥5%, <20% (n=13) (mean ± SD)										64.4 ±10.1	(60.9: 53.9~83.0)	59.6 ±12.5	(57.7: 42.8~76.7)	65.4 ±9.2	(61.5: 57.8~80.0)
5[a]	M	68	RAEB1	Int-2	VH	0	7	der(1;7)	Int	71.9		76.7		77.7	
7[a]	M	70	RAEB1	Int-2	VH	8	6	complex	Very poor	75.7		75.7		66.9	
1	F	70	RAEB2	H	VH	8.5	11	complex: del(7q),del(5q), −13	Very poor	83.3		52.9		71.3	
4	M	55	RAEB2	H	VH	0	16	complex	Very poor	80.2		71.8		80	
8	F	61	RAEB2	Int-2	VH	2.5	10.4	complex: del(5q),del(7q), del(12p)	Very poor	54.2		59.2		59.9	
9	M	23	RAEB2	Int-2	VH	3	14	NC	Good	54.6		46.3		59.3	
12	M	50	RAEB2	Int-2	VH	0	14.6	NC	Good	57.1		ND		ND	
15[a]	F	80	RAEB2	H	Int	0	10.5	NC	Good	53.9		53.4		59.4	
21[a]	M	71	RAEB2	Int-2	VH	0	19	del(6q), −Y	Int	59.7		42.8		52.8	

(*Continued*)

Table 1 (Continued).

UPN	Sex	Age (y)	FAB	IPSS	IPSS-R	PB blasts (%)	BM blasts (%)	Karyotype	New cytogenetic scoring	Methylation index (%)*					
										Whole blood		N		MNC	
26[a]	M	55	RAEB2	H	VH	14	17	complex	Very poor	63.6		ND		ND	
30[a]	M	63	RAEB2	H	VH	0	15	del(20q), +2, +8	Int	61.5		ND		ND	
118	M	76	RAEB2	Int-2	VH	3.5	8	der(1;7)	Int	60.1		57.7		61.5	
125	M	78	RAEB2	Int-2	VH	0	10	NC	Good	60.9		ND		ND	
III. Bone Marrow Blasts >20% (n=6) (mean ± SD)										66.6 ±8.0	(64.8: 57.3~80.1)	62.0 ±10.1	(61.5: 47.3~73.7)	59.3 ±7.5	(59.0: 48.2~68.4)
3	F	42	Post-MDS AML			12	29	+9		66.2		58.6		57.5	
6	M	58	Post-MDS AML			25	32.5	der(1;7)		63.3		61.5		59	
13[a]	M	70	Post-MDS AML			0	20.5	NC		61.9		47.3		48.2	
22	M	77	Post-MDS AML			5	43.2	complex: del(5q), del (20q), −Y		57.3		68.7		63.2	
27[a]	F	62	Post-MDS AML			1	28.8	complex		80.1		ND		ND	
131	M	67	Post-MDS AML			0.5	21	+8, −15		70.5		73.7		68.4	
Controls (healthy volunteers; n=13)										82.8 ±5.9	(84.1: 71.8~90.1)	79.7 ±9.3	(71.8: 48.2~88.0)	70.0 ±8.3	68.8 (59.8~86.4)

Notes: *Methylation index (%) is shown as mean ± standard deviation(S.D.). Median, minimum and maximam are shown in parentheses.
F, female; M, male; IPSS, International Prognostic Scoring System; IPSS-R, revised IPSS. [a]Thirteen patients treated with AZA whose methylation index was subsequently measured.
Abbreviations: L, low; H, high; VH, very high; Int, intermediate; N, neutrophils; MNC, mononuclear cells; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; PB, peripheral blood; BM, bone marrow; NC, normal karyotypes; Methylation index,percent of methylated DNA in the sample measured by a single molecule methylation assay.

