MiRNA-181b-5p Modulates Cell Proliferation, Cell Cycle, and Apoptosis by Targeting SSX2IP in Acute Lymphoblastic Leukemia

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Objective: Accumulating evidence indicates that miRNAs can negatively influence the expression of their downstream genes, thereby affecting the development of human cancers. The pathogenesis of acute lymphoblastic leukemia (ALL) is complex and more biomarkers and functional molecules need to be found. We attempted to reveal the specific mechanisms and functions of miRNA-181b-5p in ALL and investigated the effects of the miRNA-181b-5p/SSX2IP axis on ALL.

Materials and Methods: Bioinformatics analyses were initially performed to screen out differentially expressed miRNAs in ALL and determine the research subject. qRT-PCR and western blotting were applied to evaluate the expression levels of target genes. Cell function experiments and mouse experiments were conducted to analyze the roles of the target genes in ALL.

Results: miRNA-181b-5p was highly and differentially expressed in ALL and may target SSX2IP. The upregulation of miRNA-181b-5p and downregulation of SSX2IP were observed in ALL cells. miRNA-181b-5p could control multiple pathological processes of ALL, including cell proliferation, the cell cycle, and apoptosis, and miRNA-181b-5p could also facilitate tumor growth in vivo.

Conclusion: miRNA-181b-5p promoted the malignant progression of ALL by downregulating SSX2IP. The miRNA-181b-5p/SSX2IP axis may be a promising target for intervention against the malignant behaviors of ALL.

Keywords: Acute lymphoblastic leukemia, miRNA-181b-5p/SSX2IP axis, Proliferation, Cell cycle

Amaç: Biriken kanıtlar, miRNA’ların hedef genlerinin ekspresyonunu olumsuz yönde etkileyebileceğini ve böylece insan kanserlerinin gelişimini etkileyebileceğini göstermektedir. Akut lenfoblastik lösemide (ALL) patogenezi karmaşıktır, daha fazla biyobelirteç ve fonksiyonel molekülün bulunmasına ihtiyaç vardır. ALL'de miRNA-181b-5p’nin spesifik mekanizmalarını ve fonksiyonlarını ortaya çıkarmayı ve miRNA-181b-5p/SSX2IP eksemnin ALL’deki etkilerini araştırık.

Gereç ve Yöntemler: İlk olarak bioinformatik analizler ile ALL’de farklı ifade edilen miRNA’ları taraflı ve araştırma konusunu belirledik. Hedef gen analıtım seviyelerini değerlendirerek için qRT-PZR (qRT-PzR) ve western blot yaklaşımları kullanıldı. ALL’deki hedef genlerin rollerini belirlemek için fonksiyonel çalışmalar ve fare deneyleri yapıldı.

Bulgular: miRNA-181b-5p, ALL’de yüksek düzeyde ifade edilen SSX2IP genini hedefleyebilir. ALL hücrelerinde miRNA-181b-5p anlatımı artarken ve SSX2IP geninin baskılılığı gözlemlendi. MiRNA-181b-5p, hücre proliferasyonu, hücre düğümlüğü ve apoptoz dahil olmak üzere ALL’de birçoq patolojik süreç kontrol edebilir ve miRNA-181b-5p ayrıca in vivo olarak tümör büyümesini kolaylaştırabilir.

Sonuç: miRNA-181b-5p, SSX2IP genini baskılayarak ALL’lerinimini destekledi. miRNA-181b-5p/SSX2IP eksenleri, ALL’innin habis davranışlarına karşı müdahale için umut verici bir hedef olabilir.

Anahtar Sözcükler: Akut lenfoblastik lösemi, miRNA-181b-5p/SSX2IP eksenleri, Proliferasyon, Hücre düğümlüğü
Introduction

Acute lymphoblastic leukemia (ALL) is a malignant neoplastic disease caused by the abnormal hyperplasia of B-lymphoblastic or T-lymphoblastic cells in bone marrow [1]. ALL accounts for about 20% of all leukemias in adults and over 70% in children, with childhood (0-9 years old) being the peak of onset [2]. The cure rate of pediatric ALL has reached nearly 90% [3]. Unfortunately, the cure rate for adult ALL patients is only 30%-40% [4]. Moreover, adult ALL patients are prone to relapse and chemotherapy resistance [5], and they have poor prognoses, with a 5-year overall survival rate of only 25% [6]. In recent years, great progress has been made in the study of the pathogenesis, refinement of prognosis, and development of new therapies for ALL [5], but great challenges are still faced in its treatment.

