

Original Article

MicroRNA-185 contributes to DNA hypomethylation of CD4+ T cells in pregnant patients with systemic lupus erythematosus by targeting DNA methyltransferase 1

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Abstract: Recent studies have shown that abnormal DNA methylation in CD4+ T cells plays essential roles in the development of systemic lupus erythematosus (SLE). Accumulated data suggest that miR-185 serves as critical regulators to control the methylation process in a variety of tumors. However, the role of miR-185 in T cell aberrant DNA hypomethylation of SLE still remains unclear. Therefore, this study was conducted to investigate whether miR-185 plays a role in the pregnancy associated with SLE. Our results revealed that miR-185 was significantly upregulated in CD4+ T cells from patients with pregnancy complicated with SLE and its degree of overexpression was inversely correlated with DNA methyltransferase 1 (DNMT1) mRNA levels. Target prediction analysis and dual luciferase reporter assays confirmed that DNMT1 was a direct target of miR-185. Furthermore, overexpression of miR-185 in CD4+ T cells from healthy donors led to the DNA hypomethylation and up-regulation of genes encoding CD11a and CD70, and inhibition of miR-185 expression in CD4+ T cells from patients with SLE caused reverse effects. This study indicated that miR-185 contributes to DNA hypomethylation of CD4+ T cells in pregnancies in patients with systemic lupus erythematosus by targeting DNA methyltransferase 1. Thus, miR-185 may represent a potential therapeutic target for SLE intervention.

Keywords: MicroRNA-185, CD4+ T cell, methylation, systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by elevated autoantibodies against nuclear antigens and excessive inflammatory responses affecting multiple organs, which occurs in pregnancy. SLE predominantly affects females (~90% of cases), and is more prevalent in individuals of African descent [1]. Although genetic, racial, hormonal, and environmental factors contribute to the development of SLE, the exact etiology of SLE remains unclear at present [2, 3].

An increasing number of studies have shown that epigenetic factors, especially abnormal DNA methylation patterns in CD4+ T cells, play essential roles in the development of this disease [4]. DNA methylation, one of the epigenetic marks, plays a crucial role in the regulation

of X-chromosome inactivation [5], transposon silencing [6], biotic stress response [7], gene expression [8], and genomic imprinting [9] in both plants and animals. Epigenetics refers to the study of heritable changes in gene function that occur without a change in the DNA sequence, and DNA methylation is the most prevalent and best-described epigenetic modification in the field [10-12]. It is generally considered that DNA methylation represses the expression of relevant genes, whereas DNA demethylation actually results in transcriptional activation. However, mechanisms underlying the abnormal DNA methylation status in SLE CD4+ T cells have not been fully elucidated so far.

MicroRNAs (miRNAs) are a class of endogenous small noncoding RNAs with 18-22 nt in length. Mature miRNAs bind to the 3'-UTR of target

MicroRNA-185 contributes to DNA hypomethylation in pregnant patients with SLE

mRNAs and repress translation of target mRNAs or induce degradation of target mRNAs [13]. miRNAs are involved in diverse biological processes, including development, cancer, autoimmunity, and so on [14-16]. Dysregulation of miRNAs by several mechanisms has also been described in various disease states, including SLE [17, 18]. And, miRNAs can regulate DNA methylation by targeting the regulators of DNA methylation machinery. Recent study showed that miR-185 can directly target DNMT1, thereby leading to a reduction in global DNA methylation (GDM) and regulating the expression of the promoter-hypermethylated genes in glioma cells [19]. However, whether and how miR-185 could regulate DNMT1 expression and affect the genomic DNA methylation, contributing to the development of SLE, has not been systemically explored.

The primary objective of the present study was to elucidate the effects and underlying molecular mechanisms of miRNAs in the aberrant DNA methylation of SLE T cells. Considering the important roles of miR-185 in the aberrant DNA methylation, we investigated whether miR-185 contributes to the development of SLE through affecting the genomic DNA methylation.

