MicroRNA-31 is a potential biomarker for screening B-lymphoblastic leukemia in children

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Abstract. The present study aimed to investigate the expression and significance of microRNA-31 (miR-31) in children with acute B-lymphoblastic leukemia (B-ALL). Bone marrow specimens and peripheral blood were collected from children with B-ALL (n=38) and healthy controls (n=18). Total RNA was extracted and the expression levels of miR-31 were measured using quantitative PCR. In addition, a receiver operating characteristic curve was generated, and the area under the curve (AUC) was calculated to evaluate the diagnostic value of miR-31 for the development of B-ALL. miR-31 expression was significantly lower in the B-ALL group compared with in the control group (P<0.05). Additionally, the expression levels of miR-31 in the B-ALL group before treatment were markedly lower than in the B-ALL group after treatment, and miR-31 expression was significantly lower after 30 days of treatment compared with after 12 weeks of treatment. Furthermore, miR-31 expression in the group of children ≥10 years of age was higher than that in the group of children <10 years of age. Furthermore, the expression levels of miR-31 were higher in the low-risk group compared with in the medium- and high-risk groups (P<0.05). When the cutoff value was set at 1.8, the AUC of miR-31 for B-ALL diagnosis was 0.915 (95% CI, 0.828-1.000; P<0.0001), with a sensitivity and specificity of 80.8 and 100%, respectively. In conclusion, miR-31 may exert an anticancer role in B-ALL in children and may be a potential marker to assist in diagnosis and prognostic prediction.

Introduction

Acute lymphoblastic leukemia (ALL) is a heterogeneous hematological malignancy characterized by the abnormal proliferation of primitive and immature cells in the bone marrow (1). ALL is the most common hematologic malignancy in children, accounting for 29%, and it is relatively rare in adults (2,3). Out of all children with ALL, 85% have B-lymphoblastic leukemia (B-ALL) (4). It is suggested that the development of chemoresistance is a key regulator for relapse among children (4,5). Therefore, further understanding of the pathogenesis of B-ALL is of great significance to improve the survival rate of patients (6).

French-American-British (FAB) (7) and Morphology-Immunology-Cytogenetics-Molecular Biology (MICM) (8) classification are the two main diagnostic methods for acute leukemia (9). The former is easy to perform and does not require expensive instruments and equipment, but it is strongly subjective and has low classification accuracy, which makes it impossible to achieve standardization and automation (10,11). The MICM typing method, which was introduced by the World Health Organization, is more comprehensive than the simple morphological typing method (10,11). However, the performance of MICM is cumbersome and expensive. The MICM and FAB morphological types require lumbar puncture to obtain test samples, which increases the pain and economic burden of the patient (10,11). Therefore, searching for biomarkers for noninvasive diagnosis and typing of acute leukemia has become a research focus (12).

The discovery of microRNAs (miRNAs/miRs) represents a milestone in furthering the understanding of human cancer (13,14). miRNAs have been demonstrated to be influential factors in the pathogenesis of B-ALL (15,16). Numerous studies have identified roles of miRNAs in the diagnosis, treatment, classification, prognosis and risk assessment of tumors (16-18). Studies have revealed that miRNAs are involved in the occurrence and development of acute leukemia (18,19). For example, miR-652-3p levels are significantly increased in the bone marrow of patients with ALL compared with in healthy controls, and miR-652-3p enhances the progression of ALL by decreasing apoptosis and reducing sensitivity to chemotherapeutic drugs (18). In addition, miR-9 inhibits ALL cancer cell proliferation and cell cycle progression by targeting neuropilin-1 (19). A previous study demonstrated that miR-31 expression is decreased in chronic myeloid leukemia (CML) cells compared with in controls (20). In adult T cell leukemia, polycomb proteins lead to miR-31 downregulation in an epigenetic manner, thereby activating the NF-κB signaling

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pathway and inducing apoptosis resistance (21). At present, to the best of our knowledge, no publication has described miR-31 expression in patients with B-ALL. Therefore, the present study aimed to explore whether miR-31 is involved in the progression of B-ALL.

In the present study, miR-31 expression was evaluated in children with B-ALL before and after treatment, and the possible mechanism of miR-31 in children with B-ALL was explored.

