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Methylation level of Rap1GAP and the clinical significance in MDS

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Abstract. Previous studies on the pathogenesis of myelodysplastic syndrome (MDS) have identified multiple associated gene mutations, including mutations of tetmethylcytosinedioxygenase 2, isocitrate dehydrogenase [NADP(+)] 1 cytosolic, isocitrate dehydrogenase [NADP(+)] 2 mitochondrial and additional sex combs like 1 transcriptional regulator, all of which may be considered epigenetic regulators. Furthermore, mutations of RAS type GTPase family genes have been identified in 10-15% patients with MDS. The authors' previous study on the gene expression profile of cluster of differentiation 34^+ cells using microarray analysis identified elevated expression of RAP1GTPase activating protein 1 (Rap1GAP) in patients with MDS compared with that in non-malignant blood diseases (NM) control group. To further investigate the mechanism of increased Rap1GAP expression, the methylation pattern of the promoter of this gene was determined in 86 patients with MDS (n=29), acute myeloid leukemia (AML) (n=31) or NM (n=26) using bisulfite-specific polymerase chain reaction and DNA sequencing. The results demonstrated that the methylation of Rap1GAP occurred in all 29 patients with MDS at multiple CpG sites. The methylation level of Rap1GAP in patients with MDS was decreased compared with that in patients with NM. Significant differences at 4CpG sites (5,7,8 and 12) of Rap1GAP promoter were identified between MDS and NM. Furthermore, based on the present clinical records of the patient cohort, the methylation status of Rap1GAP promoter did not appear to be associated with the clinicopathological characteristics of patients with MDS, including age, gender and International Prognosis Score System. The difference in methylation level at CpG site 8 of Rap1GAP promoter was identified to be significantly increased in patients with MDS-refractory anemia with ring sideroblasts compared with that in the MDS-refractory cytopenia with multilineage dysplasia or MDS-unclassified groups. The results of the present study suggest that patients with MDS exhibit a lower overall methylation level within Rap1GAP promoter compared with patients with NM or AML. In addition, the methylation level at the four identified CpG sites can distinguish between MDS and NM.

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Introduction

Myelodysplastic syndrome (MDS) constitutes a heterogeneous group of clonal myeloid disorders characterized by refractory cytopenias and dysplastic changes in ≥2 hematopoietic cell lineages, frequently representing an intermediate disease stage prior to progression to acute myeloid leukemia (AML) (1). MDS progresses to AML in ~30% of patients following multiple intervals of time from diagnosis. Therefore, the early diagnosis and treatment of MDS may aid in improving the survival rate of patients with MDS and AML. The pathogenesis of MDS remains unclear with no validated biomarkers for early diagnosis of MDS.

Numerous genetic and epigenetic alterations occur during the pathogenesis of MDS (2,3). Among these alterations, methylation of the tumor suppressor gene promoter results in gene silencing, which often take place during the early stages of tumor development (4). These aberrant DNA methylations of tumor suppressor genes may be used as diagnostic markers for MDS. Thus, defining altered gene expression and understanding the underlying molecular mechanism of MDS are required.

RAP1GTPase activating protein 1 (Rap1GAP) is one of the genes thought to be involved in hematopoietic regulation. The authors' previous study confirmed that the mRNA and protein expression of Rap1GAP in the majority of patients with MDS were significantly higher compared with those with AML and non-malignant blood diseases (NM) (5,6). However, the mechanistic basis of Rap1GAP upregulation in patients with MDS remains unclear.

In the present study, the methylation levels and pattern in the transcriptional regulation region (TRR) of the Rap1GAP gene

were detected in the bone marrow mononuclear cells (BMNCs) cells of patients with MDS, NM and AML to assess the altered methylation levels of the Rap1GAP gene, and to analyze its possible association with clinicopathological characteristics of patients with MDS.

Materials and methods

Cells and patient specimens. The present study included patients with MDS, AML and NM. In total, 5 human leukemia cell lines (SKM-1, K562, SHI-1, U937 and HL-60) were used as the control of Rap1GAP methylation level, including SKM-1, a cell line established from a patient with MDS-refractory anemia with excess blasts-II with progression to AML. All cell lines were stored at and supplied by the Cell Bank of Jiangsu Institute of Hematology (The First Affiliated Hospital of Soochow University, Collaborative Innovation Center of Hematology, Soochow University, Key Laboratory of Thrombosis and Hemostasis of Ministry of Health, Suzhou, China). Cells were cultured in RPMI-1640 (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) or Iscove's modified Dulbecco's medium (Hyclone; GE Healthcare Life Sciences) with 10% fetal bovine serum (Bovogen Biologicals, Keilor East, Victoria, Australia) at 37˚C in a humidified atmosphere with 5% CO_2. Cells were passaged every 3 days for 24 days. BMNC samples from 86 patients were obtained from The First Affiliated Hospital of Soochow University (Suzhou, China) between August 2010 and December 2011. The samples were stored at -80˚C following separation. These samples were collected from 29 pathologically proven cases of MDS, 31 cases of AML and 26 cases of NM. Clinicopathological data, including age, gender, clinical classification and International Prognostic Scoring System (IPSS) (7), were collected from the medical records of patients (Table I). The present study was approved by the Ethics Committee of The First Affiliated Hospital of Soochow University and written informed consent was obtained from all participants.