workstation (Magtration System 6GC; Precision System Science, Chiba, Japan) and an EZ1 DNA Blood 350 µl Kit (Qiagen, Valencia, CA, USA) according to the manufacturers' instructions. To obtain a mononuclear lymphocyte-enriched fraction, heparinized peripheral blood cells were separated on a Ficoll-Hypaque density gradient. BM mononuclear cells containing blast fractions were also collected as described above. To collect a neutrophil-rich fraction, buffy coat was mixed with 0.2% methylcellulose to sediment the red blood cells. The leukocyte-enriched part was collected, washed with phosphate-buffered saline (PBS; pH 7.4), and resuspended in PBS and Hank's balanced salt solution containing divalent cations (HBSS; Gibco, Grans Island, NY, USA).[18]

Methylation index measured by single-molecule methylation analysis

To determine global DNA methylation levels, we performed SMMA as previously described.[16] The principle of this assay is based on two methodologies. One is the use of high-affinity methyl-CpG–binding domain protein 2 (MBD2) to bind methylated DNA, and the other is the use of fluorescence correlation spectroscopy, which determines the interaction of MBD2 with methylated DNA in the sample. We used 500 ng genomic DNA digested with *Mse* I (New England Biolabs, Beverly, MA, USA). The digested DNA was quantified by NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA).[16] To calculate the methylated DNA in the sample, we generated a standard curve using TAMRA-labeled MBD2, unlabeled MBD2, 0% methylated DNA (negative control), and 100% methylated DNA (positive control).[16] Using this standard curve, we were able to assess the genome-wide DNA methylation status of whole blood, neutrophil-enriched fractions, and mononuclear fractions; the methylation level was expressed as the MI, which indicates the percentage of methylated DNA in the sample.

Next-generation sequencing analysis

Next-generation sequencing (NGS) analysis was performed as reported previously.[19] Briefly, genomic DNA was extracted using a Gentra PureGene Cell Kit (Qiagen, Hilden, Germany). Whole exonic regions of 50 genes were amplified from 40 ng genome DNA from each patient sample using the GeneRead DNAseq Targeted Panel V2 (Human Myeloid Neoplasms Panel; Qiagen). We constructed a barcoded Illumina DNA library using the GeneRead DNA Library I Core Kit (Qiagen) and GeneRead Adapter I Set 12-Plex Kit (Qiagen); sequencing was performed on a MiSeq system (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. High-probability oncogenic mutations were extracted from annotated data in the available databases (ClinVar: https://www.ncbi.nlm.nih.gov/clinvar/and COSMIC: http://cancer.sanger.ac.uk/cosmic). All other non-polymorphous synonymous/non-synonymous mutations and intronic mutations were extracted individually by filtered population frequencies (<1%) using a sequencing data analysis tool. (VariantStudio, Illumina)

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Mann–Whitney *U* and chi-square tests were used to determine statistical significance for comparisons between the control and test groups. Multiple groups were compared using one-way analysis of variance (ANOVA). GraphPad Prism software (version 5c for Macintosh; GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis.

Results

Genome-wide DNA methylation status for untreated MDS

We first measured the genome-wide DNA methylation status of healthy volunteers. In healthy subjects, there was no significant difference in the MI between whole blood and neutrophils (82.8±5.9 vs 79.7±9.3; P=0.315); however, the MI of mononuclear cells was significantly lower than that of whole blood (P=0.0001) or neutrophils (P=0.0087). We therefore measured the methylation status of whole blood in 34 patients and of neutrophils and mononuclear cells in 28 patients when separated samples were available. The MI values of each fraction for all patients analyzed are shown in Table 1. To estimate the influence of blasts in the sample, MDS patients were subdivided into MDS-I (BM blasts <5%) and MDS-II (BM blasts >5%) groups (Table 1 and Figure 1A, B, C). A significant reduction in MI for the whole blood DNA sample was evident for patients with MDS-I (P=0.0001), MDS-II (P<0.0001), and post-MDS AML (P=0.0001) (one-way ANOVA; P<0.0001) compared with normal controls (Figure 1A). There was no significant difference in MI among the three groups, indicating that it did not reflect the percentage of blasts in the samples. This was also evident in DNA from neutrophil fractions (one-way ANOVA; P=0.0008) (Figure 1B), but not from mononuclear cell fractions (one-way ANOVA; P=0.0713) (Figure 1C). For instance, the MI of neutrophils was significantly lower in patients with