MicroRNAs (miRNAs) are small non-coding nucleotides that exert imperative functions in various biological processes [7,8]. Studies have demonstrated that some miRNAs are abnormally expressed in ALL, and they could be used for the diagnosis and treatment of the disease. Rashed et al. [9] revealed that miRNAs could be used for the diagnosis of pediatric ALL. miRNA-223, miRNA-128a, miRNA-128b, miRNA-155, miRNA let-7b, and miRNA-24 can be used to identify ALL and acute myeloid leukemia (AML) [9]. Yang and Sheng [10] reported that miRNA-101 inhibited T-cell ALL by targeting the CXCR7/STAT3 axis. Qi et al. [11] found that miRNA-410 affected the malignant biological behaviors of pediatric ALL by targeting FKB5 and the Akt signaling pathway. Su et al. [12] revealed a novel mechanism of the miRNA-181 family in normal hematopoietic stem cells and AML development, suggesting a possibility of inhibiting the expression of miRNA-181 to treat AML. However, miRNA-181b-5p has rarely been investigated in ALL-related studies.

It has been reported that miRNA-181b-5p is overexpressed in various types of cancers and has a tumorigenic effect [13,14,15]. In a recent study, miRNA-181a-5p carried by serum extracellular vesicles promoted the progression of ALL by influencing the cell cycle and inhibiting apoptosis of cancer cells in cases of ALL [1]. Therefore, in the present study, we attempted to uncover the specific mechanisms and functions of miRNA-181b-5p in ALL and investigated the effects of the miRNA-181b-5p/SSX2IP axis on ALL with in vivo and in vitro experiments.

Materials and Methods

Bioinformatics Methods

The ALL-related miRNA expression chip GSE56489 was obtained from the Gene Expression Omnibus (GEO) database. Gene differential analysis was conducted to determine the target mRNA using the “limma” package with normal samples as controls. |logFC| > 1.5 and adj. p-value < 0.05 were used as the selection standards for differentiating miRNAs. The TargetScan database, miRDB database, and miRTarBase database were used to predict the downstream targets of the target miRNA. Meanwhile, differential expression analysis of genes in ALL were conducted in TCGA. Next, the genes downregulated in ALL were taken to overlap with the predicted results. Finally, the targets with the most significant differences in expression were identified for analysis.

Cell Culture and Transfection

ALL cell lines including Jurkat (BNCC338495), BALL-1 (BNCC102176), HPBALL (BNCC342583), Nb4 (BNCC341963), and CEM/C1 (BNCC100321) and the normal human peripheral blood mononuclear cell line PBMC (BNCC341622) were supplied by the BeNa Culture Collection (BNCC, Beijing, China). All of them were maintained in a humid thermostatic incubator containing 5% CO₂ at 37 °C. The medium used was RPMI-1640 (HyClone, Logan, UT, USA) with 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA), 100 U/mL streptomycin (Gibco), and 100 U/mL penicillin (Gibco). The medium was replaced regularly.

GenePharma (Shanghai, China) synthesized the miRNA-181b-5p mimic, miRNA-181b-5p inhibitor, small interfering RNA targeting SSX2IP, and corresponding negative controls (NCs). The miRNA-181b-5p agomir and NC agomir were procured from RiboBio (Guangzhou, China) for mouse experiments. Transfection was performed with Lipofectamine 3000 reagent (Life Technologies Corporation, Carlsbad, CA, USA).