DNA extraction and bisulfite modification of DNA. BMNCs were separated from the bone marrow of patients using Ficoll-Hypaque (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 800 x g for 20 min at room temperature. Genomic DNA from MNCs was extracted using EpiTectFast DNA Bisulfite kit™ (Qiagen, Inc., Valencia, CA, USA). The concentration of DNA extraction was measured using SkanIt software (version 3.2; Thermo Fisher Scientific, Inc.) and the mass of DNA was measured, for which the $A_{260}/A_{280}$was 1.8. Genomic DNA (1 μg) was treated using the EpiTectFast DNA Bisulfite kit according to the manufacturer's protocol. The bisulfite modified DNA was subsequently suspended in 20 μl deionized water and used immediately or stored at -80˚C.

Bisulfite-specific polymerase chain reaction (BSP) and DNA sequencing. The primers used to detect the methylation of the RAP1GAP gene promoter TRR were designed to specifically amplify bisulfite-converted RAP1GAP DNA. The primers were custom synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) with the following sequences: Forward, 5'-AGTTGTTTAGTTTAGAGATAAAGTTTAAGAG-3' and Reverse, 5'-ACAACCCAACTATCCAAACA-3'. A total of 2 μl bisulfite-converted DNA (0.1 μg) from each sample was subjected to PCR analysis in a 25 μl volume containing 1X PCR buffer, 2 mmol/l $MgCl_2$, 2.5 mmol/l dNTP, 1 mmol/l primer, and 800 U/l EX TaqHS DNA polymerase (Invitrogen; Thermo Fisher Scientific, Inc.). The reaction mixture was preheated at 95˚C and then amplified (35 cycles of 95˚C for 30 sec, 52˚C for 30 sec, 72˚C for 45 sec), with a final extension of 10 min at 72˚C. The PCR products were treated using 2% agarose gel electrophoresis and a DNA fragment purification kit (Axygen; Corning Life Sciences, Hangzhou, China) according to the manufacturer's protocol, then subjected to cloning into the pMD-18-T vector (Takara Biotechnology Co., Ltd., Dalian, China). The purified DNA (4.5 μl) was incubated with the pMD-18-T vector in a water bath overnight at 16˚C, then cultured in lysogeny broth medium (Jiangsu Institute of Hematology) at 37˚C with *Escherichia coli* H5a (Jiangsu Institute of Hematology), with colonies forming after 24 h. Following the cloning, 10-18 clones from each sample were randomly selected for DNA sequencing.

Sequencing data analysis. Sequencing analysis was performed by Shanghai BioSune Co., Ltd. (Shanghai, China). Based on the data from BSP PCR-based sequencing analysis, the methylation level of each CpG site in a given sample was calculated as follows: Cpeak height/(C height + T height). The percentage of methylation of each CpG site in a given sample was calculated as follows: Number of methylated CpG sites/total number of observed sequenced clones. The percentage of the region methylation in a given sample was the average of each CpG sites methylation percentage in the DNA region.

Statistical analysis. Statistical analyses were performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA) and GraphPad Prism software (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA). The median ± 2nd and 3rd quartiles were assessed to analyze differences in the percentage of the region methylation among MDS, AML and NM samples. Variance of the variables among groups was calculated using nonparametric tests (Wilcoxon-Mann-Whitney U-test and Kruskal-Wallis test) in SPSS 17.0. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Methylation of the RAP1GAP gene in cells and MNCs of patients with MDS. Firstly, the methylation status of 20 CpG sites in the promoter region of *RAP1GAP* gene in 5 human leukemia cell lines was examined. The methylation level of RAP1GAP in SKM-1 cells was significantly lower compared with that of the other 4 leukemia cell lines, which was consistent with the clinical data of the present study (Fig. 1). Furthermore, a statistically significant difference in the methylation level of RAP1GAP was detected between SKM-1 cells and all other cell lines (Fig. 1). According to the National Center for Biotechnology Information Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) database, the *RAP1GAP* gene was analyzed in 29 MDS, 31 AML and 26 NM samples using Methyl Primer Express analysis software (version 1.0; Thermo Fisher Scientific, Inc.). CpG islands were found

Table I. Clinicopathological characteristics of patients with MDS.

