

ARTICLE



MYELODYSPLASTIC NEOPLASM

SLIT2 promoter hypermethylation-mediated *SLIT2-IT1/miR-218* repression drives leukemogenesis and predicts adverse prognosis in myelodysplastic neoplasm

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Epigenetic modifications have been found to play crucial roles in myelodysplastic neoplasm (MDS) progression. Previously, we investigated genome-wide DNA methylation alterations during MDS evolution to acute myeloid leukemia (AML) by next-generation sequencing (NGS). Herein, we further determined the role and clinical implications of an evident methylation change in CpG islands at the *SLIT2* promoter identified by NGS. First, increased *SLIT2* promoter methylation was validated in 11 paired MDS/AML patients during disease evolution. Additionally, *SLIT2* promoter methylation was markedly increased in MDS/AML patients compared with controls and was correlated with poor clinical phenotype and outcome. Interestingly, *SLIT2* expression was particularly upregulated in AML patients and was not correlated with *SLIT2* promoter methylation. However, the *SLIT2*-embedded genes *SLIT2-IT1* and *miR-218* were downregulated in AML patients, which was negatively associated with *SLIT2* promoter methylation and further validated by demethylation studies. Functionally, *SLIT2-IT1/miR-218* overexpression exhibited antileukemic effects by affecting cell proliferation, apoptosis and colony formation in vitro and in vivo. Mechanistically, *SLIT2-IT1* may function as a competing endogenous RNA by sponging *miR-3156-3p* to regulate *BMF* expression, whereas *miR-218* may directly target *HOXA1* in MDS progression. In summary, our findings demonstrate that *SLIT2* promoter hypermethylation is associated with disease evolution in MDS and predicts poor prognoses in both MDS and AML. Epigenetic inactivation of *SLIT2-IT1/miR-218* by *SLIT2* promoter hypermethylation could be a promising therapeutic target in MDS.

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INTRODUCTION

Myelodysplastic neoplasm (previously as myelodysplastic syndromes, MDS) represents a heterogeneous cluster of disorders characterized by ineffective hematopoiesis and peripheral blood cytopenias with a high risk of transformation into acute myeloid leukemia (AML) [1, 2]. MDS-derived AML (secondary AML, sAML) has unique clinical and biological characteristics with an extremely poor prognosis [3, 4]. It has been widely acknowledged that cytogenetic and genetic abnormalities play vital roles in MDS pathogenesis, and have been proven to be clearly associated with MDS diagnosis and prognosis [5, 6]. In addition, several genetic events, such as *inv(3)*, *FLT3* and *RAS* mutations, have also been identified to contribute to the progression of MDS [7–9]. However, cytogenetic and genetic aberrations cannot completely account for all patients with disease evolution in clinics. Recently, an increasing number of studies have demonstrated the crucial role

of the epigenetic dysregulation of cancer-associated genes in MDS pathogenesis [10]. Hypermethylation of CpG islands at the promoter region of tumor suppressor genes (TSGs) and their consequent gene silencing have been revealed in MDS progression [11]. Previously, our research team reported the role of several single-gene methylation changes, including *DLX5*, *SOX30*, *ID4*, and *GPX3*, in predicting the prognosis and risk of AML transformation for patients with MDS [12–15].

However, studies examining single-gene methylation could not reflect the whole-genome changes during MDS progression, and methylation in key genes may be omitted. Consequently, we previously investigated genome-wide DNA methylation changes during MDS progression to AML by reduced representation bisulfite sequencing (RRBS) and confirmed that genomic DNA hypermethylation changes were common phenomena during MDS progression [16]. When analyzing the RRBS data in detail, an

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evident methylation change in CpG islands at the *SLIT2* promoter attracted our attention.

The SLIT/ROBO signaling axis, first established in axon guidance, has been implicated in the regulation of physiological processes, including cell proliferation, cell motility and angiogenesis [17, 18]. Accumulating studies have demonstrated that SLIT/ROBO signaling pathways, particularly the SLIT2/ROBO1 pathway, play significant roles in tumorigenesis, cancer progression and disease metastasis by modulating several oncogenic signaling pathways [19, 20]. Mutations in *ROBO1* and *ROBO2* (*ROBO1/2*) were discovered to be linked to the pathogenesis and progression of MDS, indicating a vital role of the SLIT2/ROBO pathway in MDS development [21]. Moreover, epigenetic inactivation of *SLIT2* by DNA hypermethylation has been reported in several human cancer types [19, 20]. However, little is known about the pattern and role of *SLIT2* promoter methylation in MDS progression. The current study was intended to investigate the pattern of *SLIT2* promoter methylation and further elucidate its clinical relevance and biological role in patients with MDS.

MATERIALS AND METHODS

Patients and samples

First, a total of 11 paired MDS/sAML patients were enrolled in the validation of the methylation sequencing data. The detailed information of the 11 paired MDS/sAML patients is summarized in Table S1. Second, another independent cohort of 112 patients with MDS, 211 patients with AML and 51 healthy volunteers was also included in the investigation of the clinical significance of *SLIT2* promoter methylation. Patient treatment and sample collection are detailed in the Supplementary Methods.

RNA isolation, reverse transcription and RT-qPCR

RNA isolation, reverse transcription and real-time quantitative PCR (RT-qPCR) are detailed in the Supplementary Methods.

DNA isolation, bisulfite modification, RT-qMSP and BSP

DNA isolation, bisulfite modification, real-time quantitative methylation-specific PCR (RT-qMSP) and bisulfite sequencing PCR (BSP) are detailed in the Supplementary Methods.

Cell culture, transfection and reagents

Human leukemic cell-lines [SKM-1 (MDS-derived), HEL and HL60] culture, lentivirus and small interfering RNA (siRNA) transfection and recombinant human SLIT2-N protein used in the study are detailed in the Supplementary Methods.

Epigenetic regulation studies

Epigenetic regulation studies are detailed in the Supplementary Methods.

Western blot

The methods for western blotting are detailed in the Supplementary Methods.

Cell apoptosis, proliferation, cell cycle, colony formation and differentiation assays

Analysis of cell apoptosis, cell proliferation, cell cycle, cell differentiation and colony formation are detailed in the Supplementary Methods.

Xenograft mouse model

All animal experiments were further approved by the Committee on the Ethics of Animal Experiments of Jiangsu University. A xenograft mouse model was constructed based on the severe immune-deficient NCG (NOD/ShiLtJGpt-Prkdcem26Cd52Il2rgem26Cd22/Gpt) mouse, which is detailed in the Supplementary Methods.

RNA sequencing analysis

The methods for RNA sequencing are detailed in the Supplementary Methods.

Fluorescence in situ hybridization (FISH)

The methods for FISH are detailed in the Supplementary Methods.

RNA immunoprecipitation (RIP) assay

The methods for RIP are detailed in the Supplementary Methods.

Luciferase reporter assays

The methods for luciferase reporter assays are detailed in the Supplementary Methods.

Bioinformatics analyses

The public datasets and online tools used in this study are detailed in the Supplementary Methods.

