Methylation of the promoter region of the *MTRR* gene in childhood acute lymphoblastic leukemia

JUN BAI[1,2], LIJUAN LI[1], YI LI[2], QUNFEI CHEN[1], LIANSHENG ZHANG[1] and XIAODONG XIE[2]

[1]Department of Hematology, Gansu Provincial Key Laboratory of Hematology, Second Hospital of Lanzhou University; [2]Key Laboratory of Preclinical Study for New Drugs of Gansu Province, School of Basic Medical Sciences, Lanzhou University, Lanzhou, Gansu 730030, P.R. China

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Abstract. Epigenetic analysis of the association between the methylation status of the promoter region of the *MTRR* (5-methyltetrahydrofolate-homocysteine methyltransferase reductase) gene and the risk of acute lymphoblastic leukemia (ALL) in children plays an important role in the early diagnosis, assessment of the malignant degree, treatment and evaluation of the risk of relapse and prognosis of the disease. In the present study, RT-qPCR was used to detect the mRNA levels of the *MTRR* and *MTHFR* (methylenetetrahydrofolate reductase) genes in the bone marrow of 20 ALL patients and 20 age- and sex-matched controls with normal bone marrow. The methylation pattern of the *MTRR* promoter region in eligible DNA samples was quantitatively analyzed using MALDI-TOF MS. The results indicated that the mRNA expression level of *MTRR* in the bone marrow from children with ALL was lower than that in the control samples (P<0.05), but no significant difference was detected in the *MTHFR* gene between the two groups (P>0.05). According to the risk classification of ALL in children with high, medium and low risk, the low-risk group had a higher methylation rate of CpG_6 compared to the medium-risk group. However, the medium-risk group had a higher CpG_46.47 methylation rate compared to the low-risk group. The methylation rates of CpG_26 and CpG_46.47 in the high-risk group were higher than these rates in the low-risk group, while the CpG_42.23.44 methylation rate was lower in the high-risk group than in the low-risk group (P<0.05). The methylation rates at CpG_1, CpG_10, CpG_48 sites, score and the average methylation rate in the ALL-H (high) group ($\geq 50x10^9$/l) were lower than these in the ALL-NH (not high) group ($<50x10^9$/l) and the control group (P<0.05). We conclude that abnormal *MTRR* mRNA expression and the methylation of the *MTRR* promoter can be used to classify the risk of ALL in children.

Correspondence to: Dr Liansheng Zhang, Department of Hematology, Gansu Provincial Key Laboratory of Hematology, Second Hospital of Lanzhou University, 82 Cuiyingmen Road, Chengguan, Lanzhou, Gansu 730030, P.R. China
E-mail: zhanglsh@lzu.edu.cn

Professor Xiaodong Xie, Key Laboratory of Preclinical Study for New Drugs of Gansu Province, School of Basic Medical Sciences, Lanzhou University, 222 Tianshui South Road, Chengguan, Lanzhou, Gansu 730030, P.R. China
E-mail: xdxie@lzu.edu.cn

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Introduction

Acute lymphoblastic leukemia (ALL) is a hematological malignancy that originates from the abnormal proliferation of B-type or T-lymphocytes in the bone marrow (1). The disease manifests most commonly between 2-5 years of age (2,3) and accounts for 1/3 of all malignancies in children. Every year, 3-4/100,000 individuals are diagnosed with leukemia, and ALL accounts for 75% of all childhood leukemia cases (4). The long-term survival rate is 85-90%. ALL is the most common cancer that seriously jeopardizes the health and lives of children. More than 75% of children with ALL have genetic aberrations, including *ETV6-RUNX1* and *BCR-ABL1* gene translocation, *MLL* gene rearrangement (5-7), *ERG*, *IKZF1* and *CDKN2A/B* deletion, *CRLF2* overexpression, and *JAK1-3*, *PTEN*, *NOTCH1* and other gene mutations (8,9). In addition, it was found that epigenetics is an important aspect of transcriptional regulation of gene expression (10). At present, the occurrence of acute leukemia is the result of a combination of cytogenetics and epigenetics (11). In the development of leukemia, DNA methylation is an important epigenetic regulatory mechanism (12). The hypermethylation of CpG islands in the promoter region can downregulate genes or effect gene silencing (13). Genome-wide changes in DNA methylation and changes in specific targets cause the onset of leukemia and the appearance of specific phenotypes (11,14). The genetic and epigenetic analysis of children with ALL confirmed that changes in the methylation of DNA play a crucial role in the pathogenesis of leukemia and the mechanism of disease recurrence. DNA methylation serves as a molecular marker for predicting ALL recurrence (15-18). Although the response rate of childhood ALL treatment can reach >90%, the prognosis of ALL after relapse is very poor. Therefore, identification of new molecular markers for predicting the relapse of ALL will

greatly improve the efficacy of treatment in childhood ALL. DNA methylation is a potential therapeutic target and a potential molecular marker.

Folate metabolism is an important pathway linking epigenetics and DNA synthesis and is closely related to the occurrence of ALL (19,20). Several studies have shown that folic acid levels are significantly lower in ALL patients than in normal subjects, while homocysteine levels are significantly higher than normal (21,22). Mothers taking folic acid during pregnancy can effectively reduce the risk of ALL in children (23). ALL patients have disorders of folate metabolism. *MTRR* (5-methyltetrahydrofolate-homocysteine methyltransferase reductase) and *MTHFR* (methylenetetrahydrofolate reductase) are two of the most important genes in the folate metabolism pathway. ALL is characterized by overall hypomethylation and hypermethylation of some genes. Studies have found that low VB12 and MTRR activity are important factors for DNA hypomethylation (24). At present, studies on mRNA expression of the key enzymes MTRR and MTHFR in the folic acid metabolic pathway in children with ALL and the methylation of the gene promoter region have not been reported, nor has whether or not key enzymatic activity is reduced due to abnormal promoter methylation. The mechanism of folate metabolism is, therefore, worthy of intense study.