Materials and methods

General information. Children (n=38) with newly diagnosed B-ALL (excluding mature B-ALL), according to the MICM classification, who were admitted to Hongqi Hospital Affiliated to Mudanjiang Medical University between April 2017 and December 2017, were designated as the B-ALL group. Inclusion criteria were as follows: Infants diagnosed as B-ALL by MICM classification and peripheral blood collected prior to chemotherapy. Exclusion criteria were as follows: History of malignant blood disease, immune diseases and other tumors, and history of severe infection, trauma or surgery prior to admission. All patients met the criteria set by the National Conference on Pediatric Leukemia (9). There were 20 male patients and 18 female patients, with an average age of 5.6 years (range, 1.2-16.5), in the B-ALL group. Among the patients, 26 were <10 years old and 12 were ≥10 years old. A risk assessment (22) was carried out, in which the risk of leukemia was stratified according to the age of pediatric patients at initial diagnosis, peripheral leukocyte count, abnormalities in cytogenetics and molecular biology, whether they were sensitive to prednisone, bone marrow remission status at 33 days and the minimal residual disease at 33 days. Based on the aforementioned assessment, 17 cases were classified as low risk, 10 as medium risk and 11 as high risk (including four cases of recurrence, 2 in medium risk and 2 in high risk). The treatment regimen was based on the Chinese Children's Leukemia Group (CCLG)-2008 protocol (modified Berlin-Frankfurt-Münster ALL-95 protocol) (23). Patients were followed up until December 31, 2018, and the median follow-up time was 14.7 months (range, 3.2-19.8 months). The 14 month-survival rate for patients with pediatric B-ALL was 84.3%. Healthy controls (n=18) treated at the Physical Examination Center of Hongqi Hospital Affiliated to Mudanjiang Medical University, due to suspicion of B-ALL that was not further confirmed, were designated as the control group. The control group were recruited between April 2017 and December 2017, including 10 male patients and 8 female patients, with an average age of 6.2 years (range, 1.5-16.4).

Therapeutic regimen. All pediatric patients were treated according to the CCLG-2008-ALL protocol; chemotherapy regimens with different intensities were administered according to the different risk degrees, as follows (23): i) Remission induction: Pretreatment with prednisone for 7 days, vincristine, daunorubicin, L-asparaginase or pegaspargase, dexamethasone (VDLD) regimen; ii) early intensive treatment: Cyclophosphamide, cytarabine, 6-mercaptopurine (6-MP) or thioguanine (CAM) program (low risk group, one round; medium and high risk groups, two rounds); iii) consolidation therapy: Low risk and medium risk groups, HD-methotrexate (MTX) + 6-MP program (2.0 g/m^2 MTX in low risk group; 5.0 g/m^2 MTX in medium risk group); high risk group, two rounds of treatment of the aforementioned regimen; iv) delayed enhancement: The same VDLD and CAM programs as aforementioned [two rounds of delayed enhancement for medium risk group (one round of maintenance chemotherapy between the two rounds of delayed enhancement)]; and v) maintenance treatment: 6-MP + MTX. The single or triple chemotherapy drugs were regularly injected intrathecally to prevent central nervous system leukemia. All patients responded well to the therapeutic regimen.

Reverse transcription-quantitative PCR (RT-qPCR). A total of 5 ml peripheral blood samples and 3 ml bone marrow were collected prior to treatment, and subsequently 30 days and 12 weeks after treatment completion. In the control group, 5 ml peripheral blood samples and 3 ml bone marrow were collected at admission at -20˚C. Heparin anticoagulant was added to the bone marrow, and Ficoll-Hypaque lymphocyte separating liquid was added. Subsequently, mononuclear cells from bone marrow were separated by density-gradient centrifugation at 2,000 x g at 4˚C for 20 min.

Total RNA was isolated from the whole blood samples (5 ml; collected in tubes containing EDTA) or mononuclear cells using RNAVzol LS (Vigorous Biotechnology Beijing Co., Ltd.), according to the manufacturer's protocol. The concentration and purity of RNA samples were determined by measuring the optical density (OD) 260/OD280.

