

Original Article

Correlation between miR-126 expression and DNA hypomethylation of CD4+ T cells in rheumatoid arthritis patients

Ge Yang¹, Daoquan Wu¹, Guang Zeng¹, Ou Jiang¹, Pingzong Yuan¹, Shenjie Huang¹, Jing Zhu², Jie Tian², Yaguang Weng², Zihua Rao¹

¹Department of Clinical Laboratory, Affiliated Second People's Hospital of Luzhou Medical College, Neijiang 641000, China; ²Key Laboratory of Diagnostic Medicine Designated by Chinese Ministry of Education and School of Diagnostic Medicine, Chongqing Medical University, Chongqing 400016, China

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Abstract: It has been known that the occurrence of rheumatoid arthritis (RA) was closely correlated with DNA hypomethylation in CD4+ T cells, in which DNA methyltransferase plays a certain role. This study therefore investigated the effect of miR-126 on CD4+ T cell subgroup in RA patients and the alternation of DNA hypomethylation, in an attempt to provide new sights into the pathogenesis and treatment of RA. CD4+ T cells separated from RA patients were transfected with miRNA (miR)-126 expression vector or miR-126 inhibitor expression vector. The expression levels of CD11a, CD70 and DNMT1 mRNA were examined by real-time PCR. Protein levels of CD11a and CD70 were tested by flow cytometry while DNMT1 protein level was quantified by Western blotting. DNA was modified by sodium bisulfite and was sequenced for the methylation status of promoters of CD11a and CD70 genes. Both mRNA and protein expressions of CD11a and CD70 genes in CD4+ T cells were elevated by miR-126 transfection, along with decreased DNMT1 protein level but not mRNA level. The methylation degree of promoters of both CD11a and CD70 genes were significantly depressed after miR-126 transfection. The transfection by miR-126 inhibitor effectively reversed such effects. In RA patients, elevated miR-126 may promote the expression of CD11a and CD70 via the induction of hypomethylation of gene promoters by depressing DNMT1 protein levels.

Keywords: Rheumatoid arthritis, miRNA-126, CD4+ T cells, DNA methylation

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that is commonly occurred and severely affected patients' healthy. RA has the clinical feature as non-septic proliferative synovitis and may compromise the integrity of bone and cartilage tissues, thus causing articular dysfunctions [1]. Previous studies have established the role of epigenetics in the occurrence and development of various autoimmune diseases [2-4]. Classical epigenetic mechanisms include chromatin rearrangement, histone modification and DNA methylation [5, 6]. Among all these regulatory mechanisms, DNA methylation is crucial for gene expressional regulation and is closely correlated with the occurrence and progression of various diseases.

Previous studies have reported the hypomethylation at whole-genomic level of T cells in autoimmune disease patients, and the negative relationship between DNA methylation level and the disease activity [7, 8]. The application of DNA methylation inhibitor to induce the hypomethylation of T cell DNA *in vitro* and further introduction into host mice can lead to lupus-like autoimmune response or the occurrence of lupus [9-12], suggesting the important role of DNA methylation alternation of T cells in the pathogenesis of autoimmune diseases [9, 11-14]. The suppression of DNA methylation level may also activate gene expression including CD11a, Perforin, CD70 and CD40 ligands [15-18]. Various factors including environment and age may all affect the occurrence of RA via regulating DNA methylation level [19]. All these

abovementioned studies suggest the close relationship between DNA methylation and autoimmune diseases. As one of commonly occurred autoimmune diseases, RA is also correlated with DNA methylation. For example, the overexpression of interleukin-8 (IL-8) in CD4+ T cells in RA patients was dependent on the hypomethylation of gene promoter [20]. The methylation status in FOXP3 gene promoter region in CD4+/CD25+ T cell sub-populations is closely related with RA occurrence [21]. The molecular mechanism underlying pathologic hypomethylation of CD4+ T cells in RA patients, however, has not been fully illustrated yet.

