

RESEARCH ARTICLE

Epigenetic silencing of *E-cadherin* gene induced by lncRNA MALAT-1 in acute myeloid leukaemia

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Abstract

Background: Epigenetic abnormalities in acute myeloid leukaemia provide us with a target for novel therapeutic strategies. The aim of the study was to verify the epigenetic regulatory mechanism of *E-cadherin* gene silencing induced by long non-coding RNA MALAT-1 in AML.

Methods: Expression of MALAT-1, *E-cadherin*, *EZH2*, *SUZ12* and *EED* genes in AML patients was detected by RT-qPCR. After MALAT-1 silencing in AML cell lines, levels of the *E-cadherin*, *EZH2*, *SUZ12*, *EED*, *DNMT1*, *DNMT3A* and *DNMT3B* genes and encoded proteins were detected by RT-qPCR and Western blotting. The level of CpG island methylation and trimethylation modification of histone H3K27 in the promoter region of *E-cadherin* was detected by pyrosequencing and ChIP-qPCR. RIP-qPCR was used to detect the interaction between MALAT-1 and proteins.

Results: MALAT-1, *EZH2* and *EED* gene expression was markedly increased in AML patients with *E-cadherin* down-regulation. A positive correlation between *EZH2* or *SUZ12* and MALAT-1 expression was observed. After MALAT-1 silencing, the expression of *E-cadherin* was up-regulated, whereas the expression of *EZH2*, *SUZ12*, *DNMT1*, *DNMT3A* and *DNMT3B* was down-regulated. Results of Western blotting were consistent with those of RT-qPCR. Methylation levels of *E-cadherin* in AML patients were higher than that in normal controls, which appeared to increase with age. Methylation of the CpG island and H3K27 trimethylation of *E-cadherin* were decreased after MALAT-1 silencing. RIP-qPCR suggested that MALAT-1 might be enriched by *EZH2* and *SUZ12*.

Conclusion: Our findings verified that MALAT-1 might lead to the transcriptional silencing of *E-cadherin* gene through the trimethylation of H3K27 mediated by recruiting *EZH2* and *SUZ12*.

KEYWORDS

acute myeloid leukaemia, *E-cadherin*, epigenetic silencing, MALAT-1

1 | INTRODUCTION

Acute myeloid leukaemia (AML) is the most common type of acute leukaemia in adults. The disease progresses rapidly, and the incidence of AML shows an increasing trend with age.¹ According to data from the National Cancer Institute, it is estimated that approximately 19,940 patients were diagnosed with AML in 2020, of which approximately 11,180 died in the United States.² In recent years, with the continuous optimization of chemotherapy, supportive treatment and bone marrow transplantation, the complete remission rate and disease-free survival of AML have been improved to some extent. However, in the end, up to 60–80% of patients experience recurrence, and the prognosis after recurrence is very poor, which seriously threatens the survival of patients. Finding new therapeutic targets and drugs for AML remains a major obstacle to overcome.

With the in-depth study of malignant tumours, it has been found that epigenetic regulation is involved in the occurrence, development and prognosis of AML.¹ Epigenetics is the study of heritable phenotypic changes that do not involve alterations in the DNA sequence. Epigenetics mainly involves DNA methylation, histone modification (methylation, acetylation, phosphorylation, ubiquitination, etc.), chromatin remodelling, RNA editing (non-coding RNA, microRNA, antisense RNA, etc.), maternal effects and other regulatory mechanisms. These regulatory mechanisms interact with each other and are involved in multiple stages of individual growth, development and reproduction, as well as the occurrence and development of diseases.

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1) is an important member of lncRNA family. It is closely related to tumour proliferation, invasion, metastasis and other biological behaviours.³ MALAT-1 has two main functions, alternative splicing or gene transcription regulation.^{4,5} It has been confirmed in some studies on tumours that MALAT-1 can bind to the subunits enhancer of zeste homolog 2 (EZH2) and SUZ12 of polycomb repressive complex 2 (PRC2), thereby mediating the silencing of downstream genes.^{6–8} Hirata et al.⁶ found that in renal cell carcinoma, MALAT-1 inhibits the expression of *E-cadherin* through the EZH2-mediated trimethylation of histone H3K27 in the promoter region of the encoding gene. A study by Fan et al.⁷ found that MALAT-1 in bladder cancer cells associates with the PRC2 subunit SUZ12, resulting in the down-regulation of E-cadherin protein, as well as the up-regulation of N-cadherin protein and fibronectin expression. In a study of prostate cancer, Wang et al.⁸ found that MALAT-1 binds to EZH2 protein to further regulate the expression of the tumour suppressor gene *DAB2IP*. Therefore, we speculate that the binding of MALAT-1 to histone modification complexes might be common in tumours and that through this mechanism, the transcription of many genes is regulated.

The *E-cadherin* gene is an important tumour suppressor, and its encoded protein can inhibit the invasion, proliferation and metastasis of epithelial tumour cells, such as liver cancer, breast cancer, ovarian cancer, gastric cancer and prostate cancer, among others. Epithelial-mesenchymal transition (EMT) is mainly caused by the

loss of E-cadherin expression, and EMT is regarded as an important event in the metastasis and diffusion of tumour cells.⁹ In the course of EMT, cell contacts lose stability owing to epigenetic mechanisms.¹⁰ Further, low expression of E-cadherin in some tumours is related to DNA methylation and histone modification.^{10,11} However, there are still few studies on the role of E-cadherin in AML, especially with respect to its epigenetic regulation.