Case	Sex	Age	Karyotype	WHO classification	IPSS
1	M	77	Normal	MDS-RCMD	0
2	F	66	Normal	MDS-RCMD	0
3	M	39	46,XY, 3q+,inv(9)(p12q13)	MDS-RAEB1	1.5
4	M	35	47,XY, +8	MDS-RCMD	0.5
5	F	75	Normal	MDS-RCMD	0.5
6	F	59	Normal	MDS-RCMD	0.5
7	M	61	Normal	MDS-RCMD	0.5
8	M	59	Normal	MDS-RCMD	0.5
9	F	60	Normal	MDS-RCUD	0.5
10	M	25	Normal	MDS-RAEB1	1
11	M	79	Normal	MDS-RARS	0.5
12	F	64	Normal	MDS-RAEB2	2
13	M	84	Normal	MDS-RAEB2	2
14	F	62	46,XX, del(2)(p15),?der(5)del(5)(q31)t(2;5)(p15;q31)[8]	MDS-RCMD	1.5
15	M	78	Normal	MDS-RAEB1	0.5
16	F	65	46,XX, 5q-,20q-[9]/46,XX[1]	MDS-U	1
17	M	73	47,XY, +8[8]/46,XY[2]	MDS-RAEB2	2
18	F	79	46,XX, del(5),del(13),del(20)	MDS-U	1
19	M	66	Normal	MDS-RCMD	1
20	M	21	47,XY, +8[9]/46,XY[1]	MDS-RAEB1	1
21	M	85	45,X-Y[3]/46,XY[17]	MDS-RCMD	1
22	F	50	Normal	MDS-RCMD	0.5
23	F	53	Normal	MDS-RAEB1	0
24	M	77	46,XY, der(18)[8]/46,XY[4]	MDS-RCMD	1
25	F	64	Normal	MDS-RARS	1
26	M	17	46,XY, 20q-[9]/46,XY[4]	MDS-RCMD	0
27	M	20	46,XY, der(7)t(1;7)[4]/46,XY[6]	MDS-RCMD	1.5
28	F	38	Normal	MDS-RAEB2	1.5
29	M	65	47,XY, +8[2]/45,X,-Y/46,XY[12]	MDS-RCMD	0.5

WHO, World Health Organization; IPSS, International Prognostic Scoring System; M, male; F, female; RARS, refractory anemia with ring sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; RAEB, refractory anemia with excess blasts; U, unclassified; MDS, myelodysplastic syndrome.

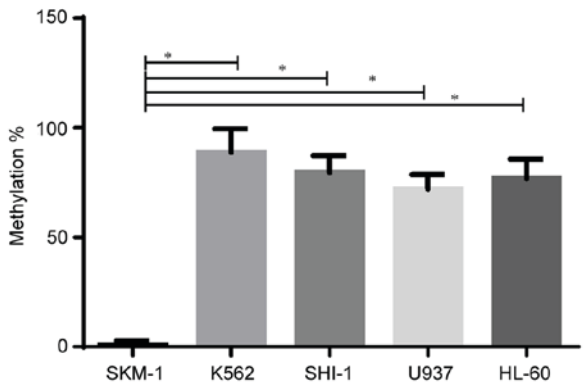

Figure 1. RAP1GAP gene methylation analysis in 5 leukemia cell lines (SKM-1, K562, SHI-1, U937 and HL-60). The methylation level of RAP1GAP in SKM-1 cells was significantly decreased compared with that in all other leukemia cell lines. Statistically significant differences in the methylation level of RAP1GAP were detected between SKM-1 cells and all other cell lines (*P<0.05). RAP1GAP, RAP1GTPase activating protein 1.

upstream of the transcriptional start site (designated as '0') between -680 and -398 bp. The structure of the RAP1GAP gene is presented in Fig. 2, indicating the position of the CpG island containing 20 CpG sites.