Statistics

SPSS 22.0 and GraphPad Prism 5.0 were utilized for statistical analysis. Comparisons of continuous variables in the two groups were conducted by independent samples *t*-test, paired samples *t*-tests, or Mann–Whitney's *U*-tests, whereas categorical variables were compared by Pearson chi-square/Fisher exact tests. Spearman's correlation analysis was performed to analyze the correlation between *SLIT2*-*IT1* promoter methylation and *SLIT2*/*SLIT2*-*IT1*/*miR*-218 expression. The prognostic effects of *SLIT2* promoter methylation on leukemia-free survival (LFS) and overall survival (OS) were determined through Kaplan–Meier analysis and Cox regression analysis. A two-tailed *P* < 0.05 was defined as statistically significance in all analyses.

RESULTS

Identification and validation of *SLIT2* promoter methylation as a frequent event during MDS progression

Previously, we conducted RRBS in 4 paired samples of MDS/sAML patients and identified a series of genome-wide DNA methylation changes during MDS progression [16]. Herein, we further validated a cancer-associated CpG island methylation located at the *SLIT2* promoter in 11 paired MDS/sAML patients together with 2 controls by both RT-qMSP and BSP. The methylation density of *SLIT2* promoter was relatively low in controls (Fig. S1A, B), but *SLIT2* promoter methylation density/level was significantly increased in sAML stage compared with MDS stage and controls (Fig. S1C–M and Fig. 1A). Additionally, *SLIT2* promoter methylation level was heavily correlated with *SLIT2* promoter methylation density ($R = 0.837$, $P < 0.001$, $n = 22$), which indicated that RT-qMSP was an effective methodology for detecting *SLIT2* promoter methylation in larger samples.

Next, *SLIT2* promoter methylation was also detected in a cohort of 112 newly diagnosed MDS and 211 newly diagnosed AML patients by RT-qMSP. Significantly increased methylation levels of *SLIT2* promoter were observed in both MDS and AML patients as compared with healthy volunteers (Fig. 1B). However, although *SLIT2* promoter methylation level in AML patients seemed to be higher than that in MDS patients, no remarkable difference was found (Fig. 1B). Moreover, other independent cohorts of AML derived from public datasets also confirmed the phenomenon of *SLIT2* promoter hypermethylation in AML (Fig. 1C), in accordance with our results. Finally, we detected *SLIT2* promoter methylation in 20 AML patients after complete remission (CR) matched to primary diagnosed AML. As expected, *SLIT2* promoter methylation level was dramatically decreased in the CR time compared with the primary diagnosis time (Fig. S2).

Clinical implication and prognostic value of *SLIT2* promoter methylation in MDS and AML patients

First, MDS cases were divided into two groups [hypermethylated (>1.056) and non-hypermethylated (<1.056)] according to the cutoff point of 1.056 (means \pm 4 SD in controls). The comparison of clinicopathological features between the two groups for MDS was shown in Table S2. Notably, *SLIT2* promoter hypermethylation

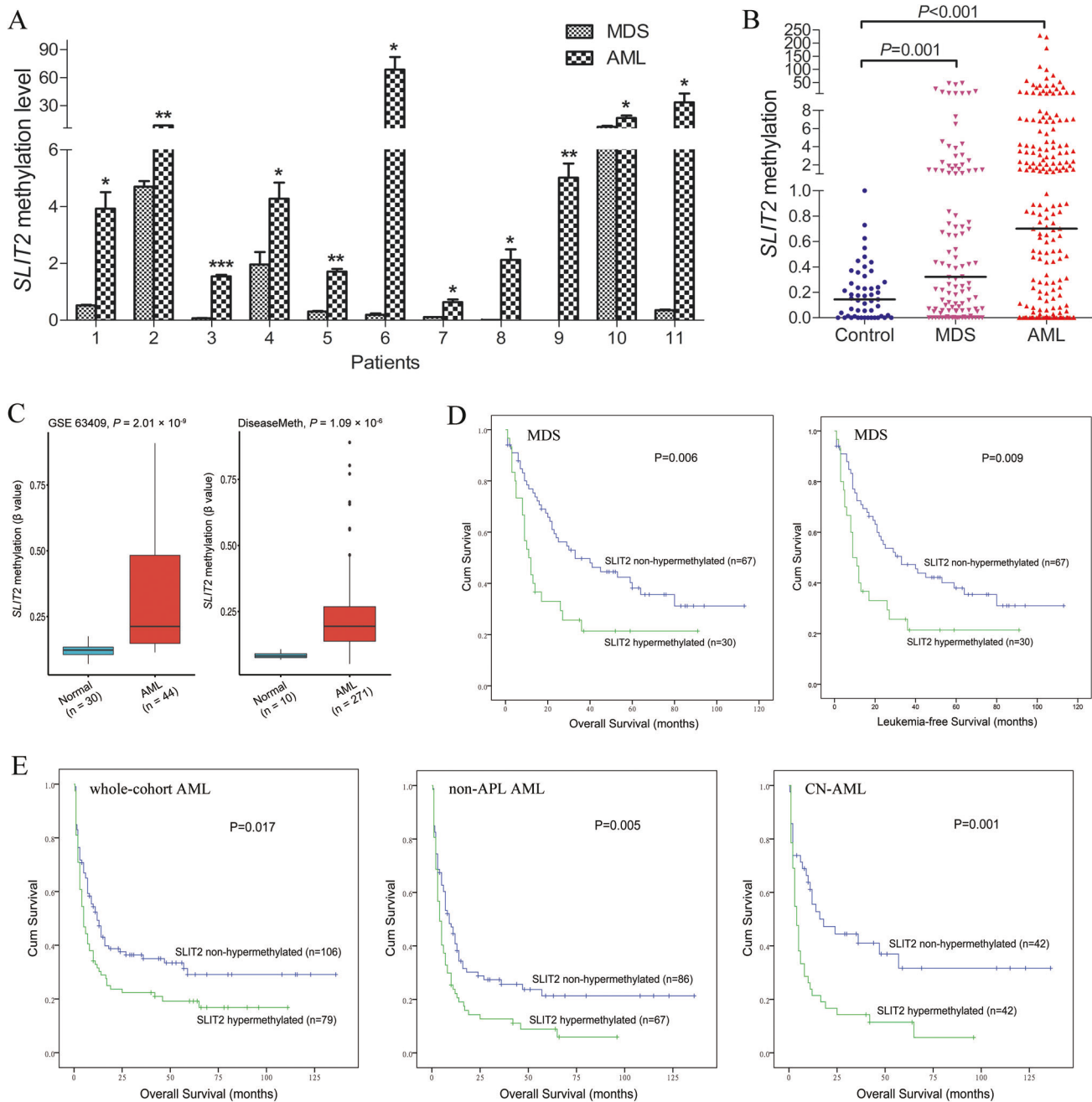
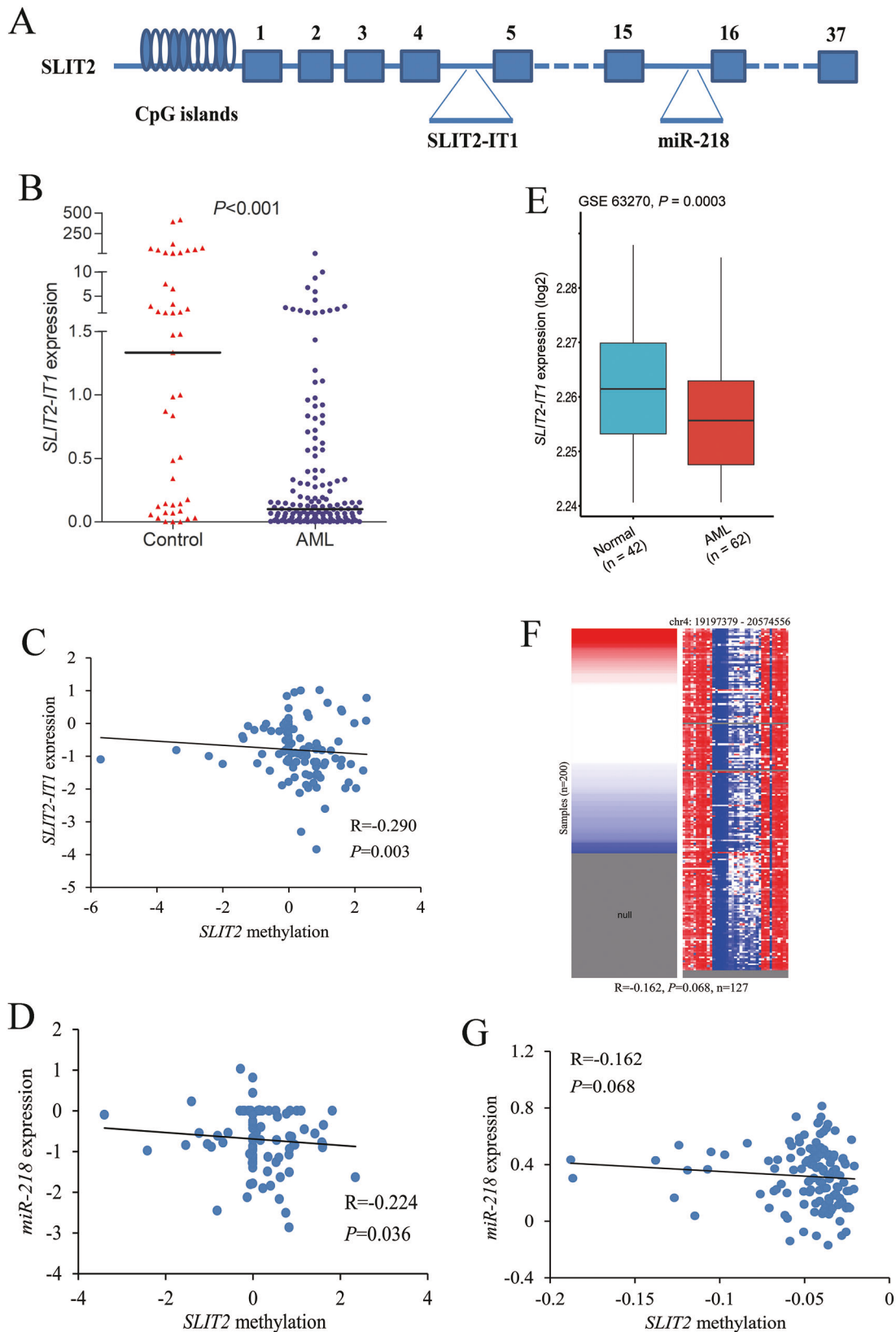