We hoped to determine whether MALAT-1 forms an RNA-induced gene silencing complex or RNA-induced transcriptional gene silencing complex, with the participation of epigenetic regulatory proteins, by binding to different histone modification complexes, in the AML cell lines (U-937 and THP-1). We attempted to explore the possible mechanism underlying the methylation of the *E-cadherin* gene promoter region and/or histones. At the same time, the clinical significance of MALAT-1 and E-cadherin in AML patients was further verified using clinical specimens. We anticipated that this research might reveal the epigenetic regulatory mechanism of MALAT-1-mediated target gene silencing and would further clarify the relationship between these mechanisms and the development of AML. Accordingly, this study might provide new targets for the treatment of AML.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

In total, 51 bone marrow samples were acquired from patients with newly diagnosed AML (27 males and 24 females with a median age of 61 years; range, 32–86 years) at the Union Hospital of Fujian Medical University (Fuzhou, China), who were diagnosed between May 2013 and November 2016. The diagnosis was established according to the World Health Organization diagnostic criteria.¹² Patients who underwent chemotherapy or radiotherapy prior to the study were excluded. According to the French–American–British classification, 51 samples were classified, including two samples as M0, four samples as M1, 15 samples as M2, five samples as M3, two samples as M4, 22 samples as M5 and one sample as M6. Twenty-five healthy bone marrow donors (13 males and 12 females) served as healthy controls. Informed consent was obtained from patients and healthy individuals before the use of samples, and the study protocol was approved by the Ethics Committee at the hospital. All patients enrolled in the study underwent bone marrow puncture under sterile conditions, and 3–5 ml bone marrow fluid was reserved.

2.2 | Cell culture

The AML cell lines U-937 and THP-1 were obtained from the cell bank of the National Collection of Authenticated Cell Cultures (Shanghai, China). Cell lines were maintained in RPMI-1640 medium (Hyclone, USA) containing 10% foetal bovine serum (Gibco, USA) and maintained at 37°C in a humidified incubator containing 5% CO₂. Cells in logarithmic growth were used for further study.

2.3 | Vector construction and cell transfection

Lentiviral vectors encoding MALAT-1 small interfering RNA (si-MALAT-1) or scrambled negative control (si-NC) were designed and synthesized by Genechem (Shanghai, China). Cells were transfected with si-MALAT-1 and si-NC lentivirus according to the manufacturer's instructions.

2.4 | RNA extraction and real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using the RevertAid First-Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA). An ABI7500 real-time PCR System (ABI, USA) and a FastStart Universal SYBR Green Master kit (Roche, USA) were employed to investigate reverse transcription products using *GAPDH* as the internal control. RT-qPCR was performed according to a previous description.¹³ The relative expression ratio was calculated using the $2^{-\Delta\Delta C_t}$ method. The primer sequences of genes are shown in Table 1.

2.5 | Western blotting analysis

The cells were lysed using RIPA protein extraction reagent (Beyotime, China) supplemented with protease inhibitors (Lulong Biotech, China); then, 80 μ g of protein extraction was separated by 10% SDS-PAGE, transferred onto PVDF membranes (Millipore, USA) and incubated with the following antibodies: *GAPDH* (Abcam, USA), *EED* (Millipore, USA), *DNMT1*, *DNMT3A*, *DNMT3B*, *EZH2*, *SUZ12* and *E-cadherin* (Cell Signaling Technology, USA). Next, the membranes were incubated with secondary antibodies (Cell Signaling Technology, USA), and the signals from membranes were detected with West Pico Chemiluminescent substrate (Thermo Fisher Scientific, USA). Band intensities were analysed with the ImageJ software (National Institutes of Health Bethesda, USA). The results were normalized to the expression of *GAPDH*.

2.6 | Pyrosequencing analysis

CpG methylation of the promoter region of the *E-cadherin* gene was quantified by pyrosequencing. The EpiTect Bisulfite Kit (Qiagen, Germany) was used for bisulphite conversion of genomic DNA. The PyroMark Gold Q96 Reagents and the PyroMark Q96 ID instrument (Qiagen, Germany) were used for pyrosequencing according to the manufacturer's instructions. Seven total CpG sites were analysed per sample.

Pyro Q-CPG (Biotage, SWEDEN) was used to analyse the methylation status of each Site. Pyrosequencing primers were designed

TABLE 1 Primer sequences for RT-qPCR

Gene	Primer sequence (5'→3')	Product size (bp)
MALAT-1	Forward primer: AAAGCAAGGTCTCCCCACAAG	71
	Reverse primer: GGTCTGTGCTAGATCAAAAGGCA	
GAPDH	Forward primer: CCCCTTCATTGACCTCAACTACAT	135
	Reverse primer: CGCTCCTGGAAGATGGTGA	
EZH2	Forward primer: GGAACAACGCGAGTCGG	102
	Reverse primer: CTGATTTACACGCTTCCGC	
SUZ12	Forward primer: GCATTGCCCTTGGTGTACTC	214
	Reverse primer: TGGTCCGTGCGACTAAAA	
EED	Forward primer: ATCCGGTTGTTGCAATCTTA	218
	Reverse primer: TTTGATCTCTTGGATGGAA	
<i>E-cadherin</i>	Forward primer: TACTATGATGAAGAAGGAGG	262
	Reverse primer: CGGAACCGCTTCCTTCATAG	
DNMT1	Forward primer: CCGAGTTGGTGATGGTGTGTAC	324
	Reverse primer: AGGTTGATGTCTGCGTGGTAGC	
DNMT3A	Forward primer: TATTGATGAGCGCACAAAGAGAGC	111
	Reverse primer: GGGTGTTCCAGGGTAACATTGAG	
DNMT3B	Forward primer: GACTCGAAGACGCACAGCTG	98
	Reverse primer: CTCGGTCTTTGCCGTTGTTATAG	

using PYROMARK assay design software 2.0 (Qiagen, Germany). The primers for the analysis of *E-cadherin* CPG regions were as follows (5' biotin modification): FORWARD, 5'-GGGTTGGGATTAGAA TTTAGTGGAAATTA-3'; REVERSE, 5'-ATTCACCTACCCACCACAACC AATCAACAA-3' and S, 5'-ATTTTAGGTTAGAGGGTTAT-3'.