MicroRNA (miRNA) is an endogenous small (21~25 nt) RNA molecule with hairpin-like structure, and can suppress the expression of target gene(s) via binding onto target mRNA, thus degrading mRNA and impeding further translation. Recent studies have shown the participation of miRNA in various immune responses [22] and its crucial role in the pathogenesis of autoimmune diseases [23]. In systemic lupus erythematosus (SLE) patients, the expression of miR-126 in CD4+ T cells showed negative relationship with protein expression level of DNMT1, and suppressing methylation level of CD70 and CD11a genes [24]. The abnormal expression of miR-126 also has been found in RA patients [25]. This study constructed miR-126 over-expression vectors and miR-126 inhibitor-expression vector, both of which were further transfected into CD4+ T cells of RA patients. The role of miR-126 on DNA methylation in RA was then determined by analyzing the expression of CD11a, CD70 and DNMT1 expression, along with DNA methylation level of CD11a and CD70 genes, in an attempt to provide new sights for RA pathogenesis and novel treatment strategy.

Materials and methods

CD4+ T cells separation and transfection

Peripheral blood samples (5 mL) were collected from RA patients and were separated for peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation method. CD4+ T cell sub-population was selected by magnetic activated cell sorting (MACS) method. In brief, cell suspensions were firstly washed in PBS, followed by 300 g centrifugation for 10 min. 10^7 cells were mixed with 80 μ L buffer and 20 μ L

MACS CD4+ beads, followed by 4°C incubation for 15 min. After gentle washing, cells were centrifuged again for re-suspension and were then removed for LS separation column in a Midi MACS apparatus (Miltenyi Biotec, Germany). CD4+ T cells were eluted by 5 mL elution buffer. Purity and viability of PBMCs were determined by flow cytometry and trypan blue staining, respectively. Cells were frozen at -80°C for further use.

CD4+ T cells were transfected with psilencer-miR-126, psilencer-negative (Affiliated Second people's Hospital of Luzhou medical college, China), miR-126 inhibitor (5'-UCGUA CCGUG AGUAA UAAUG CG-3') and scrambled oligonucleotide vectors (Qiagen, US) with the help of Amaxa T transfection kit (Qiagen, US). In brief, CD4+ T cells were aliquot into four tubes (each containing 5×10^6 cells) for centrifugation (200 g, 10 min) and resuspension in 100 μ L transfection buffer. After 20 min incubation at room temperature, four different vectors (psilencer-miR-126, psilencer-negative, miR-126 inhibitor and scrambled oligonucleotide) were separately added into these four tubes. After electroporation in the cube, cells were gently transferred into 12-well plate which contained 2 mL T cell culture medium (containing 10% fetal bovine serum and 5% penicillin-streptomycin compound). After incubation at 37°C for 6 hours, the plate was centrifuged at 140 g for 8 min, with re-suspension of T cells in fresh culture medium. After another 24-hour incubation, cell suspension samples were collected from pmaxGFP transfected cells for calculating transfection efficiency. In general, one fixed view was firstly counted for total cell number under a light-field inverted microscope. Fluorescent microscope was then used to count the number of fluorescent cells. Transfection efficiency was calculated by the ratio of fluorescent cells against total cells in the same field.

Real-time PCR

Real-time fluorescent quantitative PCR was used to quantify the mRNA levels of DNMT1, CD11a, CD70 and miR-126, along with house-keeping genes β -actin and 18s rRNA. The reaction system included 10 μ L SYBR Green RT-PCR master mix (Invitrogen, US), forward and reverse primers (1 μ L each), total RNA (3 μ L)

Table 1. Nested PCR primer sequence

Target gene	Primer	Sequence (5'->3')
CD11a (1 st round)	Forward	GGTGAATTCTTAAGGTTAGGAGTTTAAAGTTTAGT
	Reverse	CAATCTAGAACTACAACCTAAAAAATTAATTCG
CD11a (2 nd round)	Forward	GTTGAATTCGGTATATATGGTGAATTTACTTTTAT
	Reverse	CACTCIAGTACAACAAACATCCAAAAATATAAAAAT
CD70 (1 st round)	Forward	GGTGAATTCTTAAGGTTAGGAGTTCAAGTTTAGT
	Reverse	CAACCTAGAACTACACATTCATTAATAAATAAAT
CD70 (2 nd round)	Forward	GTTGAACTCGGTTAACTGGTGAATTTTATTTTCAT
	Reverse	CACTCTAGATACAACAAACATCCAAAAATTAATAAAT