In these 20 CpG sites, the majority of the CpG sites were slightly methylated or unmethylated in all samples, including 8 CpG sites, which exhibited statistically significant differences among MDS, AML and NM samples. The results of the current study confirmed that the methylation of Rap1GAP in patients with MDS was 100%, distributing over various CpG sites in different patients. The overall methylation level in patients with MDS was decreased compared with that in patients with NM. The methylation pattern in 20 CpG sites differed among NM, MDS and AML. A significant difference in 4 CpG sites was identified in the patients with NM compared with those with MDS. These preliminary results suggest that the methylation state of the whole region, rather than the methylation state of a single CpG site, is associated

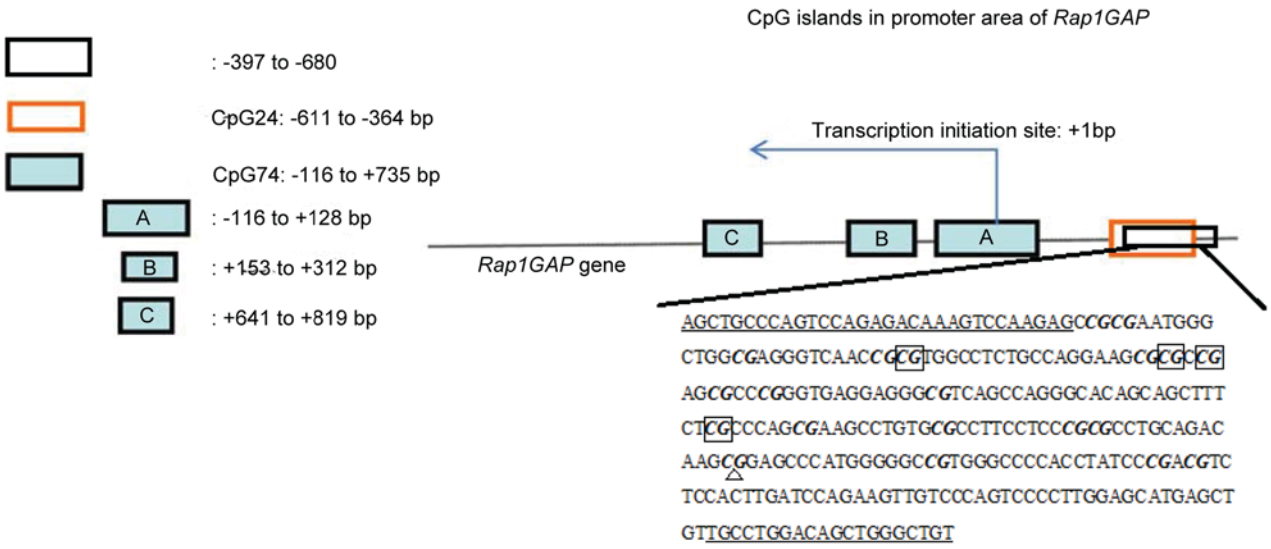

Figure 2. Illustration of the RAP1GAP gene transcriptional regulation region and topology of the BSP primer. The black bar indicates the analyzed region. The bold italic 'CG' indicates the location of 20 CpG island sites. The underlined sequences indicate the BSP primers. Black rectangles indicate the 4 CpG sites that were identified to be significantly different between myelodysplastic syndrome and patients with NM. The triangle indicates the CpG site that was identified to be significantly different between acute myeloid leukemia and NM patients. RAP1GAP, RAP1GTPase activating protein 1; BSP, bisulfite-specific polymerase chain reaction; NM, non-malignant blood disease.

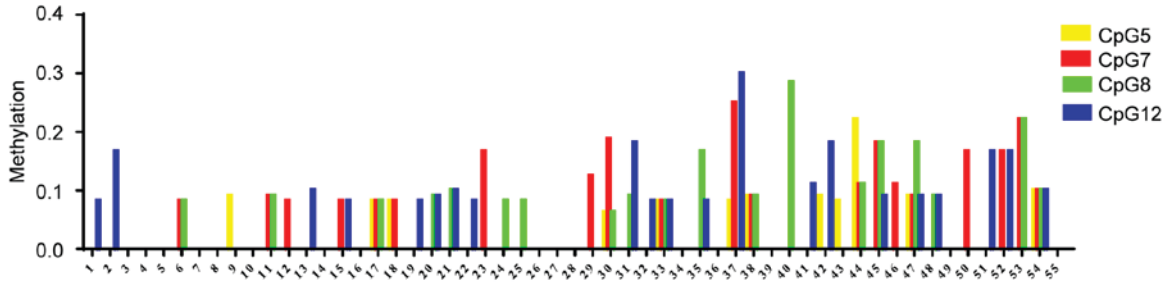

Figure 3. Rap1GAP methylation analysis in 29 patients with MDS (no. 1-29) and 26 non-malignant blood diseases (no. 30-55). Total methylation of the 4 CpG sites (5, 7, 8, 12) of Rap1GAP promoter in patients with MDS was decreased compared with that in patients with non-malignant blood disease. RAP1GAP, RAP1GTPase activating protein 1; MDS, myelodysplastic syndrome.

with the sample groups. Statistical analysis of the data was subsequently performed.

