Down-Regulated hsa-miR-190b-5p is Correlated With BCL11A Expression in Pediatric β-Thalassemia

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Abstract

Background: Transcription factor BCL11A is a key regulator of hemoglobin switching in adult β-thalassemia. Several microRNAs (miRNAs) involve in the pathology of β-thalassemia by regulations of BCL11A. However, the expressions and regulators of BCL11A in pediatric β-thalassemia were unclear.

Methods: 18 pediatric β- thalassemia patients and 11 healthy controls were selected in this study. We applied reverse transcript quantitative real time PCR (RT-PCR) to analyze the expression levels of hsa-miR-190b-5p and γ-globin in pediatric β-thalassemia patients and luciferase activity assay to find out the direct regulations of BCL11A . Correlation between hsa-miR-190b-5p and biochemical indicators, BCL11A was assessed by the Pearson's correlation test.

Results: The expression levels of γ-globin in pediatric β-thalassemia patients were significantly increased. Moreover, the expression levels of hsa-miR-190b-5p were significantly down-regulated in pediatric β-thalassemia patients. Furthermore, hsa-miR-190b-5p was negatively correlated with BCL11A expression in pediatric β-thalassemia patients. Through luciferase activity assay, we found that hsa-miR-190b-5p was directly interacted with BCL11A 3'UTR 499-506 regions.

Conclusion: Our results suggested that hsa-miR-190b-5p played key roles in regulating of BCL11A expression, which might provide novel therapies in pediatric patients with β-thalassemia .

Introduction

β-thalassemia is an autosomal recessive hereditary anemia, characterized by the disruption of β-globin expression. β-thalassemia is highly prevalent in Mediterranean countries, including the Middle East, Central Asia, India and Southern China [1,2]. The phenotypes of β-thalassemia are variable ranging from severe anemia to clinically asymptomatic individuals because of the wide spectrum of mutations in a homozygous or compound heterozygous state [3,4]. Current treatments of β-thalassemia include regular transfusions and hematopoietic stem cell allogenic transplant. However, there are many challenges and limitations in the currently available conventional therapies[5-7]. Epigenetic manipulations and genomic editing are novel therapeutic approaches [8-11]. An increased knowledge about the pathophysiology of β-thalassemia may lead to alternative treatments.

The decreasing of β-globin in β-thalassemia compensatory reactivates γ-globin expression and fetal hemoglobin (HbF) synthesis. Transcription factor B-cell lymphoma/leukemia 11A (BCL11A) is a key regulator of hemoglobin switching and HbF silencer in adult. BCL11A directly binds to the promoter regions of HbF and inhibits its expression [12,13]. Also, in β-thalassemia patients, the expression levels of BCL11A are decreased to reactivate HbF synthesis. Now, BCL11A represents an important therapeutic target of β-thalassemia [14]. Some transcription factor, like Krueppel-like factor 1 (KLF1) regulate the development of β-thalassemia through the regulations of BCL11A. However, the expressions and regulators of BCL11A in pediatric β- thalassemia are unclear.

MicroRNAs (miRNAs) are a category of conserved, small non-coding RNA molecules (21–25 nucleotides in length) [15,16]. Studies show that miRNAs play potential regulatory roles in β-thalassemia by interaction with BCL11A [17-19]. For instance, increased expression of miR-30a was associated with decreased BCL11A expression and elevated γ-globin and HbF levels in adult β-thalassemia [17]. Another study showed that the coding mRNA sequence of BCL11A can be targeted by miR-210 and miR-486-3p in adult β-thalassemia [18,19]. However, in pediatric β-thalassemia, miRNAs regulated the expression levels of BLC11A are unclear.