Patients and methods

Subjects

Children with ALL and healthy controls. In the present study, 20 subjects with ALL hospitalized at the Department of Hematology, Second Hospital of Lanzhou University (Lanzhou, China) from June 2016 to June 2017 and 20 healthy controls were recruited. The children with ALL were younger than 14 years of age, with an average age of 6.2 years, and the ratio of male to female patients was 1.4:1. We obtained complete clinical data, including bone marrow morphology, leukemia immunophenotyping, leukemia micro-residue detection, karyotype analysis and identification of leukemia-related genes. Clinical diagnosis, categorization and prognosis of ALL were based on the criteria from the 2014 (Fourth Revision) edition of the Chinese Journal of Pediatrics (Children with Acute Lymphoblastic Leukemia) (25). Of the 20 patients, there were 4 cases of T-cell acute lymphoblastic leukemia (T-ALL) and 16 cases of B-cell acute lymphoblastic leukemia (B-ALL). Among them, 16 samples were newly diagnosed and 4 samples were previously diagnosed as ALL recurrence. EDTA anticoagulated bone marrow specimens were acquired from the 20 patients.

The control group consisted of 20 samples of peripheral blood and normal EDTA anticoagulated bone marrow samples taken between June 2016 and June 2017 from healthy subjects. The average age among the control group was 5.8 years and the male to female ratio was 1.3:1. The karyotype analysis of the control group subjects was normal, and all members of the control group were less than 14 years of age. All patient and control samples were obtained after obtaining signed informed consent forms. This project was approved by the Ethics Committee of Lanzhou University Basic Medical College (201400105).

Extraction of genomic DNA from bone marrow smear samples. A total of 116 samples were collected from the patient and the control groups. Unstained bone marrow smear samples (n=83) were collected at room temperature from the newly diagnosed/relapsed children with ALL. Among these 83 samples, there were 14 cases of T-ALL and 69 cases of B-ALL. The control group consisted of 33 samples of peripheral blood and normal bone marrow.

MALDI-TOF MS detection of promoter methylation of the MTRR gene in bone marrow from ALL patients and control. A total of 88 samples meeting the criteria of DNA quality were tested by MALDI-TOF MS.

A total of 55 patients with ALL were recruited for the methylation analysis in the present study. More than 80% leukemia cells were present in the newly diagnosed and relapsed bone ALL marrow cases, including 37 newly diagnosed cases and 18 relapsed patients. The relapsed patients were not the same patients as the newly diagnosed patients. At the time of initial diagnosis, the bone marrow smear had been kept for a long time and the extracted DNA was severely degraded, so no subsequent methylation testing was performed. Among the 55 samples, 11 were T-ALL samples and 44 were B-ALL samples. Baseline characteristics of the study population are shown in Table I.

Methods

Cryopreservation of bone marrow samples. Approximately 1-2 ml of EDTA anticoagulated fresh bone marrow sample was collected and placed in a 15-ml sterilized V-bottom centrifuge tube. A total of 5 ml saline was added to the tube; 5 ml of lymphocyte separation solution was placed into another 15-ml centrifuge tube, and the diluted bone marrow sample was added carefully along the tube wall to the top of the lymphocyte separation layer. The specimen was centrifuged at 1,000 x g for 20 min. After centrifugation, the middle white membrane layer was carefully transferred to another clean centrifuge tube. Saline (10 ml) was added and the sample was centrifuged again at 1,000 x g for 8 min. The pellet was suspended in 10 ml saline, washed and centrifuged at 1,000 x g for 8 min. The cells (pellet) were washed a final time with 10 ml saline. The cells were resuspended in 500 μl of TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and mixed with a pipette tip and stored at -80˚C.

mRNA extraction. The frozen bone marrow samples were removed from the -80˚C refrigerator. Briefly, 500 μl of TRIzol suspended cells were thawed and 400 μl of chloroform was added to the tube. The mixture was shaken for 30 sec, and centrifuged at 12,000 x g at 4˚C for 15 min. After centrifugation, 400-500 μl of the upper aqueous layer was transferred into a 1.5-ml RNase-free EP tube. An equal volume of isopropanol was added, mixed gently and allowed to stand for 10 min at room temperature. The sample was centrifuged again at 12,000 x g at 4˚C for 10 min. The supernatant was removed and 75 μl of 75% ethanol (mixed with DEPC water) was added to the pellet, shaken vigorously to dislodge the pellet, and allowed to stand for 5 min. The sample was centrifuged again at 7,500 x g at 4˚C for 5 min. The liquid was discarded, using care not to disturb the pellet, and the tube was allowed to

Table I. Baseline characteristics of the study population (ALL patients, N=55).

Parameters	Data n (%)
Sex	
Male	32 (58.2)
Female	23 (41.8)
Therapeutic response	
Relapse	18 (32.7)
Persistant remission	37 (67.3)
Risk classification	
High-risk	26 (47.3)
Medium-risk	16 (29.1)
Low-risk	13 (23.6)
Peripheral white blood cells counts	
$\geq 50x10^9/l$	12 (21.8)
$<50x10^9/l$	43 (78.2)
Immune classification	
T-ALL	11 (20.0)
B-ALL	44 (80.0)

The presence of bone marrow leukemia cells was higher than 80% in ALL samples. ALL, acute lymphoblastic leukemia; T-ALL, T-cell acute lymphoblastic leukemia; B-ALL, B-cell acute lymphoblastic leukemia.

air-dry at RT for 20-30 min. The RNA concentration and purity were determined on a NanoDrop 2000 Protein Nucleic Acid analyzer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). RNA quality requirements were an OD_{260}/OD_{280} in the range of 1.8-2.1.