Fig. 1 *SLIT2* promoter methylation was significantly increased during MDS progression and correlated with poor clinical outcome. **A** Alterations of *SLIT2* promoter methylation level in 11 paired patients of MDS/sAML determined by RT-qMSP. **B** *SLIT2* promoter methylation level in MDS and AML patients compared with healthy controls determined by RT-qMSP. **C** *SLIT2* promoter methylation was further analyzed in AML in public datasets. Left: GSE63409, Right: DiseaseMeth version 2.0. **D** The effect of *SLIT2* promoter methylation on survival in MDS patients. Left: Overall survival, Right: Leukemia-free survival. **E** The impact of *SLIT2* promoter methylation on overall survival of AML patients. Left: Whole-cohort AML, Middle: Non-APL-AML, Right: CN-AML. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

is dramatically correlated with poor karyotype (21% vs. 4%, *P* = 0.007). Moreover, although a significant difference between the two groups was not observed in the distribution of all subtypes of International Prognostic Scoring System (IPSS) risks, *SLIT2* promoter hypermethylation was markedly associated with Int-2/High risks compared with Low/Int-1 risks (42% vs. 24%, *P* = 0.039). Kaplan-Meier analysis showed that patients with *SLIT2* promoter hypermethylation presented a markedly shorter OS and LFS times (Fig. 1D). However, based on the Cox regression analysis, the impact of *SLIT2* promoter methylation on OS and LFS in MDS patients was not statistically significant (Table S3).

Next, the comparison of clinical characteristics and laboratory features between the two groups for AML is presented in Table S2. Importantly, patients with *SLIT2* promoter hypermethylation had a significantly lower CR rate among whole-cohort AML and non-acute promyelocytic leukemia (non-APL) AML, with a trend, and among cytogenetically normal AML (CN-AML) (Table S2). Additionally, *SLIT2* promoter hypermethylated cases presented significantly shorter OS times among whole-cohort AML, non-APL AML and CN-AML (Fig. 1E). However, based on Cox regression analysis, the impact of *SLIT2* promoter methylation on OS in non-APL AML and CN-AML patients was not statistically significant (Table S4).



Transcriptional regulatory effects of *SLIT2* promoter methylation on *SLIT2*/*SLIT2-IT1*/*miR-218* expression

Since aberrant promoter hypermethylation is associated with gene silencing, we further detected *SLIT2* transcript levels by

RT-qPCR in 107 AML patients and 28 controls with available RNA samples. Surprisingly, *SLIT2* expression was strikingly upregulated in AML patients (Fig. S3A). Moreover, *SLIT2* promoter methylation level was not correlated with *SLIT2* transcript level (Fig. S3B). The

Fig. 2 Transcriptional regulatory effects of *SLIT2* promoter methylation on noncoding RNAs *SLIT2-IT1*/*miR-218* expression in leukemogenesis. **A** Diagram of the structure of *SLIT2* with its internal genes and CpG islands. **B** *SLIT2-IT1* expression in AML patients in our cohort. **C** The association between *SLIT2* promoter methylation and *SLIT2-IT1* expression in AML patients in our cohort. **D** The association between *SLIT2* promoter methylation and *miR-218* expression in AML patients in our cohort. **E** *SLIT2-IT1* expression in AML patients in GSE63270 dataset. **F** *miR-218* expression and *SLIT2* methylation at different regions in AML patients in TCGA dataset. Left: *miR-218* expression. Right: *SLIT2* methylation in HM450k. Each row represents *miR-218* expression in the same patient and *SLIT2* methylation at different sites. **G** The association between *SLIT2* promoter methylation and *miR-218* expression in AML patients in TCGA dataset.

same results were also observed in other independent cohorts of AML derived from public datasets (Fig. S3C, D). Functional studies demonstrated that human recombinant *SLIT2* protein induced the increased proliferation and decreased apoptosis rate in SKM-1 and HEL cells (Fig. S3E, F). All these results suggested that *SLIT2* promoter methylation does not directly affect *SLIT2* expression during MDS progression.