Protein level assay

Flow cytometry was used to detect the protein expression level of CD70 and CD11a in CD4+ T cells. In brief, 45 hours after transfection, cells (2×10⁶) along with blank controls were firstly centrifuged at 2 000 rpm for 10 min, rinsed in PBS, and added with FITC-CD11a or FITC-CD70 conjugated anti-

bodies (eBioscience, US). After mixture and washing, cells were fixed by 0.5 mL paraformaldehyde and loaded on flow cytometry for detection.

Western blotting was used to detect the content of DNMT1 protein in CD4+ T cells. In brief, total proteins were firstly extracted from CD4+ T cells using total protein extraction kit (Qiagen, US) following manual instruction. Purified proteins were separated by SDS-PAGE and were transferred to nitrocellulose membrane, which was blocked in defatted mild powders by 1.5 hours at room temperature. After the incubation with mouse anti-human DNMT1 or anti-β-actin antibodies (1:200, eBioscience, US). Goat anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) was added for a further 2-hour incubation at room temperature. The chromogenic reaction was initiated by ECL reagent and was stopped by PBST buffer.

DNA methylation assay

Bisulfite modification and sequencing method was used to detect the DNA methylation level of CD11a and CD70 genes. Firstly, genomic DNA was extracted from human CD4+ T cells by DNA extraction kit (Boao Biotech, China). Total DNA was then modified by bisulfite using EpiTechR kit (Qiagen, US) following the manual instruction. The target DNA fragment was then amplified by nested PCR using primers as shown in **Table 1**. In 1st round, the reaction mixture included forward and reverse primers (10 ng/μL), dNTP (1.0 μL), Taq polymerase (0.2 μL) and DNA templates. The reaction began with 95°C pre-denature for 5 min, followed by 30 cycles each containing 95°C denature for 30 sec, 60°C annealing for 30 sec and 72°C elongation for 30 sec, and ended with 72°C elongation for 5 min. 2nd PCR reaction mixture consist-

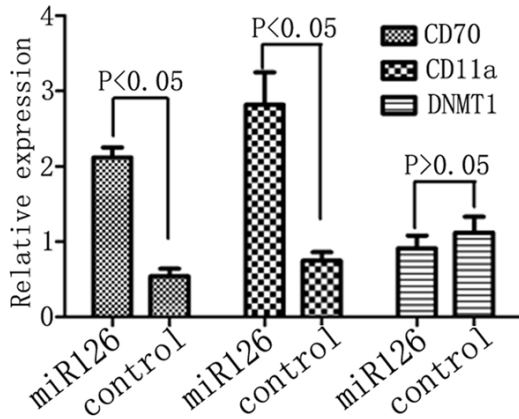


Figure 1. mRNA levels of transfected cells.

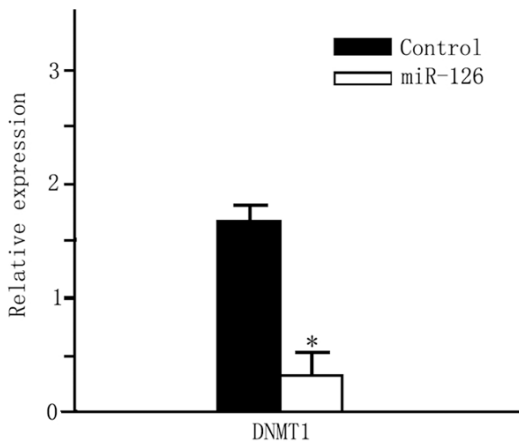


Figure 2. DNMT1 protein level. *P<0.05 compared to the control group.

and enzyme-free water. The reaction parameters were: 50°C *in vitro* reverse transcription for 20 min, 95°C pre-denature for 15 min, followed by 40 cycles each containing 94°C denature for 15 sec, 55°C annealing for 30 sec and 72°C elongation for 30 sec.