Previously, we analyzed the abnormal regulated miRNAs in pediatric β-thalassemia by miRNA sequencing and suggested that hsa-miR-190b-5p might participate in pediatric β-thalassemia by targeting on BCL11A [20]. In this study, we further analyzed the expression levels of hsa-miR-190b-5p in pediatric β-thalassemia patients by reverse transcript quantitative real time PCR (RT-PCR) and validated the direct regulations of BCL11A by hsa-miR-190b-5p in pediatric β-thalassemia.

Materials And Methods

Study participants

This study was approved by the Ethics Review Committee of Fujian Province Maternity and Child Health Hospital (approval no. 201, 2018). Written informed consent was obtained from all participants following a detailed description of the purpose of the study. 18 pediatric β- thalassemia patients and 11 healthy controls were selected in this study. All subjects had no genetic relationship. Blood cell parameters were analyzed on a Sysmex XN-3000 automatic hematology analyzer (Sysmex; Shanghai, China) and the hemoglobin components and levels were analyzed using an automated capillary electrophoresis system (CapillaryS 2, software version 6.2; Sebia, Paris, France). Biochemical indicators were determined by the chemiluminescent microparticle immunoassay (CMIA) (Abbott; ARCHITECT ci16200, USA). 5ml peripheral blood was collected from selected subjects, using PAXgene Blood RNA Kit and was used for further RNA isolation.

Molecular analysis for the thalassemia genotypes

Genomic DNA was extracted from the peripheral blood samples using a genomic DNA isolation kit (Qiagen) following the manufacturer's instructions. The three common deletional α-thalassemia (– SEA /αα,-α 3.7 /αα, and -α 4.2 /αα) were detected using Gap-PCR with the thalassemia gene detection kit (Shenzhen Yishengtang Biological Products Co., Ltd)[21]. Point mutations in the three nondeletional α-thalassemia (α CS α/αα,α QS α/αα,α WS α/αα), and the 17 common β-thalassemia were detected using reverse dot blot hybridization (RDB) with the thalassemia gene detection kit(Shenzhen Yishengtang Biological Products Co., Ltd)[22] following the manufacturer's instructions.

RNA extraction

Total RNA from peripheral blood was isolated using a miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol[20]. The concentration and purity of the RNA samples was assessed as 260/280 nm and 260/230 nm ratios, respectively, and were measured by a Bio Photometer MULTISKAN GO (Thermo, America).

RT-PCR

cDNA for evaluation of hsa-miR-190b-5p and U6 was synthesized with a Mir-X™ First Strand Synthesis kit according to the manufacturer’s instructions (Takara Bio, Inc., Japan) and cDNA for evaluation of mRNA (β-actin, γ-globin and BCL11A) synthesized with a PrimeScript™ RT-PCR kit according to the manufacturer’s instructions (Takara Bio, Inc., Japan).The following primers were used for qPCR: BCL11A sense: 5'- ACAGGAACACATAGCAGATAAAC -3' and antisense: 5'- TATTCTGCACTCATCCCAGG -3'; β-actin sense: 5'- GCACAGAGCCTCGCCTT -3' and antisense: 5'- GTTGTCGACGACGAGCG -3'; U6 sense: 5'- CTCGCTTCGGCAGCACA -3' and antisense: 5'- AACGCTTCACGAATTTGCGT -3'; The hsa-miR-190b-5p primers was designed and purchased from Takara (Takara Bio, Inc., Japan). Quantitative real-time PCR (qRT-PCR) was performed using the StepOne Real-time PCR System (Applied Biosystems, USA). β-actin was the internal control in mRNA quantification and U6 was the internal control in miRNA quantification. Relative expression was calculated using the comparative cycle threshold method and the internal control genes. Three

negative controls (no template cDNA) were also run with every experimental plate to assess specificity and to rule out contamination. The real-time PCR reactions were performed in three times for both target and internal control genes.