qRT-PCR

TRIzol reagent (Gibco) was applied for total RNA isolation. The PrimeScript RT Kit (TaKaRa, Tokyo, Japan) was used to synthesize cDNA. The mRNA expression level was studied using a Synergy (SYBR) Premix ExTaq Quantitative PCR Kit (Thermo Fisher Scientific, Waltham, MA, USA) and LightCycler instrument (Roche, Basel, Switzerland). U6 and GAPDH served as internal controls. The relative expressions of miRNA-181b-5p and SSX2IP were quantified by 2-ΔΔCt method. The primers for qRT-PCR were acquired from Shanghai Biotechnology Co., Ltd. (Shanghai, China). The primers used were as follows: miRNA-181b-5p: forward primer 5’-CCAGCTGGGGTCCTAGAATGGA-3’, reverse primer 5’-CAACTGGTGTCGTGGAGTCGGC-3’; GAPDH: forward primer 5’-ATGCCCTCCAGGACGAGAAG-3’, reverse primer 5’-GTTTGTTCGGAGATGAT-3’.
After being blocked for 1 h at room temperature, the membrane was subjected to incubation with primary antibodies and a secondary antibody. The primary antibodies were SSX2IP (AB199425, 1:10,000, Abcam, Cambridge, UK) and GAPDH (AB9485, 1:2500, Abcam, UK). The secondary antibody was goat anti-rabbit IgG H&L (HRP, AB6721, 1:2000, Abcam). Tris buffer saline/Tween-20 was then applied to rinse the membrane three times. A chemiluminescence system was applied to analyze the proteins (Bio-Rad, Hercules, CA, USA).

Dual-Luciferase Reporter Gene Detection

According to TargetScan (http://www.targetscan.org/), SSX2IP is one of the targets of miRNA-181b-5p. Therefore, we used the mutant (MUT) and wild type (WT) of the SSX2IP 3' UTR to treat pmirGLO vectors (Promega, Madison, WI, USA). The recombined pmirGLO vectors were then co-transfected with the miRNA-181b-5p mimic into BALL-1 cells to detect the relative luciferase activity.

CCK-8 and Colony Formation Assays

To evaluate the cell viability of ALL cells, a CCK-8 assay was performed. Cells were harvested 24, 48, and 72 h after transfection and then subjected to the CCK-8 assay. Subsequently, cell viability was analyzed by CCK-8 (Dojindo, Kumamoto, Japan).

A colony formation assay was performed to analyze cell proliferation. At 48 h after transfection, 500 cells were placed in 6-well plates. The cells were incubated at 37 °C for about 2 weeks, followed by fixation with 20% methanol solution and staining with 0.5% crystal violet solution. Finally, colony counting was conducted.

Flow Cytometry

Apoptosis and cycle analyses of ALL cells after transfection were performed by flow cytometry. For apoptosis evaluation, the cells were cultured with 5 µL of annexin V-fluorescein isothiocyanate and 5 µL of propidium iodide (PI) in darkness. For cell cycle assessment, the cells were incubated with PI (20 mg/mL) in darkness. A flow cytometer (BD Biosciences, San Jose, CA, USA) and FACScan instrument (Becton, Franklin Lakes, NJ, USA) were utilized to analyze the cells.

Mouse Subcutaneous Tumor Formation

To verify the effect of miRNA-181b-5p on ALL tumors in vivo, female BALB/c mice (SLAC Animal Center, Shanghai, China) aged 4–6 weeks were selected and randomly divided into two groups. After the BALL-1 cells were incubated with NC agomir or miRNA-181b-5p agomir (RiboBio, Guangzhou, China) for 3 days, a subcutaneous tumorigenesis test was performed on the mice. Tumor volume was evaluated once a week and tumor growth curves were plotted. The formula for this was: volume = length x width² x 0.5. Five weeks later, the mice were euthanized and the tumors were isolated and weighed.

Statistical Analysis

Data were processed using SPSS 20.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA), and they were shown as means ± standard deviations. Values of p<0.05 as calculated by Student’s t-test indicated statistically significant differences. The data evaluated with t-tests were normally distributed. Each experiment was repeated three times.