Materials and methods

Subjects

A total of 20 pregnancy patients with SLE (mean age: 31.90±10.72) were included in the study. 20 subjects (mean age: 31.21±11.40) were used as healthy controls. The normal controls were matched with the patients for age, sex, and race. All patients fulfilled the American College of Rheumatology classification criteria for SLE. SLE activity was assessed with the SLE Disease Activity Index (SLEDAI). All participants are from Chinese Han population. The participants with concurrent infections were excluded from the study. The study was approved by the Research Ethics Board of Hebei Medical University. Peripheral blood samples were collected from each subject in tubes containing acid citrate dextrose formula A.

T cells isolation, culture and transfection

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density-gradient centrifugation (Eppendorf, Germany). CD4+

T cells were isolated by positive selection using magnetic beads (Miltenyi Biotec, Germany) and the purity was evaluated by flow cytometry (mean purity: 95.5±1.21%; Becton Dickinson, USA). The cells were then cultured in AIM V serum free medium (Life Technologies, USA). For transfection, CD4+ T cells were transiently transfected with FAM labeled-miRNA using Human T cell Nucleofector Kit and Amaxa nucleofection technology (V-24 program), according to the manufacturer's instructions. The DNMT1 mRNA and protein expression were analyzed 48 h postnucleofection and levels of methylation sensitive genes were performed 72 h postnucleofection.

Real-time PCR

Total RNA was extracted from cell lines using TRIzol reagent (Life Technologies) following the manufacturer's instructions. Reverse transcription of mRNA and miRNAs were performed using PrimeScript RT Master Mix and SYBR PrimeScript miRNA RT-PCR Kits, respectively (Takara Biotechnology, Dalian, China). Real-time PCR was performed using two-step Stemaim-it miR qRT-PCR Quantitation Kit (SYBR Green) (Novland, Shanghai, China) on BIO-RAD IQ5 real-time PCR instrument. All reactions were conducted in triplicate. Quantitative normalization was performed on U6 and β -actin for miRNA detection. The relative expression levels were calculated using $2^{-\Delta\Delta Ct}$ methods. The primers are as follows: DNMT1 (forward 5'-CG-GTTCTTCCTCCTGGAGAATGTCA-3', reverse 5'-C-ACTGATAGCCCATGCGGACCA-3'), β -actin (forward 5'-GCACCACACCTTCTACAATGAGC-3', reverse 5'-GGATAGCACAGCCTGGATAGCAAC-3'). And the primers for miR-185 and U6 were obtained from Genepharma, Shanghai, China.

Protein extraction and Western blot analysis

Cells were lysed on ice for 30 min in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Igepal CA-630, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.1% SDS) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). Lysates were cleared by centrifugation at 4°C for 10 min at 10,000 × g. Equal amount of protein was separated on 10% SDS-PAGE gels and transferred electrophoretically to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% (w/v) nonfat milk in Tris-buffered saline with