2.7 | Chromatin immunoprecipitation (ChIP)-qPCR analysis

ChIP assays were performed as described previously,¹⁴ and an EZ-Magna ChIP™ A/G One-Day Chromatin Immunoprecipitation Kit (Millipore, USA) was used according to the manufacturer's instruction. According to the provided protocol, cell harvesting,

sonication, chromosome immunoprecipitation, washing of the precipitated complex and de-crosslinking were strictly performed. An H3 trimethyl Lys 27 antibody (Millipore, USA) was used in the experiments. Quantification of immunoprecipitated DNA was detected by qPCR with a SYBR Green Master kit (Roche, USA). The primers for ChIP-qPCR to detect *E-cadherin* were designed using Beacon Designer 7 software (PREMIER Biosoft, USA) as follows: forward, CAAGAACACACCATTGCACT; and reverse, TGCCAGTCTCTGTGCTAAGC. The ChIP data were calculated as a percentage relative to the input DNA, as described previously.¹⁴

2.8 | RNA immunoprecipitation (RIP)-qPCR analysis

RIP experiments were performed according to a previous description,¹⁴ and a Magna RIP™ RNA-Binding Protein Immunoprecipitation kit (Millipore, USA) was used according to the manufacturer's instructions. EED (Millipore, USA), EZH2, SUZ12, DNMT1, DNMT3A, DNMT3B and control IgG (Cell Signaling Technology, USA) antibodies were used for RIP assays. The RNA immunoprecipitation fraction was digested with DNase, and cDNA was generated using the RevertAid First-Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA). A final analysis was performed using qPCR and shown as the fold enrichment of MALAT1.

2.9 | Statistical analysis

Data are expressed as mean ± SD and were analysed with SPSS 20.0 Statistical Software (IBM, Armonk, NY, USA) and GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA). Results of lncRNA MALAT-1 expression in clinical samples were assessed by non-parametric Mann-Whitney U test. Three independent experiments were performed for all measurements. The significance of mean differences between two groups in the cellular experiments was calculated by unpaired two-tailed Student's *t*-tests. The correlation between gene expressions was analysed by Spearman's correlation analysis. $P < 0.05$ was deemed statistically significant.

3 | RESULTS

3.1 | Expression of MALAT-1, E-cadherin, EZH2, SUZ12 and EED in AML patients and healthy controls

mRNA expression levels were evaluated by RT-qPCR. Compared with those in healthy controls, expression levels of MALAT-1 were higher in AML patients (Figure 1A), whereas the expression of *E-cadherin* was lower (Figure 1B). Moreover, the expression of *EZH2* (Figure 1C) and *EED* (Figure 1D) was also higher in AML patients. However, the expression of *SUZ12* in AML patients was not significantly different from that in healthy controls (Figure 1E). The correlation between MALAT-1 and

EZH2, *SUZ12* and *EED* gene expression was analysed by Spearman's correlation analysis. The results showed that the expression levels of *EZH2* and *SUZ12* were positively correlated with MALAT-1 in AML patients (*EZH2*: $r = 0.475$, $P = 0.001$; *SUZ12*: $r = 0.349$, $P = 0.0235$; Figure 1F,G), and there was no significant correlation between *EED* and MALAT-1 ($r = 0.07893$, $P = 0.6106$; Figure 1H).

3.2 | Expression of E-cadherin, EZH2, SUZ12, EED, DNMT1, DNMT3A and DNMT3B genes in AML cell lines after MALAT-1 silencing

We silenced MALAT-1 expression in the U-937 and THP-1 cell lines with small interfering RNA (Figure 2A). After MALAT-1 silencing, expression of the *E-cadherin* gene in U-937 and THP-1 cells was up-regulated (Figure 2B), whereas the levels of *EZH2*, *SUZ12*, *DNMT1*, *DNMT3A* and *DNMT3B* were down-regulated to varying degrees (Figure 2C,D,F-H), but there was no significant difference in *EED* (Figure 2E).

3.3 | Protein expression of EZH2, SUZ12, EED, DNMT1, DNMT3A, DNMT3B and E-cadherin in AML cell lines after MALAT-1 silencing

After MALAT-1 silencing, the expression of *EZH2*, *SUZ12*, *DNMT1*, *DNMT3A* and *DNMT3B* proteins in U-937 and THP-1 cells decreased, whereas that of *E-cadherin* protein increased (Figure 3A-C). There was no significant difference in *EED* protein levels between the two groups (si-NC group and si-MALAT-1 group) in U-937 and THP-1 cells (Figure 3A-C).

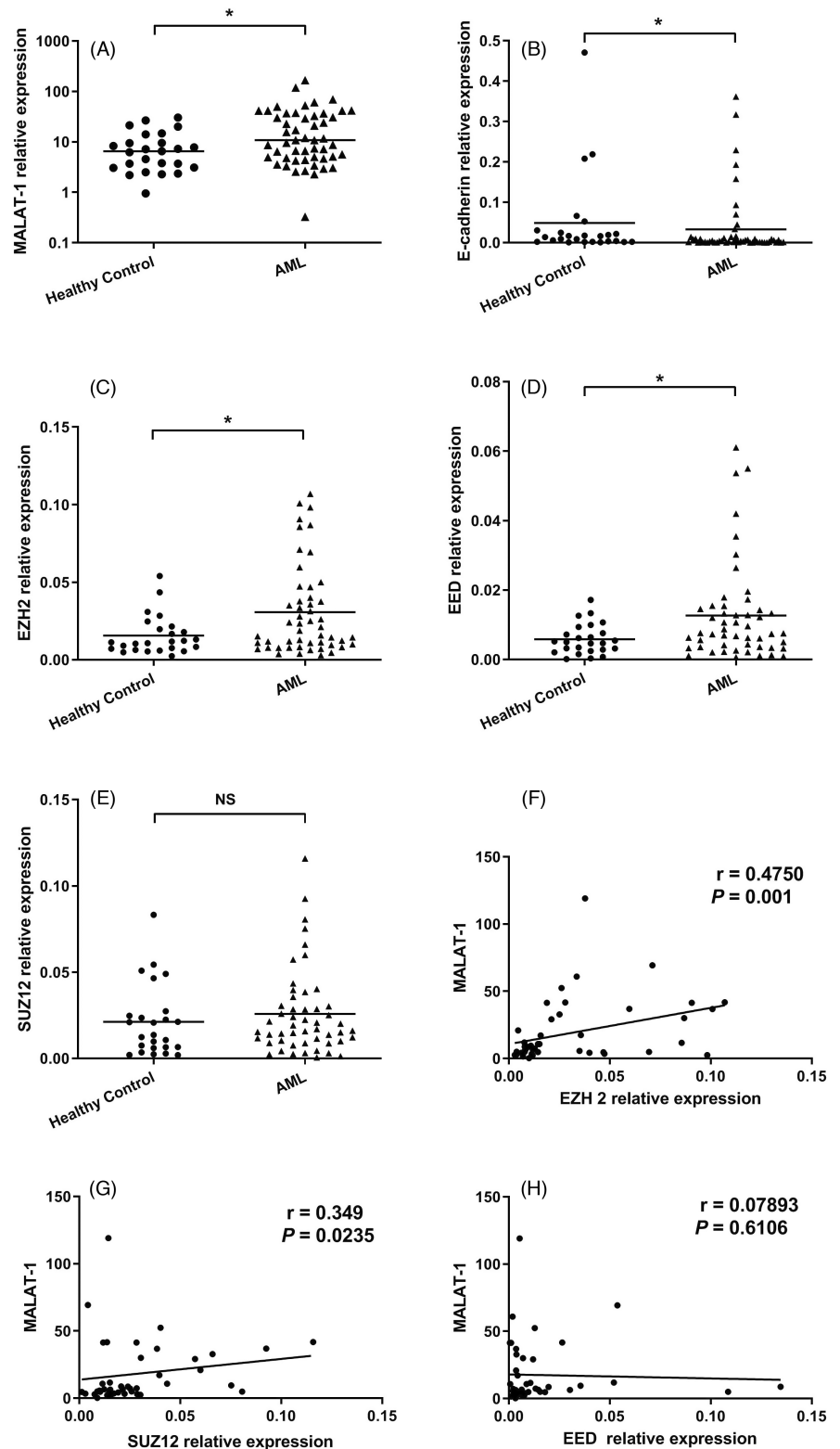
3.4 | Methylation modification of CpG island in the promoter region of the E-cadherin gene in AML patients and normal controls