To explore the regulatory mechanism of *SLIT2* promoter methylation in MDS progression, we carefully studied the gene structure of *SLIT2* and found that two noncoding genes, *SLIT2-IT1* and *miR-218*, were contained in *SLIT2* (Fig. 2A). We hypothesized that the expression of two noncoding genes might be regulated by *SLIT2* promoter methylation. To test our hypothesis, we first detected the expression of *SLIT2-IT1* and *miR-218* in AML. As expected, both *SLIT2-IT1* (Fig. 2B) and *miR-218* transcripts were significantly decreased in AML patients [22]. Moreover, the *SLIT2* promoter methylation level exhibited a weak negative correlation with both the *SLIT2-IT1* and *miR-218* transcript levels (Fig. 2C, D). Similar results were also observed in other independent cohorts of AML derived from public datasets (Fig. 2E–G). Next, we performed demethylation studies in both SKM-1 and HL60 cells to further validate our hypothesis. As expected, with the reduced density of *SLIT2* promoter methylation after 5-aza-dC treatment, *SLIT2-IT1* and *miR-218* expression was restored in a dose-dependent manner (Fig. S4A, B). Taken together, *SLIT2* promoter methylation may mainly regulate the expression of the noncoding genes *SLIT2-IT1* and *miR-218* rather than *SLIT2* during MDS progression.

The antileukemic effects of *SLIT2-IT1* in vitro and in vivo

To investigate the potential role of *SLIT2-IT1* in leukemogenesis in vivo, we first examined *SLIT2-IT1* expression in eight leukemic cell lines. The results of RT-qPCR showed that *SLIT2-IT1* transcript level was decreased in all eight leukemic cell lines compared with controls (Fig. S5A). Thus, we performed gain-of-function experiments in both SKM-1 and HL60 cells in vitro. The overexpression efficacy of *SLIT2-IT1* in SKM-1 and HL60 cells was confirmed by RT-qPCR (Fig. S5B). *SLIT2-IT1* overexpression in both SKM-1 and HL60 cells suppressed proliferation together with G0/G1 arrest, promoted apoptosis and inhibited colony formation (Fig. 3A, B and Fig. S5C, D). However, both the erythroid and granular differentiation-related markers, such as CD235, CD15 and CD11b, were not affected after *SLIT2-IT1* overexpression in either SKM-1 or HL60 cells.

We next constructed xenograft mouse models by tail vein injection of SKM-1/*SLIT2-IT1* and control SKM-1/NC cells in NCG mice to determine the role of *SLIT2-IT1* in vivo. The experimental procedures are briefly shown in Fig. 3C. First, the progression of tumor load in mice was monitored every week for four weeks using bioluminescence imaging. The results showed that the tumor load of SKM-1/*SLIT2-IT1* group mice was conspicuously lower than that of SKM-1/NC group mice (Fig. 3D and Fig. S6A). In addition, *SLIT2-IT1* overexpression prolonged the survival time of mice (Fig. 3E). Representative results of the organs, such as the liver, spleen and ovary, with tissue sections between the two groups are shown in Fig. S6B, C. Furthermore, bone marrow (BM) blasts of serious mice between the two groups were also observed. The percentage of BM blasts in SKM-1/NC group mice was 60.5%, whereas in SKM-1/*SLIT2-IT1* group mice, it was 15% (Fig. S6D).

Molecular mechanism of *SLIT2-IT1* in leukemogenesis

To investigate how *SLIT2-IT1* exerts its function, we first predicted its subcellular localization by IncLocator. *SLIT2-IT1* was predicted to be located mainly in the cytoplasm of all the available cell types. Further lncRNA-FISH assays also confirmed the location of *SLIT2-IT1* in the cytoplasm of SKM-1 cells (Fig. 4A). We suspected that *SLIT2-IT1* might act as a competing endogenous RNA (ceRNA) to exert its function. Since Ago2 is a core component of the RNA-induced silencing complex (RISC) that is involved in miRNA-mediated mRNA destabilization or translational repression, we next conducted RIP assays using an anti-Ago2 antibody, which demonstrated that endogenous *SLIT2-IT1* was preferentially enriched in Ago2-RIPs compared with control IgG-RIPs (Fig. 4B). All these findings support the hypothesis that *SLIT2-IT1* may function through the ceRNA network to play its biological role during leukemogenesis.

Given the results above, we predicted miRNAs potentially binding with *SLIT2-IT1* by the publicly available online tools (LncBase v.2, RegRNA 2.0 and lncRNASNP2), and the results of the Venn diagram indicated that only *miR-3156-3p* was shared in all three online tools (Fig. 4C and Table S5). By dual-luciferase reporter assays, overexpression of *miR-3156-3p* significantly reduced the luciferase activity of the wild-type *SLIT2-IT1* vector, but not the mutated *SLIT2-IT1* vector (Fig. 4D). Moreover, *miR-3156-3p* was also enriched in Ago2-RIPs compared with control IgG-RIPs by RIP assays (Fig. 4B). However, overexpression of *SLIT2-IT1* did not change the level of *miR-3156-3p* in either SKM-1 or HL60 cells. Similarly, although *miR-3156-3p* expression was distinctly increased in AML ($P=0.005$), we did not observe an obvious association between *SLIT2-IT1* and *miR-3156-3p* expression in AML patients ($R=-0.090$, $P=0.361$). These results suggested that *SLIT2-IT1* might only “sponge” *miR-3156-3p* but could not degrade its expression during leukemogenesis.

To investigate the downstream target of *SLIT2-IT1*/*miR-3156-3p*, we first performed RNA sequencing in SKM-1 cells before and after *SLIT2-IT1* overexpression. A total of 568 gene transcripts presented an increased pattern (SKM-1/NC vs. SKM-1/*SLIT2-IT1* cells) (Table S6). Combined with the predicted mRNAs potentially binding with *miR-3156-3p* by the publicly available online tools (miRDB, TargetScan 7.2 and miRWalk), we screened a total of 13 mRNAs that may act as direct targets of *SLIT2-IT1*/*miR-3156-3p* during leukemogenesis (Fig. 4E and Table S7). Because *BMF* (*BCL2* modifying factor) is an apoptosis-related gene in accordance with the proapoptotic effects of *SLIT2-IT1*, we selected it for further validation and analysis. By dual-luciferase reporter assays, overexpression of *miR-3156-3p* significantly reduced luciferase activity of the wild-type 3'-untranslated region (3'-UTR) of the *BMF* vector but not the mutated 3'-UTR of the *BMF* vector (Fig. 4F). Moreover, *SLIT2-IT1* overexpression and *miR-3156-3p* downregulation significantly induced the increased expression of *BMF* in both SKM-1 and HL60 cells, which were detected by both RT-qPCR and western blotting (Fig. 4G, H). In clinical samples, *BMF* expression was markedly decreased in AML patients (Fig. S7A) and exhibited a weak negative correlation with *miR-3156-3p* expression (Fig. S7B) but a weakly positive association with *SLIT2-IT1* expression (Fig. S7C). Therefore, we concluded that *SLIT2-IT1* might act by “sponging” *miR-3156-3p* to regulate *BMF* expression in leukemogenesis.