MicroRNA-185 contributes to DNA hypomethylation in pregnant patients with SLE

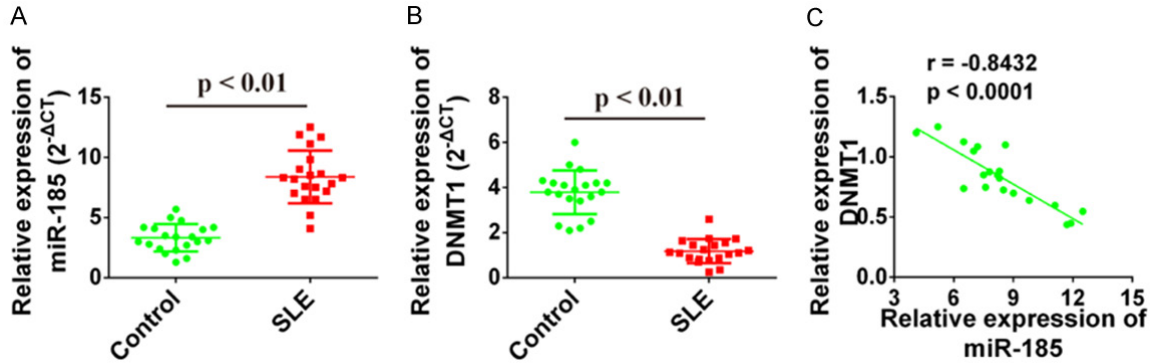


Figure 1. Expression of miR-185 and DNMT1, and the correlation analysis between the expression of miR-185 and DNMT1 mRNA in CD4⁺ T cells from pregnancy patients with SLE. A, B. The expression levels of miR-185 and DNMT1 were analyzed by real time PCR in PBMCs from 20 pregnancy patients with SLE as compared to healthy donor, ($P < 0.01$). C. Pearson's correlation was performed to analyze the correlations between expression of miR-185 and DNMT1 mRNA levels. ($r = -0.8432$; $P < 0.0001$).

0.1% Tween-20. The membranes were incubated overnight with primary Abs and 1 h at room temperature with secondary Ab conjugated with HRP. Then the bands were visualized with ECL technology (Fujifilm LAS-3000, Japan). Antibodies anti-DNMT1, anti-CD11a, anti-CD70 and anti- β -actin were purchased from Abcam (USA). Relative expression levels were quantified using Quantity One software (Bio-Rad).

Luciferase reporter assay

To determine whether DNMT1 is a direct target of miR-185, we cloned the miR-185-predicted target sequence that was contained within its 3'-untranslated region (3'-UTR) downstream of a luciferase gene in the p-miR-reporter luciferase plasmid (Biotech, Shanghai). The lysate was assayed using Dual-Glo luciferase assay system (E1960; Promega, USA) and was measured by a luminometer (EG&G Berthold, Wildbad, Germany).

DNA extraction and global DNA methylation detection

Genome DNA was extracted from CD4⁺ T cells by using All Prep DNA/RNA/Protein Mini kit (Qiagen, Germany) following the manufacturer's protocol. The global methylation levels of DNA in CD4⁺ T cells were evaluated by the MethyflashTM DNA Methylation Quantification Kit (Epigentek, USA) according to the instructions. The proportion of methylated nucleotide in the global DNA was shown in the results (methylation %).

Bisulfite sequencing

Genomic DNA was isolated from the cells after miRNA transfection using the QIAamp DNA Blood Midi Kit (Qiagen, Valencia, CA) and was then treated with bisulfite using the Imprint DNA Modification Kit (Sigma-Aldrich). The bisulfite conversion-based PCR Primers were designed with the MethPrimer program: CD70, forward 5'-GAGGTTATGAATTTGGGAGGATAT-3' and reverse 5'-TCCCATCTCAACCTTTTACATAATTA-3'; CD11a forward 5'-GTTATTTAGGTAGGAGTGTAGTGGTA-3', reverse 5'-ACAAATCAACTAAAA-TCAAAAATTC-3'. The PCR products were purified using the Wizard DNA Clean-up System (Promega), and then cloned into the pGEM-T Easy Vector I (Promega). Eight independent clones for each sample were picked, and the T7 and Sp6 primers were used to sequence inserted fragments.

Statistical analysis

All data are. All the results were presented as the mean \pm SD. Data from different groups were compared with each other by using one-way analysis of variance followed by the Bonferroni post hoc test. Only a P -value < 0.05 was considered statistically significant.

Results

miR-185 expression showed negatively correlation with DNMT1 expression in CD4⁺ T cells from pregnancy patients with SLE

To investigate the biological role of miR-185, we firstly detected the expression of miR-185 in