Based on pyrosequencing, compared with that in normal controls, methylation of the CpG island in the promoter region of the *E-cadherin* gene was significantly higher in AML patients (Figure 3D). A positive correlation between methylation levels of *E-cadherin* and the age of AML patients was observed ($r = 0.3281$, $P = 0.0187$; Figure 3E).

3.5 | Methylation level of CpG island in the promoter region of E-cadherin gene in AML cell lines after MALAT-1 silencing

After MALAT-1 silencing, methylation of the CpG island in the promoter region of the *E-cadherin* gene in U-937 (Table 2) and THP-1 (Table 3) cells was decreased, and the difference was statistically significant ($*P < 0.05$) (Figure 4).

FIGURE 1 Expression of MALAT-1, *E-cadherin*, *EZH2*, *SUZ12* and *EED* in AML patients and healthy controls. RT-qPCR was used to detect the expression of MALAT-1(A), *E-cadherin*(B), *EZH2*(C), *EED*(D) and *SUZ12*(E) in AML patients and healthy controls. Correlation between MALAT-1 expression and *EZH2* (F), *SUZ12* (G) and *EED* (H) levels in patients with AML. * $P < 0.05$, NS: not statistically significant



3.6 | Trimethylation modification of histone H3K27 (H3K27me3) in the *E-cadherin* gene promoter region after MALAT-1 silencing

Gel electrophoresis was used to analyse the products of sonication. The size of DNA fragments was mostly concentrated in the

200- to 1000-bp range (Figure 5A), which was the best fragment size for ChIP. The results of ChIP-qPCR showed that after silencing MALAT-1, the expression of *E-cadherin* gene promoter region-related histone H3K27me3 in U-937 and THP-1 cells was down-regulated, and the difference was statistically significant (Figure 5B).