RT-qPCR. Total RNA was extracted from tissues and cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNA was reverse transcribed into cDNA using a Reverse Transcription reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). qPCR was performed using the SYBR-Green PCR kit (Takara Biotechnology Co., Ltd.). The PCR reaction conditions were as follows: Preliminary denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 20 sec. The relative quantification ($2^{-\Delta\Delta Cq}$) method was used for calculating fold change (26). The primer sequences are shown in Table II.

Detection of methylation of the MTRR gene by MALDI-TOF MS Potential CpG island forecast. Using the NCBI database (https://www.ncbi.nlm.nih.gov/), the sequence of the transcription initiation site of the *MTRR* gene was identified from the upstream 5,000 bp to the downstream 1,000 bp. The CpG island online prediction website (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/) was used to predict the sequence of the potential CpG island (Fig. 1A). After prediction, a total of 691 bp of CpG sites were found in 4335-5025, but 7 sites could not be detected due to sequencing problems. Forty-one CpG sites could be detected, and the actual coverage rate was 85.4%. The 5' primer sequence was aggaagagagTGG TAGAGTTAGGGTTTAAATTTAGGT, and 3' primer sequence was cagtaatacgactcactatagggagaaggctATACCCAA AACCAATAAAAAACTCC.

Extraction of genomic DNA from bone marrow smear (phenol-chloroform method). Extraction of gDNA from the bone marrow smears was performed using the method previously described by Smobook and Russell (27). Briefly, the bone marrow smears were selected from samples whose length of the blood film was not <1 cm. The blood film was carefully scraped into a 1.5-ml centrifuge tube with a sterile, clean disposable blade. Sterilized ultrapure water (1 ml) was added and mixed by inversion. The sample was centrifuged at 12,000 x g for 10 min and the supernatant was discarded. Residual water was removed by blotting with absorbent paper. A total of 300 μl of leukocyte lysate, 50 μl SDS and 10 μl proteinase K were added to the sample and the tube was placed in a 37˚C water bath for 14-16 h.

After the digestion was completed, the same volume of phenol was added, fully mixed and centrifuged at 12,000 x g for 10 min and absorbed. The supernatant was placed into the new centrifuge tube. The step was repeated once, and then the same volume of chloroform-isoamyl alcohol mixture (24:1) was added, gently reversed 5 min, centrifuged at 12,000 x g for 10 min, and the upper supernatant was added to the new centrifuge tube. One milliliter of anhydrous ethanol on ice that was precooled in advance was added, and then 1/10 volume of sodium acetate was added, reversed and mixed, and kept for 5 min at -20˚C, centrifuged at 12,000 x g for 10 min, and the supernatant was discarded. The DNA precipitate was washed with 70% ethanol, and the DNA was collected by centrifugation at 12,000 x g for 5 min. The supernatant was discareded, then the open tubes were maintained on the bench until any remaining ethanol had evaporated. The DNA was dissolved in 50 μl of TE (pH 8.0), by rocking it gently overnight at 4˚C. The DNA concentration and purity were determined on a NanoDrop 2000 Protein Nucleic Acid analyser (NanoDrop Technologies; Thermo Fisher Scientific, Inc.).

Purification and bisulfite modification. CT conversion reagents were prepared by adding 750 μl of water and 210 μl of M-Dilution buffer to the CT conversion reagent and shaking the mixture vigorously at room temperature for 10 min. The preparation was performed in the dark. The M-WASH concentrated solution (6 ml) was diluted with 24 ml of 100% ethanol and mixed well. The procedure of DNA sulfite treatment and Agena MassArray System Methylation Detection Amplification PCR reaction (Agena Bioscience, San Diego, CA, USA) were carried out as Popp *et al* described (28). Briefly, the PCR amplification reaction procedure was performed with the following parameters: Temperature time cycle: 94˚C for 4 min, followed by 45 cycles of 94˚C for 20 sec, 60˚C for 30 sec and 72˚C for 1 min and a final 72˚C for 3 min. The PCR products were stored at 4˚C. SAP enzyme digestion reaction, T-cut/RNase A digestion reaction and resin purification were performed following the Veriti 384-Well thermal cycler (Life Technologies; Thermo Fisher Scientific, Inc.).

Quantitative MassARRAY analysis of gene methylation status. A Nanodispenser was used to distribute 22 μl of the

Table II. Primer sequences of *MTRR*, *MTHFR* and *β-actin*.