MicroRNA-185 contributes to DNA hypomethylation in pregnant patients with SLE

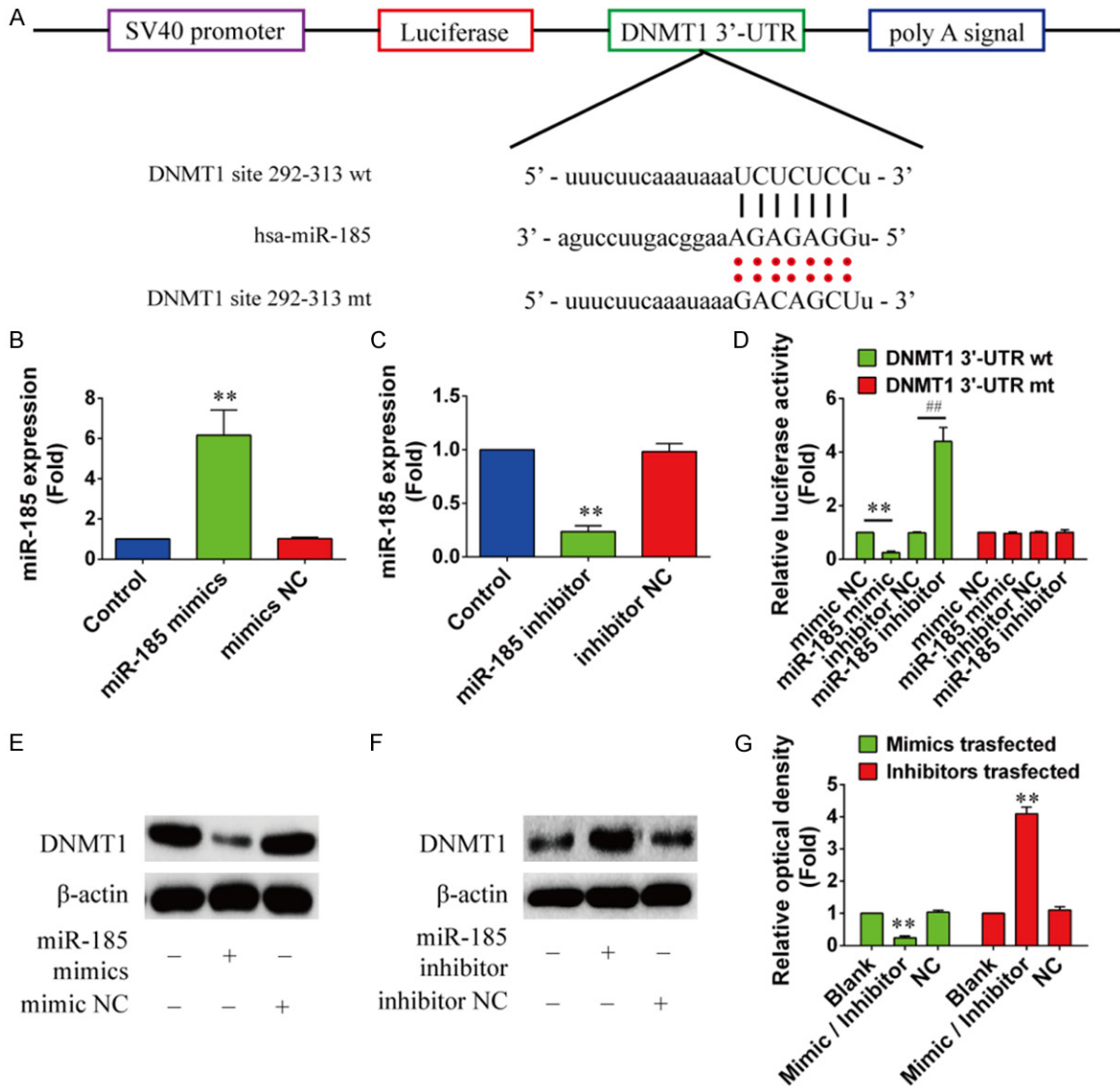


Figure 2. miR-185 directly binds and downregulates DNMT1. A. Schema of the firefly luciferase reporter constructs for the DNMT1, indicating the interaction sites between miR-185 and the 3'-UTRs of the DNMT1. B and C. The expression of miR-185 after treatment with miR-185 mimic or miR-185 inhibitor (n = 6). D. Luciferase activities. CD4+ cells were co-transfected with firefly luciferase constructs containing the DNMT1 wild-type or mutated 3'-UTRs and miR-185 mimic, mimic NC, miR-185 inhibitor or inhibitor NC, as indicated (n = 6). E and F. protein expression of DNMT1 after treatment with miR-185 mimic or miR-185 inhibitor (n = 6). G. Quantifying Western blot using Band-Scan 5.0 software. All data represent the mean ± SD results of three independent experiments. **P < 0.01.