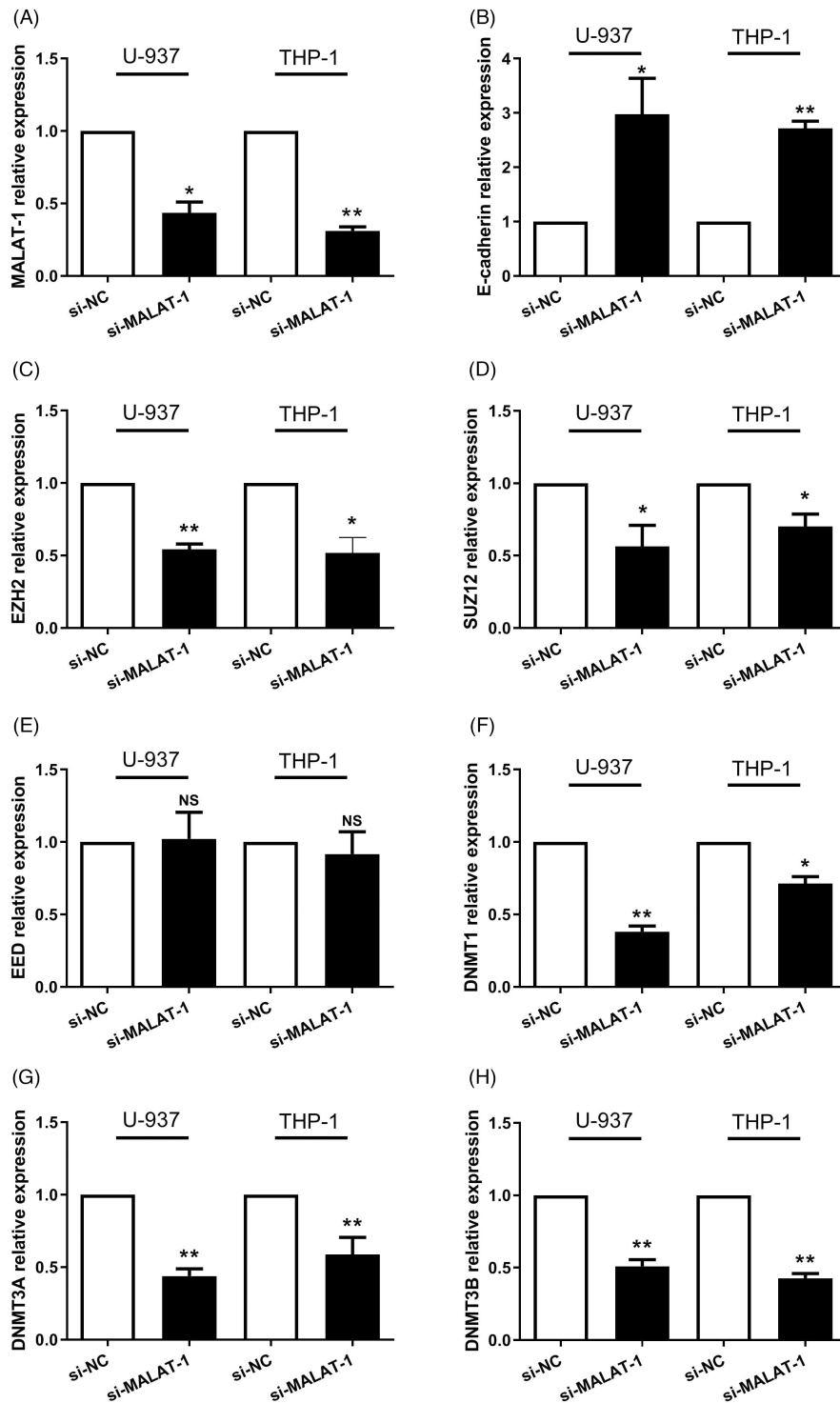


FIGURE 2 Expression levels of MALAT-1, *E-cadherin*, *EZH2*, *SUZ12*, *EED*, *DNMT1*, *DNMT3A* and *DNMT3B* genes in U-937 and THP-1 cells after MALAT-1 silencing. After MALAT-1 was silenced in U-937 and THP-1 cell lines (A), expression levels of *E-cadherin* (B), *EZH2* (C), *SUZ12* (D), *EED* (E), *DNMT1* (F), *DNMT3A* (G) and *DNMT3B* (H) genes were detected by RT-qPCR. * $P < 0.05$, ** $P < 0.01$ and NS: not statistically significant

3.7 | Interaction between MALAT-1 and EZH2, SUZ12, EED, DNMT1, DNMT3A and DNMT3B proteins in AML cell lines

The results of RIP-qPCR assay in U-937 and THP-1 cells showed that MALAT-1 in U-937 cells could be enriched by corresponding antibodies to EZH2 and SUZ12 proteins, and the difference was statistically significant compared with levels in the negative control group (Figure 5C,D). However, when using EED, DNMT1, DNMT3A and

DNMT3B antibodies for RNA immunoprecipitation analysis, compared with levels in the negative control group (IgG), no significant differences were detected in the enrichment of MALAT-1 (Figure 5E–H).

4 | DISCUSSION

At present, the treatment of acute myeloid leukaemia is still based on traditional chemotherapy and haematopoietic stem cell

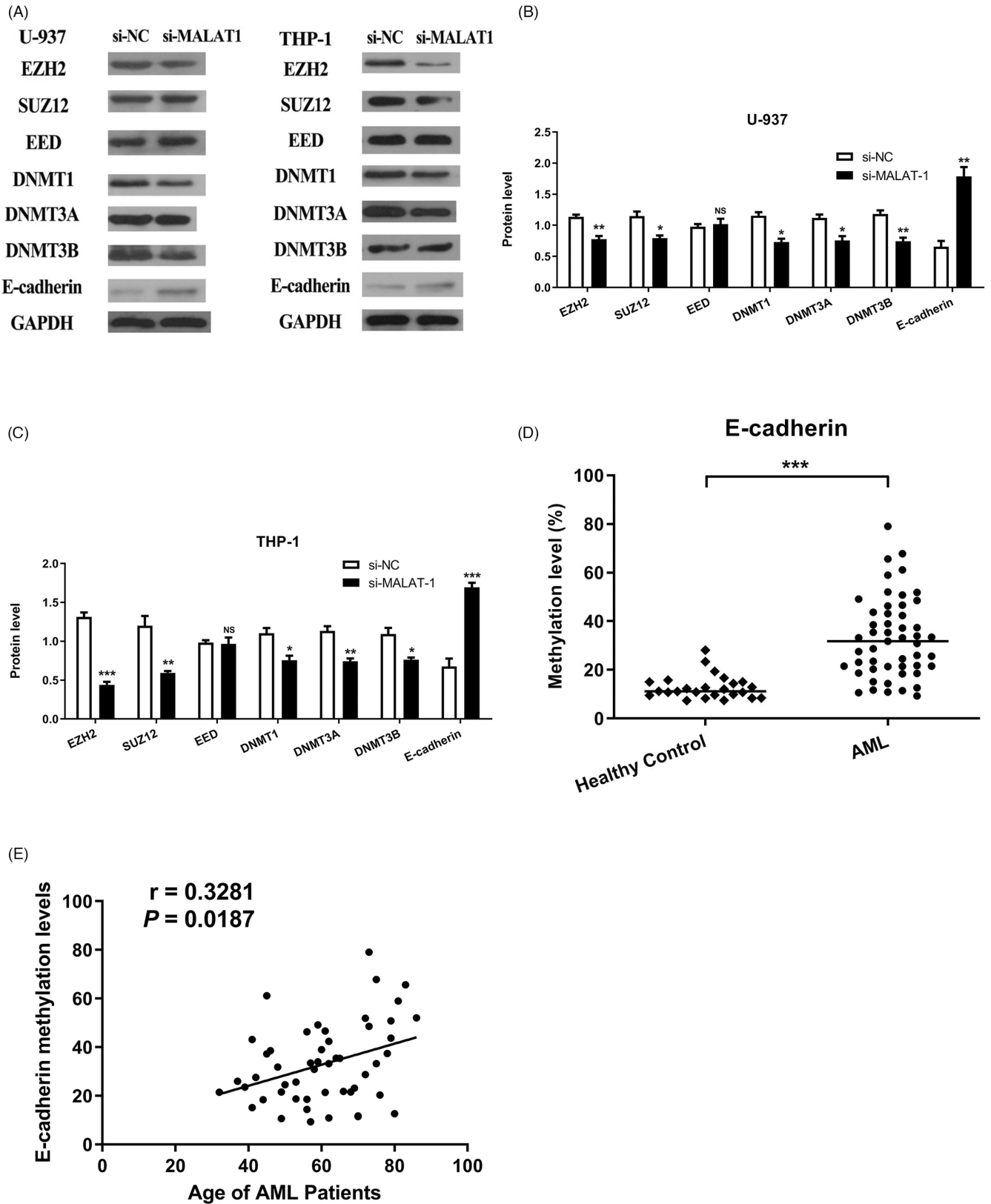


FIGURE 3 Protein expression of EZH2, SUZ12, EED, DNMT1, DNMT3A, DNMT3B and E-cadherin in U-937 and THP-1 was detected by Western blotting after MALAT-1 silencing (Figure 3A–C). Methylation modification of CpG island in the promoter region of the *E-cadherin* gene in AML patients and healthy controls (Figure 3D). Correlation between methylation levels of *E-cadherin* gene and the age of AML patients (Figure 3E). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and NS: not statistically significant