A total of 1 μg RNA was reverse transcribed using Moloney murine leukemia virus RT enzyme (Applied Biosystems; Thermo Fisher Scientific, Inc.) with specific primers. The temperature protocol used for RT was as follows: 72˚C for 10 min, 42˚C for 60 min, 72˚C for 5 min and 95˚C for 2 min. To quantify the relative expression levels, qPCR was performed using SYBR Green Supermix (Bio-Rad Laboratories, Inc.) in an iCycleriQ real-time PCR detection system (Bio-Rad Laboratories, Inc.). PCR amplification was performed in a 10 μl reaction mixture containing 5 μl SYBR Green Supermix, 0.4 μl forward primer, 0.4 μl reverse primer, 2.2 μl double-distilled H_2O and 2 μl template cDNA. Thermocycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec, 60˚C for 1 min, and 72˚C for 15 sec. Dissolution curve with only one peak for miR-31 and U6 confirmed that there was no contamination in the experiment, which ensured the test efficiency. Relative expression was normalized to that of U6 using the $2^{-\Delta\Delta Cq}$ method (24). Primer sequences were as follows: miR-31-RT, 5'-GTCGTATCCAGTGCAGGGTCC GAGGTATTCGCACTGGATACGACAGCTATG-3'; U6-RT, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACAAAATG-3'; miR-31, forward 5'-GCGCAGGCA AGAUGCUGGC-3'; U6, forward 5'-GCGCGTCGTGAAGCG TTC-3'; and universal reverse primer, 5'-GTGCAGGGTCCG AGGT-3'.

Statistical analysis. Data are presented as the means ± SD. Each experiment was performed in triplicate. SPSS version 13.0 (SPSS, Inc.) was used to perform statistical analysis. A two-tailed unpaired Student's t-test was used for comparisons of two groups. One-way ANOVA followed by the Tukey post hoc test was used for comparisons of more than two groups. A receiver operating characteristic (ROC) curve for diagnostic

B-ALL was generated, and the area under the curve (AUC) was calculated. The AUC range is 0.5-1; it is generally considered that the AUC has low diagnostic value when it is 0.5-0.7, medium diagnostic value when it is 0.7-0.9 and high diagnostic value when it is >0.9. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-31 expression is decreased in the peripheral blood of patients with B-ALL. The expression levels of miR-31 in patients with B-ALL before treatment were compared with those of patients in the healthy control group. The results revealed that miR-31 expression in the peripheral blood of patients with B-ALL was 0.33±0.21, and that in the healthy control group was 1.00±0.36 (Fig. 1A; P<0.001). The present study further evaluated the expression levels of miR-31 according to white blood cell (WBC) count at initial diagnosis, by dividing patients into WBC <50x10^9/l and WBC ≥50x10^9/l groups. As shown in Fig. 1B, the expression levels of miR-31 were significantly reduced in patients with B-ALL with WBC ≥50x10^9/l (0.51±0.28; n=23) compared with in patients with WBC <50x10^9/l (1±0.54; n=15; P<0.001).

miR-31 may be used to differentiate patients with B-ALL from healthy controls. Subsequently, a ROC curve was plotted for miR-31 expression and the diagnosis of B-ALL, and the AUC was calculated. The present study revealed that when the cutoff value was 0.18, the AUC of miR-31 for the diagnosis of B-ALL was 0.915 (95% CI, 0.828-1.000; P<0.0001), with a sensitivity and specificity of 80.8 and 100%, respectively (Fig. 2).

miR-31 expression is lower in patients <10 years old. The present study further divided the patients with B-ALL according to age, including 26 patients who were younger than 10 years and 12 who were older than 10 years. As shown in Fig. 3, the expression levels of miR-31 before treatment in the peripheral blood (0.44±0.12; n=26) and mononuclear cells (0.38±0.15; n=26) were markedly lower in the group of children <10 years old compared with in the group of children >10 years old (1±0.42; n=12; and 1±0.38; n=12, respectively).

miR-31 expression is increased in patients with B-ALL after treatment. Furthermore, the present study compared the expression levels of miR-31 before treatment (n=38) and after treatment for 30 days (n=38) or 12 weeks (n=36). A total of 36 patients were analyzed in the 12 weeks group as two patients succumbed to the disease. qPCR analysis demonstrated that miR-31 expression in peripheral blood was 0.33±0.21 before treatment, whereas miR-31 expression was 0.68±0.17 after treatment for 30 days and 0.92±0.28 after treatment for 12 weeks (Fig. 4A). Additionally, the data demonstrated that miR-31 expression was lowest in mononuclear cells in the group before treatment (0.28±0.15). Conversely, miR-31 expression gradually increased in the mononuclear cells after treatment for 30 days (0.62±0.16) and 12 weeks (0.87±0.23; Fig. 4B).