CD4+ T cells from 20 pregnancy patients with SLE and 20 healthy controls using qRT-PCR. As indicated in **Figure 1A**, miR-185 expression showed significant upregulation in CD4+ T cells from pregnancy patients with SLE as compared to that in healthy CD4+ T cells (P < 0.01), while inverse results was observed for DNMT1 concentration (P < 0.01; **Figure 1B**). Furthermore, Pearson's correlation analysis showed a strong negative relationship between miR-185 and DNMT1 expression (R = -0.8432, P < 0.0001;

Figure 1C). These results implied that miR-185 might regulate DNMT1 expression in CD4+ T cells from SLE patients.

DNMT1 as a target of miR-185

To test whether DNMT1 is a direct target of miR-185, we constructed a firefly luciferase reporter containing the potential binding site for miR-185 in DNMT1 3'-UTR (**Figure 2A**). The constructs were then co-transfected into CD4+ T

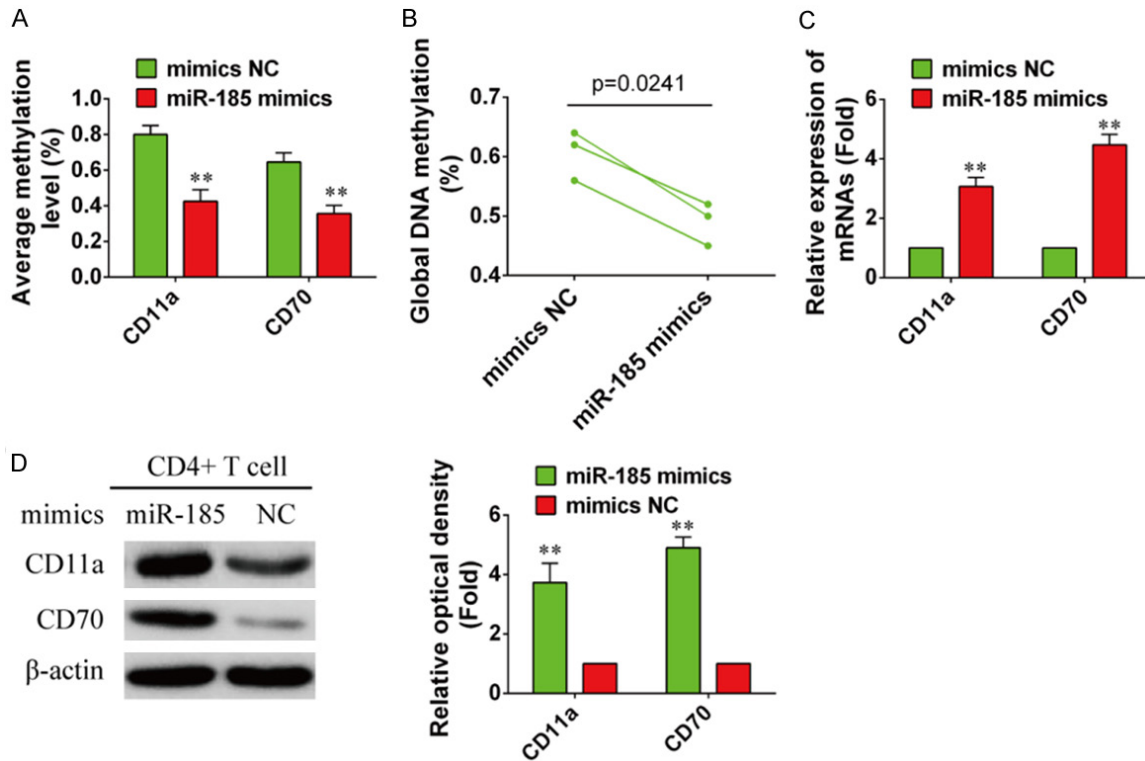


Figure 3. Overexpression of miR-185 promotes hypomethylation of global DNA and CD11a and CD70 promoter DNA, leads to increase CD11a and CD70 expression. CD4+ T cells from pregnancy patients with SLE were transfection with miR-185 mimics or negative control. A. The average methylation levels of CD11a and CD70 promoter regions were significantly decreased in SLE CD4+ T cells 72 h after transfection with miR-185 mimics or negative control. B. The global DNA methylation status was significantly decreased in SLE CD4+ T cells 72 h after transfection with miR-185 mimics or negative control. C, D. The relative mRNA and protein levels of CD11a and CD70 in SLE CD4+ T cells were determined in miR-185 mimics and negative control transfected CD4+ T cells. All data represent the mean ± SD results of three independent experiments. **P < 0.01.