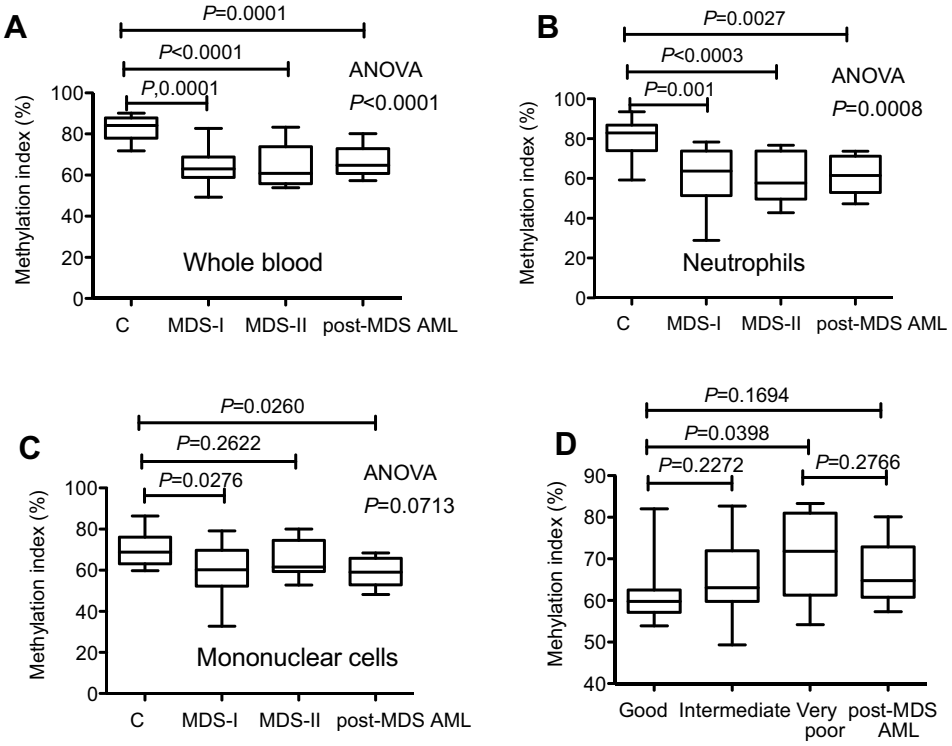

Figure 1 Methylation index (MI) measured using a single-molecule methylation assay (SMMA). The percent of methylation DNA in the sample is shown. (**A**). MI of whole blood. (**B**). MI of the neutrophil fraction. (**C**). MI of mononuclear cells. (**D**). MI is significantly increased in MDS patients with a very poor cytogenetic score according to the Revised International Prognostic Scoring System.

Abbreviations: MDS, myelodysplastic syndrome; post MDS-AML, post myelodysplastic acute myeloid leukemia.

MDS-I (P=0.001), MDS-II (P<0.0003), and post-MDS AML (P=0.0027) (Figure 1B), whereas that of mononuclear cells was not significantly decreased in patients with MDS-II (P=0.2622) (Figure 1C). There was no consistent pattern for MI levels found among MDS subtypes (P=0.3064), IPSS categories (P=0.2827), or IPSS-R categories (P=0.1940); however, we found that the MI was significantly higher for MDS patients in the very poor cytogenetic category according to the IPSS-R compared with patients in the good cytogenetics category (Figure 1D). We did not identify any correlation between the MI and hematologic features. No correlation was evident between the MI and neutrophils (P=0.6775) or mononuclear cells (P=0.9965) (data not shown).