Gene name	Forward primers	Reverse primers
MTRR	GTGCCTGCTTGTTGGATCTC	AGCCAGCCTGTACATACTCC
MTHFR	CCATCAACTCACAGCCCAAC	AGTTCAGGGGCATTGGTGAT
β-actin	CTCCATCCTGGCCTCGCTGT	GCTGTCACCTTCACCGTTCC

Figure 1. *MTRR* gene CpG island prediction and sequence information map. (A) Multiple CG sites were predicted after fragments were generated by digestion. The results show the average degree of methylation at the sites. Yellow is labeled as a detectable CG site and gray is an undetectable site. The sequence is a forward sequence. The circle represents a CpG site. The blue-labeled CpG sites could be detected while the red-labeled CpG sites were unable to be detected due to sequencing issues. (B) MALDI-TOF-MS provided the methylation intensity of each CpG site of the gene. Each sample in the Figure contains a certain number of CpG sites, and each CpG site shows the difference in methylation intensity with a different color. The yellow-to-blue transition corresponds to sample methylation intensity gradually increasing from 0 to 100%. Each small circle of a different color corresponds to a methylation rate. Some fragments could not be displayed due to a molecular weight >7,000 Da or <1,500 Da, and some methylation mass spectral peaks overlapped and could not be analyzed; these CpG sites in these cases are shown in gray.

cleaved transcripts onto a SpectroCHIP (Agena Bioscience GmbH), which was loaded with the reaction substrate. Gene mass spectrograms were acquired by MassARRAY using MALDI-TOF MS and analyzed using EpiTYER® software (v1.05) (Agena Bioscience). The methylation test results of the MALDI-TOF-MS mass spectrometer provided the methylation intensity of each CpG site of the gene, as shown in Fig. 1B. Each sample in the Figure contained a certain number of CpG sites, and each CpG site with a difference in methylation intensity is shown as a difference in color. The yellow to blue change corresponds to a sample methylation intensity gradually increasing from 0 to 100%, and each small circle of a different color corresponds to a methylation rate. In fact, not all fragments could be analyzed, nor the methylation status displayed. Some fragments could not be displayed due to excessive or too small molecular weights (>7,000 Da or <1,500 Da), and some methylation mass spectral peaks overlapped and could not be separated and analyzed. In these cases, the CpG sites were excluded (gray).

CpG methylation and total methylation rate were added as the total methylation value. The specific evaluation method was performed according to Table III.

Statistical analysis. The ΔCq value of each gene was calculated, and the Cq value of each gene was subtracted from the Cq value of the internal reference β-actin. The data of the case group were analyzed using the $2^{-\Delta\Delta Cq}$ method. Statistical analysis was performed using the SPSS v22.0 software (IBM Corp., Armonk, NY, USA). For methylation data analysis, an independent sample t-test or Mann-Whitney U test was used for comparison between the two groups. One-way analysis of variance (ANOVA) was used for comparison between the three groups. When compared between the two groups in these three groups, the variance homogeneity test was performed first.

Table III. Evaluation criteria of the gene methylation.

CpG methylation number (%)	Score	Methylation rate (%)	Score	Total score
<50	1	≤4	1	2
<50	1	4-10	2	3
<50	1	≥10	3	4
≥50	2	≤4	1	3
≥50	2	4-10	2	4
≥50	2	≥10	3	5

The Student-Newman-Keuls (SNK) method was used to test the difference between the two groups if the variables passed the variance homogeneity test. The Dunnett's T2 method was used to test the difference between the two groups for the variables did not pass the variance homogeneity test. P<0.05 was considered to indicate a statistically significant result.

Results

Expression of MTRR/MTHFR mRNA in bone marrow from ALL patients and healthy children. RT-qPCR was used to detect the expression of *MTRR* gene mRNA in bone marrow samples from 20 children with ALL and healthy controls. Fig. 2 shows the extracted RNA electrophoresis. After agarose gel electrophoresis, three bands of 28S, 18S and 5S were detected. The mRNA expression level of *MTRR* in the bone marrow from children with ALL was much lower than that in the healthy controls (P<0.05; Fig. 3A). However, no significant difference in the *MTHFR* mRNA level in the bone marrow was detected between children with ALL and the healthy controls (P>0.05; Fig. 3B).

Identification of genomic DNA extracted from bone marrow smears. Total genomic DNA from the bone marrow smears was extracted using the phenol-chloroform method. The results are shown in Fig. 4. There was a total of 55 samples in the first batch (Fig. 4A) and 61 samples in the second batch (Fig. 4B), showing good extraction results. The concentration of extracted DNA, A260/A280 ratio and A260/A230 bone marrow smear-sample preservation times were measured and analyzed (data not shown). Eighty-eight of the total 116 samples were qualified for further experiments, and 28 failed because of degradation and the lack of major bands. We found that the bone marrow smear-sample preservation time was critical. When the sample preservation time was <2 years, 98.86% (87/88) of the samples met the criteria of 260/230 ratio >0.6, the 260/280 ratio from 1.5 to 2.0, sample concentration >20 ng/μl, and total DNA >1 μg. However, when the samples were preserved >2 years, only 3.57% (1/28) of the samples met the criteria (Fig. 4).

Methylation of MTRR promoter by MALDI-TOF MS

Methylation of MTRR promoter region in bone marrow from children with ALL and control samples. The sulfite treatment of 88 samples of genomic DNA extracted from bone marrow smears was used to perform PCR amplification and

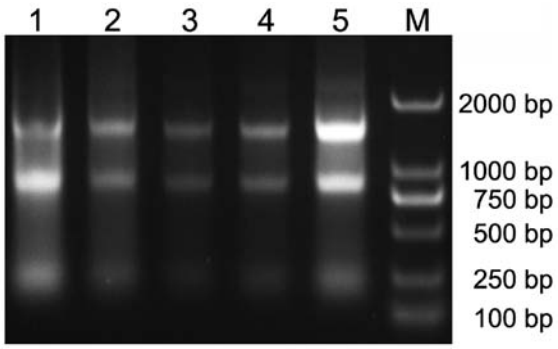

Figure 2. Extracted RNA electrophoresis and RT-qPCR amplification curves and dissolution profiles. RNA electrophoresis in 1.5% of agrose gel. Three bands of 28S, 18S and 5S were detected. Lane 1, sample 1; lane 2, sample 2; lane 3, sample 3; lane 4, control 1; lane 5, control 2; lane M, DNA marker.