cells together with the miR-185 mimic, its inhibitor or negative control, and luciferase activity was measured 48 h after transfection. Along with significant changes in the expression level of miR-185 (Figure 2B and 2C), we found that miR-185 significantly repressed the activity of reporter vectors harboring wt 3'-UTRs of DNMT1, whereas the mutations of potential miR-185 binding sites in these 3'-UTR regions largely abrogated the inhibitory effects of miR-185 (Figure 2D).

To further confirm the inhibitory regulation of miR-185 on DNMT1 expression, we performed Western blot analysis. We found that overexpression of miR-185 significantly reduced the protein levels of DNMT1, whereas knockdown of miR-185 promoted the levels of DNMT1 (Figure 2E-G). Collectively, these data indicate that the up-regulated miR-185 expression is related to low levels of DNMT1 expression,

which may be associated the development of SLE and support the notion that miR-185 directly targets DNMT1 mRNA, thereby regulating the expression of DNMT1 in CD4+ T cells.

Over-expression of miR-185 reduces global DNA methylation and induces the expression of CD11a and CD70

In lupus, the inhibition of DNMT1 results in passive DNA hypomethylation and the overexpression of methylation-sensitive genes like CD70 and LFA-1 (CD11a) in CD4+ T cells. CD70, a T cell costimulatory molecule encoded by the TNFSF7 gene, is overexpressed in CD4+ T cells from patients with lupus and increases B cell costimulation and subsequent Ig overproduction [20]. CD11a is overexpressed in subsets of autoreactive T cells in patients with lupus and also contributes to the development of lupus [21].