Results

MiRNA-181b-5p Is Highly Expressed in ALL

Analysis of the ALL-related miRNA expression chip (GSE56489) obtained from the GEO database revealed that miRNA-181b was significantly upregulated in tissues in the presence of ALL (Figure 1A). Since miRNAs generally play regulatory roles as mature molecules, we evaluated miRNA-181b-5p expression and found that it was remarkably upregulated in the five considered ALL cell lines compared to the normal PBMC cell line (Figure 1B). Among the five ALL cell lines, BALL-1 showed the

Figure 1. miRNA-181b-5p expression is stimulated in acute lymphoblastic leukemia (ALL). A) miRNA-181b expression in ALL according to GEO database; B) miRNA-181b-5p expression in each cell line. *: p<0.05.
highest miRNA-181b-5p expression; thus, it was selected for subsequent experiments.

**Low Expression of miRNA-181b-5p Reduces ALL Cell Proliferation and Affects Cell Cycle and Apoptosis**

To explore the functions of miRNA-181b-5p in ALL cells, we produced BALL-1 cells with low expression of miRNA-181b-5p. We confirmed that our inhibition treatment was successful (Figure 2A). We then evaluated parameters including cell proliferation, cell cycle, and cell apoptosis. According to the results, the proliferative ability of cells with miRNA-181b-5p inhibition was decreased, and the proportion of G0/G1 cells was higher than that in the NC group (Figures 2B-2E). Correspondingly, the apoptosis rate of the cells was also higher. Overall, the cell functional assays suggested that miRNA-181b-5p could affect the growth and development processes of ALL cells.

**miRNA-181b-5p Downregulates SSX2IP**

To study the action mechanism of miRNA-181b-5p, we predicted its downstream target genes using the TargetScan, miRDB, and miRtarBase databases, and 19 potential target genes were found (Figure 3A). At the same time, differential expression analysis was conducted for genes in ALL in TCGA. The genes obtained in this way were overlapped with the predicted results. Among the intersecting results, PNRC2, SCD, and SSX2IP had significant expression changes in ALL (Table 1). It has been reported that SSX2IP is a target in immunotherapy for B-cell ALL [16] and that it is also related to the survival rates of AML patients [17]. This indicates that SSX2IP may play a key role in ALL. Therefore, we chose SSX2IP as the downstream target of miRNA-181b-5p for further study.

Based on the expression data of SSX2IP in TCGA, we found that SSX2IP expression was remarkably reduced in ALL patients (Figure 3B). qRT-PCR analysis showed that SSX2IP expression in all five ALL cell lines was lower than that in the PBMC cell line (Figure 3C). In addition, the TargetScan database showed us the binding site of miRNA-181b-5p and SSX2IP (Figure 3D), and the binding relationship between miRNA-181b-5p and SSX2IP was verified by dual-luciferase reporter detection. Compared to the NC mimic group, the miRNA-181b-5p mimic significantly inhibited the luciferase activity of WT SSX2IP but did not affect the luciferase activity of MUT SSX2IP (Figure 3E). Meanwhile, qRT-PCR and western blotting indicated that the SSX2IP mRNA and protein levels were lower in the miRNA-181b-5p mimic group than in the NC group (Figures 3F and 3G). From these results, we concluded that miRNA-181b-5p suppressed the expression of SSX2IP in ALL.