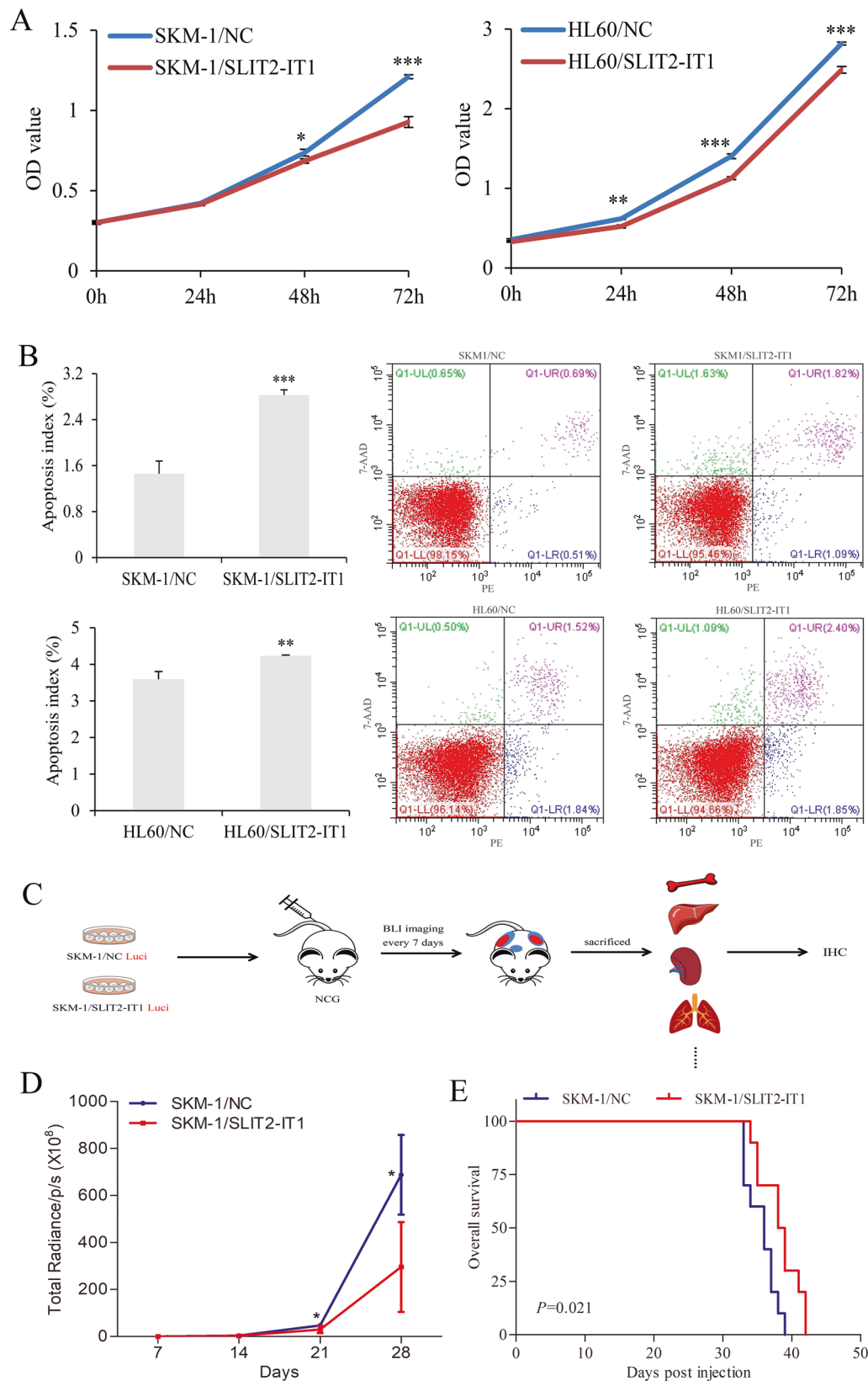
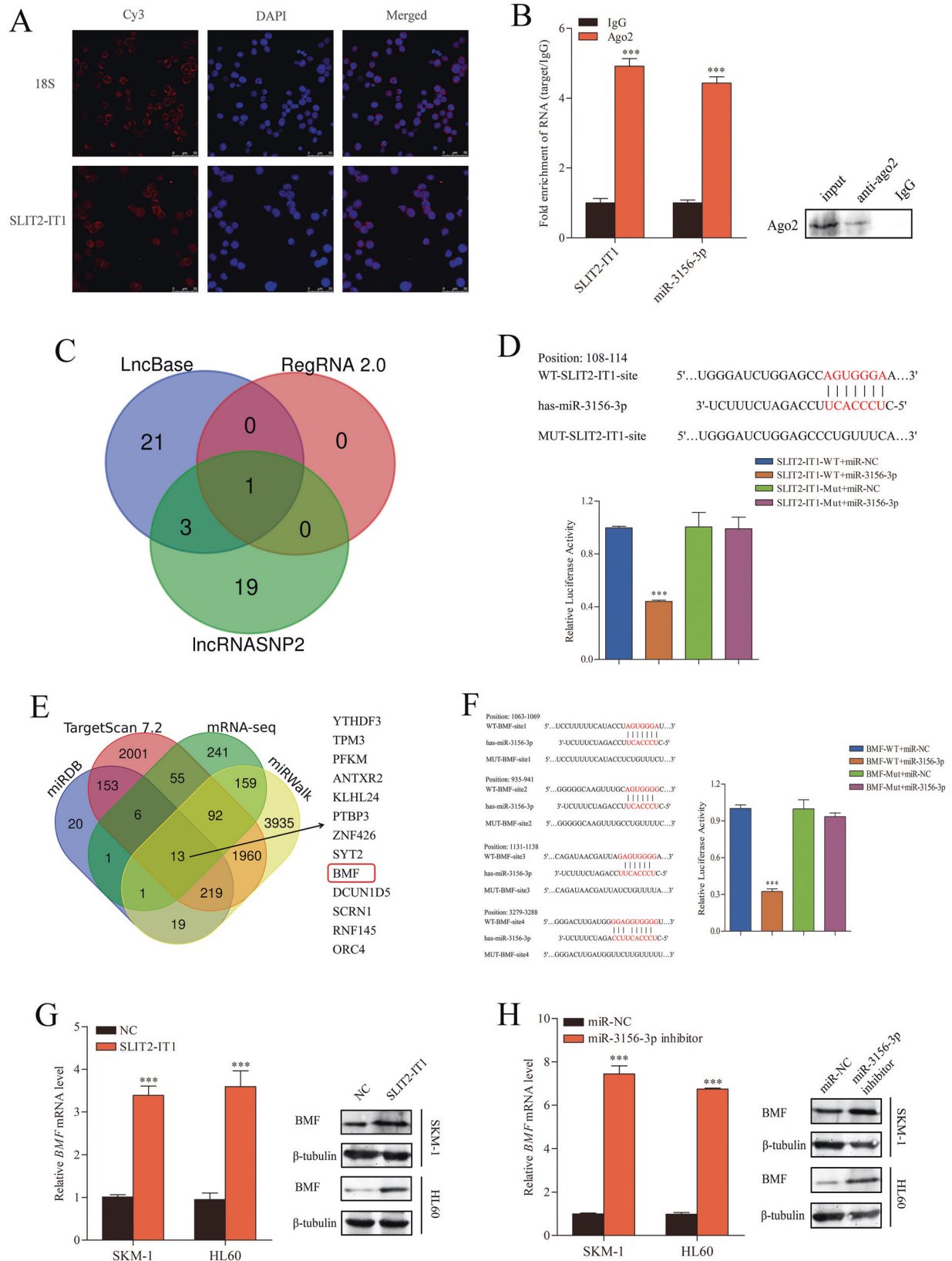