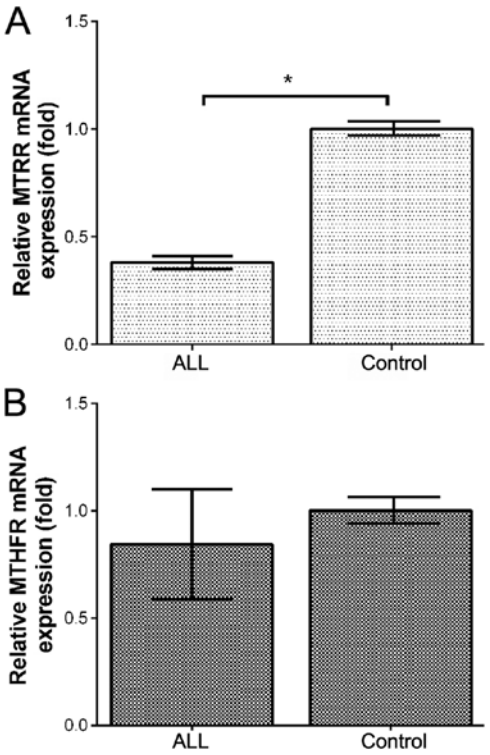

Figure 3. Expression of *MTRR*/*MTHFR* mRNA in bone marrow from children with ALL and controls. The average mRNA expression of (A) MTRR and (B) MTHFR in bone marrow from 20 ALL patients and from healthy controls. *P<0.05. ALL, acute lymphoblastic leukemia.

followed by gel electrophoresis to analyze the PCR products. The 88 amplified samples were qualified to perform further MALDI-TOF MS analysis. The comparison of methylation in the *MTRR* gene promoter region was performed on bone marrow from 55 children with ALL and 33 health controls. Most of the CpG units had a low level of methylation. None of them conformed to the normal distribution, and, thus, the Mann-Whitney U test was used. The results (Table IV) showed that there were no significant differences in the methylation rates, 41 total methylation sites, score, average methylation rates, or overall methylation levels of the 41 CpG sites in the promoter region of the *MTRR* gene between ALL patients and healthy controls (P>0.05).

Table IV. Methylation differences in CpG loci of the *MTRR* promoter region in bone marrow from children with ALL and healthy controls.

	Methylation rate		
MTRR CpG loci	ALL (n=55)	Controls (n=33)	P-value
CpG_1	7.47±9.579	5.59±6.962	0.391
CpG_2.3	1.59±1.645	2.76±3.113	0.065
CpG_6	3.67±2.358	3.76±2.747	0.896
CpG_7.8	8.67±7.096	10.45±9.128	0.522
CpG_9	1.47±2.433	1.48±1.920	0.775
CpG_10	7.47±9.579	5.59±6.962	0.391
CpG_11.12.13.14	2.84±2.641	3.14±2.532	0.541
CpG_15.16.17	2.45±2.467	2.59±1.783	0.378
CpG_18.19.20	6.73±4.086	5.59±3.708	0.305
CpG_21	3.55±6.776	2.66±4.654	0.937
CpG_22.23.24.25	1.45±1.276	2.07±1.412	0.052
CpG_26	0.20±0.499	0.86±2.722	0.696
CpG_27	9.63±14.915	8.03±14.647	0.436
CpG_28.29	1.59±1.645	2.76±3.113	0.065
CpG_30.31	2.86±1.443	2.76±1.976	0.366
CpG_32.33.34	9.33±3.526	9.59±3.841	0.815
CpG_35.36.37	2.49±2.575	2.69±2.892	0.878
CpG_42.43.44	1.10±1.141	1.83±2.221	0.354
CpG_46.47	0.86±1.339	0.72±0.996	0.930
CpG_48	3.04±2.189	3.24±2.340	0.590
Methylation sites	11.57±1.720	12.21±2.932	0.188
Average methylation rate	3.92±1.303	3.91±1.638	0.788
Total methylation level	3.08±0.449	3.00±0.655	0.547
Score	1.90±0.306	1.83±0.384	0.372

ALL, acute lymphoblastic leukemia.

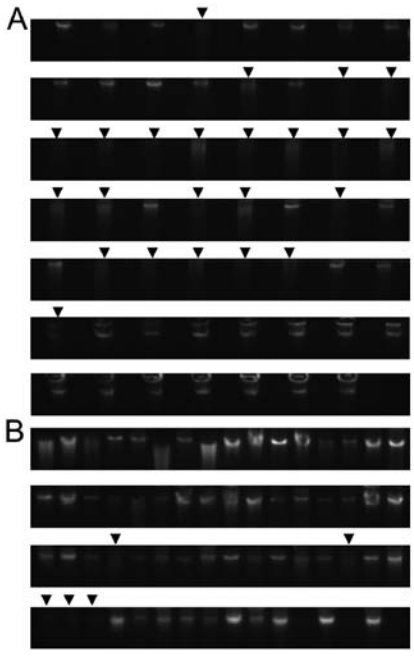

Figure 4. Electrophoresis results of genomic DNA extracted from bone marrow smear samples. (A) The first batch of 55 samples. (B) The second batch of 88 samples. The black arrow denotes unqualified sample because of the low concentration, no major band, or degradation (failed to qualify in this study).

Methylation rates of 41 CpG sites in the promoter regions of MTRR genes were found in male and female children with ALL, as well as in normal boy and girl controls. There was no statistically significant difference in the rate of totalization and methylation (P>0.05) (data not shown).