| comcom_diff_taget | comMean | treatMean | logFC | Adj. p–value |
|-------------------|---------|-----------|-------|--------------|
| TNPO1             | 14.3797 | 7.933507259 | -0.858 | 1.82122E-26 |
| SSX2IP            | 12.78611| 1.688415953 | -2.92083 | 4.74317E-24 |
| RAP1B             | 46.2277 | 24.00835897 | -0.94522 | 1.51545E-23 |
| CPoX              | 16.37416| 6.818546676 | -1.26388 | 1.01728E-28 |
| GSKIP             | 6.053779| 2.66389213 | -3.67188 | 8.03102E-33 |
| PNRC2             | 36.32635| 2.850199534 | -3.67188 | 4.74317E-34 |
| NAA50             | 23.16551| 19.40662498 | -0.25543 | 2.90172E-06 |
| G3BP2             | 26.30422| 16.51538886 | -0.67148 | 5.18361E-26 |
| MAP2K1            | 17.51483| 13.85582545 | -0.33808 | 9.82229E-15 |
| ZFAND6            | 25.12043| 8.266799222 | -1.60346 | 4.8602E-34 |
| OSBP3             | 4.796917| 2.665294915 | -0.84781 | 2.27912E-21 |
| SLC25A37          | 45.23746| 21.64716273 | -1.06331 | 9.76349E-23 |
| SCD               | 99.37347| 6.85796556 | -5.73925 | 4.74317E-34 |
| LRRCD8            | 11.08662| 6.068351658 | -0.86944 | 2.22615E-26 |
| SEC24C            | 22.05439| 13.40716328 | -0.71806 | 1.66228E-26 |
| HSP90B1           | 186.6431| 72.97065086 | -1.35489 | 1.91456E-31 |
| PDIA6             | 72.71965| 36.12152409 | -1.00949 | 9.14591E-29 |
| IPO5              | 21.95025| 15.59886555 | -0.4928 | 8.6644E-22 |
| KPNB1             | 79.48164| 25.22399437 | -1.65582 | 4.74317E-34 |
Figure 2. miRNA-181b-5p affects the biological functions of acute lymphoblastic leukemia cells. A) Effect of miRNA-181b-5p inhibition treatment in cell lines. B) Proliferation ability of cells after miRNA-181b-5p inhibition treatment. C) Colony-forming ability of cells after miRNA-181b-5p inhibition treatment. D) Changes in cell cycle in each group. E) Cell apoptosis in each group. *: p<0.05.
miRNA-181b-5p Affects Proliferation, Apoptosis, and Cell Cycle of ALL Cells by Regulating SSX2IP Expression

To confirm whether SSX2IP is a functional target of miRNA-181b-5p, we established three groups of transfected cells: inhibitor-NC+si-NC, miRNA-181b-5p inhibitor+si-NC, and miRNA-181b-5p inhibitor+si-SSX2IP. First, we verified the SSX2IP expression in each transfection group by qRT-PCR and western blotting (Figures 4A and 4B). According to the results, the mRNA and protein levels of SSX2IP expression were notably higher in the miRNA-181b-5p inhibitor group, which was reversed after SSX2IP downregulation. Next, we detected cell proliferation by performing CCK-8 and colony formation assays, and we analyzed the cell cycle and apoptosis using flow cytometry. Compared with the NC group, inhibiting the miRNA-181b-5p expression led to cell proliferation activity repression, colony formation ability reduction, cell cycle arrest in the G0/G1 phase, and an increased rate of apoptosis (Figures 4C-4F). The effect of miRNA-181b-5p inhibitor on cellular function was reversed when SSX2IP was downregulated. These experiments proved that miRNA-181b-5p could affect a series of processes of BALL-1 cells by regulating SSX2IP expression. In addition, when SSX2IP expression was directly inhibited, the effect of the low miRNA-181b-5p level on cell functions was reversed.
miRNA-181b-5p Overexpression Promotes Tumorigenicity of ALL Cells

To verify the in vivo functions of miRNA-181b-5p, BALL-1 cells transfected with miRNA-181b-5p agomir or NC agomir were injected into the mice to establish xenograft or metastasis mouse models. The mouse models were observed every 7 days and weights were recorded. Compared to the NC group, the miRNA-181b-5p antagonor promoted tumor formation and tumor growth in mice remarkably (Figure 5A). After 35 days, the tumor size and weight in the NC agomir group were notably lower than those in the overexpression group (Figure 5B). To observe the proliferation and apoptosis of cells in tumor tissues more clearly, we tested the tumor tissues of the mice with immunohistochemical detection of Ki-67 and TUNEL staining. The results indicated that the positive cells in the miRNA-181b-5p agomir transfection group were significantly increased, while the numbers of apoptotic cells were significantly reduced (Figure 5C).

In addition, we also evaluated the miRNA-181b-5p and SSX2IP expressions in the miRNA-181b-5p agomir and NC agomir groups. The results showed that the expression of miRNA-181b-5p was significantly increased in the agomir group compared to the NC group, while the expression of SSX2IP was reduced. These findings suggest that miRNA-181b-5p may regulate SSX2IP expression, thereby modulating the tumorigenicity of ALL cells.
groups and found that the SSX2IP level was relatively lower in tumor tissues with relatively higher miRNA-181b-5p expression (Figures 5D and 5E). This finding supported the conclusion that the high miRNA-181b-5p expression in ALL cells could indeed downregulate SSX2IP expression and facilitate cancer cell growth.