TABLE 2 Methylation status of CpG island in the promoter region of *E-cadherin* gene in U-937 cells after MALAT-1 silencing (* $P < 0.05$)

E-cadherin	Pos. 1	Pos. 2	Pos. 3	Pos. 4	Pos. 5	Pos. 6	Pos. 7		
Sample ID	Meth. (%)	Meth. (%)	Meth. (%)	Meth. (%)	Meth. (%)	Meth. (%)	Meth. (%)	Number of included CpGs	Mean
U-937 NC	93.45	94.8	94.16	96.22	85	99.73	100	7	94.77
U-937 KD	82.33	86.03	89.52	85.1	81.75	91.14	95.43	7	87.33*

TABLE 3 Methylation status of CpG island in the promoter region of *E-cadherin* gene in THP-1 cells after MALAT-1 silencing

E-cadherin	Pos. 1	Pos. 2	Pos. 3	Pos. 4	Pos. 5	Pos. 6	Pos. 7		
Sample ID	Meth. (%)	Meth. (%)	Meth. (%)	Meth. (%)	Meth. (%)	Meth. (%)	Meth. (%)	Number of included CpGs	Mean
THP-1 NC	88.33	92.36	92.64	99.82	85.62	96.82	100	7	93.66
THP-1 KD	80.59	81.27	85.53	91.32	79.2	90.04	93.42	7	85.91*

Abbreviations: Pos.1-7, position 1st-7th for pyrosequencing analysis; Meth(%), percentage of methylation.

transplantation. However, owing to many factors such as disease recurrence, poor tolerance and lack of a donor source, treatment still faces severe challenges. Based on SEER Program data (Surveillance, Epidemiology, and End Results) from 2011 to 2017, the 5-year survival rate for AML patients is less than 30%.

With the acceleration of global population ageing, the number of elderly patients with acute leukaemia is gradually increasing. Owing to the lack of effective treatment measures, the prognosis of these patients is even less optimistic. Although traditional high-dose chemotherapy can achieve complete remission in 40–80% of patients, older patients (age 70 years or older) cannot benefit from chemotherapy because of their poor tolerance. The survival time of elderly patients receiving intensive chemotherapy is only 4.6 months, and the 1-year survival rate is less than 30%.^{15,16} The median age of AML patients in the United States was 68 years according to the NIH (National Institutes of Health) SEER database. The median age of AML patients included in this study was 61 years, which was similar to that of American patients. It can be seen that the prevalence of AML was mainly observed in middle-aged and elderly individuals. The increase in age might cause epigenetic changes in haematopoietic cells and eventually lead to the occurrence of AML.¹ AML in the elderly is often accompanied by gene mutations and epigenetic abnormalities, which urges us to explore a new way to treat acute leukaemia from the perspective of epigenetics.

Epigenetic regulatory mechanisms include DNA methylation, histone methylation and ncRNA, among others. There are three known transferases involved in regulating DNA methylation (DNMT1, DNMT3A and DNMT3B), and they are overexpressed in human tumour cells to varying degrees, which is related to the silencing of tumour suppressor genes.^{17,18} PRC2 is a well-studied histone methylation modification complex, and its three core subunits include EZH2, SUZ12 and EED (embryonic ectoderm development). As the most important subunit of the PRC2 complex, EZH2 is mainly involved in the trimethylation of H3K27, whereas SUZ12 and EED subunits are mainly involved in maintaining the stability of the complex.

