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ORIGINAL ARTICLE

Overexpression of the EZH2, RING1 and BMI1 genes is common in myelodysplastic syndromes: relation to adverse epigenetic alteration and poor prognostic scoring

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Abstract Epigenetics refers to the study of clonally inherited changes in gene expression without accompanying genetic changes. Previous research on the epigenetics of myelodysplastic syndromes (MDS) mainly focused on the inactivation of tumor suppressor genes as a result of DNA methylation. However, the basic molecular pathogenesis of epigenetics in MDS remains poorly understood. Recent studies have revealed that DNA methylation and histone modification may be controlled by Polycomb-group (PcG) proteins, which may give new clues toward understanding the epigenetic mechanism of MDS. In this study, we explored for the first time the expression of PcG genes, including EZH2, EED, SUZ12, RING1, and BMI1, in various MDS subsets and acute myeloid leukemia (AML), as well as the relationship between the expression of PcG genes and epigenetic alteration and prognosis-risk scoring. Patients with MDS/AML showed overexpression of EZH2, RING1, and BMI1 genes compared to their expression levels in patients with non-clonal cytopenia diseases. The MDS patients with DNA methylation had higher EZH2 expression than those without DNA methylation. The patients who received decitabine treatment presented significantly reduced expression of EZH2 and RING1 besides decreased $p15^{INK4B}$ methylation after decitabine treatment. Moreover, overexpression of EZH2, RING1, and BMI1 was always linked to poor prognostic scoring. In conclusion, overexpression of the EZH2, RING1, and BMI1 genes is common in MDS and indicate poor prognosis. The products of these genes might participate in epigenetic regulation of MDS. These studies may also contribute to our understanding of the effective mechanism of decitabine.

Keywords Myelodysplastic syndrome · Polycomb · EZH2 · RING1 · BMI1 · Methylation

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Myelodysplastic syndromes (MDS) are a class of clonal diseases that originate from malignant hematopoietic stem/progenitor cells and have obvious heterogeneity [1]. They are characterized by abnormal maturation and differentiation of hematopoietic cells and a high risk of progression to leukemia [2]. Until now, the pathogenesis of MDS has been poorly understood because of its heterogeneity and complexity. However, it is well accepted that the pathogenesis of MDS is generally triggered by internal factors (epigenetic and genetic changes) and external factors (microenvironment changes and immunological anomalies). In recent years, decitabine and 5-azacytidine, which are considered to be epigenetic agents, have been successfully used to treat patients with various MDS subsets. The success of these treatments emphasizes epigenetic alterations as key players in the carcinogenesis of MDS [3–6].

Epigenetics refers to the study of clonally inherited changes in gene expression in the absence of accompanying genetic changes, including DNA promoter methylation and histone modification [7]. Previous research on epigenetics has mainly focused on the inactivation of tumor suppressor genes by DNA methylation [8–10]. Recent studies have revealed that transcription silencing mediated by Polycomb-group (PcG) proteins can be independent of

promoter DNA methylation [11, 12]. Moreover, PcG proteins can also directly control DNA methylation [13]. PcG proteins perform a critical role in gene regulation and are thought to coordinate DNA accessibility throughout the organism's development, repressing transcription through the regulation of DNA methylation and specific amino acid modifications in histones [14].

Biochemical purification and functional genetic studies have assigned the various PcG genes into two distinct subsets, namely Polycomb repressive complex 1 (PRC1) and Polycomb repressive complex 2 (PRC2) based on the types of repressive complexes to which they contribute [15]. PRC2 and PRC1 are considered to be involved in the initialization and maintenance of the repression of gene transcription, respectively [16]. PRC2 comprises the core components enhancer of zeste-2 (EZH2), embryonic ectoderm development (EED), and suppressor of zeste-12 (SUZ12), while PRC1 consists of ring finger protein 1 (RING1), B lymphoma Mo-MLV insertion region 1 (BMI1), and chromobox homologue 2/4/8 (CBX2/4/8) [15]. EZH2 is the catalytic subunit of PRC2. It is a highly conserved histone methyltransferase that targets lysine 27 of histone H3. This methylated H3-K27 is commonly associated with the silencing of genes involved in differentiation [17]. In addition, EZH2 is required for DNA methylation of EZH2-target promoters, serving as a recruitment platform for DNA methyltransferases [13]. SUZ12 is a recently identified PcG protein that, together with EED, is essential in maintaining the repressive function of PRC2 [18]. RING1 is thought to bind to this histone modification and catalyze the monoubiquitination of histone H2A at lysine 119. The H2AK119 ubiquitination likely increases chromatin compaction, and thus, interferes with the access or action of transcription factors [19]. BMI1 is mostly detected in primitive CD34+CD45−CD71−bone marrow (BM) cells and takes part in HSC proliferation and self-renewal [20].

Methylation of tumor-suppressor genes, such as p15, DAPK, and SOCS-1, is common in MDS patients, along with elevated levels of DNA methyltransferase (DNMTs) [21–24]. However, epigenetic studies on PcG genes in MDS are still rare. Considering that PcG genes serve as upstream regulators of DNA methylation and histone modification, research on PcG gene expression and function should shed light on the epigenetic mechanism in the pathogenesis of MDS. In this study, we explored the expression of PcG genes, including EZH2, EED, SUZ12, RING1, and BMI1, in various MDS subsets and AML patients, and we examined the differential expression of PcG genes in patients with or without $p15^{INK4B}$ methylation. Second, we observed the influence of PcG genes expression on different prognostic risk groups. Finally, the impact of decitabine treatment on the expression of PcG genes was also investigated.

Methods

Patients

MDS was diagnosed in accordance with the minimum diagnostic criteria established by the Conference on MDS (Vienna, 2006) [25]. The classification and prognostic risk scoring of MDS were performed according to the WHO criteria [26] and the International Prognostic Scoring System (IPSS) [27]. AML was classified according to the FAB criteria [28]. The definition of non-MDS diseases was based on clinical characteristics, morphological changes, special biochemical indicators, and response to treatment. All MDS and AML patients underwent only supportive care prior to recruitment into this study. All subjects were given informed consent. The research was approved by the Ethics Committee of the Sixth Hospital affiliated with Shanghai Jiao Tong University, and all patient-relevant research strictly abided by the Declaration of Helsinki.