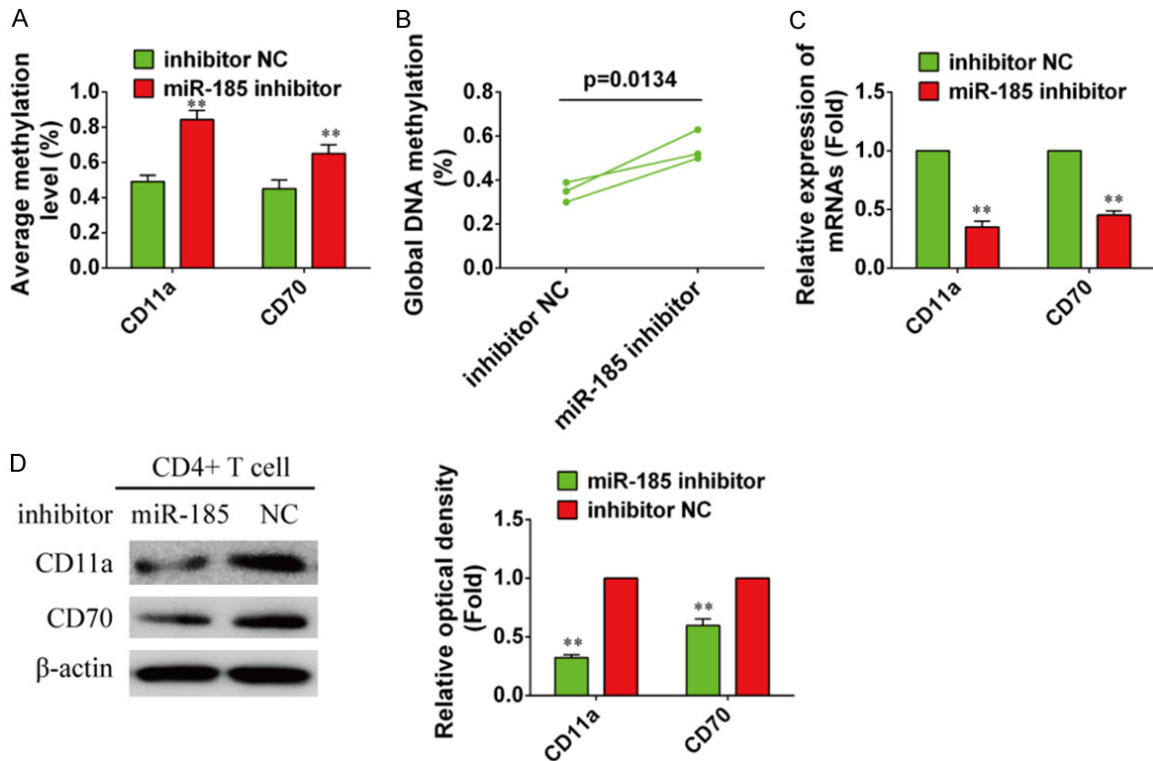


Figure 4. Inhibition of miR-185 reverses global DNA and CD11a and CD70 promoter demethylation, and suppresses CD11a and CD70 overexpression. CD4+ T cells from pregnancy patients with SLE were transfected with miR-185 inhibitor or negative control. A. The average methylation levels of CD11a and CD70 promoter regions were significantly increased in SLE CD4+ T cells 72 h after transfection with miR-185 inhibitor or negative control. B. The global DNA methylation status was significantly increased in SLE CD4+ T cells 72 h after transfection with miR-185 inhibitor or negative control. C, D. The relative mRNA and protein levels of CD11a and CD70 in SLE CD4+ T cells were determined in miR-185 inhibitor and negative control transfected CD4+ T cells. All data represent the mean ± SD results of three independent experiments. **P < 0.01.

Given that low levels of miR-185 were associated with higher levels of DNMT, we investigated whether the enforced expression of miR-185 could functionally modulate DNA hypermethylation and overexpression of CD70 and CD11a in CD4+ T cells. To test the hypothesis, we transfected CD4+ T cells with miR-185 mimic or negative control, respectively, and then DNA methylation levels, mRNA and protein levels of CD11a and CD70 were measured after 72 h transfection. As shown in **Figure 3A**, upregulation of miR-185 reduces the levels of global DNA methylation, and also increased the mRNA (**Figure 3C**) and protein levels (**Figure 3D**) of CD11a and CD70. Furthermore, reports have shown that the promoters of CD70 and CD11a were demethylated in response to DNMTs inhibitors, in T cells from patients with lupus. In this study, we used bisulfite sequencing to determine the methylation status of the CD70 and CD11a promoter posttransfection of

CD4+ T cells with miR-185. We found that the methylation levels of CD11a and CD70 promoters were significantly decreased (**Figure 3B**), consistent with the increased expression of mRNA and protein of both genes.

Knockdown of miR-185 potentially alleviated the hypomethylation in CD4+ T cells from pregnancy patients with SLE

The results above demonstrated that miR-185 up-regulation inhibited DNMT1 expression and induced hypomethylation of global DNA and promoters of CD11a and CD70 and caused overexpression of the two genes in mRNA and protein levels. Therefore, we investigated whether the manipulation of miR-185 levels could alleviate the hypomethylation of CD4+ T cells from pregnancy patients with SLE. We transfected SLE CD4+ T cells with miR-185 inhibitor. As expected, we found that the meth-

ylation levels of CD11a and CD70 promoter DNA (Figure 4A) and global DNA (Figure 4B) were markedly increased ($P < 0.05$) and CD11a and CD70 mRNA (Figure 4C) and protein levels (Figure 4D) were significantly reduced ($P < 0.05$) 72 h posttransfection with miR-185 inhibitor in CD4+ T cells from patients with SLE. These results implied that the manipulation of miR-185 levels could potentially alleviate the DNA hypomethylation of CD4+ T cells in pregnancy patients with SLE.