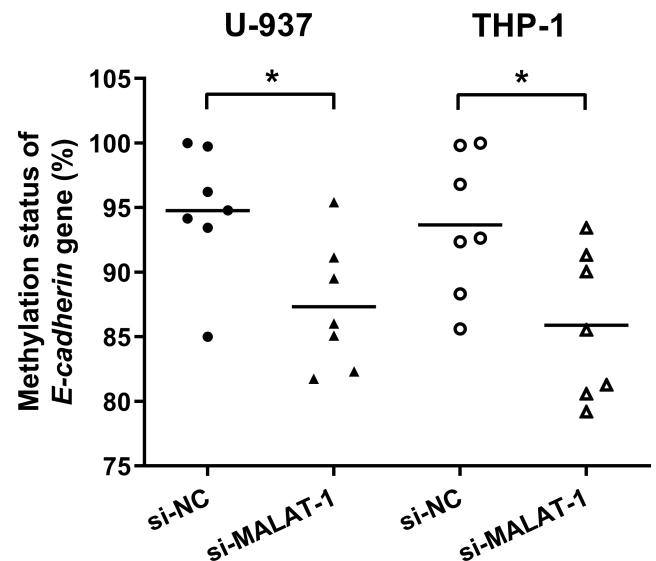
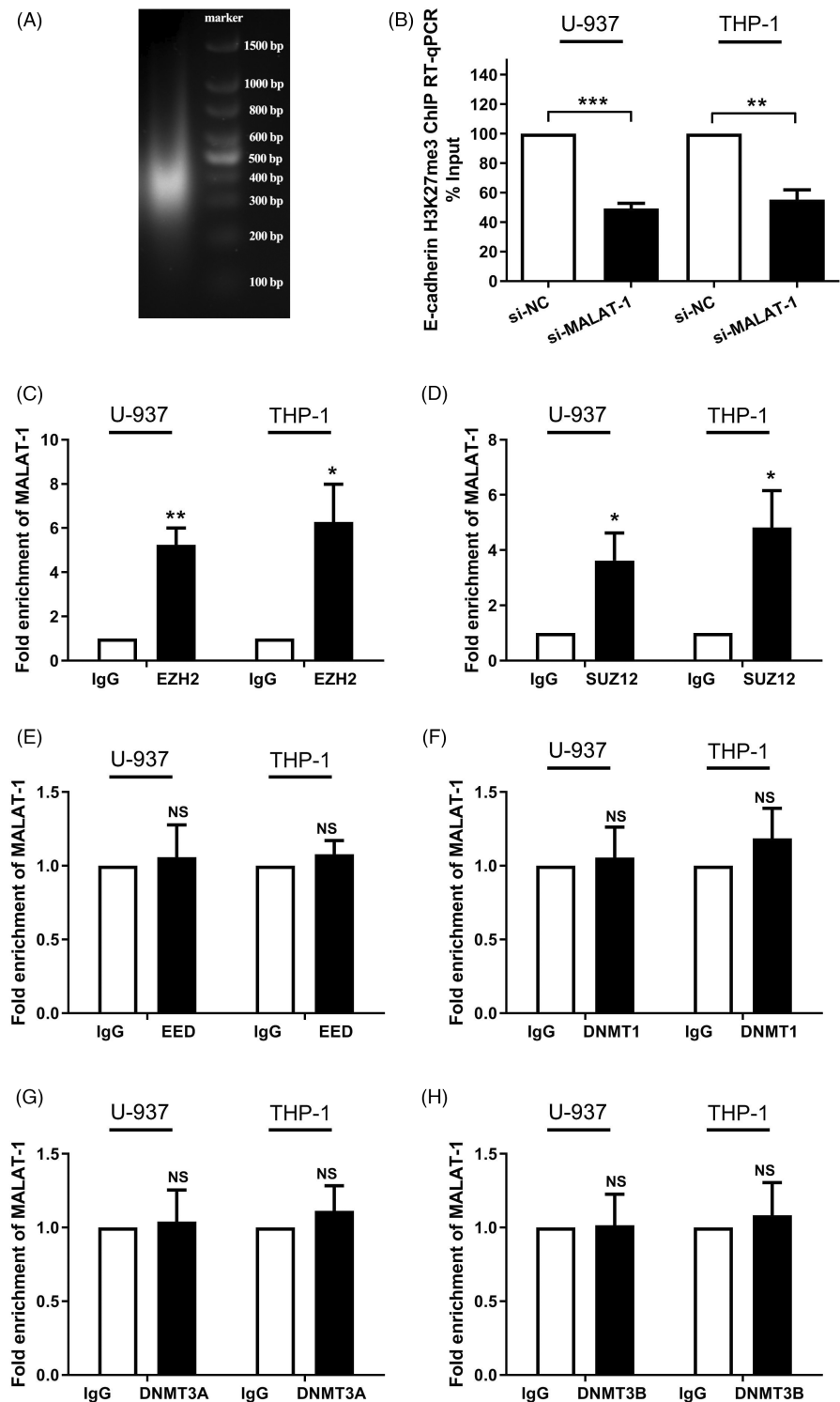


FIGURE 4 Methylation status of CpG island in the promoter region of *E-cadherin* gene in U-937 and THP-1 cells after MALAT-1 silencing * $P < 0.05$

lncRNA accounts for the majority of ncRNAs and can act as signals, inducers, guiders or scaffolds to regulate the biological processes in cells.¹⁹ It has been confirmed that some lncRNAs can regulate the transcription of downstream genes by combining different histone modification complexes to mediate the methylation of target gene promoter regions or histones.²⁰ The lncRNA HOTAIR acts in a manner similar to a scaffold, and it promotes the methylation of histone H3 lysine K27 (H3K27) by binding to the PRC2 complex, whereas binding to histone demethylase 1 (LSD1) of the REST/CoREST complex mediates the demethylation of histone H3 lysine K4 (H3K4).²¹ The lncRNA H19 enriches histone H3 lysine K9 (H3K9) histone methyltransferases SUV39H1 and SETDB1 through methyl-CpG-binding domain 1 (MBD1), which mediates the transcriptional silencing of target genes.²⁰ lncRNA GCInc1 acted as a modular scaffold to recruit the WDR5 histone

FIGURE 5 (A) Electrophoretic diagram of sonication products. (B) Expression of E-cadherin gene promoter region-related histone H3K27me3 in U-937 and THP-1 cells after MALAT-1 silencing. Detection of the interaction between MALAT-1 and EZH2 (C), SUZ12 (D), EED (E), DNMT1 (F), DNMT3A (G) and DNMT3B (H) proteins in U-937 and THP-1 cells by RIP-qPCR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and NS: not statistically significant



methyltransferase and KAT2A histone acetyltransferase and regulate the transcription of target genes.²² DNA methylation, histone modification, and lncRNAs might also interact to form a complex cellular gene regulatory network.

Our previous study showed that MALAT-1 is highly expressed in AML-M5 patients and related to poor prognosis for these individuals. Silencing MALAT-1 in U-937 and THP-1 cells could inhibit cell proliferation and induce apoptosis.¹³ This prompted us to explore

the underlying role of MALAT-1 in epigenetic regulation. To probe into this mechanism, we confirmed that the expression of *EZH2* and *EED* was increased in AML patients compared with normal control levels, and there was a positive correlation between either *EZH2* or *SUZ12* and MALAT-1. The expression of *SUZ12* in AML patients was not higher than that in the normal control, whereas the results suggested that the median expression level was slightly higher in AML patients (0.01702661 for AML vs 0.01357691 for NC, $2^{-\Delta\Delta Ct}$

method), and the lack of statistical significance may be related to the insufficient amount of clinical specimens in our opinion. After MALAT-1 silencing, expression of the *E-cadherin* gene was up-regulated in AML cell lines; however, the expression of *EZH2*, *SUZ12*, *DNMT1*, *DNMT3A* and *DNMT3B* genes was down-regulated, and no significant difference was observed in *EED*. The results of Western blotting were consistent with those of RT-qPCR. Therefore, we speculate that MALAT-1 might be involved in the occurrence and development of AML by mediating the methylation of DNA and/or histone, which might involve the regulation of some oncogenes or tumour suppressor genes.