Statistical analysis results. The associations between the methylation degree of each of the 6 CpG sites and tumor types were analyzed. Firstly, the hypomethylated level in 4 CpG sites (CpG5, CpG7, CpG8 and CpG12) were identified to be significantly different between patients with MDS and those with NM ($P<0.05$; Fig. 4), and was significantly different in one site (CpG17) between patients with AML and those with NM ($P=0.023$; data not shown). Compared with AML, the methylation level of patients with MDS is lower in four CpG sites (CpG5, CpG7, CpG8 and CpG12) ($P<0.05$; Fig. 4). Based on these results, it was demonstrated that the methylation level at four recurring sites (CpG5, CpG7, CpG8 and CpG12) was significantly different among the groups, which may correlate with the progression of MDS to AML.

A previous study reported the methylation score as a novel variance to measure and compare the methylation status of the CpG sites in the differentially methylated region of each patient (8). As the methylation score was more stable and reproducible for the evaluation of DNA methylation status (8), methylation scoring was performed on 4 CpG sites (5, 7, 8 and 12) using GraphPad Prism 6. A marked difference in methylation scores was identified among patient groups (Fig. 5). It is hypothesized that the methylation score reflects the methylation status for more CpG sites, and could be more helpful for diagnosis and risk stratification.

Clinical association analysis. The mechanism underlying the hypomethylation in promoter of Rap1GAP remains unclear. To access the clinical significance of these findings, the association between the methylation status in promoter of Rap1GAP in patients with MDS, and clinical parameters, including age, gender, clinical classification and IPSS was further analyzed (Table I). As older patients with MDS possess poorer prognoses, reduced disease-free survival (DFS) and reduced overall survival (OS) times (3 and 8.5 months, respectively) (9) and the majority of patients with high-risk MDS and AML are >60 years (10), the age threshold was set at 60. Based on the present clinical data, no significant differences in age, gender and IPSS were identified between