Fig. 3 *SLIT2-IT1* exerted antileukemic effects in vitro and in vivo. **A** *SLIT2-IT1* overexpression suppressed proliferation ability in SKM-1 and HL60 cells. Left: SKM-1 cells; Right: HL60 cells. **B** *SLIT2-IT1* overexpression promoted apoptosis in SKM-1 and HL60 cells. Upper: SKM-1 cells; Down: HL60 cells. **C** Schematic diagram of the in vivo experiment. **D** The tumor load of SKM-1/SLIT2-IT1 group mice was significantly lower than that of SKM-1/NC group mice via bioluminescence imaging weekly in vivo. **E** *SLIT2-IT1* overexpression prolonged the survival time of mice by survival analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



Finally, we determined whether silencing *BMF* expression could reverse the antileukemic effects of *SLIT2-IT1* overexpression in MDS to further verify the association between *SLIT2-IT1* and *BMF*. The knockdown of *BMF* in SKM-1 and HL60 cells as well as SKM-1/

SLIT2-IT1 and HL60/SLIT2-IT1 cells was performed using siRNA interference, and the efficiency was confirmed by RT-qPCR and western blot (Fig. S7D). As expected, silencing *BMF* expression in both SKM-1 and HL60 cells also exhibited antiproliferative effects

Fig. 4 *SLIT2-IT1* may function as a competing endogenous RNA by “sponging” *miR-3156-3p* to regulate *BMF* expression in leukemogenesis. **A** The location of *SLIT2-IT1* at the cytoplasm detected by RNA-FISH. Red: *SLIT2-IT1/18S* probe labeled by Cy3; Blue: the nucleus stained by DAPI. *18S* was used as an internal control, mainly located in the cytoplasm. **B** The detection of target genes *SLIT2-IT1/miR-218* by RT-qPCR and RIP efficiency by western blot after AGO2-RIP assays. **C** Venn diagram of the predicted targets of *SLIT2-IT1* using three bioinformatics websites (LncBase v.2, RegRNA 2.0 and LncRNASNP2). **D** Dual luciferase experiment of *miR-3156-3p* binding to *SLIT2-IT1*. Upper: predicted binding site of *SLIT2-IT1* and *miR-3156-3p*; Down: the luciferase activity of *SLIT2-IT1* binding to *miR-3156-3p*. **E** Venn diagram of the predicted targets of *miR-3156-3p* using bioinformatics websites (miRDB, TargetScan 7.2 and miRWalk) and mRNA-seq. **F** Dual luciferase experiment of *BMF* binding to *miR-3156-3p*. Left: predicted binding sites of *BMF* and *miR-3156-3p*; Right: the luciferase activity of *BMF* binding to *miR-3156-3p*. **G** *BMF* expression after *SLIT2-IT1* overexpression in SKM-1 and HL60 cells. **H** *BMF* expression after *miR-3156-3p* knockdown in SKM-1 and HL60 cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

together with G2/M phase promotion, proapoptotic and anticolony formation role, and partially reversed the effects caused by *SLIT2-IT1* overexpression in both SKM-1/*SLIT2-IT1* and HL60/*SLIT2-IT1* cells (Fig. S7E–H). In summary, all these results together confirmed the crucial role of the *SLIT2-IT1/miR-3156-3p/BMF* axis in MDS progression.

The anti-leukemia effects of *miR-218* in vivo and in vitro

Similarly, we first detected *miR-218* expression in eight leukemic cell lines. The level of *miR-218* in all leukemic cell lines was lower than that in controls (Fig. S8A). Consequently, gain-of-function experiments were also performed in both SKM-1 and HL60 cells in vitro. SKM-1 and HL60 cells stably overexpressing *miR-218* were established and analyzed by RT-qPCR (Fig. S8B). Overexpression of *miR-218* resulted in reduced proliferation ability together with G0/G1 arrest, increased apoptosis and decreased colony formation among both SKM-1 and HL60 cells (Fig. 5A, B and Fig. S8C, D). Moreover, the erythroid differentiation-related marker CD235a was markedly increased after *miR-218* overexpression only in HL60 cells (Fig. S8E), whereas the granular differentiation-related markers CD15 and CD11b were not affected in either SKM-1 or HL60 cells.

Next, the potential role of *miR-218* in vivo was also determined by constructing xenograft mouse models in NCG mice. Bioluminescence imaging showed that the tumor load of SKM-1/*miR-218* group mice was markedly lower than that of SKM-1/*miR-NC* group mice during 4 weeks of monitoring (Fig. S9A, B). Tumor invasion appeared in the ovaries of mice only in the SKM-1/*miR-218* group (Fig. S9C). However, acute leukemia occurred in the two groups of mice, since the percentage of BM blasts in representative SKM-1/*miR-NC* group mice was 43.5%, whereas in SKM-1/*miR-218* group mice, it was 78% (Fig. S9D).

Molecular mechanism of *miR-218* in leukemogenesis

To explore the potential molecular mechanism of *miR-218* in leukemogenesis, we first performed RNA-sequencing in SKM-1 cells before and after *miR-218* overexpression. A total of 49 genes presented a decreased pattern (SKM-1/*miR-NC* vs. SKM-1/*miR-218* cells) (Table S8). Moreover, combined with the predicted mRNAs potentially binding with *miR-218* by the publicly available online tools (miRDB, TargetScan 7.2, microT-CDS and miRWalk), the results of the Venn diagram showed that only one mRNA, i.e., *HOXA1*, may act as a direct target of *miR-218* during leukemogenesis (Fig. 5C and Table S9). To verify the results, dual-luciferase reporter assays revealed that overexpression of *miR-218* markedly reduced the luciferase activity of the wild-type 3'-UTR of the *HOXA1* vector but not the mutated 3'-UTR of the *HOXA1* vector (Fig. 5D). Moreover, overexpression of *miR-218* greatly decreased the expression of *HOXA1* in both SKM-1 and HL60 cells, which was detected by both RT-qPCR and western blotting (Fig. 5E). Additionally, *HOXA1* expression was markedly increased in AML patients (Fig. S10A) and exhibited a nearly negative correlation with *miR-218* expression (Fig. S10B). An independent cohort of AML derived from public datasets also showed a nearly negative association between *miR-218* and *HOXA1* expression (Fig. S10C).

To further confirm the association of *miR-218* with *HOXA1* and explore the role of *HOXA1* in reversing the anti-leukemia effects caused by *miR-218* overexpression in MDS, we next performed rescue experiments by upregulating *HOXA1* expression in SKM-1/*miR-218* and HL60/*miR-218* cells. The *HOXA1* overexpression efficiency was confirmed by RT-qPCR and western blotting (Fig. S10D). As expected, *HOXA1* overexpression in both SKM-1/*miR-218* and HL60/*miR-218* cells reversed the antileukemic effects by facilitating proliferation together with promoting S phase, inhibiting apoptosis and promoting colony formation (Fig. S10E–H). Taken together, these results suggest that *miR-218* functions by targeting *HOXA1* in MDS progression.