Methylation of the MTRR promoter region in bone marrow from children with T-ALL, B-ALL and healthy control groups. Methylation analysis of the 41 CpG loci in the *MTRR* gene was performed to compare 44 cases of children with B-ALL, 11 children with T-ALL and 33 health controls. The results showed that there were no significant differences in the methylation rates, overall methylation sites, score, average methylation rates or overall methylation levels of CpG loci in the *MTRR* gene (P>0.05) (data not shown).

Methylation of the MTRR gene promoter region in bone marrow from children with high, medium and low risk of ALL. After grading the risk of 55 ALL patients, there were 26 children in the high-risk, 16 in the medium-risk and 13 in the low-risk groups. In the comparison of each two-group pairing, the CpG_6 methylation rate was higher in the low-risk group than in the medium-risk group, while the methylation rate of CpG_46.47 was lower in the low-risk group than in the

Table V. Analysis of methylation differences of CpG loci in the *MTRR* gene promoter region in bone marrow from high-, medium- and low-risk ALL patients.

	P-value		
MTRR CpG site	H-risk (n=26)/M-risk (n=16)	H-risk (n=26)/L-risk (n=13)	M-risk (n=16)/L-risk (n=13)
CpG_1	0.491	0.797	0.737
CpG_2.3	0.450	0.822	0.360
CpG_6	0.319	0.060	0.014[a]
CpG_7.8	0.933	0.905	0.978
CpG_9	0.710	0.716	0.999
CpG_10	0.491	0.797	0.737
CpG_11.12.13.14	0.174	0.343	0.776
CpG_15.16.17	0.630	0.470	0.872
CpG_18.19.20	0.120	0.861	0.097
CpG_21	0.761	0.982	0.822
CpG_22.23.24.25	0.573	0.554	0.941
CpG_26	0.477	0.042[a]	0.093
CpG_27	0.800	0.636	0.546
CpG_28.29	0.450	0.822	0.360
CpG_30.31	0.192	0.608	0.132
CpG_32.33.34	0.179	0.080	0.876
CpG_35.36.37	0.488	0.243	0.661
CpG_42.43.44	0.897	0.049[a]	0.096
CpG_46.47	0.561	0.038[a]	0.012[a]
CpG_48	0.471	0.176	0.503
Methylation sites	0.674	0.705	0.944
Average methylation rate	0.628	0.340	0.213
Total methylation level	0.845	0.264	0.082
Score	0.931	0.868	0.947

ALL, acute lymphoblastic leukemia. [a]P <0.05, significant difference.

medium-risk group (P<0.05). The CpG_26 and CpG_46.47 methylation rates were higher in the high-risk group than in the low-risk group, while the CpG_42.23.44 methylation rate was lower in the high-risk group than in the low-risk group (P<0.05). There was no significant difference in CpG locus or methylation between the high-risk group and the medium-risk group (P>0.05) (Table V and Fig. 5A).

Methylation of the MTRR promoter region in bone relapse and non-relapse groups in children with ALL. Methylation analysis was performed on the 41 CpG sites in the promoter region of *MTRR* in 18 patients presenting with relapse and 37 patients without relapse. After normal distribution analysis of the methylation rate data of each point, CPG32/33/34 showed a normal distribution (P>0.05), and the Student's t-test was applied. The other groups did not demonstrate a normal distribution (P<0.05) and the Mann-Whitney U test was used. There were no significant differences in the 41 total methylation sites, score, average methylation rates, or overall methylation levels of the 41 CpG sites in the promoter region of the *MTRR* gene in recurrent and non-recurrent children (P>0.05) (data not shown).

Methylation of the MTRR promoter region in bone marrow from ALL children with peripheral white blood cells ≥50x10^9/l and peripheral blood leukocytes <50x10^9/l. Based on studies included in the Interpretation of the Recommendations for the Diagnosis and Treatment of Children with Acute Lymphocytic Leukemia (Four Revisions) (25) and Genetic Basis of Acute Lymphoblastic Leukemia (29), peripheral white cell count is an important predictor of prognostic stratification. Peripheral white blood cells ≥50x10^9/l suggest a poor prognosis. Therefore, patients having peripheral white blood cells ≥50x10^9/l were defined as the ALL-H (ALL-High) group, and those with peripheral white blood cells <50x10^9/l were defined as ALL-NH (ALL-Not High) group.

Methylation analysis was performed on the 41 CpG sites in the promoter region of *MTRR* in bone marrow from 12 ALL-H children, 43 ALL-NH children and 33 healthy controls. We found that the average methylation rates and mean methylation rates of the CpG_1, CpG_10, CpG_48 sites and score in the ALL-H patients were lower than in the ALL-NH patients and healthy controls (P<0.05). However, there were no significant differences in CpG locus, score, or methylation between the ALL-NH patients and the healthy controls (Fig. 5B and Table VI).