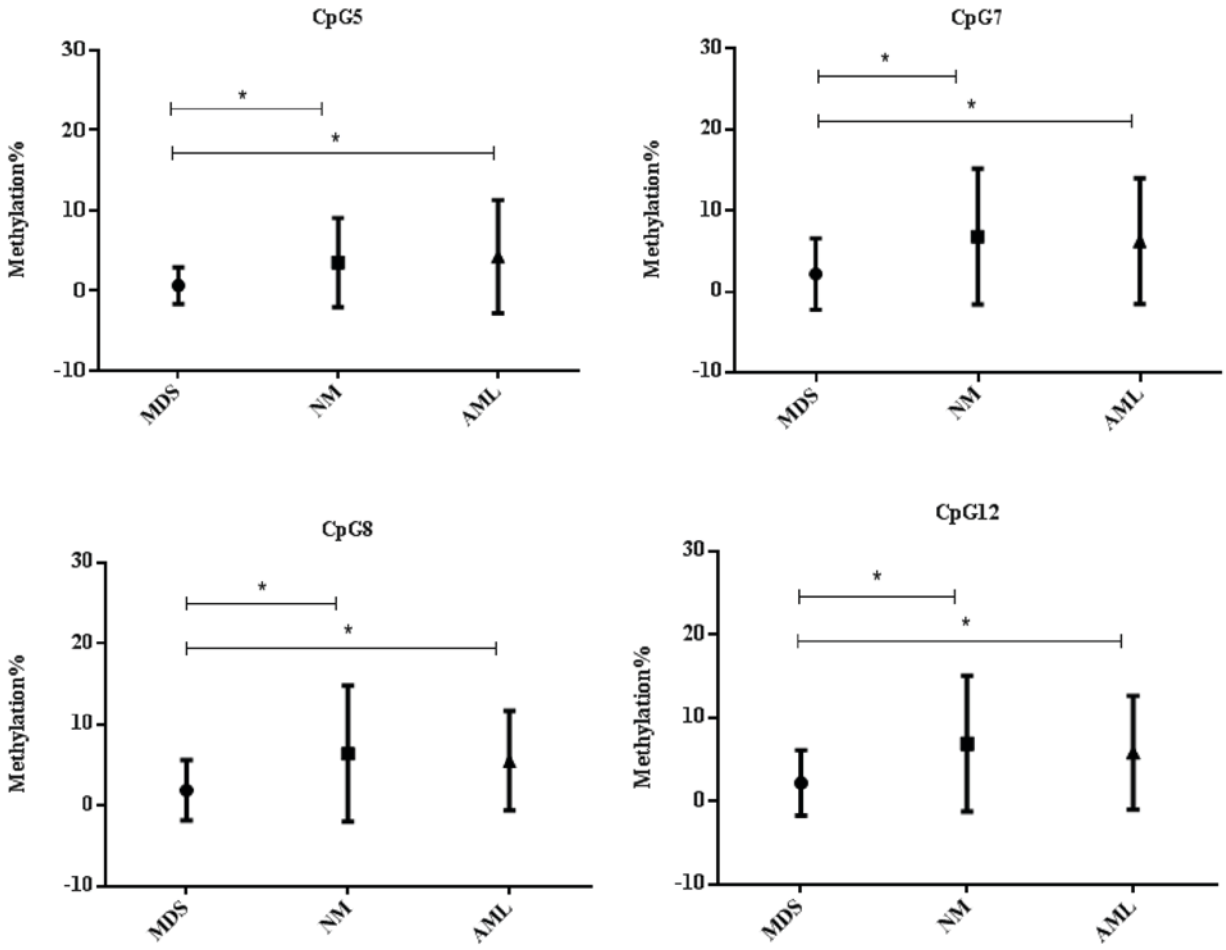

Figure 4. Statistical analysis of individual CpG site methylation degree. Data are presented as the median ± 2nd and 3rd quartilesof the percentage of methylation at each CpG site in the MDS, NM and AML groups. The methylation levels at these CpG sites were statistically significant between MDS and NM or AML (*P<0.05). NM, non-malignant blood disease; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia.

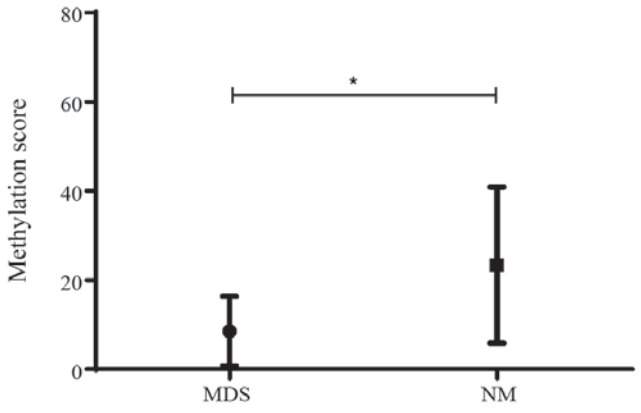

Figure 5. Statistical analysis of the average methylation of 4 CpG sites. Data are presented as the median ± 2nd and 3rd quartilesof the methylation score obtained by adding the values of the CpG sites (5, 7, 8 and 12) for each patient. The average methylation score for patients with MDS was significantly decreased compared with those with NM (*P<0.05). MDS, myelodysplastic syndrome; NM, non-malignant blood disease.

patient groups with different methylation statuses (Table II). However, according to the 2008 World Health Organization classification of MDS (11), only the difference in methylation level at CpG site 8 of Rap1GAP promoter was identified to be significantly increased in patients with MDS-refractory anemia with ring sideroblasts (RARS) compared with that in the MDS-refractory cytopenia with multilineage dysplasia (RCMD; P=0.033) or MDS-unclassified (U; P=0.034) groups (Fig. 6). A previous study reported that 40-60% of all patients with MDS possess identifiable karyotypic abnormalities (12) and that cytogenetic subgroups of outcome are associated with prognosis (13). In the present cohort, 13 patients exhibited abnormal karyotypes, accounting for 45% of all patients in the sample, which is consistent with the previous study described. However, the majority of the karyotypes identified in these patients were not beneficial as prognostic indicators in MDS according to IPSS (14). Furthermore, it was revealed that the methylation level of CpG11 was significantly different between patients with the normal and abnormal karyotypes, which may be associated with prognosis in MDS (P=0.0429; Fig. 7).

Discussion

Rap1GAP codes for an enzyme that catalyzes the hydrolytic switch from ATP to ADP bound to Rap1, a member of small G proteins. The intrinsic GTPase activity enhanced by Rap1GAP inactivates Rap1GTP, influencing a variety of essential biological processes in eukaryotic cells, including cell proliferation,

Table II. Clinical data analysis of MDS.