DISCUSSION

The mechanisms underlying the progression of MDS to AML are poorly understood. To date, chromosomal abnormalities, such as -7/7q-, +8, 11q-, i(9q) and complex karyotypes, as well as genetic mutations, including *FLT3*, *RAS*, *TP53*, *DNMT3A*, *TET2*, *IDH1/2*, *EZH2* and *ASXL1*, are considered as progression-related drivers in MDS [5–7, 23]. However, these alterations cannot be observed during the overall process of MDS and are still unable to explain the mechanism underlying disease progression. Recently, epigenetic alterations, especially DNA methylation, were shown to be involved in MDS progression [9]. Ying et al. indicated that abnormal methylation existed in every sample, on average affecting 91/1505 CpG loci in early MDS and 179 of 1505 loci after blast transformation [24]. Moreover, our previous study also confirmed DNA hypermethylation changes during MDS progression [16]. Following this study, we further validated that *SLIT2* promoter hypermethylation was correlated with disease progression in MDS evolution. Notably, a recent study by whole-exome sequencing and targeted sequencing revealed that *ROBO1/2* mutations were related to disease progression in MDS [21]. Moreover, *ROBO1/2* mutations resulted in the loss of antiproliferative and proapoptotic effects in leukemia cells [21]. Similarly, epigenetic inactivation of *SLIT/ROBO* pathway genes was also shown to be associated with disease progression in human cervical cancer [25]. These results provide new insights into possible molecular mechanisms in disease progression and suggest that *SLIT/ROBO* hypermethylation may serve as a potential biomarker in risk prediction.

In this study, the clinical significance of *SLIT2* promoter hypermethylation was also revealed. *SLIT2* promoter methylation was associated with advanced MDS stage and predicted adverse clinical outcomes. Moreover, *SLIT2* promoter hypermethylation in AML was associated with poorer chemotherapy response and survival. From the results above, it is reasonable to deduce that monitoring the *SLIT2* promoter methylation level could be used as an important predictor of disease prognosis and evaluation of curative effects. Previous studies have also pointed out the correlation between *SLIT2* dysregulation and prognosis in solid tumors. Tseng et al. revealed that lower levels of *SLIT2* expression, regulated by promoter hypermethylation, predicted worse survival and postoperative recurrence of lung cancer and diffuse large B-cell lymphoma patients [26, 27]. Likewise, *SLIT2* underexpression

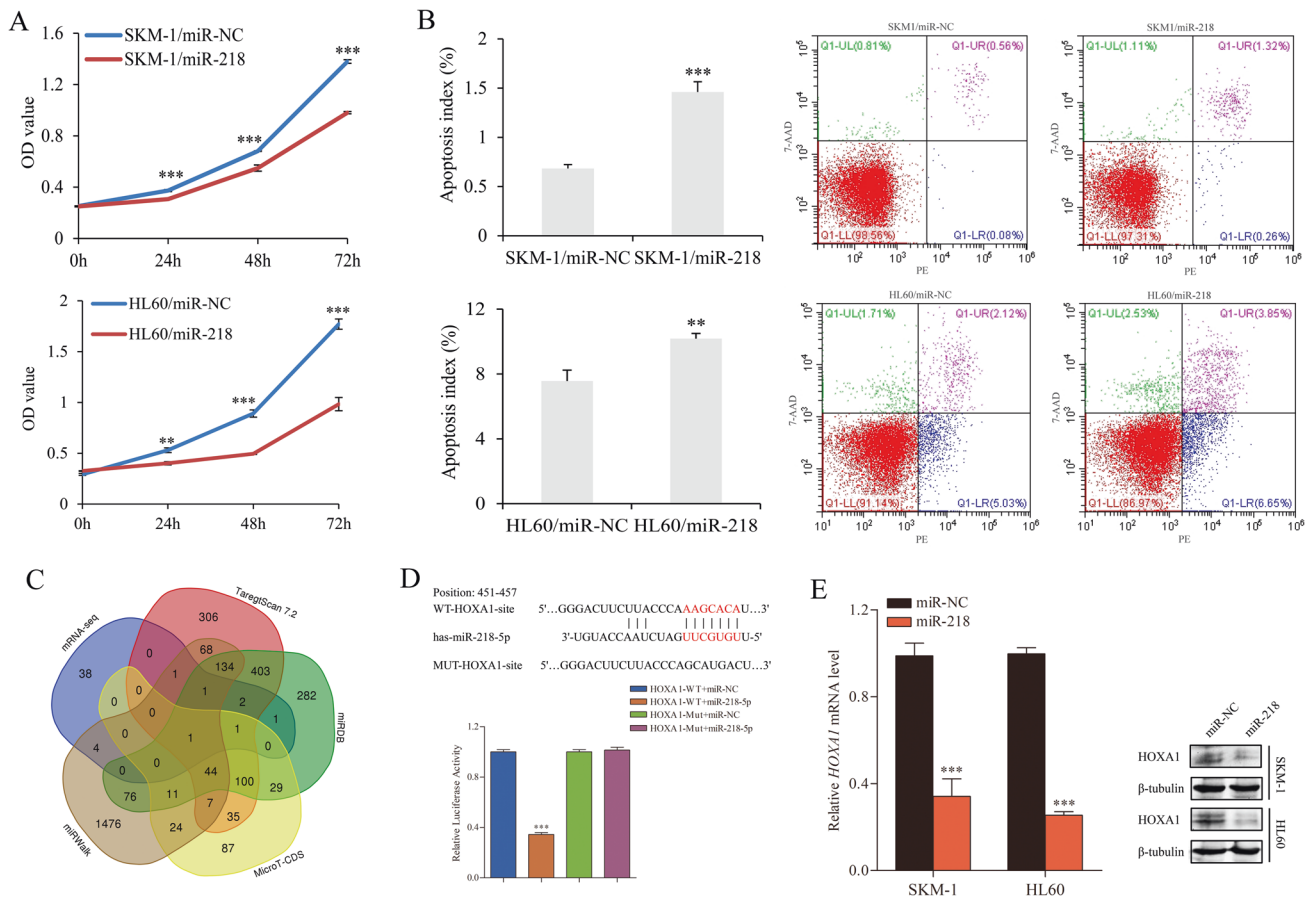


Fig. 5 *Mir-218* elaborated antileukemic effects in vitro and in vivo by targeting *HOXA1*. **A** *Mir-218* overexpression suppressed proliferation ability in SKM-1 and HL60 cells. Upper: SKM-1 cells; Down: HL60 cells. **B** *Mir-218* overexpression inhibited colony formation in SKM-1 and HL60 cells. Upper: SKM-1 cells; Down: HL60 cells. **C** Venn diagram of the prediction results of bioinformatics websites (miRDB, TargetScan 7.2, microT-CDS and miRWalk) and mRNA-seq. **D** Dual luciferase experiment of *HOXA1* binding to *miR-218*. Upper: predicted binding sites of *HOXA1* and *miR-218*; Down: the luciferase activity of *HOXA1* binding to *miR-218*. **E** *HOXA1* expression after *miR-218* overexpression in SKM-1 and HL60 cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

correlated with poor OS and disease-free survival was also reported in uterine cervical carcinoma and esophageal squamous cell carcinoma [28, 29]. Overall, further studies with larger samples will yield valuable data to confirm the role of *SLIT2* promoter methylation with prognosis and disease surveillance before *SLIT2* promoter hypermethylation could be used for designing epigenetic-based therapy in the treatment of advanced-stage MDS.