Fluorescence quantitative reverse transcription-polymerase chain reaction analysis

Bone marrow mononuclear cells (BMNCs) were separated by centrifugation over a Ficoll-hypaque gradient (Lymphoprep TM, Oslo, Norway). Isolated BMNCs were washed twice with phosphate-buffered saline (PBS). Total RNA from 5×10^6 cells was extracted and purified using an RNeasy Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocols. The cDNA was prepared using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada) according to the manufacturer's protocols. The specific primer pairs were designed with the aid of the Primer Synthesis Service (Sangon Biotech, ShangHai, China). Sense and anti-sense primers for amplification of the EZH2, EED, SUZ12, RING1, and BMI1 genes were designed and constructed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The sequences and Tm values of all the primers, as well as the lengths of the PCR products, can be seen in Table.1.

Real-time monitoring of the PCR reactions was performed using the LightCycler™ system (Roche Applied Science, Indianapolis, IN) and Real Master Mix SYBR Green (TianGen Biotech, Beijing, China). Monitoring was performed as follows: a master mixture containing 2 μl of cDNA for each gene, 9 μl of Real Master SYBR Green mix, and 0.2 μl of primers (10 μmol/L) was prepared on ice. The final volume was then adjusted to 20 μl with deionized water. After the reaction mixture was loaded into a glass capillary tube, PCR was carried out under the following cycling conditions: initial denaturation at 95°C for 60 s, followed

Table 1 Primer sequences for quantitative reverse transcription polymerase chain reaction and methylation analysis

Gene	Origin	F/R	Sequence	Tm value	Product length
qRT-PCR					
BMI1	Human	F	5′-AATCTAAGGAGGAGGTGA-3′	63°C	120 bp
		R	5′-AAACAAGAAGAGGTGGA-3′		
RING1		F	5′-GCCAGACGAGGTATGTGAAGA-3′	63°C	258 bp
		R	5′-TGTAGATGGTGTACTGCTTTTCACT-3′		
EZH2		F	5′-TTGTTGGCGGAAGCGTGTAAAATC-3′	63°C	117 bp
		R	5′-TCCCTAGTCCCGCGCAATGAGC-3′		
SUZ12		F	5′-GATAAAAACAGGCGCTTACAGCTT-3′	56°C	98 bp
		R	5′-AGGTCCCTGAGAAAATGTTTCGA-3′		
EED		F	5′-GGATTTCTGGCAAAAGATGCTT-3′	56°C	87 bp
		R	5′-AGCAGCACCACATTTATGATGAGT-3′		
GAPDH		F	5′-GCACCGTCAAGGCTGAGAAC-3′	60°C	139 bp
		R	5′-GTGGTGAAGACGCCAGTGGA-3′		
MS-PCR					
p15-methylation		F	5′-GCGTTCGTATTTTGCGGTT-3′	55°C	148 bp
		R	5′-CGTACAATAACCGAACGACCGA-3′		
p15-unmethylation		F	5′-TGTGATGTGTTTGTATTTTGTGGTT-3′		154 bp
		R	5′-CCATACAATAACCAAACAACCAA-3′		

by 40 cycles of denaturation at 95°C for 20 s, annealing at 56°C or 63°C for 30 s and extension at 72°C for 30 s. To test the specificity of the PCR, the reaction products were subjected to melting curve analysis with the LightCycler system and to conventional agarose gel electrophoresis to rule out the synthesis of unspecific products. The relative gene expression levels were calculated as the difference of the C_T values of EZH2, EED, SUZ12, RING1, and BMI1, and the housekeeping gene GAPDH as a control (ΔC_T). For all patients sampled, the relative expression of the PcG genes were calculated relative to the mean expression levels of these genes in the healthy volunteers (Δ-ΔC_T). Table 3 shows the individual Δ-ΔC_T values of all MDS samples analyzed.

Methylation-specific polymerase chain reaction

A 1-μg sample of DNA in a volume of 100 μl was treated with bisulfite using EZ DNA Methylation™ Kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. The modified DNA was subjected to two separate PCRs. MS-PCR primers were designed to amplify the methylated (M) or unmethylated (U) alleles (Table.1). DNA from healthy individuals was used as a negative control. Methylated DNA (CpGenome universal methylated DNA; Chemicon International) was used as a positive control. The 10-μl PCR mixture contained 80 μg of bisulfite-treated DNA, 1 unit of AccuPrime Taq DNA Polymerase (Invitrogen, Paisley, UK), and 2.5 mmol/L of each primer. The MS-PCR cycling conditions used for the p15 promoter were: 95°C for 3 min, 40 cycles of 95°C for 30 s, annealing at 60°C for 2 min, and 72°C for 1 min followed by a final extension at 72°C for 5 min. The PCR products were electrophoresed on 10% polyacrylamide gels and visualized by ethidium bromide staining and ultraviolet transillumination. The results from duplicate experiments were used to determine the methylation status.

Decitabine treatment

Thirteen MDS patients, including 1 case with RARS, 5 cases with RCMD, 1 case with RCMD-RS, 2 cases with RAEB-1, and 4 cases with RAEB-2, received decitabine treatment. Each course of treatment consisted of 20 mg/m^2 administered intravenously over 1 h daily for 5 days, and the courses were given every 4 weeks. The response to treatment was assessed according to the International Working Group (IWG) criteria [29].The relative expression of the PcG genes from the 13 patients were detected at the beginning of decitabine treatment and after two course of treatment, respectively. Detailed treatment information and relative expression of PcG genes before and after treatment are shown in Table 2.