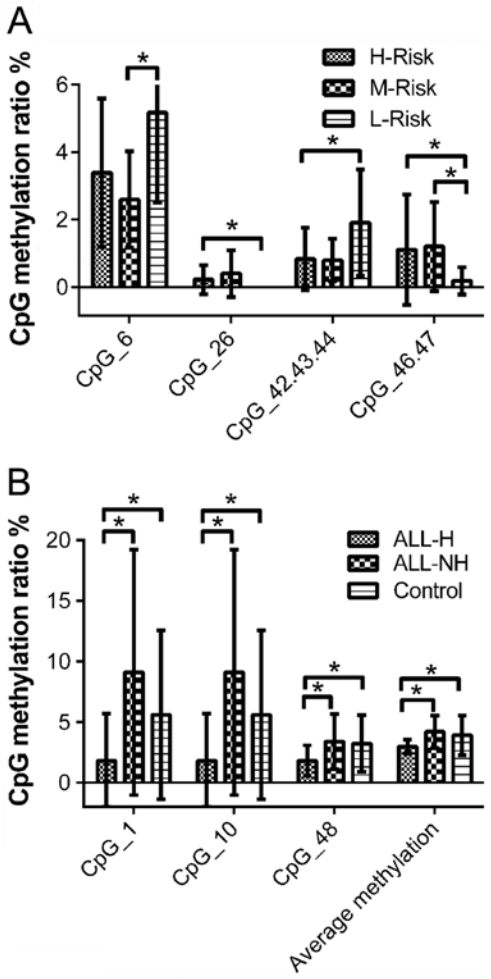

Figure 5. Analysis of differential methylation histogram of CpG locus in the *MTRR* gene. (A) Methylation rates of CpG_6, CpG_26, CpG_42.43.44 and CpG_46.47 in bone marrow from children with high- and medium-risk ALL and from control samples. (B) Methylation rate of CpG_1, CpG_10, CpG_48 and average methylation rates in bone marrow from ALL-H, ALL-NH and healthy controls. *P<0.05. ALL, acute lymphoblastic leukemia; ALL-H, ALL-High; ALL-NH, ALL-Not High.

Discussion

Acute lymphoblastic leukemia (ALL) accounts for 80% of all cases of childhood leukemia. Therefore, ALL is the most commonly diagnosed tumor that endangers the health of children worldwide. Moreover, the proportion of bone marrow leukemia cells is >80% in ALL. The high proportion of leukemia cells is highly appropriate for methylation research. Some studies have shown that low expression levels of folate and elevated levels of homocysteine are noted in ALL (21,22), but the mRNA levels of *MTRR* and *MTHFR* genes in ALL have not been reported. In the present study, we found that only the mRNA level of *MTRR* in children with ALL was lower than that in the healthy controls, but no significant difference in the *MTHFR* gene was detected between the two groups. The reason for the low level of folate is probably due to the inactivation of *MTRR* with the low transcription level of MTRR. In general, MTR and VB12 jointly maintain the activity of MTR. After MTR is inactivated, the ability of THF to be reduced to 5mTHF, which provides one carbon unit to methionine, and then to SAM for methylation donation, is insufficient to make up the methylation body. Most tumors show a decrease in the overall gene methylation levels and an increase in the methylation levels of specific genes. The decrease of intracellular methylation donors results in a decrease in the methylation level of the genome. MTHFR has a strong compensatory feedback regulation (30). When the body is deficient in folic acid and HCY is accumulated, it compensates for the synthesis of 5mTHF to supplement THF and MET required for DNA synthesis and methyl-donor to alleviate the accumulation of HCY. The high feedback capacity of MTHFR may account for why the mRNA level of this gene is not significantly different between the ALL and control groups. Similar results have been found in preeclampsia studies (31). The level of MTRR expression in patients with preeclampsia is significantly lower than that noted in healthy controls, but MTHFR expression is not significantly different between patients and controls. After eclampsia, it is difficult to survive. More than 90% of ALL occurs in infants and young children. Some studies have confirmed that infant ALL originates before birth and embryos with prenatal exposure to toxic compounds may suffer genetic changes during the developmental process (32). Therefore, the *MTRR* gene plays an important role in the development of the fetus and children. Immunostaining of tissue microarrays showed that 517 human tumor tissues had different levels of MTRR protein expression, most of which were expressed at low levels, suggesting that *MTRR* also plays an important role in tumorigenesis (33). The decrease of mRNA levels of *MTRR* leads to a deficiency of the MTRR enzyme and a sufficient amount of MTRR cannot be activated, which may be the cause of the decrease of folate and the accumulation of HCY in cases of ALL.

Wong *et al* (34) confirmed that archived bone marrow smear samples were suitable for methylation sequencing. Court *et al* (35) reported that no differences in gene mutation analysis and polymorphism screening were detected from DNA extracted from bone marrow smears with phenol/chloroform/isoamyl alcohol compared to DNA extracted from frozen-preserved cells using a commercial extraction kit. We used MALDI-TOF MS to detect the methylation of 48 CpG sites in the promoter region of the *MTRR* gene (4335-5025 position 691 bp) in bone marrow from children with ALL and from healthy controls. Among them, 7 CpG sites could not be detected due to sequencing problems. Forty-one CpG sites were identified, and the number of methylation sites, average methylation rate, and overall methylation levels were evaluated. The results showed that there were no significant differences in the CpG locus or methylation levels of the *MTRR* gene in the bone marrow from children with ALL compared with the controls, suggesting that the differences in the downregulation of the *MTRR* gene mRNA levels were not caused by methylation, but may be due to other mRNA level regulatory factors, such as miRNA, lncRNA, transcription factors, RNA binding proteins, post-transcriptional regulation, or abnormal chromatin remodeling. Notably, there were no significant differences in the methylation or methylation status of the *MTRR* gene promoter between the relapsed and non-relapsed ALL patients and no significant differences were found in T-ALL and B-ALL compared to the healthy controls. Therefore, *MTRR* cannot be used as a molecular indicator for the diagnosis, immunophenotyping and assessment of recurrence for children with ALL.

Table VI. Analysis of methylation status of CpG loci in the MTRR gene promoter region in bone marrow from ALL-H, ALL-NH and healthy controls.