Cell Culture and Transfection

Human 293T cells was purchased from Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; GibcoBRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (GibcoBRL, Gaithersburg, MD, USA). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO2.Hsa-miR-190b-5p mimics/inhibitors and the corresponding negative control were purchased from Ribobio (RiboBio Co. Ltd., Guangzhou, China). Cells were transfected at a concentration of 50 nM using Lipofectamine 6000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Plasmids and Luciferase Reporter Assay

Wild type or mutant human BCL11A 3 $UTRagmentsweresynthesizedbySangonBiotech(Shanghai, Ch \in a), respectively. Mutant humanBCL11A3$ UTR fragments included BCL11A 3 $UTR499 - 506ACATATCA > TGTATAGT$ and $BCL11A3$UTR 2553-25599 (ACATATC>TGTATAG) were divined by using miRDB. These fragments were inserted into the multiple cloning sites of pmiR-RB-REPORT™. For the luciferase reporter assay, 293T cells were co-transfected with the reporter vectors and miRNA mimics (hsa-miR-190b-5p mimic or negative control (NC) mimic). After 48h, cells were collected and measured by using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the kit instructions.

Statistical analysis

The normality of the sample distributions was calculated by the Kolmogorov-Smirnoff test. Because the samples had normal distribution, we used the t-test and one-way ANOVA to assess the data differences between the pediatric β-thalassemia patients and normal children and the data from different groups detected by Luciferase Reporter Assay. Correlation between hsa-miR-190b-5p and biochemical indicators, BCL11A was assessed by the Pearson's correlation test. SPSS version 20.0 was used for statistical analysis. p values less than 0.05 were considered statistically significant.

Results

1. The characteristics in pediatric β-thalassemia patients

Totally, 18 pediatric β-thalassemia patients and 11 healthy controls were studied. The genotypes of 18 pediatric β-thalassemia patients were shown in Table 1. All the 18 pediatric β-thalassemia patients were with genetic mutations of β-globin. The mutations of β-globin gene were concentrated in $\beta^{CD41-42}$ and $\beta^{IVS-2-654}$ regions.

The hematological parameters of 11 normal children and 18 pediatric β-thalassemia patients were presented in Table 2. The mean age of the normal children (5.7±1.8 years) was not significantly different from the pediatric β-thalassemia patients (6.2±3.3 years). Compared with normal children, the mean level of red blood cells (RBC), hemoglobin, mean corpuscular hemoglobin (MCH) and hemoglobin A (HbA) was significantly lower in the β-thalassemia patients. On the contrary, the mean level of HbF in pediatric β-thalassemia (15.2±18.2%) was significantly higher than normal children (0.4±0.7%) ($p < 0.05$).

Table 1 The genotypes of pediatric β- thalassemia patients

Case no.	Age (years)	Sex	Genotype
1	5	Female	$\beta^{IVS\text{-}2\text{-}654(C\rightarrow T)}/\beta^{CD41\text{-}42(-TCTT)}$
2	6	Female	$\beta^{CD41\text{-}42(-TCTT)}/\beta^{CD17(A\rightarrow T)}$
3	5	Male	$\beta^{CD17(A\rightarrow T)}/\beta^{CD71\text{-}72(+A)}$
4	7	Male	$\beta^{IVS\text{-}2\text{-}654(C\rightarrow T)}/\beta^{-28(A\rightarrow G)}$
5	12	Female	$\beta^{IVS\text{-}2\text{-}654(C\rightarrow T)}/?$
6	1	Female	$\beta^{IVS\text{-}2\text{-}654(C\rightarrow T)}/\beta^{CD17(A\rightarrow T)}$
7	1	Male	$\beta^{IVS\text{-}2\text{-}654(C\rightarrow T)}/\beta^{CD41\text{-}42(-TCTT)}$
8	9	Male	$\beta^{CD41\text{-}42(-TCTT)}/\beta^{CD41\text{-}42(-TCTT)}$
9	5	Male	$\beta^{CD41\text{-}42(-TCTT)}/\beta^{CD17(A\rightarrow T)}$
10	8	Female	$\beta^{IVS\text{-}2\text{-}654(C\rightarrow T)}/\beta^{CD17(A\rightarrow T)}$
11	10	Female	$\beta^{CD41\text{-}42(-TCTT)}/\beta^{CD17(A\rightarrow T)}$
12	9	Male	$\beta^{-28(A\rightarrow G)}/\beta^{-28(A\rightarrow G)}$
13	3	Male	$\beta^{CD17(A\rightarrow T)}/\beta^{CD17(A\rightarrow T)}$
14	11	Male	$\beta^{CD41\text{-}42(-TCTT)}/\beta^{CD17(A\rightarrow T)}$
15	7	Female	$\beta^{CD17(A\rightarrow T)}/\beta^{-28(A\rightarrow G)}$
16	4	Female	$\beta^{IVS\text{-}2\text{-}654(C\rightarrow T)}/\beta^{CD41\text{-}42(-TCTT)}$
17	2	Male	$\beta^{CD41\text{-}42(-TCTT)}/\beta^{-28(A\rightarrow G)}$
18	7	Male	$\beta^{CD41\text{-}42(-TCTT)}/\beta^{CD41\text{-}42(-TCTT)}$