**Discussion**

In this work, we investigated the expression and mechanism of miRNA-181b-5p in ALL. Through bioinformatics analysis, we found that miRNA-181b expression was remarkably increased in ALL patients. We verified its expression in various cell lines, and the results agreed with the outcomes of bioinformatics analysis. Studies have shown that esophageal squamous cell carcinoma (ESCC) cells secreting EVs rich in miRNA-181b-5p can participate in angiogenesis in ESCC metastasis, providing promising diagnostic biomarkers or drug targets for ESCC patients [15]. Migration and invasion inhibitory protein suppresses the epithelial-mesenchymal transition and cell invasion via the miRNA-181a/b-5p-KLF17 axis in prostate cancer [18]. In addition, other mature bodies of this miRNA-181 have also been reported in cases of ALL. miRNA-181a is remarkably upregulated in ALL and associated with minimal residual disease; thus, it can be used as a marker in childhood ALL [19]. The upregulation of miRNA-181a-5p has a direct influence on WIF1 in ALL cells, indicating that miRNA-181a-5p-mediated Wnt signaling activation might be related to ALL pathogenesis [20]. These results suggested that miRNA-181 is highly expressed in ALL cells and affects the molecular regulatory mechanism of ALL. Thus, we verified the role of miRNA-181b-5p in ALL cells. After inhibiting miRNA-181b-5p in ALL cells, we found that cell proliferation was reduced and the apoptosis rate was greatly increased. As indicated by in vivo experiments, forced
expression of miRNA-181b-5p also promoted the formation of subcutaneous tumor in mice.

We also analyzed the downstream targets of miRNA-181b-5p and found that SSX2IP showed the most significant expression difference among the downregulated genes of ALL, and qRT-PCR verified the low expression of SSX2IP in ALL patients. It has been reported that SSX2IP expression reaches its peak on the surface of myeloid leukemia cells during mitosis [21]. Moreover, SSX2IP is preferentially recognized by AML serum compared with normal donor serum. qRT-PCR results demonstrated that 33% of AML patients are able to express SSX2IP while normal donor hematopoietic samples have no SSX2IP expression [22]. In ALL, on the basis of the targeted binding site of miRNA-181b-5p and SSX2IP as predicted by bioinformatics, dual-luciferase reporter detection confirmed the inhibitory role of the miRNA-181b-5p mimic for luciferase activity, indicating the existence of a targeted binding relationship between miRNA-181b-5p and SSX2IP. qRT-PCR and western blotting experiments also showed that miRNA-181b-5p overexpression could downregulate SSX2IP expression. In subsequent rescue experiments, we observed that silencing SSX2IP could reverse the inhibitory effect of low miRNA-181b-5p level on ALL cell proliferation. All of the above results indicated that SSX2IP was targeted by miRNA-181b-5p and could affect cell proliferation and the cell cycle in ALL.

Conclusion

The data obtained in this study proved that miRNA-181b-5p plays roles of facilitating cell proliferation and colony formation, affecting the cell cycle, and inhibiting cell apoptosis in the progression of cases of ALL. Moreover, these effects are likely to be achieved through downregulation of SSX2IP expression. This provides a solid foundation for further research on the diagnosis and molecularly targeted therapy of ALL. However, the roles of the miRNA-181b-5p/SSX2IP axis in vivo and related downstream pathways have not been reported. Therefore, the pathogenesis of ALL remains to be further elucidated.

Ethics

Ethics Committee Approval: The study was approved by the ethics committee of Guizhou Provincial People's Hospital. The methods were carried out in accordance with the approved guidelines.

Informed Consent: This study involved no human participants so informed consent was not applicable.

Authorship Contributions

Design: X.F., J.S., L.W., P.G., J.C.; Data Collection or Processing: X.F.; Literature Search: Y.H.
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