E-cadherin is one of the most widely studied tumour suppressor genes. We screened *E-cadherin* as a potential target gene regulated by 'MALAT-1, DNMTs and PRC2'. Silencing of *E-cadherin* results in the suppression of cell-cell adhesion, which has been regarded as an important step in the EMT process.⁹ Loss of the *E-cadherin* gene can also induce abnormal cell proliferation and lead to tumour invasion and metastasis.¹¹ The CpG island in the promoter region of the *E-cadherin* gene in AML patients and cell lines was found to be methylated, resulting in gene silencing.²³ Hypermethylation of the *E-cadherin* gene in AML patients is related to poor prognosis.²⁴ Our observation that methylation of the CpG island in *E-cadherin* gene was higher in AML patients than that in normal controls is in good agreement with previous studies.^{23,25} Moreover, a positive correlation between methylation levels of *E-cadherin* and the age of AML patients was observed. AML mainly affects middle-aged and elderly people, and the increase in age could cause epigenetic silence of tumour suppressor genes such as *E-cadherin* and eventually lead to the occurrence of AML. However, previous studies did not further clarify the specific mechanism leading to the methylation and silencing of the *E-cadherin* gene. In the current study, by the ChIP-qPCR assay, we found that the level of H3K27me3 in the promoter region of *E-cadherin* was decreased in U-937 and THP-1 cells after MALAT-1 silencing. MALAT-1 and PRC2 might thus form a histone methylation modification complex and participate in the aforementioned regulatory mechanism. To investigate the components of the modification complex, RIP-qPCR assay was further used to explore the interaction between MALAT-1 and EZH2, SUZ12, EED, DNMT1, DNMT3A and DNMT3B proteins. The results in U-937 and THP-1 cells showed that compared with those in the negative control group (IgG), MALAT-1 could be enriched by antibodies targeting EZH2 and SUZ12 proteins. However, when using EED, DNMT1, DNMT3A and DNMT3B antibodies for RIP analysis, there was no significant difference. Results revealed the interaction between MALAT-1 and EZH2 or SUZ12, suggesting that MALAT-1 might bind these two subunits to form the main component of the MALAT-1-PRC2 complex, ultimately causing the methylation of histones in the promoter region of downstream target genes. Our experimental results correspond to previous findings of Hirata⁶ and Fan.⁷ Polycomb reactive complex 2 (PRC2) mainly consists of three primary subunits: EZH2, SUZ12 and EED. PRC2 complex plays a critical role in transcriptional regulation, and the binding of EED to trimethylated H3K27 further enhanced the activity of EZH2.²⁶ EED plays an essential role in stabilizing and

activating PRC2 for maintaining H3K27 methylation.²⁷ Loss of EED impaired normal differentiation of haematopoietic stem cells.²⁸ Electrophoretic mobility shift assay by Wu et al²⁹ indicated that the PRC2 subunits EZH2 and EED are necessary and sufficient for binding to lncRNA HOTAIR. RIP-qPCR analysis in our study verified that lncRNA MALAT-1 regulated the transcriptional silencing of *E-cadherin* gene through recruiting EZH2 and SUZ12, suggesting that the EED subunit might not significantly contribute to the PRC2 interaction with MALAT-1. In this perspective, it also could be the reason why no significant correlation between *EED* gene expression and MALAT-1 was observed in AML clinical specimens. Similarly, EED showed no significant changes at the gene and protein levels after MALAT-1 silencing in our research. Although no direct interaction between EED protein and MALAT-1 was observed, the role of EED in epigenetic modification in AML could not be ignored, which needs to be further studied.

Previous studies have confirmed that the synergistic effect of DNA methyltransferase and the PRC2 complex is needed to realize the regulation of some genes.^{30,31} The PRC2 complex recruits DNA methyltransferases to inhibit transcription in the germinal centre (GC) reaction.³⁰ A study by Richard et al found the synergistic activation effect of the tumour suppressor genes (CDKN1A and FBXO32) by combining the inhibitors of DNA methylation and histone methylation in leukaemic cells.³² Despite the fact that no direct interaction between DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) and MALAT-1 was observed in our RIP-qPCR analysis, the synergy of DNA methyltransferase and PRC2 in gene transcription regulation still requires further research in AML.

Epigenetic modifications are reversible to some degree and therefore allow opportunities for therapeutic intervention. Recently, epigenetic research has promoted the development of a variety of targeted drugs in haematologic malignancies. The antineoplastic effects of the DNA methyltransferase inhibitors decitabine and azacitidine on myelodysplastic syndrome (MDS) and AML have been gradually affirmed.^{33,34} Further, the histone deacetylase inhibitors romidepsin and vorinostat show certain efficacy in the treatment of lymphoma.³⁵ Panobinostat has also been approved by the US FDA and EU for the treatment of multiple myeloma.^{36,37} MAK683, an EED inhibitor, is currently under a Phase I/II clinical trial (NCT02900651) for patients with diffuse large B-cell lymphoma (DLBCL).³⁸ Phase II trials have shown high objective response rates for patients with relapsed/refractory follicular lymphoma without major toxicity, leading to EZH-2 inhibitor tazemetostat approval by the US FDA in 2020.^{39,40} Epigenetic targeted drugs show impressive anticancer activity and provide a novel and safe strategy for the treatment of haematologic malignancies.

In summary, our study demonstrated that MALAT-1 might form a MALAT-1-PRC2 complex by binding EZH2 and SUZ12, inducing trimethylation modification of histone H3K27 in the promoter region of *E-cadherin*, ultimately leading to gene silencing. MALAT-1 might induce CpG island methylation at the *E-cadherin* promoter region by affecting the expression of DNMT1, DNMT3A and DNMT3B, to

regulate the expression of this marker. However, the specific mechanism remains to be further studied. Obviously, treatment of AML still faces great obstacles; thus, this study might facilitate the development of AML pathogenesis and therapeutic research.

CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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