Statistical analysis

All statistical analyses were performed using the SPSS 11.0 System. Multiple pairwise comparisons were made using a one-way analysis of variance (ANOVA) test. The chi-squared test ($R \times C$) was applied between different groups of positive cases. Pearson correlation analysis was used for numeric type tests. Spearman correlation analysis was used

Table. 2 Information of decitabine treatment and the expression change of PcG genes before and after decitabine treatment

No.	Diagnosis	IPSS	p15 methylation		Relative expression of PcG genes (folds)[a]					
					EZH2		BMI1		RING1	
			Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
3	RARS	Low	NO	NO	1.43	2.09	2.29	1.06	1.63	1.45
8	RCMD	Int-1	YES	YES	3.51	4.85	2.95	3.50	2.21	2.78
10	RCMD	Int-1	YES	NO	2.67	2.89	0.73	−0.81	0.16	−0.85
29	RCMD	Int-1	YES	YES	4.76	4.11	1.92	2.03	2.01	1.87
36	RCMD	Int-1	YES	NO	4.43	2.89	2.55	4.34	2.80	2.01
40	RAEB-1	Int-1	YES	NO	3.49	2.90	1.89	1.07	2.90	1.85
42	RAEB-1	Int-1	YES	NO	3.22	1.59	2.39	−0.26	3.54	0.89
43	RCMD-RS	Int-2	YES	NO	4.24	1.94	3.02	1.80	2.40	1.03
45	RCMD	Int-2	YES	NO	7.20	4.00	2.42	1.22	3.60	0.79
47	RAEB-2	Int-2	YES	YES	5.15	3.04	4.81	1.93	4.30	2.56
48	RAEB-2	Int-2	YES	YES	3.28	2.61	1.90	2.33	3.20	0.88
53	RAEB-2	Int-2	YES	YES	6.00	4.62	3.74	4.24	3.89	3.86
54	RAEB-2	High	YES	YES	7.09	3.44	6.18	2.21	4.12	2.78

[a] Relative expression of PcG genes from the patients who received decitabine treatment was analyzed at the beginning of decitabine treatment and after two courses of treatment, respectively

for ranking correlation tests. $P<0.05$ was considered statistically significant.

Results

Patients' characteristics

A total of 54 MDS patients, including 32 males and 22 females, were involved in this study. Their median age was 64 years (16–85 years). They were classified as RA ($n=2$), RARS ($n=2$), RCMD ($n=31$), RCMD-RS ($n=1$), RAEB-1 ($n=11$), RAEB-2 ($n=5$), and MDS-U ($n=2$). Risk assessment was conducted by the IPSS. Thirty-two MDS patients, with IPSS scores ≤1.0, were defined as the low-risk MDS group and 22 MDS patients with IPSS scores >1.0 was defined as the high-risk MDS group. Detailed information about these patients is shown in Table.3. Fifteen cases of AML comprised the AML group, classified according to FAB criteria as M1 ($n=3$), M2 ($n=2$), M3 ($n=3$), M4 ($n=4$), M5 ($n=1$) and M6 ($n=2$). These patients included nine males and six females, and their median age was 55 years (33–81 years). The non-clonal cytopenia group contained 30 cases, including 8 patients with megaloblastic anemia, 10 patients with iron-deficiency anemia, 3 patients with hemolytic anemia, 3 patients with anemia of renal disease and 6 patients with idiopathic thrombocytopenic purpura. In addition, 25 healthy volunteers with a median age of 58 years (21–86 years) were included in the study as a baseline reference for calculating the relative expression of PcG genes.

Expression of PRC2 genes (EZH2, EED, and SUZ12) in MDS, AML, and non-clonal cytopenia patients

The expression levels of EZH2 in the low-risk MDS, high-risk MDS, AML and non-clonal cytopenia groups were 3.6±0.2, 5.4±0.3, 5.2±0.2, and 1.8±0.2-fold (mean±SE) relative to the values in healthy volunteers, respectively (Fig. 1a). Significant differences were observed among the four groups ($F=53.703$, $P<0.001$). Although EZH2 expression was significantly higher in the high-risk MDS and AML groups than in the low-risk MDS group ($P<0.001$, $P<0.001$), the expression of the gene in the low-risk MDS group was still obviously higher than in the non-clonal cytopenia group ($P<0.001$). The EED expression levels in the low-risk MDS, high-risk MDS, AML, and non-clonal cytopenia groups were −1.9±0.4, −1.4±0.7, −0.9±0.6, and −1.4±0.5-fold relative to the values in healthy volunteers, respectively ($F=1.962$, $P=0.105$) (Fig. 1b). No significant difference was observed between the four groups. SUZ12 expression levels in the low-risk MDS, high-risk MDS, AML, and non-clonal cytopenia groups were 1.5±0.2, 1.2±0.5, 0.8±0.6, and 1.0±0.4-fold of the values in healthy volunteers, respectively ($F=0.720$, $P=0.542$) (Fig. 1c). No significant difference was observed between the four groups.

Table. 3 Clinical characteristics and relative expression of PcG genes in MDS patients