Table 2 The clinical characteristics of normal children and pediatric β- thalassemia patients

Hematological parameter	Normal(n=11)	pediatric β-thalassemia(n=18)
Sex (male/female)	9/2	10/8
Age (years)	5.7±1.8	6.2±3.3
RBC($\times 10^{12}$ /L)	4.7±0.4	2.5±0.5*
HB (g/L)	131.7±6.0	60.6±12.3*
MCV (fL)	82.2±3.2	79.1±8.0
MCH (pg)	28.2±1.3	25.3±2.6*
HbA (%)	96.1±1.2	81.0±18.1*
HbA_2 (%)	2.8±0.2	3.0±0.6
HbF (%)	0.4±0.7	15.2±18.2*

RBC: Red blood cells, Hb: hemoglobin,, MCV mean corpuscular volume, MCH mean corpuscular hemoglobin, HbA: hemoglobin A, HbA_2 hemoglobin A_2,HbF: fetal hemoglobin. Data expressed as mean ± SD. The significant data were shown in values with *p < 0.05 using independent Student's t test when compared to normal children

2. Down-regulated hsa-hsa-miR-190b-5p in pediatric β- thalassemia patients

Previously, we analyzed the abnormal regulated hsa-miR-190b-5p in pediatric β-thalassemia by miRNA sequencing in 5 pediatric β-thalassemia patients [34]. Expression levels of hsa-miR-190b-5p in 18 β-thalassemia patients were further studied. Compared with the normal children, hsa-miR-190b-5p was significantly down-regulated in pediatric β-thalassemia patients ($p < 0.001$) (Fig. 1). Moreover, the expression level of γ-globin in 18 pediatric β-thalassemia patients was significantly increased ($p < 0.001$), compared with normal children (Fig. 1).

3. Correlation between hsa-miR-190b-5p and biochemical indicators in pediatric β- thalassemia patients

To further analyze the roles of hsa-miR-190b-5p in the development of β-thalassemia, correlations between hsa-miR-190b-5p and biochemical indicators in pediatric β- thalassemia patients were determined. Our data showed that the expression level of hsa-miR-190b-5p was significantly negative correlated with the serum alkaline phosphatase (ALP) ($r=-0.968$, $p=0.007$) in pediatric β-thalassemia patients (Table3). However, total bilirubin (TBIL), direct bilirubin (DBIL), urea, creatinine and glomerular filtration rate (GFR) had no significant correlations with hsa-miR-190b-5p expression in pediatric β-thalassemia patients (Table3).