Sequential analysis of methylation index for AZA-treated patients

Based on the results obtained from various sample fractions in the current study, we concluded that MI measurements in whole blood were the most robust and reliable for estimating genome-wide DNA methylation. Since MI was highly variable among the patients, regardless of the proportion of blasts, we analyzed the MI sequentially for 13 patients treated with at least four courses of AZA (Table 1). The clinical response to AZA and a summary of the mutation analysis related to myeloid neoplasia are shown in Table 2. Detailed information from the NGS analysis is shown in Table S1. Although the number of patients in the current study is small, six patients showed hematological improvement (unique patient number [UPN] 5, 7, 13, 15, 18, and 30). Among these six, five patients (all except for UPN 30) developed resistant to AZA and exhibited secondary failure after several courses of treatment. The remaining seven patients did not respond to AZA. TET2 mutation was detected in four patients, including two AZA responders, whereas TP53 mutation was detected in another four patients including one AZA responder.

To address the question of whether the MI before AZA administration could be a predictive marker for AZA response, we first compared the MI between responders and non-responders; however, there was no significant difference in the MI (66.6±6.3 vs 67.4±9.5; P=0.8689). A transient reduction in the MI at day 7 after AZA administration was noted in the majority of patients, but there was no significant difference in the MI between responders and non-responders (55.0±14.1 vs 52.0±16.2; P=0.7289).

Table 2 Clinical response to AZA in 13 patients whose methylation index was subsequently measured

UPN	Sex	Age	Dx	IPSS	IPSS-R	AZA cycles	Clinical response	Outcome	Mutations*
5	M	68	RAEB1	Int-2	H	10	HI-P, HI-E → relapse	Dead	DNMT3A, TET2, EZH2
7	M	70	RAEB1	Int-2	H	4	HI-E → relapse	Dead	TP53
10	M	60	RA	Int-1	L	9	failure	Alive	not detected
13	M	70	post-MDS AML			15	HI-P, HI-N→relapse→HI-P, HI-E,HI-N	Alive	not detected
15	F	80	RAEB2	H	Int	12	HI-P→relapse	Alive	RAD21, RUNX1
18	M	75	RCMD	Int-2	H	6	HI-P, HI-E→relapse	Alive	DNMT3A, KMT2A
20	M	62	RCMD	Int-1	Int	5	Failure	Alive	TET2, EZH2
21	M	71	RAEB2	Int-2	H	4	Failure	Alive	EED, TP53
25	M	72	RCMD	Int-2	Int	9	Failure	Alive	DNMT3A, TET2, SETBP1, KRAS
26	M	55	RAEB2	H	vH	5	failure	Dead	TP53
27	F	62	post-MDS AML			4	failure	Dead	SH2B3, TP53, STAG2
30	M	63	RAEB2	H	vH	6	HI-E, HI-N	Alive	TET2, RUNX1
31	M	66	RCMD	Int-2	Int	6	failure	Alive	ASXL1

Note: *Detailed results of mutation analysis using a the GeneRead DNAseq Targeted Panel V2 (Human Myeloid Neoplasms. Panel, QIAGEN) are shown in Supplementary file 1.
Abbreviations: UPN, unique patient number; IPSS, International Prognostic Scoring System; IPSS-R, IPSS revised; AZA, 5-azacitidine; Dx, diagnosis; HI-P, hematological improvement in platelets; HI-E, HI in erythrocytes; HI-N, HI in neutrophils

We then focused on the sequential change in the MI for each case. Because the MI for each patient was highly variable, we attempted to calculate the normalized rate rather than the MI at a single point. The restoration rate of MI at day 28 (the day of next AZA treatment) is expressed as follows: ΔMI^{day28} ($MI^{day28} - MI^{day0}$). The ΔMI^{day28} for AZA responders was significantly higher than that of non-responders (P=0.0352) (Figure 2A). In addition, ΔMI^{day120} was significantly higher than that of non-responders (P=0.0251) (Figure 2B). It is notable that ΔMI^{day120} was significantly higher than ΔMI^{day28} for AZA responders, although there was no significant difference in ΔMI at day 28 and day 120 for non-responders (Figure 2C and D). This indicates that, in responders, the genome-wide DNA methylation level is gradually normalized after four courses of AZA. In contrast, there were no significant differences in ΔMI^{day7} ($MI^{day7} - MI^{day0}$) (P=0.6915), ΔMI^{day14} ($MI^{day14} - MI^{day0}$) (P=0.937), or ΔMI^{day21} ($MI^{day21} - MI^{day0}$) (P=0.6623) between AZA responders and non-responders. This indicates that the genome-wide DNA methylation level for responders tended to normalize before the next AZA administration (on day 28), whereas that for non-responders did not.