	CpG5		CpG7		CpG8		CpG12	
	Methylation, % (OR)	P-value	Methylation, % (OR)	P-value	Methylation, % (OR)	P-value	Methylation, % (OR)	P-value
Age, years		0.1643		0.6817		0.5659		0.3163
<60	0.0 (0.0)		0.0 (0.0)		0.0 (0.0)		0.0 (0.0)	
≥60	0.0 (0.0)		0.0 (8.3)		0.0 (8.3)		0.0 (8.3)	
Sex		0.4592		0.2562		0.2540		0.2726
Male	0.0 (0.0)		0.0 (4.15)		0.0 (8.3)		0.0 (8.3)	
Female	0.0 (0.0)		0.0 (8.3)		0.0 (0.0)		0.0 (0.0)	
IPSS		0.7727		0.8219		0.2988		0.2502
Low	0.0 (12.5)		0.0 (0.0)		0.0 (14.6)		0.0 (0.0)	
Int-1	0.0 (0.0)		0.0 (8.3)		0.0 (8.3)		0.0 (8.3)	
Int-2	0.0 (0.0)		0.0 (8.3)		0.0 (0.0)		0.0 (0.0)	
High	-		-		-		-	

The methylation percentage is presented as the median (quartiles)=M (OR); OR, 75th percentile to 25th percentile. OR, odds ratio.

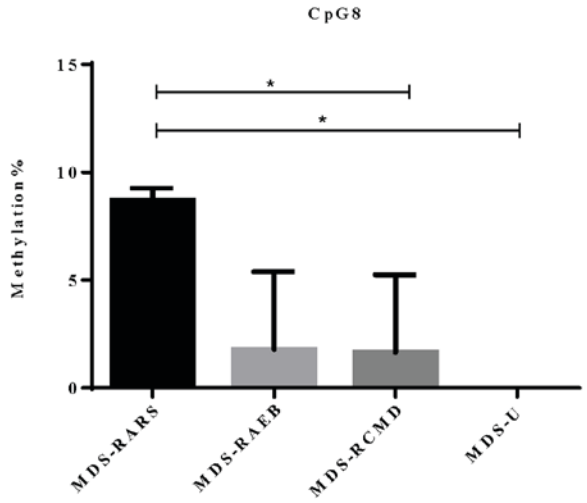

Figure 6. Analysis of individual CpG site methylation degree according to the 2008 World Health Organization classification of MDS. Data are presented as the median ± second and third quartiles. The methylation level at CpG site 8 of Rap1GAP promoter was identified to be significantly increased in patients with MDS-RARS compared with that in the MDS-RCMD or MDS-U groups (*P<0.05). RAP1GAP, RAP1GTPase activating protein 1; RARS, refractory anemia with ring sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; RAEB, refractory anemia with excess blasts; U, unclassified; MDS, myelodysplastic syndrome.

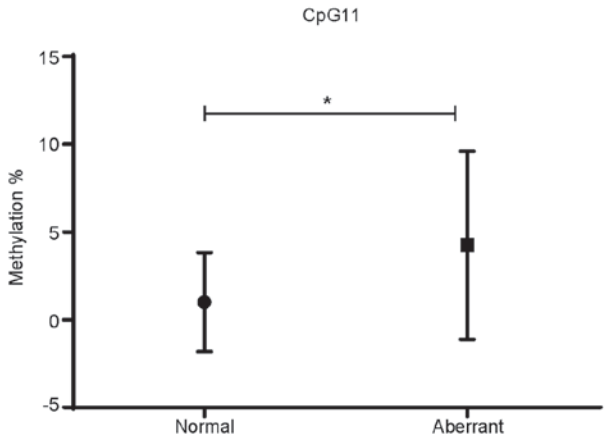

Figure 7. Methylation levels of cytogenetic subgroups (normal and aberrant) in patients with myelodysplastic syndrome. Data are presented as the median ± 2nd and 3rd quartiles of the percentage of methylation. CpG11 site methylation was significantly decreased in the normal compared with the aberrant group (*P<0.05).

adhesion and migration via specific signal molecules, including BRAF, and ERK (15). A previous study on hematopoietic cells reported multiple functions for Rap1, including its involvement in the maturation of megakaryocytes, and the adhesion of leukocytes and T lymphocytes (15).

Rap1GAP has been identified as a putative tumor suppressor gene in pancreatic cancer. The downregulation of Rap1GAP expression is associated with pancreatic cancer progression, which may function through the modulation of integrin activity (16). Rap1GAP-knockdown in human colon cancer cells results in more aggressive migratory and invasive properties compared with normal colon cancer cells (17). Similar results were demonstrated in melanoma whereby the overexpression of Rap1GAP in melanoma cells inhibited cell proliferation and survival (18). In thyroid cancer Rap1GAP expression is decreased due to promoter hypermethylation, and progressively higher frequencies of promoter hypermethylation are identified in the more aggressive types of thyroid cancer, thus promoting thyroid cancer cell invasion (19). In addition, upregulation of Rap1GAP expression was demonstrated to inhibit focal adhesion formation and decrease melanoma cell migration (18). Altered expression of Rap1GAP in multiple types of cell may also be associated with the pathology of multiple diseases. For example, the monocytes of patients with chronic lymphocytic leukemia exhibiting increased Rap1GAP expression exerted impaired phagocytosis compared with those exhibiting decreased Rap1GAP