Table 3 Correlation between hsa-miR-190b-5p and biochemical indicator in pediatric β- thalassemia patients

		TBIL	DBIL	ALP	Urea	Creatinine	GFR
hsa-miR-190b-5p	Coefficient	-0.420	-0.801	-0.968	-0.984	-0.590	0.506
	P value	0.482	0.104	0.007**	0.114	0.410	0.663

TBIL: total bilirubin, DBIL: direct bilirubin, ALP: alkaline phosphatase, GFR: glomerular filtration rate. *p<0.05

4.Correlation between hsa-miR-190b-5p and BCL11A in pediatric β- thalassemia patients

BCL11A is a key regulator of the reactivation of γ-globin in β-thalassemia. To study the correlations between hsa-miR-190b-5p and BCL11A, we further detected the BCL11A mRNA expressions in 18 pediatric β-thalassemia patients. We found that the expression of hsa-miR-190b-5p had a negative correlation with BCL11A expression in pediatric β-thalassemia patients (r=-0.522, p=0.026) (Fig. 2A). However, in normal children, the expression of hsa-miR-190b-5p had no correlation with BCL11A expression (r=0.053, p=0.877) (Fig. 2B).

5. hsa-miR-190b-5p interacts with the 3'UTR of BCL11A

Using luciferase assay, we determined the direct binding of hsa-miR-190b-5p in the 3'UTR regions of BCL11A. Schematic representation of the dual-luciferase reporter was shown in Fig3A. BCL11A 3'UTR region harbored two hsa-miR-190b-5p putative binding sites, BCL11A 3'UTR 499-506 and BCL11A 3'UTR 2553-2559 regions (Fig3B). Dual-luciferase plasmid with BCL11A 3'UTR or mutated BCL11A 3'UTR and hsa-miR-190b-5p were co-transfected into human 293T cells. Compared with the non-targeting oligonucleotide negative control, hsa-miR-190b-5p transfection significantly inhibited the luciferase activity, suggested the suppression of BCL11A by hsa-miR-190b-5p (Fig3C). Moreover, when the BCL11A 3'UTR 499-506 regions were mutated (Mut1), the luciferase activity was significant increased, compared with wild type BCL11A 3'UTR ($p<0.01$) (Fig3C). However, when the BCL11A 3'UTR 2553-2559 regions were mutated (mut2), the luciferase activity was not significant different with wild type BCL11A 3'UTR ($p<0.01$) (Fig3C). Those results suggested that hsa-miR-190b-5p was bound to the BCL11A 3'UTR 499-506 regions.

Discussion

Hsa-miR-190b is abnormally expressed in multiple types of cancer, for instance, in breast cancer, endometrial cancer, lung cancer, gastric cancer, bladder carcinoma and colorectal cancer [23-28] and involves in promoting tumor growth [29,30], inhibiting tumor growth [31,32], differentiation[33], autophagy [34,35] and tumor prognosis [36]. Furthermore, hsa-miR-190b is up-regulated in obese adolescents with insulin resistance and participates in down-regulation of HLA-DRA expression [37]. However, there has been limited study of the roles of hsa-miR-190b in pediatric β- thalassemia

In this study, we collected 18 pediatric β-thalassemia patients and 11 normal children. Expression level of γ-globin in pediatric β-thalassemia patients was significantly increased in β-thalassemia patients. Consistent with our previous study, expression level of hsa-miR-190b-5p was significantly down-regulated in pediatric β- thalassemia patients, compared with normal children[20]. Moreover, hsa-miR-190b-5p was significant negative correlation with serum ALP levels in pediatric β-thalassemia patients. Our data suggested that hsa-miR-190b may be involved in the pathology of pediatric β- thalassemia.