Discussion

Genome-wide DNA hypomethylation and hypermethylation in the promoter region of tumor suppressor genes are hallmarks of cancer.[20,21] Several attempts have been made to determine the association between clinical response and the effect of HMA. Although many gene-specific methylations have been found to be associated with leukemia progression[22] or effectiveness of HMA treatment, including AZA, it is likely that the effect of AZA is not restricted to aberrantly hypermethylated regions of single genes.[9,10] Shen et al proposed a methylation prognostic model for predicting response to therapy by analyzing the promoter region in 10 selected genes rather than in single genes.[23] In contrast, Grövdal et al recently demonstrated that AZA induced genome-wide DNA hypomethylation in progenitor cells obtained from MDS patients. A clear decease in active chromatin was observed, suggesting that molecular mechanisms underlying AZA resistance are more complex than reactivation of the silenced genes.[11] We previously showed marked genome-wide DNA demethylation together with constitutive activation of the ATM/BRCA1 pathway in AZA-resistant human leukemia cell lines.[24] We also found aberrant histone modification in two AZA-resistant human leukemia cell lines.[25] Therefore, we considered that the architecture of genomes, such as in genome-wide DNA demethylation, may play some role in the clinical efficacy of AZA in MDS patients.

To date, the analysis of DNA methylation has focused on the promoter region of genes, even when a comprehensive analysis was performed using the methylation array. Genome-wide DNA methylation can be measured by several methods,