Although a few studies have illustrated the correlation between *SLIT2* promoter methylation and expression in several types of cancers [27, 30], the current study did not observe a significant correlation of *SLIT2* promoter methylation with *SLIT2* expression in AML. These results indicated that *SLIT2* expression was not directly regulated by *SLIT2* promoter methylation, and other mechanisms may play a more important role in the regulation of *SLIT2* expression in AML. For instance, *SLIT2* expression was also identified to be regulated by several miRNAs, such as *miR-424*, *miR-27-3p*, and *miR-224-3p* [31–33]. Interestingly, a few studies have shown the potential tumor suppressor roles of *SLIT2* in MDS and AML [21, 34, 35]. However, the limitation in the study by Golos et al. was that they were mainly based on bioinformatics analysis and used very few clinical samples for validation [34]. In Xu et al.'s study, the control group seemed to be inappropriate when examining the biological function of *SLIT2* in MDS [21]. Critically, a recent study revealed that *SLIT2* can promote or suppress cancer progression depending on its cellular source [36]. Our study observed a significant negative association of *SLIT2* promoter

methylation with *SLIT2* embedded gene *SLIT2-IT1* and *miR-218* expression in AML, which was further confirmed by demethylation studies. Although it was the first report regarding *SLIT2-IT1* dysregulation in human cancers to date, several studies have reported the epigenetic mechanism regulating *miR-218* by DNA methylation in various types of human cancers. Altogether, these results suggested that *SLIT2* promoter hypermethylation mediated *SLIT2-IT1/miR-218* repression plays a significant role in MDS progression.

The tumor suppressor roles of *SLIT2-IT1* and *miR-218* in MDS/AML have been proven in this study and support the phenomenon of *SLIT2* promoter hypermethylation in MDS/AML. Although this was the first study to disclose the biological function of *SLIT2-IT1* in human cancer, the potential role of *miR-218* has been widely investigated. Lu et al. reviewed that *miR-218* acted as a tumor suppressor by targeting a variety of oncogenes related to proliferation, apoptosis and invasion in diverse human cancers [37]. Specifically, in myeloid leukemia, *miR-218* by targeting *RET* proto-oncogene played a crucial role in AML with translocation (8;16)(p11;p13) and MYST3-CREBBP rearrangement [38]. Moreover, *miR-218* inhibited cell growth by targeting the *BMI-1* in APL [39]. Li et al. reported that *miR-218* inhibited erythroid differentiation by targeting *ALAS2* in K562 cells [40]. All these results were in accordance with our study and supported that *miR-218* plays a tumor suppressor role during leukemogenesis.

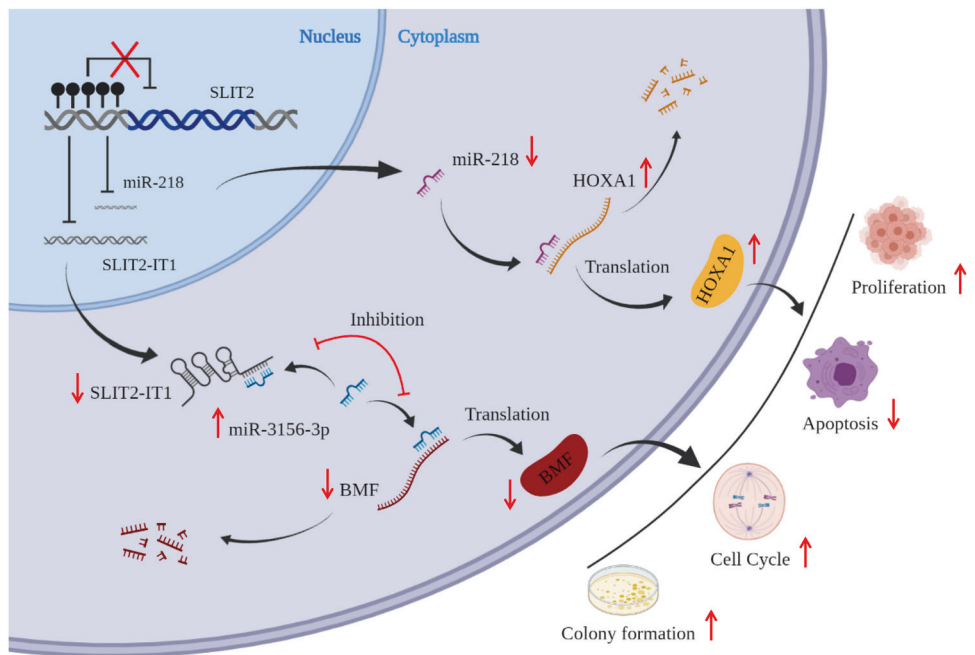


Fig. 6 The schematic diagram of the regulatory mechanism and role of *SLIT2* promoter hypermethylation during MDS transformation. *SLIT2* promoter hypermethylation regulate the expression of the noncoding gene *SLIT2-IT1*/miR-218 rather than *SLIT2* during MDS progression. *SLIT2-IT1* functioned through the ceRNA network by “sponging” *miR-3156-3p* to regulate *BMF* expression in leukemogenesis, whereas *miR-218* functioned by targeting *HOXA1* expression. Functional studies revealed the antileukemic effects of *SLIT2-IT1*/miR-218 by affecting proliferation, apoptosis, cell cycle and colony formation in leukemogenesis.

It is emerging that the biological roles of lncRNAs are dependent on their unique subcellular localizations [41]. Cytoplasmic lncRNAs are known to interact with miRNAs as ceRNAs in the regulation of mRNA translation or stability and influence signal transduction pathways [42]. Moreover, miRNAs negatively regulate genes by targeting the 3'-UTR of mRNAs, thereby facilitating translational silencing or degradation of the targeted genes [43]. In this study, we identified and verified that *SLIT2-IT1* is located in the cytoplasm of cells. Not surprisingly, our study supported the hypothesis that *SLIT2-IT1* functioned through a ceRNA network by “sponging” *miR-3156-3p* to regulate *BMF* expression in leukemogenesis. A previous investigation reported downregulation of the proapoptotic gene *BMF* in AML, in accordance with our study [44]. Moreover, *HOXA1*, as a target gene of *miR-218*, was also identified to be overexpressed in AML [45]. Collectively, these results support the biological network of *SLIT2-IT1* and *miR-218* in MDS/AML biology with antileukemic activity.

In summary, our findings demonstrated that *SLIT2* promoter hypermethylation was correlated with disease evolution in MDS and predicted adverse prognosis in both MDS and AML. *SLIT2* promoter methylation may mainly regulate the expression of the noncoding gene *SLIT2-IT1*/miR-218 rather than *SLIT2* during MDS progression. Functional studies revealed the antileukemic effects of *SLIT2-IT1*/miR-218 in MDS and AML. Mechanistically, *SLIT2-IT1* functioned through the ceRNA network by “sponging” *miR-3156-3p* to regulate *BMF* expression in leukemogenesis, whereas *miR-218* functioned by targeting *HOXA1* expression (Fig. 6). The current study emphasized that epigenetic dysregulation of *SLIT2-IT1*/miR-218 by *SLIT2* promoter hypermethylation could be a promising therapeutic target in MDS.

DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

JQ and J-DZ conceived and designed the experiments; T-JZ and X-MW performed the experiments; Z-JX analyzed the data and provided bioinformatics analysis; YG and QY collected the clinical data; J-CM and J-L provided the technical and financial supports; T-JZ wrote the manuscript; J-DZ revised the manuscript; All authors read and approved the final manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The present study approved by the Ethics Committee of the Affiliated People's Hospital of Jiangsu University. Written informed consents were obtained from all enrolled individuals prior to their participation.

ADDITIONAL INFORMATION

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