No	Age/ sex	Diagnosis[a]	Cytogenetics	IPSS	Methylation of $p15^{INK4B}$	Relative expression of PcG genes (folds)				
						EZH2	EED	SUZ12	RING1	BMI1
1	85/F	RA	Normal	0	NO	1.43	2.92	−2.30	1.10	2.99
2	84/F	RA	Normal	0	YES	4.06	2.64	−2.09	0.43	0.91
3	78/M	RARS	Normal	0	NO	1.33	−6.18	1.62	1.63	2.29
4	66/F	RARS	Trisomy 8	0.5	YES	4.73	−3.12	2.92	2.20	3.00
5	48/M	MDS-U	Trisomy 8	0.5	NO	2.09	2.47	−1.02	0.23	0.98
6	17/M	MDS-U	Trisomy 8	0.5	NO	2.84	−1.64	1.53	1.64	1.99
7	50/M	RCMD	Normal	0.5	YES	3.46	−5.10	2.36	1.08	1.71
8	21/M	RCMD	Normal	0.5	YES	3.51	−6.41	1.92	2.21	2.95
9	26/F	RCMD	Normal	0.5	NO	3.33	1.17	4.16	2.13	3.02
10	28/F	RCMD	Normal	0.5	YES	2.67	−0.92	1.20	0.16	0.73
11	49/M	RCMD	Normal	0.5	NO	2.28	1.43	−0.54	0.58	1.18
12	44/M	RCMD	Complex	0.5	YES	3.75	−5.93	1.55	1.98	2.37
13	29/F	RCMD	Normal	0.5	YES	3.48	−5.84	1.51	0.63	1.25
14	36/F	RCMD	Normal	0.5	YES	3.18	−5.74	2.28	0.91	1.38
15	16/M	RCMD	Normal	0.5	NO	2.29	−2.72	2.26	1.77	2.37
16	34/M	RCMD	Normal	0.5	YES	4.83	−3.40	2.32	1.95	2.62
17	75/M	RCMD	Del(5q)	0.5	YES	3.16	−4.58	1.70	1.69	2.44
18	22/F	RCMD	Normal	0.5	NO	4.84	−3.26	1.91	1.62	2.09
19	27/M	RCMD	Normal	0.5	YES	2.66	−5.57	−0.23	0.82	1.39
20	42/M	RCMD	Normal	0.5	YES	4.76	−3.23	1.35	1.38	1.77
21	53/F	RCMD	Normal	0.5	YES	1.87	−4.47	1.61	0.99	1.02
22	68/M	RCMD	Normal	0.5	NO	1.05	−3.67	1.91	1.11	2.33
23	19/F	RCMD	Normal	0.5	YES	4.31	−2.08	2.18	2.42	1.99
24	45/F	RCMD	Normal	0.5	NO	2.59	−2.15	−0.12	0.23	0.41
25	23/M	RCMD	Normal	0.5	NO	5.44	−1.21	2.83	4.01	4.45
26	65/F	RCMD	Normal	0.5	YES	5.24	−1.51	3.01	2.56	3.73
27	28/M	RCMD	Normal	0.5	NO	4.70	−2.03	2.65	2.90	3.08
28	60/F	RCMD	Normal	0.5	YES	5.29	−3.85	2.41	2.20	1.95
29	27/F	RCMD	Normal	0.5	YES	4.76	−2.99	2.40	2.01	1.92
30	35/F	RCMD	Normal	0.5	NO	1.64	0.07	1.60	1.23	2.70
31	68/M	RCMD	Normal	0.5	YES	4.78	−0.26	2.18	2.12	2.89
32	55/M	RAEB-1	Normal	0.5	NO	1.75	0.94	1.06	3.90	5.02
33	62/M	RCMD	Trisomy 8, -Y	1.0	YES	6.12	1.58	0.26	1.28	4.58
34	25/M	RCMD	Trisomy 8	1.0	NO	2.35	−2.73	2.48	1.92	1.79
35	68/M	RCMD	Del(12)(20)	1.0	YES	6.49	−2.69	2.84	2.31	3.27
36	43/M	RCMD	Del(6)(20)	1.0	YES	4.43	−0.34	0.95	2.82	2.55
37	33/M	RAEB-1	Normal	1.0	YES	7.17	−0.19	3.56	3.79	4.25
38	81/M	RAEB-1	Normal	1.0	YES	3.96	0.71	0.13	1.61	3.39
39	70/M	RAEB-1	Normal	1.0	YES	4.79	0.38	2.89	3.60	6.48
40	69/M	RAEB-1	Normal	1.0	YES	3.49	0.84	1.74	2.90	1.89
41	66/F	RAEB-1	Normal	1.0	YES	3.02	−3.63	2.00	3.12	1.68
42	65/F	RAEB-1	Normal	1.0	NO	3.22	−2.41	2.63	3.54	2.39
43	81/F	RCMD-RS	Complex	1.5	YES	4.24	2.53	−2.92	2.43	3.02
44	74/M	RCMD	Complex	1.5	NO	5.60	−2.61	2.59	3.48	3.65
45	61/M	RCMD	Complex	1.5	YES	7.20	−1.82	1.39	3.60	2.42
46	56/M	RAEB-1	Add(15)	1.5	YES	4.63	−3.61	2.47	4.32	2.86
47	63/M	RAEB-2	Normal	1.5	YES	5.15	−5.06	1.82	4.31	4.81

Table. 3 (continued)

No	Age/ sex	Diagnosis[a]	Cytogenetics	IPSS	Methylation of $p15^{INK4B}$	Relative expression of PcG genes (folds)				
						EZH2	EED	SUZ12	RING1	BMI1
48	56/M	RAEB-2	Normal	1.5	YES	3.28	−2.75	1.24	3.22	1.90
49	49/F	RAEB-1	Del(17p)	2.0	YES	4.42	0.66	0.99	2.29	3.22
50	81/F	RAEB-1	Complex	2.0	YES	6.01	1.15	−1.92	5.81	6.14
51	47/F	RAEB-1	Complex	2.0	YES	5.58	−1.55	1.57	6.22	2.44
52	70/M	RAEB-2	Normal	2.0	YES	5.80	−0.84	2.62	3.91	4.50
53	69/M	RAEB-2	Normal	2.0	YES	6.00	−3.77	1.24	3.89	3.74
54	67/F	RAEB-2	Complex	3.0	YES	7.09	0.23	3.55	4.12	6.18

[a] Represents that Morphologic diagnosis at presentation in MDS according to WHO classification.

Expression of PRC1 genes (RING1 and BMI1) in MDS, AML, and non-clonal cytopenia patients

The expression levels of RING1 in the low-risk MDS, high-risk MDS, AML, and non-clonal cytopenia groups were 2.0±0.2, 4.1±0.3, 3.9±0.2, and 0.8±0.2-fold the values in healthy volunteers, respectively, (Fig. 1d). Significant differences were observed in the four groups (F=55.978, P<0.001). RING1 expression was significantly higher in the high-risk MDS and AML groups than in the low-risk MDS group (P<0.001; P<0.001). However, the RING1 expression was still significantly higher in the low-risk MDS group than in the non-clonal cytopenia group (P=0.001). BMI1 expression levels in the low-risk MDS, high-risk MDS, AML and non-clonal cytopenia groups were 2.5±0.2, 3.7±0.4, 3.4±0.3, and 1.1±0.3-fold those in healthy volunteers, respectively (Fig. 1e). Significant differences were observed among the four groups (F=31.603, P<0.001). As above, the BMI1 expression level was significantly higher in the high-risk MDS and AML groups than in the low-risk MDS group (P=0.002; P=0.009). However, the BMI1 expression level in the low-risk MDS group was still significantly higher than that in the non-clonal cytopenia group (P<0.001).