BCL11A is a key therapeutic target of β-thalassemia [14]. We found that hsa-miR-190b-5p was negatively correlated with BCL11A mRNA expression in pediatric β-thalassemia patients. Furthermore, hsa-miR-190b-5p was bound to the BCL11A 3'UTR 499-506 regions. Our results revealed a new regulator of BCL11A in pediatric β- thalassemia. In conclusion, we showed the down-regulated hsa-hsa-miR-190b-5p was correlated with BCL11A expression in pediatric β-thalassemia. Further studies about the regulation of hsa-miR-190b-5p may lead to novel therapies in pediatric patients with β-thalassemia. Moreover, additional analyses are needed to assess the detailed mechanism of hsa-miR-190b-5p in the regulation of hemoglobin switch during pediatric β-thalassemia development.

Declarations

Acknowledgments

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Authors' contributions

Meihuan Chen, Xinrui Wang and Haiwei Wang collected the blood samples performed the data analysis and wrote the manuscript. Liangpu Xu, Hailong Huang designed the study and supervised the work. Min Zhang ,Lingji Chen, Hong Chen,Yali Pan and Yanhong Zhang helped with the collection of blood samples.

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Availability of data and materials

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

Ethics approval and consent to participate

Written informed consent to participate was obtained from all of the parents of the participants in the study and the study was conducted in accordance with the Declaration of Helsinki. This study was proved by the Ethics Review Committee of Fujian Maternity and Child Health Hospital (2018-201).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicts of interest.

Footnotes

Publisher's Note

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Figures

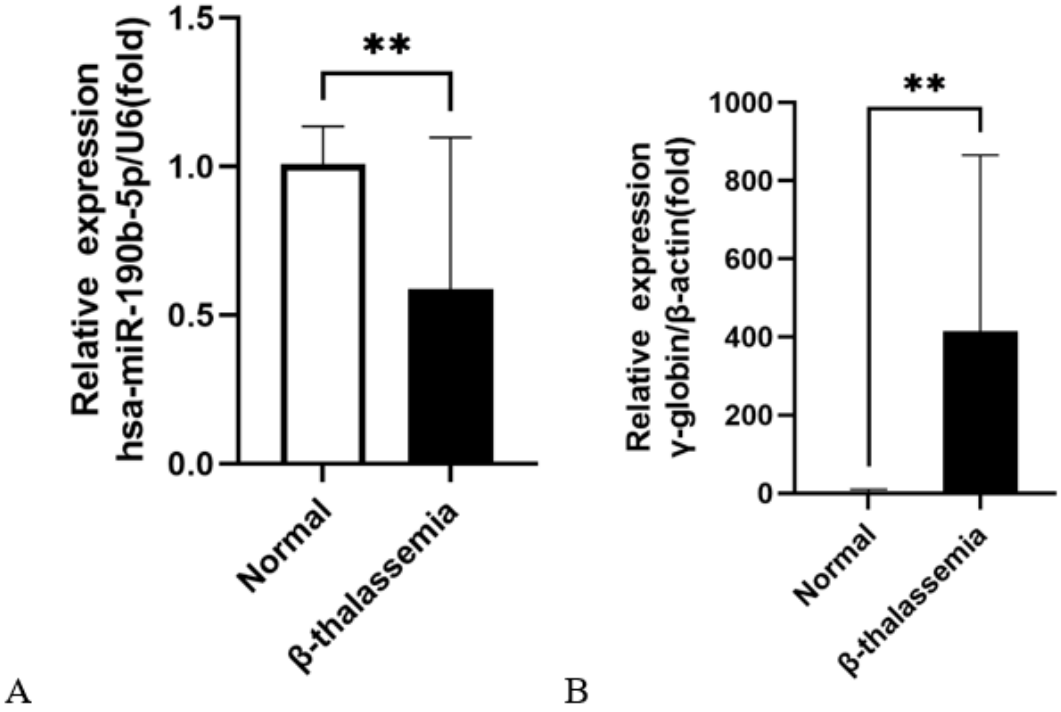

Figure 1

Analysis of hsa-miR-190b-5p and γ-globin expression in monocyte cells. A Analysis of hsa-miR-190b-5p expression in monocyte cells. B Analysis of γ-globin expression in monocyte cells. Expression of has-miR-190b-5p and γ-globin in normal children and pediatric β- thalassemia patients was carried out by qRT-PCR. Significant difference from normal individual, **p<0.01.