Lowest expression levels of miR-31 are observed in the high-risk group. Additionally, the present study evaluated the expression levels of miR-31 before treatment according to a risk assessment. The present study identified 17, 10 and 11 cases with low, medium and high risk, respectively. As shown in Fig. 5, the expression levels of miR-31 were higher in the peripheral blood (1±0.23) and mononuclear cells (1±0.28) of the low-risk group compared with in the medium-risk (0.61±0.18 and 0.58±0.13) and high-risk (0.35±0.16 and 0.28±0.10) groups. No differences were identified between male and female patients (data not shown).

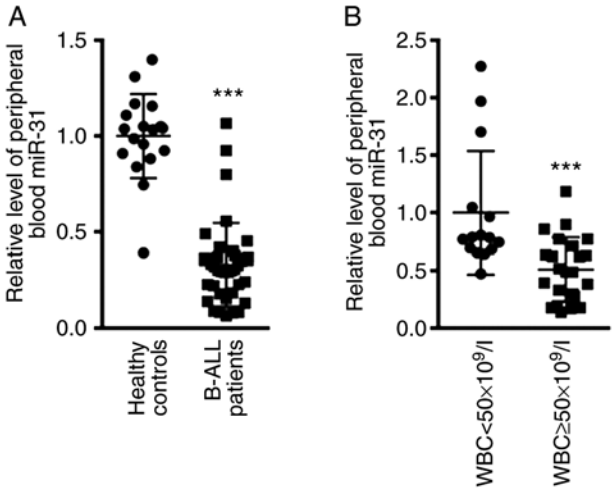

Figure 1. Quantitative PCR analysis indicates that miR-31 expression is reduced in patients with B-ALL compared with in controls. (A) miR-31 expression was decreased in the peripheral blood of patients with B-ALL compared with in healthy controls. (B) miR-31 levels were significantly reduced in patients with B-ALL with WBC ≥50x10^9/l (n=23) compared with in patients with WBC <50x10^9/l (n=15). ***P<0.001. B-ALL, B-lymphoblastic leukemia; miR-31, microRNA-31; WBC, white blood cells.

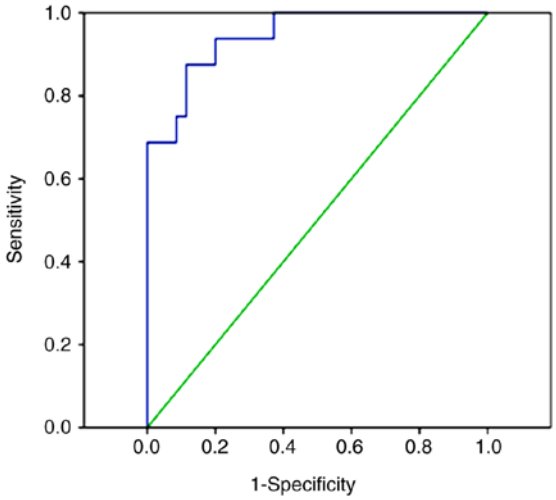

Figure 2. Receiver operating characteristic curve analysis demonstrates that microRNA-31 may differentiate patients with B-lymphoblastic leukemia from healthy controls.

Discussion

The present study first demonstrated that the expression levels of circulating miR-31 in patients with B-ALL were significantly decreased compared with those in the healthy control group, suggesting that miR-31 could be used as a diagnostic