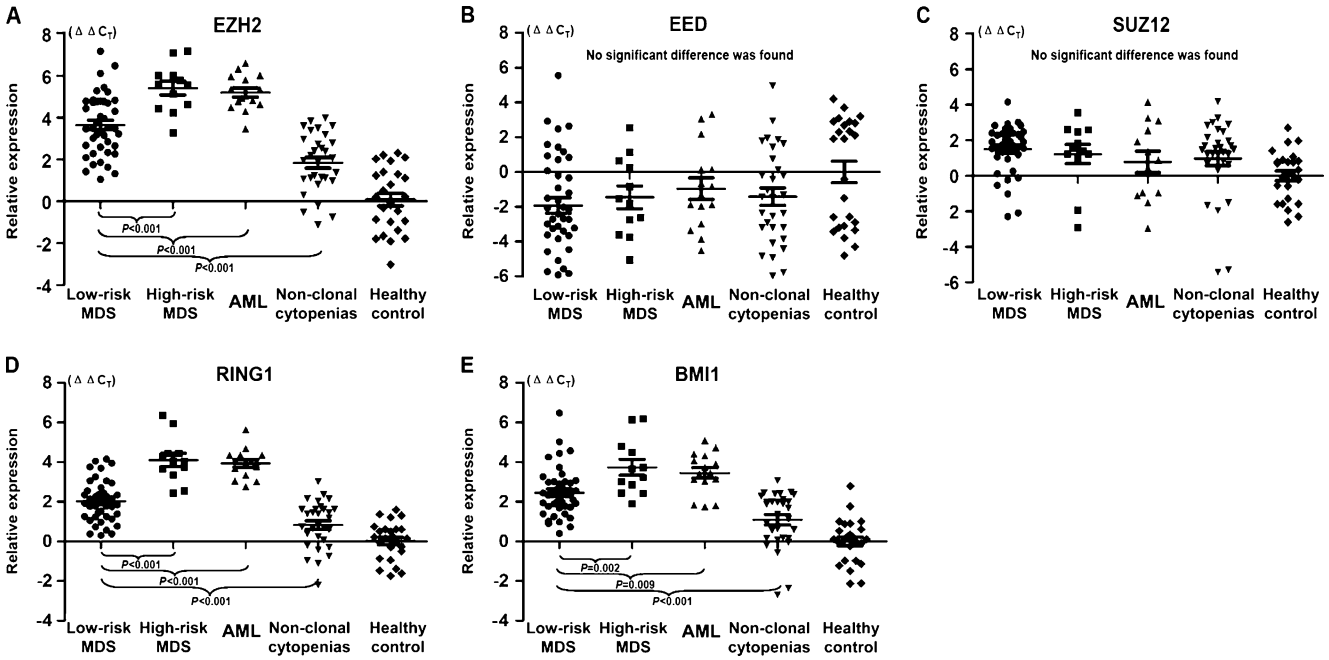

Fig. 1 Expression of PcG genes in patients with low-risk MDS, high-risk MDS, AML, and non-clonal cytopenias groups. **a** showed that relative expression of EZH2 in the low-risk MDS group was significantly lower than in the high-risk MDS group and AML group (P<0.001 and P<0.001), but higher than in the non-clonal cytopenia group (P<0.001). **b** and **c** showed that relative expression of EED and SUZ12 showed no significant difference between the four groups. **d** and **e** showed that respectively relative expression of RING1 and BMI1 in the low-risk MDS group was significantly lower than in the high-risk MDS and AML group (P<0.001 and P<0.001; P=0.002 and P=0.009), but higher than in the non-clonal cytopenia group (P<0.001; P<0.001)

Correlation of the expression of PcG genes with each other in MDS patients

In all MDS patients, the EZH2 expression level had a significant positive correlation with those of RING1 and BMI1 (r=0.590, r=0.536; P<0.001) (Fig. 2a and b). A significant positive correlation was also observed between the expression levels of BMI1 and RING1 (r=0.684, P<0.001) (Fig. 2c). Interestingly, SUZ12 expression showed a significant negative correlation with EED expression (r=-0.432, P=0.001).

Differential expression of PcG genes in MDS patients with or without methylation of the $p15^{INK4B}$ gene

Methylation of $p15^{INK4B}$ was detected in 38 of 54 (70.4%) MDS samples. Most of the positive MDS cases exhibited both methylated and unmethylated PCR products, and a small fraction of the samples from high-risk patients exhibited only the methylated product. Twenty of 32 (62.5%) patients with low-risk MDS and 20 of 22 (90.9%) patients with high-risk MDS showed methylation of $p15^{INK4B}$. This difference between the two groups was significant (P=0.019) (Fig. 3a).

For the 38 patients who presented $p15^{INK4B}$ methylation, the mean relative expression of EZH2 was 4.8±15, whereas the mean relative expression of EZH2 was 2.7±1.2 in the 16 patients without $p15^{INK4B}$ methylation. This difference between the two groups was significant (P<0.001) (Fig. 3b). The relative expression of other PcG genes, such as RING1, BMI1, EED, and SUZ12, did not differ significantly between patients with and without p15 methylation (all P>0.05) (Fig. 3).

The change in PcG genes expression before and after decitabine treatment

For the 13 MDS patients who received decitabine treatment, methylation of $p15^{INK4B}$ was detected in 10 (92.3%) patients and in 6 (46.1%) patients, respectively before and after decitabine treatment. This significant difference in $p15^{INK4B}$ methylation before and after treatment can be observed (P= 0.011) (Fig. 4a).