Discussion

In the present study, we found that the expression of miR-185 was significantly increased and showed negatively correlation with DNMT1 expression in CD4+ T cells from pregnancy patients with SLE. More importantly, we demonstrated that miR-185 directly reduced DNMT1 expression level, thereby resulted in DNA hypomethylation and overexpression of methylation-sensitive genes and mediated the pathogenesis of SLE. And, our studies provide potential novel strategies for therapeutic interventions.

DNA methylation is a fundamental determinant of chromatin structure, with potent suppressive effects on gene expression. Recent studies have shown that T-cell DNA demethylation plays an important role in the pathogenesis of SLE [22]. More evidences have found that T-cells from patients with SLE have reduced expression of DNA methyltransferases [23]. And a study from Quddus J et al. showed that DNA methylation inhibitors like 5-azacytidine can induce T-cell autoreactivity and lupus symptoms in mice [24]. Furthermore, drug-induced lupus is associated with reduced DNA methylation and aberrant expression of DNA methyltransferases [25]. DNA methyltransferases (DNMTs) are critical regulators of the status and intensity of methylation in the genome. Currently, three catalytically active DNMTs, namely DNMT1, DNMT3A, and DNMT3B, have been identified [26]. DNMT1 is constitutively expressed and responsible for the maintenance of DNA methylation during DNA replication. In this work, we used T cell samples from pregnancy patients with SLE and identified miR-185 that was significantly upregulated in CD4+ cells. Mechanistically, miR-185 was linked to DNA hypomethylation in SLE by directly inhibiting the expression of DNMT1 and therefore implicated in the pathogenesis of SLE.

Recent experimental studies have revealed that miRNAs are relevant to the pathogenesis of SLE. For example, Dai et al. firstly identified a number of deregulated miRNAs in peripheral blood mononuclear cells (PBMCs) from SLE patients compared to healthy controls [27]. Tang et al. found that miR-146a can regulate the progression of SLE by suppressing IFN signaling pathway in PBMC of SLE patients [17]. In addition, recent works also demonstrated that several miRNAs directly bind to 3'-UTR region of DNA methyltransferases (DNMT1) and inhibit its expression in lupus CD4+ T cells, such as miR-126, miR-148a and miR-21 [28, 29]. In addition, miR-29b, which is upregulated in SLE CD4+ T-cells, has been shown to inhibit SP1 expression in human T-cells, meaning that they inhibit the expression of DNMT1 and modulate DNA methylation, and further studies demonstrated that inhibition of miR-29b in the T-cells of SLE patients reversed DNA hypomethylation and the upregulation of downstream genes [30]. In this study, we found that overexpression of miR-185 in CD4+ T cells from healthy control subjects induced hypomethylation of global DNA and promoter of CD11a and CD70 genes and up-regulation of associated gene. Interestingly, knock-down of miR-185 in SLE CD4+ T cells increased the expression of DNMT1 and decreased the methylation-sensitive genes expression. These results imply that miR-185 levels may be manipulated to provide useful therapeutic interventions for SLE. Further work on knockout and transgenic animal models would help us to identify the in vivo function of miR-185 in autoimmune diseases.

In conclusion, the results of the current work demonstrated that miR-185 is involved in the pathogenesis of SLE. It could reduce DNMT1 expression and promote DNA hypomethylation in CD4+ T cells from pregnancy patients with SLE. These findings suggest that miR-185 could serve as a novel therapeutic target for the treatment of SLE, as the inhibition of miR-185 expression reversed the hypomethylation status in CD4+ T cells.

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Disclosure of conflict of interest

None.

MicroRNA-185 contributes to DNA hypomethylation in pregnant patients with SLE

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MicroRNA-185 contributes to DNA hypomethylation in pregnant patients with SLE

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