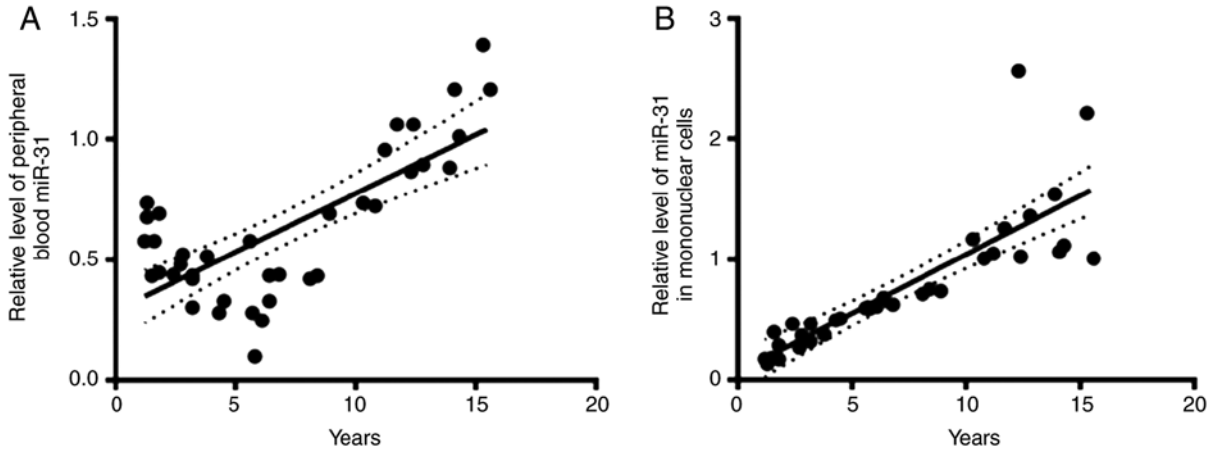

Figure 3. Lower miR-31 expression is observed in patients <10 years old compared with in patients >10 years old. Quantitative PCR analysis demonstrated that the levels of miR-31 in the (A) peripheral blood and (B) mononuclear cells were much lower in the group of children <10 years old (n=26) than those in the group of children >10 years old (n=12). miR-31, microRNA-31.

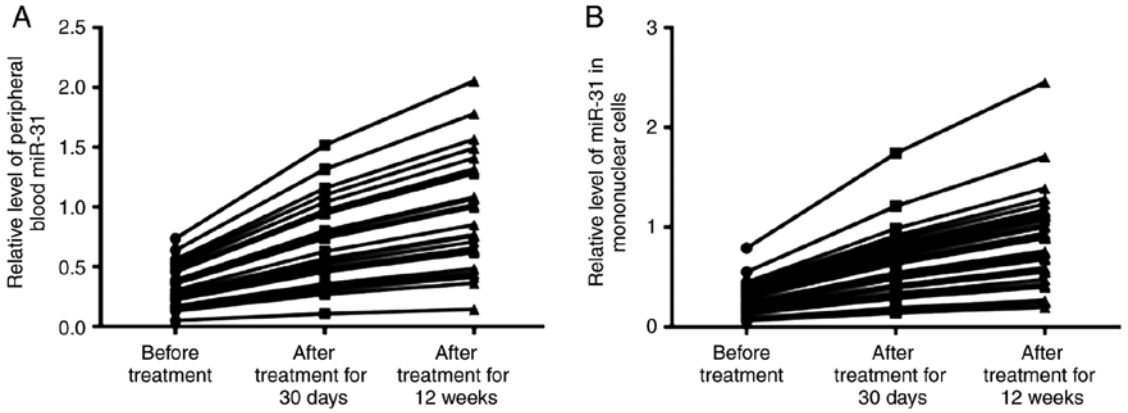

Figure 4. miR-31 expression is increased in patients with B-lymphoblastic leukemia after treatment. Quantitative PCR analysis demonstrated that miR-31 expression in the (A) peripheral blood and (B) mononuclear cells was lowest before treatment, and was gradually increased following treatment for 30 days and 12 weeks. miR-31, microRNA-31.