expression (20). Furthermore, the proliferation of T cells in contact with these monocytes was inhibited (20). The increased expression of Rap1GAP in podocytes contributes to their dysfunction and the injury underlying the pathogenesis of all proteinuric kidney diseases (21). However, certain studies have reported contradictory results regarding the expression and function of Rap1GAP in different cells (6,22). A previous study demonstrated that patients with MDS express Rap1GAP at a significantly increased level compared with that in patients with NM or *de novo* AML (6). Furthermore, Rap1GAP-transfected leukemia cells exhibited an increased apoptosis rate in response to arsenic trioxide and elevated invasion compared with non-transfected leukemia cells (22).

Kim *et al* (23) demonstrated that promoter hypermethylation of Rap1GAP in renal cell carcinoma may lead to decreased Rap1GAP expression. Zheng *et al* (19) confirmed these results in multiple melanoma tumors and cell lines using demethylating agent treatment. Although aberrant DNA methylation has been considered the dominant mechanism underlying the progression of MDS to AML, whether promoter hypomethylation is the cause of the elevated expression of Rap1GAP previously observed in the cluster of differentiation 34^+ cells of patients with MDS remains unclear (6).

To qualitatively or quantitatively analyze DNA methylation levels, various techniques can be used, including methylation-specific PCR, sequencing of certain regions of DNA fragments and high throughput genome-wide sequencing. As the distribution of CpG sites and the methylation pattern in Rap1GAP promoter is presently unknown and whole genome sequencing is unnecessary, the DNA fragments of 20 CpG sites within the Rap1GAP promoter were amplified, cloned and sequenced in the present study. The frequency of subclones in which methylation could be detected at a certain CpG site may estimate the level of methylation for that CpG site. The results of the current study demonstrated a relatively lower overall methylation level within Rap1GAP promoter in patients with MDS compared with that in patients with NM or AML. However, only 4 CpG sites were confirmed to be methylated at a significantly lower level in MDS compared with that in NM or AML. Furthermore, based on the clinical records of the patient cohort in the present study, no significant associations were identified between the methylation status of Rap1GAP promoter and the clinicopathological characteristics of patients with MDS, which included age, gender, and IPSS.

Together, the results of the current study suggest that the upregulation of Rap1GAP expression in patients with MDS is associated with a lower methylation status in the promoter region of this gene. However, further studies are required to elucidate the association between the methylation of Rap1GAP promoter and the clinical course, and prognosis of patients with MDS. Currently, demethylating agents, including decitabine, have been used for the treatment of patients with MDS based on a putative hypothesis that certain tumor suppressor genes are inactivated by the hypermethylation in their promoter leading to the initiation and progression of MDS (24). Therefore, demethylating the promoter may reactivate these tumor suppressor genes to inhibit or reverse the progression of MDS towards AML. However, in the present study, although Rap1GAP can be considered as a tumor suppressor gene in multiple tumor types, inconsistently the expression of this gene in MDS is upregulated in parallel with a lower level of methylation in promoter of this gene. This phenomenon appears to contradict the usage of demethylation agents for the treatment of MDS. As MDS is recognized as a dynamic pre-leukemic process, the epigenetic mechanism in addition to genetic alterations may serve essential roles in the initiation of MDS and its progression towards AML. The detailed function of Rap1GAP and its signaling pathway during this process remains unclear. Further studies are warranted to identify other epigenetic mechanisms in addition to promoter methylation that underlie MDS progression.

In conclusion, the present study has demonstrated that the methylation level in the 4 CpG sites distinguished MDS from the NM. Based on the clinical results of the present study, no significant differences in age, gender and IPSS were identified between patient groups with different methylation statuses. The difference in methylation level of Rap1GAP promoter was identified to be statistically significant between patients with MDS-RARS and MDS-RCMD or MDS-U, and between the normal with abnormal karyotypes. This suggests that methylation level is associated with prognosis in MDS. Methylation within Rap1GAP promoters in patients with MDS was decreased compared with that in patients with NM or AML.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZXC, YYW and SNC designed the study and gave final approval of the manuscript to be published. WJD and YY performed the experiments and aquistion of data. WJD, YY and FJ analyzed the experimental data. XFQ, JNC and WLD analyzed and interpreted the patient data regarding the hematological disease. WJD, YY and YYW wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Patient's data and sample usage protocol was approved by the Ethics Committee of The First Affiliated Hospital of Soochow University with documented consent.

Patient consent for publication

All patients and their families consent to the publication.

Competing interests

The authors declare that they have no competing interests.

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