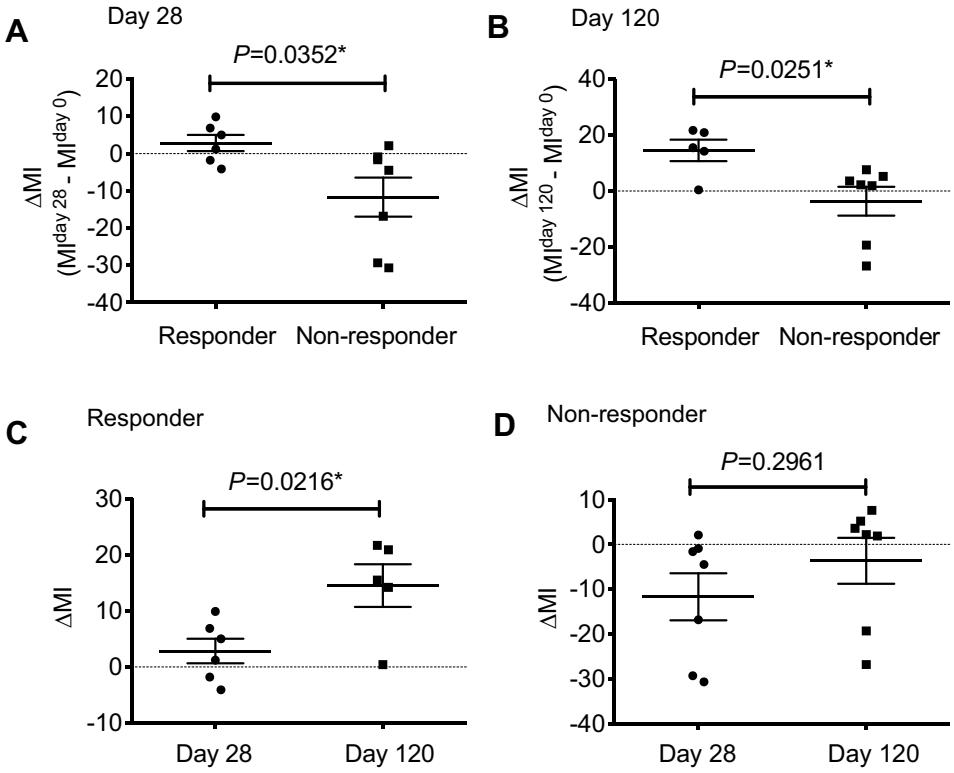

Figure 2 The restoration rate of methylation for AZA-treated patients. (**A**). ΔMI at day 28 ($MI^{day28} - MI^{day0}$) for AZA responders was significantly higher than that of non-responders (*P*=0.0352). (**B**). ΔMI at day 120 was significantly higher than that of non-responders (*P*=0.0251). (**C**). For AZA responders, ΔMI at day 120 was significantly higher than at day 28 (*P*=0.0216). (**D**). For AZA non-responders, there was no significant difference between ΔMI at day 28 and at day 120 (*P*=0.2961).
Note: *Represents a significant difference.
Abbreviation: MI, methylation index.

such as methylation-sensitive restriction enzymes, methyl-binding proteins, and anti-methylcytosine antibodies/[26,27] However, these techniques are complex and, in some cases, have been modified using bisulfate conversion. In the current study, we used an SMMA with 500 ng genomic DNA, which is less time-consuming than the methods described above: the fluorescence correlation spectroscopy measurement can be performed in 1–2 mins per assay, without bisulfate modification.[16] Our previous studies demonstrated that the MI (originally designated the SMMA index) measured by SMMA correlates with the global methylation status investigated by the DNA methylation array.[16]

The binding of MBD2 to methylated DNA occurs not only in the CpG islands and its "island shores" in the promoter area, but also in the gene body; therefore, it is likely that the MI mainly reflects genome-wide DNA methylation in the gene body. Theoretically, it would be logical to perform assays using isolated CD34-positive MDS cells. In practical terms, however, this is time-consuming and methodologically complex because the percentage of blasts varies widely among patients. We found that the MI for MDS patients was significantly lower than that of normal individuals, regardless of the percentage of blasts, but significantly higher for MDS patients with very poor cytogenetics according to IPSS-R. These results suggest that the MI involving peripheral blood may reflect epigenetic changes in MDS cells in some cases, because it depends on the cells from which the DNA originated.

Regarding promoter-associated CpG site analysis, it has been reported that the methylation dynamics of tumor suppressor genes do not predict clinical response in MDS patients treated with AZA and entinostat.[28] Similar to previous reports,[23,28] neither MI at diagnosis nor MI after AZA therapy could predict clinical efficacy in the present study. A reduction in MI was observed in the majority of patients at day 7 after AZA administration; therefore, we could not distinguish responders from non-responders at day 7. We next focused on the difference between the initial MI and that after cycle 1 of therapy (ie, ΔMI^{day28}: $MI^{day28} - MI^{day0}$). Eventually, we found that ΔMI^{day28} could represent a marker for normalization of genome-wide DNA methylation. It is

notable that the normalization of MI was more evident after cycle 4 of therapy (ΔMI^{day120}).

Histone modification in accordance with genome-wide DNA methylation may have a key role in sustaining genome architecture, which regulates transcription.[10,11,29] Therefore, a genome-wide DNA methylation assay could function as a robust technique for managing MDS patients receiving AZA treatment. In clinical practice, it would be more reliable to use the MI value in combination with the methylation prognostic model based on the promoter analysis of 10 selected genes and mutation analysis of methylation-associated genes,[23] such as TET2.[7] Because the number of MDS patients studied was too small to draw final conclusions for both primary and secondary AZA resistance, further studies using larger numbers of patients should be performed to clarify the efficacy of MI.

In conclusion, we established a genome-wide DNA methylation assay using whole blood to monitor the efficacy of AZA in MDS patients. Compared with healthy individuals, MDS patients had hypomethylated DNA, and AZA responders showed recovery of hypomethylated DNA during the next AZA administration. Therefore, it was possible to discriminate responders and primary AZA non-responders after the first cycle of therapy. Patients with secondary AZA failure showed a progressive decrease in MI, indicating that the MI recovery of peripheral blood after AZA could represent a non-invasive monitoring marker for AZA efficacy in MDS patients.