Before decitabine treatment, the relative expression levels of EZH2, RING1 and BMI1 in the 13 patients who received decitabine treatment were 4.3±0.5, 2.8±0.3, and 2.8±0.4-fold those of healthy volunteers, respectively. After two courses of decitabine treatment, the expression levels of EZH2, RING1, and BMI1 in these 13 patients were 2.9±0.3, 1.7±0.3, 2.0±0.4-fold those of healthy volunteers, respectively. The results showed that the relative expression levels of EZH2 and RING1 in these patients were significantly reduced after decitabine treatment (P=0.013; P=0.020) (Fig. 4b and c). However, decitabine treatment seemed to have no significant effect on the expression of BMI1 (P=0.06) (Fig. 4d).

The relationship of the expression of PcG genes with IPSS

For the 54 MDS cases tested in this assay, the relative expression of EZH2, RING1, and BMI1 increased with increasing IPSS scores (r=0.556, P<0.001; r=0.697, P< 0.001; r=0.480, P<0.001) (Fig. 5a, b and c). The expression of EZH2 showed a positive correlation (r= 0.326, P=0.016) with the line number of peripheral cytopenias (one IPSS indicator) (Fig. 5d). The expression of RING1 showed a significant positive correlation with the proportion of morphological marrow blasts (another IPSS indicator) (r=0.694, P<0.001) (Fig. 5e). However, the relative expressions of these PcG genes did not correlate significantly with karyotype scoring (the third IPSS indicator) (P>0.05).

Discussion

Besides the known genetic changes that occur in cancer, such as the deletion of tumor suppressor genes, amplification of

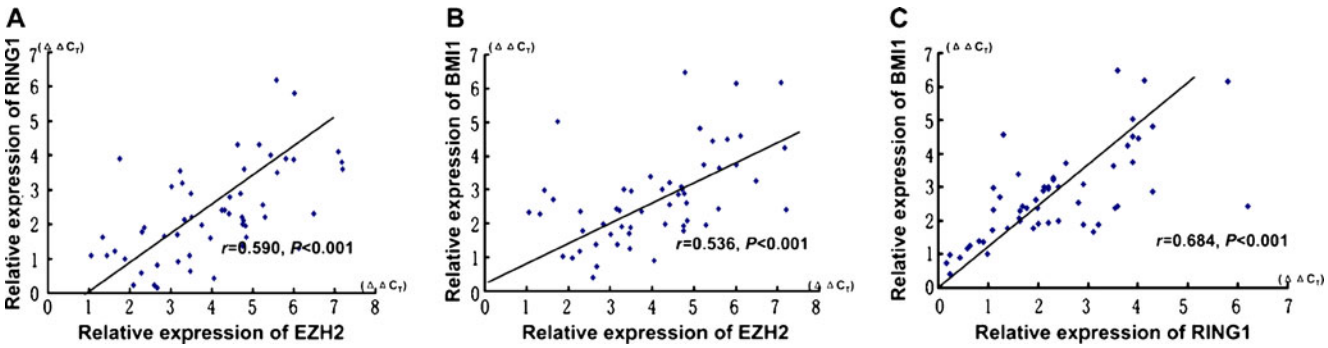

Fig. 2 Correlation of the expression of PcG genes with each other in MDS patients. **a** showed relative expression of EZH2 had a significant positive correlation with those of RING1 (r=0.590, P<0.001). **b** showed that relative expression of EZH2 also had a significant positive correlation with those of BMI1 (r=0.536, P<0.001). **c** showed that a significant positive correlation was also observed between the expression levels of RING1 and BMI1 (r=0.684, P<0.001)

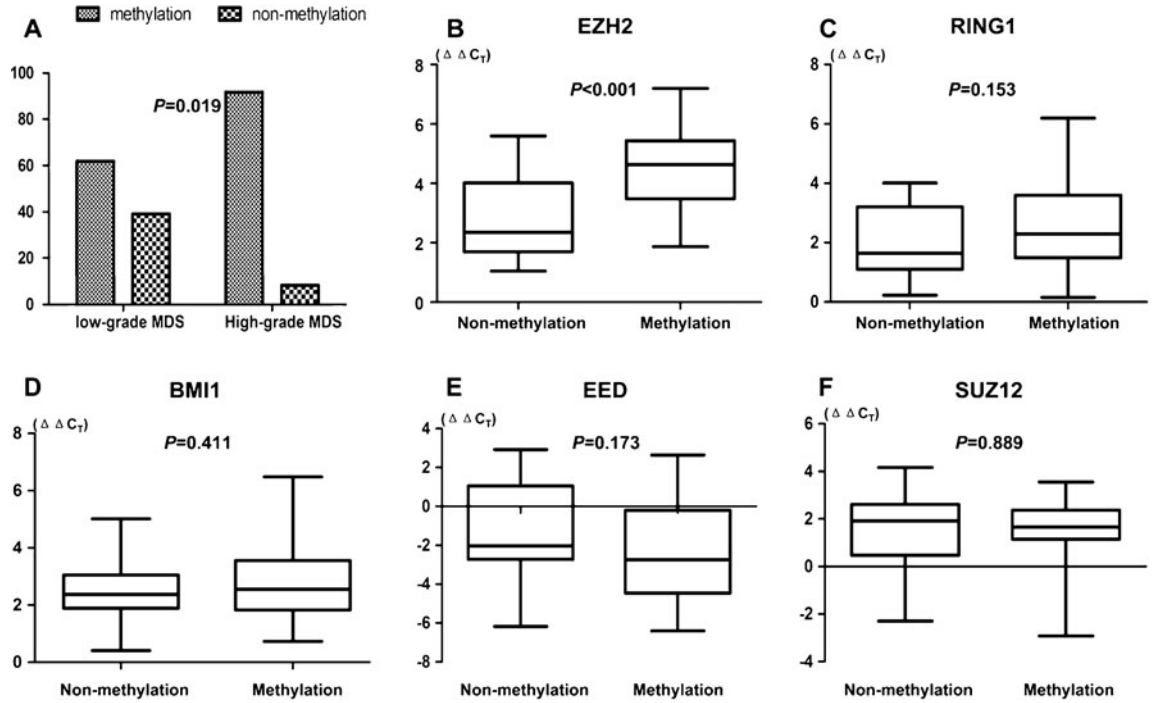

Fig. 3 Differential expression of PcG genes in MDS patients with or without methylation of the $p15^{INK4b}$ gene. **a** showed that the patients with high-grade MDS showed more frequent $p15^{ink4b}$ methylation than those patients with low-grade MDS (P=0.019). **b** showed that the relative expression of EZH2 in patients with $p15^{ink4b}$ methylation was significantly higher than in patients without $p15^{ink4b}$ methylation (P< 0.001). However, **c**, **d**, **e**, and **f** showed that the relative expression of RING1, BMI1, EED, and SUZ12, respectively, did not differ significantly between MDS patients with and without p15 methylation (all P>0.05)