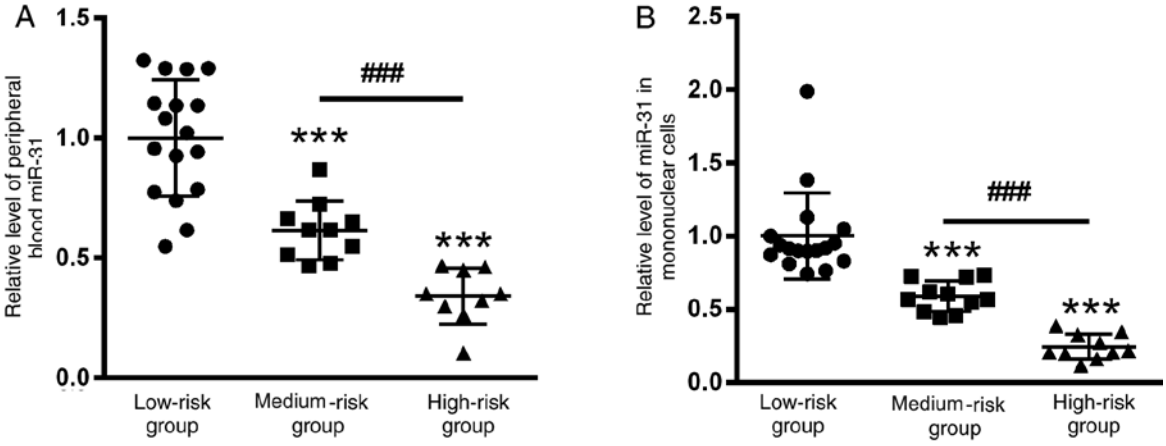

Figure 5. Lowest miR-31 expression is observed in the high-risk group. Quantitative PCR analysis revealed that the expression levels of miR-31 in the (A) peripheral blood and (B) mononuclear cells of the low-risk group were higher than those in the medium- and high-risk groups. ***P<0.001 vs. low-risk group; $^{###}$P<0.001. miR-31, miR-31. miR-31, microRNA-31.

marker in B-ALL. Subsequently, a ROC curve was plotted and the AUC was calculated. The AUC was 0.915, suggesting that miR-355 has a high diagnostic value for B-ALL. When an optimal cutoff value of 0.18 was selected, the probability of the correct diagnosis of patients with B-ALL based on miR-31 was 80.8%, while the probability of excluding B-ALL

was 100%. Therefore, decreased circulating miR-31 levels in patients with B-ALL may have a high diagnostic efficiency for B-ALL.

The expression levels of miR-31 were also compared at the different treatment stages for patients with B-ALL. Notably, miR-31 expression gradually increased with the progression of treatment stages, suggesting that miR-31 may improve the progression of B-ALL in children. The present study also revealed that the expression levels of miR-31 were higher in children with B-ALL who were >10 years old than those in children with B-ALL who were <10 years old, indicating that the expression levels of miR-31 were associated with age, and suggesting that detecting the expression levels of miR-31 may be helpful in evaluating the prognosis of children with B-ALL. The expression levels of miR-31 prior to treatment in the different risk groups were further compared. The data revealed that miR-31 expression in the high-risk group was lower than that in the medium- and low-risk groups, and the expression levels of miR-31 also differed between the medium- and high-risk groups, indicating that when miR-31 expression was lower, the prognosis of the children was worse.

However, there were some limitations of the present study. Due to limitations of time and number of specimens, it remains to be investigated as to whether miR-31 acts as an independent factor or in combination with other pathogenic factors in the pathogenesis of B-ALL. Additionally, miR-31 expression in T cell acute lymphoblastic leukemia, acute myeloid leukemia or other hematological disease samples was not analyzed. Therefore, whether miR-31 is a specific diagnostic marker for B-ALL requires further study.

It is well known that miRNAs can modulate the expression of multiple target genes, thus exerting roles in numerous signaling pathways (20). In a previous study, Rokah *et al* (20) determined potential target genes and signaling pathways that may be involved in the progression of CML. It was suggested that Casitas B-lineage lymphoma and E2F transcription factor 3 may be possible target genes of miR-31 in CML initiation and progression (20). Additionally, in ALL, reduced miR-31 expression has been demonstrated to activate the NF-κB signaling pathway and suppress cell apoptosis via inhibiting NF-κB inducing kinase (21). The underlying mechanism by which the occurrence of B-ALL was regulated via miR-31 remains to be further investigated by evaluating the role of these target genes.

In conclusion, in children with B-ALL, the expression levels of miR-31 were downregulated compared with those in the healthy controls, and this downregulation was associated with age of onset, risk and treatment stage. These findings suggested that miR-31 expression may be an important prognostic indicator in B-ALL.

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Availability of data and materials

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

Authors' contributions

YZ performed the experiments and analyzed the data. XL, LB and LL analyzed and interpreted the work. DL, XD and BW performed the reverse transcription-quantitative PCR experiments. CL designed the experiments, analyzed the data and gave the final approval of the version of the manuscript to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Hongqi Hospital Affiliated to Mudanjiang Medical University (Mudanjiang, China), and all guardians of the patients provided written informed consent for this study.

Patient consent for publication

All patients provided written informed consent for the publication of data in this study.

Competing interests

The authors declare that they have no competing interests.

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