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Disclosure

Prof. Dr. Kazuma Ohyashiki reports personal fees from Celegene KK personal fees from Nippon Shinyaku Co., Ltd, during the conduct of the study; and personal fees from Novartis Pharma KK, personal fees from Janssen Pharmaceutical K.K, personal fees from Kirin Brewery KK, personal fees from Chugai KK, personal fees from Bristrol Myere Squib KK, and personal fees from Dainipon Sumitomo, outside the submitted work. The authors report no other conflicts of interest in this work.

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Supplementary material

Table S1 Mutation analysis using the GeneRead DNAseq Targeted Panel V2 (Human Myeloid Neoplasms Panel; Qiagen)

Transcription/Other							RAS	Splicing	Chromatin Modification						DNA Methylation							Group
STAG2	RUNX1	TP53			SH2-B3	RAD21	KRAS	SETBP1	ASXL1	EED	KMT2A	EZH2			TET2				DNMT3A			Gene
X	21	17	17	17	12	8	12	18	20	11	11	7	7	7	4	4	4	4	2	2	2	Chr
123,189-,997	36,252,-940	7,5-77,-120	7,5-78,-190	7,5-77,-081	111,-884,-785	117,-866,-542	25,-380-,285	42,-531-,913	31,-023-,483	85,-988-,145	118-,37-5,2-76	148-,52-3,5-79	148-,50-7,4-36	148-,50-8,7-31	106-,19-3,7-48	106-,18-0,8-19	106-,15-5,7-55	106-,19-6,8-29	25,-469-,981	25,-467-,449	25,-463-,285	Position
mut	mut	mut	mut	mut	mut	mut	mut	mut	mut	mut	mut	mut	mut	mut	mut	inframe_del	del	mut	mut	mut	del	Variant
T406A	S141L	R27-3H	Y2-20C	E28-6V	E29-2K	G36-8V	T5-8I	G8-70S	E99-0*	W3-64R	R28-90P	Y2-92N	N6-73I	E64-5K	R14-04*	S12-84del	H2-22-Wfs	L17-21-W	F35-4S	G5-43C	R73-6Pfs	AA Influence
	8.5	24.6	15	37.3		5.6	9.5	39.7		29.2	39.4	42.3	39.6	31.7			41.4	42.1 45.5	38.2	37.7	29.6	UPN5 UPN7 UPN10 UPN13 UPN15 UPN18 UPN20 UPN21 UPN25 UPN26

(*Continued*)

Table S1 (Continued).

Transcription/Other							RAS	Splicing	Chromatin Modification						DNA Methylation							Group
STAG2	RUNX1	TP53			SH2-B3	RAD21	KRAS	SETBP1	ASXL1	EED	KMT2A	EZH2			TET2				DNMT3A			Gene
X	21	17	17	17	12	8	12	18	20	11	11	7	7	7	4	4	4	4	2	2	2	Chr
123,189-,997	36,252,-940	7,577,-120	7,578,-190	7,577,-081	111,-884,-785	117,-866,-542	25,-380-,285	42,-531-,913	31,-023-,483	85,-988-,145	118-,37-5,2-76	148-,52-3,5-79	148-,50-7,4-36	148-,50-8,7-31	106-,19-3,7-48	106-,18-0,8-19	106-,15-5,7-55	106-,19-6,8-29	25,-469-,981	25,-467-,449	25,-463-,285	Position
mut	mut	mut	mut	mut	mut	mut	mut	mut	mut	mut	mut	mut	mut	mut	mut	inframe_del	del	mut	mut	mut	del	Variant
T406A	S141L	R273H	Y220C	E286V	E292K	G368V	T58I	G870S	E990*	W364R	R2890P	Y292N	N673I	E645K	R1404*	S1284del	H2222Wfs	L1721W	F354S	G543C	R736Pfs	AA Influence
39.8	44.1	59.1			47.8				11.2						10	41.1						UP-N2 7 UP-N3 0 UP-N3 1 UP-N3 4