oncogenes, and loss of heterozygosity or gene mutations in tumor-associated genes, epigenetic lesions, such as altered DNA methylation and the misregulation of chromatin remodeling by histone modifications, have emerged as common hallmarks of many cancers [30]. Based on the above-mentioned, as well as the successful application of epigenetic agents, the studies of MDS are now focusing on its epigenetics. Methylation of tumor suppressor genes is frequently found in MDS, especially in advanced cases, and it has been considered to be a part of the pathogenesis of MDS occurrence and development; this methylation could be used as a prognostic marker for MDS [31–33]. Re-expression of tumor suppressor genes can be established by decitabine or 5′-azacytidine treatment [5, 6]. However, the more profound molecular pathogenesis of epigenetic abnormalities in MDS remains poorly understood. In particular, the expression and function of the PcG gene family as upstream epigenetic regulators are still unclear in MDS. We preliminarily explored the gene expression of the PcG gene family, including EZH2, EED, SUZ12, RING1, and BMI1 in various MDS subsets and AML. In our study, the expression levels of EZH2, RING1, and BMI1 were increased in low-

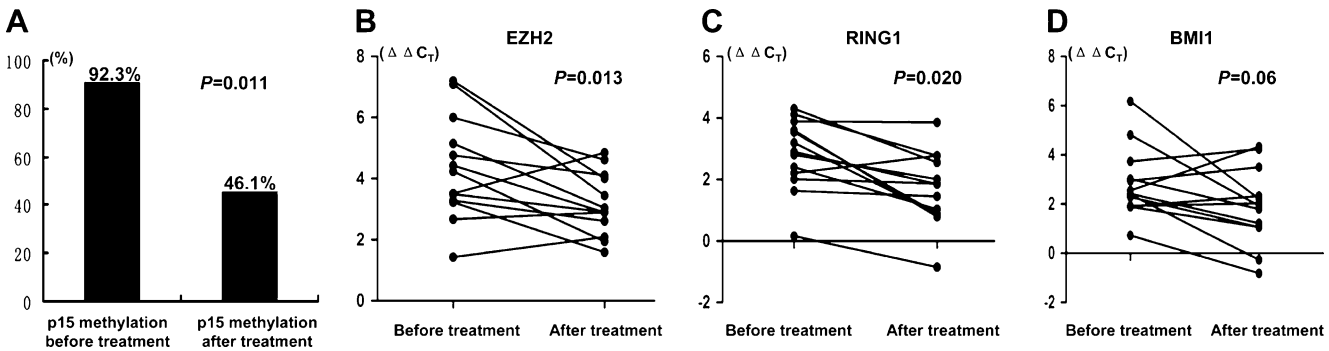

Fig. 4 The change in PcG gene expression before and after decitabine treatment. **a** showed that the incidence rate of $p15^{INK4B}$ methylation has significantly decreased after decitabine treatment. **b** showed that relative expression of EZH2 in MDS patients was significantly reduced after decitabine treatment (P=0.013). **c** showed that relative expression of RING1 in MDS patients was also significantly reduced after decitabine treatment (P=0.020). However, **d** showed that decitabine treatment seemed to have no significant effect on the expression of BMI1 (P=0.06)