	P-value		
MTRR CpG loci	ALL-H (n=12)/ALL-NH (n=43)	ALL-H (n=12)/Controls (n=33)	ALL-NH (n=43)/Controls (n=33)
CpG_1	0.001[a]	0.038[a]	0.114
CpG_2.3	0.917	0.241	0.075
CpG_6	0.071	0.186	0.703
CpG_7.8	0.463	0.292	0.501
CpG_9	0.661	0.642	0.905
CpG_10	0.001[a]	0.038[a]	0.114
CpG_11.12.13.14	0.878	0.635	0.686
CpG_15.16.17	0.488	0.647	0.607
CpG_18.19.20	0.746	0.281	0.283
CpG_21	0.801	0.813	0.495
CpG_22.23.24.25	0.987	0.223	0.063
CpG_26	0.610	0.485	0.195
CpG_27	0.364	0.674	0.490
CpG_28.29	0.917	0.241	0.075
CpG_30.31	0.403	0.541	0.991
CpG_32.33.34	0.506	0.456	0.993
CpG_35.36.37	0.545	0.812	0.650
CpG_42.43.44	0.354	0.159	0.164
CpG_46.47	0.366	0.323	0.885
CpG_48	0.034[a]	0.019[a]	0.788
Methylation sites	0.801	0.296	0.335
Average methylation rate	0.000[a]	0.012[a]	0.428
Total methylation level	0.210	1.000	0.461
Score	0.023[a]	0.023[a]	0.648

ALL, acute lymphoblastic leukemia. ALL-H, ALL-High; ALL-NH, ALL-Not High. [a]P<0.05, significant difference.

After risk classification of children with ALL, we found that risk classification can be performed on high-risk ALL and low-risk ALL by combined *MTRR* gene promoter CpG_6, CpG_26, CpG_42.23.44, and CpG_46.47 methylation markers. The reason for the high- or low-level of methylation sites in the promoter region of *MTRR* gene in high-risk and low-risk ALL groups may be due to the current risk stratification factors of ALL, including the heterogeneity of different fusion genes, heterogeneity of different leukocyte immunophenotypes, or heterogeneity of drug sensitivity in different populations. Thus, although patients were classified to the same risk category by the current risk assessment, they had different individual heterogeneity and should be subdivided according to detailed grading factors. However, there was a limited number of clinical samples available in this experiment and they could be divided only temporarily according to the current clinical risk classification. In a follow-up study, the clinical sample size should be expanded, and the correlation between the *MTRR* promoter methylation and the various types of ALL should be explored in more detail.

In the ALL-H group, the promoter region of the *MTRR* gene was demethylated mainly through the CpG_1, CpG_10 and CpG_48 sites, which reduced the overall methylation levels of the gene and activated the *MTRR* gene. This contrasts with the RT-qPCR experiment in the present study. The possible reason is that most of the fresh bone marrow samples collected by the experiment were related to ALL-NH, and there were only 2 cases of ALL-H in 20 cases of ALL. It was difficult to carry out statistical difference analysis, and more samples are necessary to verify the above hypothesis. The ALL-H group is clinically different from other types of ALL and is often classified into the medium-high risk group due to poor prognoses. However, by analyzing the methylation of the *MTRR* gene in the medium-high-risk and the low-risk groups, data (Fig. 5A) confirmed that the medium-high-risk group had greater methylation consistency. However, the differential methylation sites CpG_6, CpG_26, CpG_42.23.44, and CpG_46.47 appear to be inconsistent with the CpG_1, CpG_10 and CpG_48 sites in the ALL-H group. The reason is that the differences in the CpG_1, CpG_10 and CpG_48 sites are only seen in ALL-H, but the addition of other grading criteria increases the clinical heterogeneity of the disease, resulting in the absence of differential sites. Whether these differential sites have the potential to supplement the molecular markers of future individualized stratification based on high white blood cell counts requires validation with a larger number of clinical samples. Additionally, the treatment

plan for ALL-H is different from other ALL types. It is necessary to reduce the high load of white blood cells and prevent tumor lysis syndrome. This often results in a very high mortality rate for leukemia patients (36). Decreasing the methylation of the *MTRR* gene promoter causes activation of the gene in ALL-H. This possible mechanism provides a basis for the treatment of high leukocyte acute lymphoblastic leukemia patients by using MTRR-targeting inhibitors to reduce tumor burden during treatment.

In the present study, the phenol-chloroform extraction was highly efficient for the extraction of genomic DNA from the bone marrow smears within 2 years of acquisition. When the preservation time of the bone marrow smears was greater than 2 years, we found that most of the samples showed obvious degradation, where the main band was unclear and the genomic DNA bands were diffuse. This result indicated that the routine preservation of bone marrow smears at room temperature should not exceed 2 years in order to meet the experimental requirements. The extraction of gDNA may have been a reason for the poor concentration of samples. The present study showed, however, that bone marrow smear samples can be preserved easily for a considerable time, is convenient for large-scale and disease-related specimens to be retrospectively studied, and have great application prospects.

In conclusion, we found that the levels of *MTRR* mRNA expression were significantly lower in ALL patients than in the control samples. After stratification of risk, we found that the methylation rates of CpG_6, CpG_26, CpG_42.23.44 and CpG_46.47 sites were statistically different between the high-/medium- and low-risk groups. These sites provide new molecular markers for risk stratification of childhood ALL. The methylation rate of CpG_1, CpG_10, CpG_48 and score in the ALL-H group was lower than in the ALL-NH and control groups, suggesting a correlation with peripheral blood leukocyte proliferation. The methylation of the *MTRR* gene promoter region can be used to classify the risk of ALL in children.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

XX and LZ conceived and designed the experiments. JB and LL performed the experiments. JB, YL and QC analyzed the data and wrote the paper. JB revised the paper. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Lanzhou University Basic Medical College (201400105). All patient and control samples were obtained after obtaining signed informed consent forms.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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