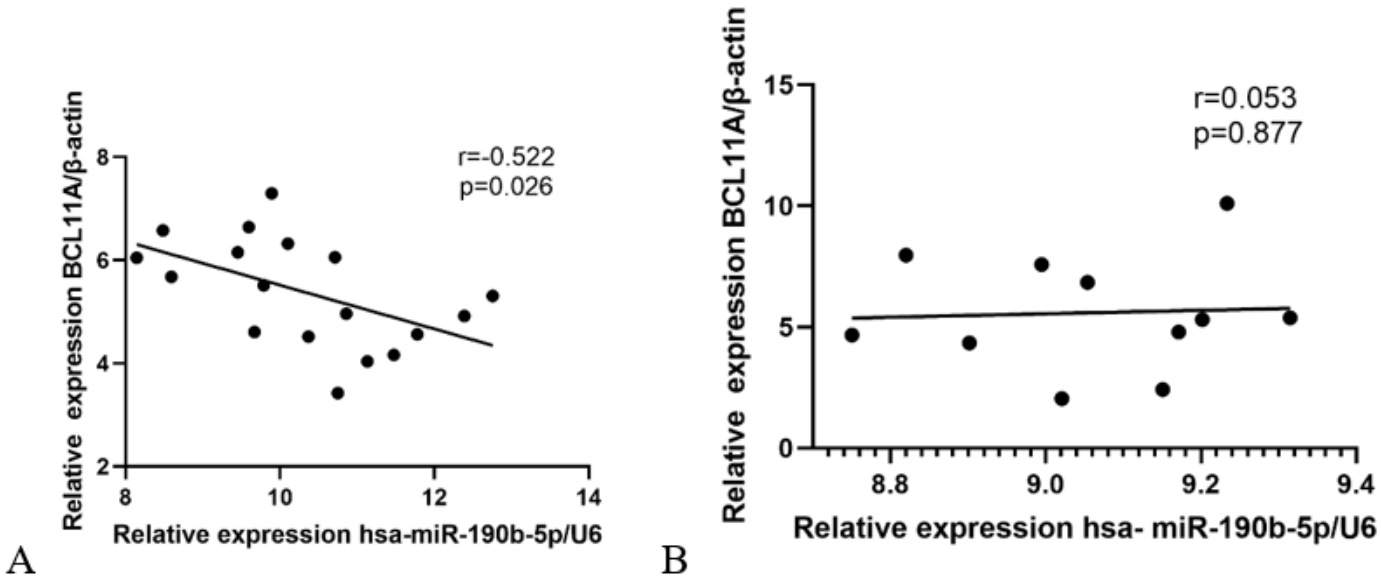

Figure 2

Correlation between hsa-miR-190b-5p and BCL11A. (A)Correlation between hsa-miR-190b-5p and BCL11A in pediatric β-thalassemia patients. (B) Correlation between hsa-miR-190b-5p and BCL11A in normal children.