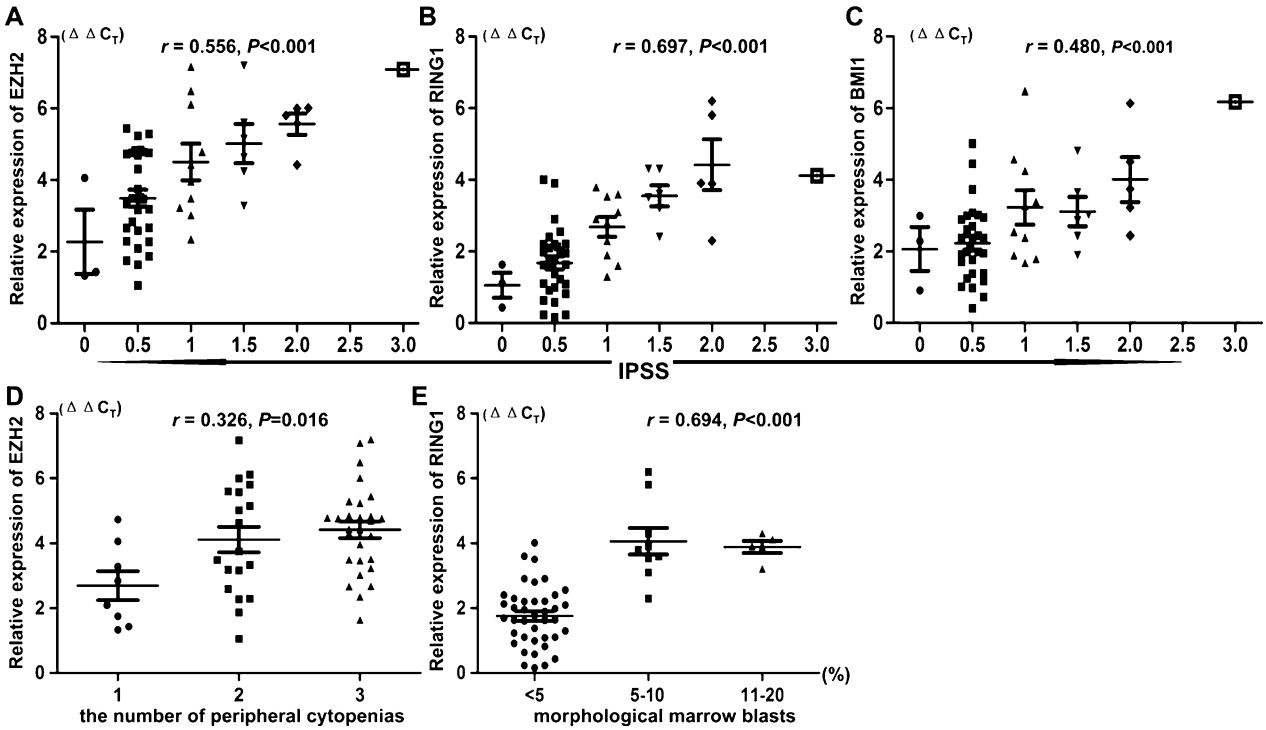

Fig. 5 The relationship of the expression of PcG genes with IPSS. **a**, **b**, and **c** showed that the relative expression of EZH2, RING1, and BMI1 increased with increasing IPSS scores (r=0.556, P<0.001; r=0.697, P<0.001; r=0.480, P=0.001). **d** showed that the relative expression of EZH2 showed a positive correlation with the number of peripheral cytopenias (r=0.326, P=0.016) (one IPSS indicator). **e** showed that the relative expression of RING1 showed a significant positive correlation with the proportion of morphological marrow blasts (another IPSS indicator) (r=0.694, P<0.001)

risk and high-risk MDS patients compared to those in non-clonal cytopenia patients. In addition, the expression levels of these genes progressively increased from low-risk MDS to high-risk MDS and to AML patients. Several studies [34–36] have shown that the overexpression of EZH2, RING1, and BMI1 reflects the degree of tumor proliferation, invasion, and metastasis in solid tumor cells. Similarly, the overexpression of EZH2, RING1, and BMI1 is associated with the level of tumor burden and disease progression in MDS patients.

We found that EZH2, RING1, and BMI1 had synchronous expression. Though the expression levels of these PcG proteins are different in various diseases, they are increased or decreased at the same time in each disease, suggesting that the three proteins are closely connected, and that the PRC1 and PRC2 families often function as a whole. In addition, it was interesting that SUZ12 and EED (both belonging to PRC2) expression levels had a significant inverse correlation in MDS patients. Previous studies have shown that SUZ12 and EED are essential cofactors for the activity of the EZH2 histone methyltransferase [18]. In our study, we found that MDS patients had either increased SUZ12 and decreased EED levels, or decreased SUZ12 and increased EED levels. However, this phenomenon is not associated with MDS subtypes and is observed even in non-clonal cytopenia patients. This finding seems to suggest that the expression levels of SUZ12 and EED genes are antagonistic and that EZH2 may need only one auxiliary factor physiologically.

As epigenetic regulators, PcG family proteins function mainly in two ways. First, they can regulate the binding between transcription factors and the upstream promoter regions of key development-associated genes (mainly tumor suppressor genes, such as p16 and p19) to silence gene transcription. Second, they can induce chromosome remodeling, which frequently leads to a transition between chromatin and heterochromatin to shut off gene transcription. The transcriptional silencing mediated by PcG proteins and DNA methylation are generally considered to be two independent processes [37]. The latest research has shown that PcG family also participates in DNA methylation, and the PcG protein EZH2 can directly induce DNA methylation independent of DNA methyltransferase [13]. So far, methylation of the tumor suppressor gene p15 is frequently found in MDS patients and is the epigenetic change that is most closely associated with the development and progression of the MDS. Our results indicated that MDS patients with p15 methylation had a significantly higher level of EZH2 expression (not other PcG genes) than those without p15 methylation in bone marrow cells, suggesting that EZH2 was involved in the methylation of

p15 in MDS patients. The overexpression of EZH2 (histone methyltransferase) and EZH2-related methylation of tumor suppressor gene may be an important epigenetic change in MDS.

Among three FDA-approved medicines for MDS treatment, 5-azacytidine and decitabine are currently used as epigenetic agents. Decitabine is a DNA de-methyltransferase that can reverse the methylation of tumor suppressor genes, thereby restoring their expression. In our study, the incidence rate of $p15^{INK4B}$ methylation was decreased after decitabine treatment. However, some research has shown that the methylation status of p15 was not associated with the efficacy of decitabine [38]. Two possible mechanisms could contribute to this phenomenon. First, as the methylation of multiple tumor suppressor genes led to epigenetic changes in MDS, monitoring p15 methylation status before and after treatment was not sufficient to accurately reflect the therapeutic effects of decitabine. Second, epigenetic changes did not result solely from the methylation of tumor suppressor genes, and demethylation drugs could also change histone methylation and ubiquitination. In our study, the expression levels of EZH2 and RING1 were significantly decreased in patients who received decitabine treatment. This finding also supported our speculation and provided us with specific clues for understanding the roles of decitabine. In addition, PcG family members are upstream genes for DNA methylation. Therefore, they may be more useful than the detection of p15 methylation status in monitoring the effects of decitabine.

PcG family members, such as EZH2, RING1, and BMI1, are essential for the self-renewal and proliferation of normal cells. However, the induced overexpression of these proteins can drive tumorigenesis. Our studies showed that the expression of EZH2, RING1, and BMI1 were positively correlated with the IPSS prognostic scoring in MDS patients, suggesting that the overexpression of each of these three genes is a negative prognostic indictorsof MDS. The EZH2 expression level was positively correlated with a reduction of peripheral blood cells, which was likely to reflect the severity of ineffective hematopoiesis of MDS. The RING1 expression was positively correlated with the proportion of bone marrow cells, reflecting the degree of tumor load in MDS patients.

In conclusion, the overexpression of EZH2, RING1, and BMI1 is common in MDS, which may account for the pathogenesis of myelodysplastic syndromes and relate to adverse epigenetic alteration and poor prognosis.

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