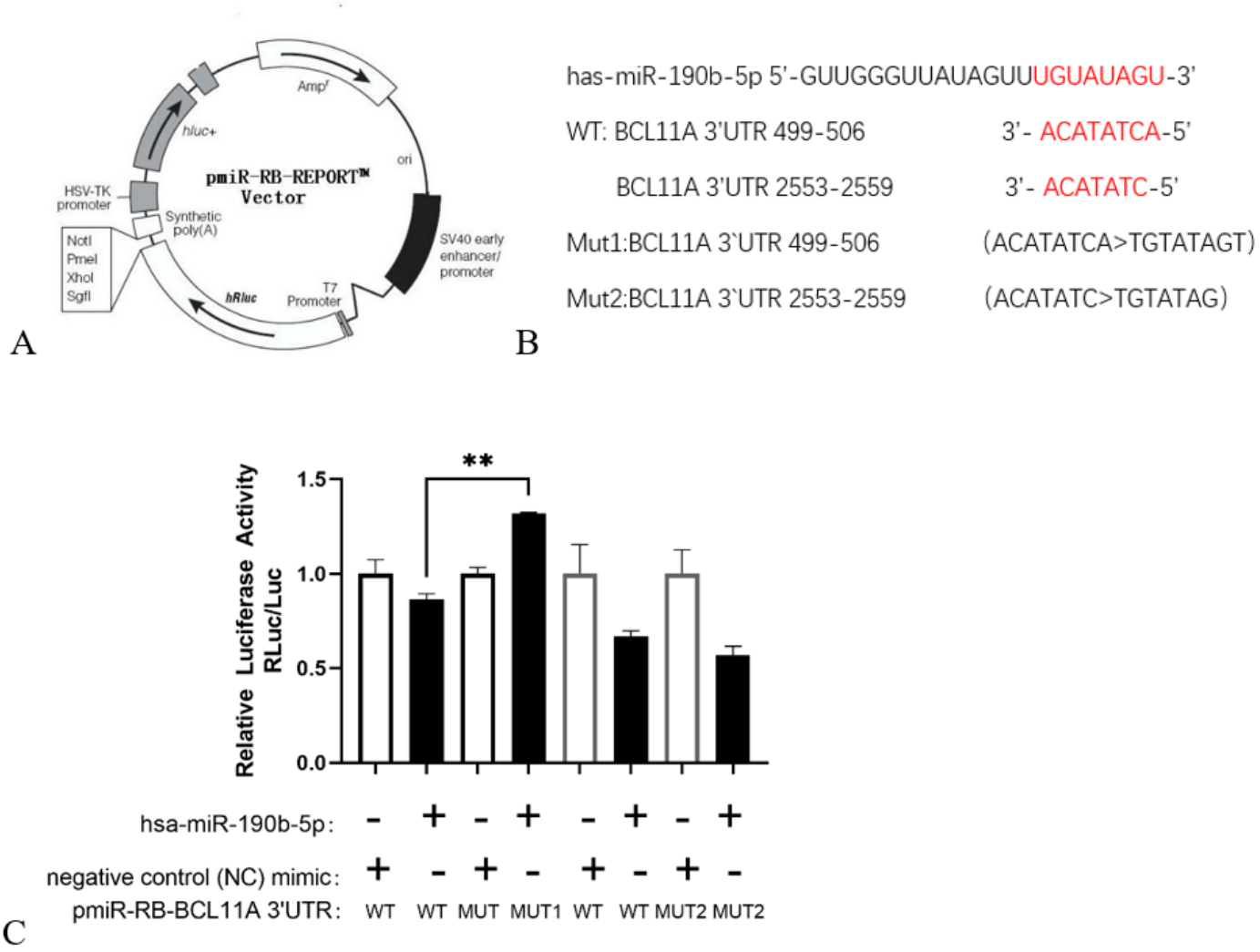

Figure 3

BCL11A is a target of hsa-miR-190b-5p. (A) Schematic representation of the plasmid of dual-luciferase reporter. The BCL11A 3'UTR or mutant BCL11A 3'UTR was fused to the Renilla luciferase gene (Rluc), with luciferase gene (Luc) is used as the endogenous control. (B) The human BCL11A 3'UTR harbored two putative binding sites (bases marked in red) complementary to hsa-miR-190b-5p. The mutant BCL11A 3'UTR regions were shown. (C) Luciferase activity in human 293T cells co-transfected with either BCL11A 3'UTR or mutated -BCL11A 3'UTR and hsa-miR-190b-5p or non-targeting oligonucleotide (NC). Firefly luciferase values normalized for renilla luciferase were presented. Mean±SD was from three independent experiments. **, $p < 0.01$.