Table 2. Expression of CD70 and CD11a in CD4+ T cells

	Cell percentage		Mean fluorescent intensity (MFI)	
	Control	miR-126	Control	miR-126
CD11a	48.33±1.04	58.83±1.15*	7.6±2.1	8.34±2.5
CD70	3.85±0.8	12.06±1.20*	17.77±2.44	18.44±2.75

Note: * $P < 0.05$ compared to the control group.

ed of forward and reverse primers (10 ng/ μ L), dNTP (1.0 μ L), Taq polymerase (0.2 μ L) and PCR products from 1st reaction (5.0 μ L). The reaction began with 95°C pre-denature for 5 min, followed by 30 cycles each containing 95°C denature for 30 sec, 60°C annealing for 30 sec and 72°C elongation for 30 sec, and ended with 72°C elongation for 5 min.

Nested PCR products were purified by Gel Extraction Kit (Qiagen, US), ligated with pGEM-T Easy Vector, and were transfected into DH5 α competent cells. After PCR identification, positive recombinant vectors were sequenced.

Statistical analysis

SPSS 15.0 software package was used to process all collected data, of which measurement data were presented as mean \pm standard deviation (SD). The comparison of means between two independent samples was finished by student t-test. Whilst non-parametric data were compared by Mann-Whitney test. A statistical significance was defined when $P < 0.05$.

Results

mRNA levels in CD4+ T cells after transfection

Using fluorescent quantitative PCR, we examined the mRNA levels of DNMT1, CD11a and CD70 in CD4+ T cells. As shown in **Figure 1**, miR-126 transfected CD4+ T cells had significantly elevated mRNA levels of CD11a and CD70 genes ($P < 0.05$) but not DNMT1, when compared to the vector control group.

Protein contents after transfection

We further utilized Western blotting method to detect the DNMT1 protein level in CD4+ T cells. Results (**Figure 2**) showed significantly decreased DNMT1 protein level in miR-126 transfected cells when compared to those were transfected with blank control vectors ($P < 0.05$).

Flow cytometry was further recruited to detect protein level of CD11a and CD70. As shown in **Table 2**, after miR126 transfection, CD4+ T cells had elevated percentages of CD11a and CD70-positive cells ($P < 0.05$). The mean fluorescent intensity (MFI), however, had no significant difference across groups ($P > 0.05$).

Methylation status of gene promoter regions

Using bisulfite modification and sequencing method, we examined the methylation condition of CD70 (TNFSF7) and CD11a (ITGAL) promoter regions. The methylation percentage of CD70 in controlled group was about 48.59% \pm 3.58%. After miR-126 transfection, the methylation level was decreased to 30.42% \pm 2.97% (**Figure 3A**). CD11a has methylation percentage at 50.19% \pm 4.12% in control ones, and only 31.56% \pm 3.68%, suggesting the significant suppressed methylation of CD11a gene after miR-126 transfection (**Figure 3B**).

mRNA level after miR-126 inhibitor transfection

Compared to those transfected with scrambled oligonucleotide, CD4+ T cells transfected with miR-126 inhibitor showed significantly suppressed CD11a and CD70 mRNA levels (**Figure 4**, $P < 0.05$).

Protein level after miR-126 inhibitor transfection

The protein expression level of DNMT1 was significantly elevated after transfection with miR-126 inhibitor in CD4+ T cells from RA patients, when compare to those transfected with scrambled oligonucleotide (**Figure 5**, $P < 0.05$). The percentage of CD11a and CD70 positive cells in CD4+ subpopulation of T cells was significantly decreased after miR-126 inhibitor transfection ($P < 0.05$) while the mean fluorescent intensity (MFI) remained unchanged (**Table 3**, $P > 0.05$).

Gene promoter methylation level after miR-126 inhibitor transfection

In scrambled control cells, the methylation of CD70 gene was 44.86% \pm 3.42%. After miR-126 inhibitor transfection, the methylation level was

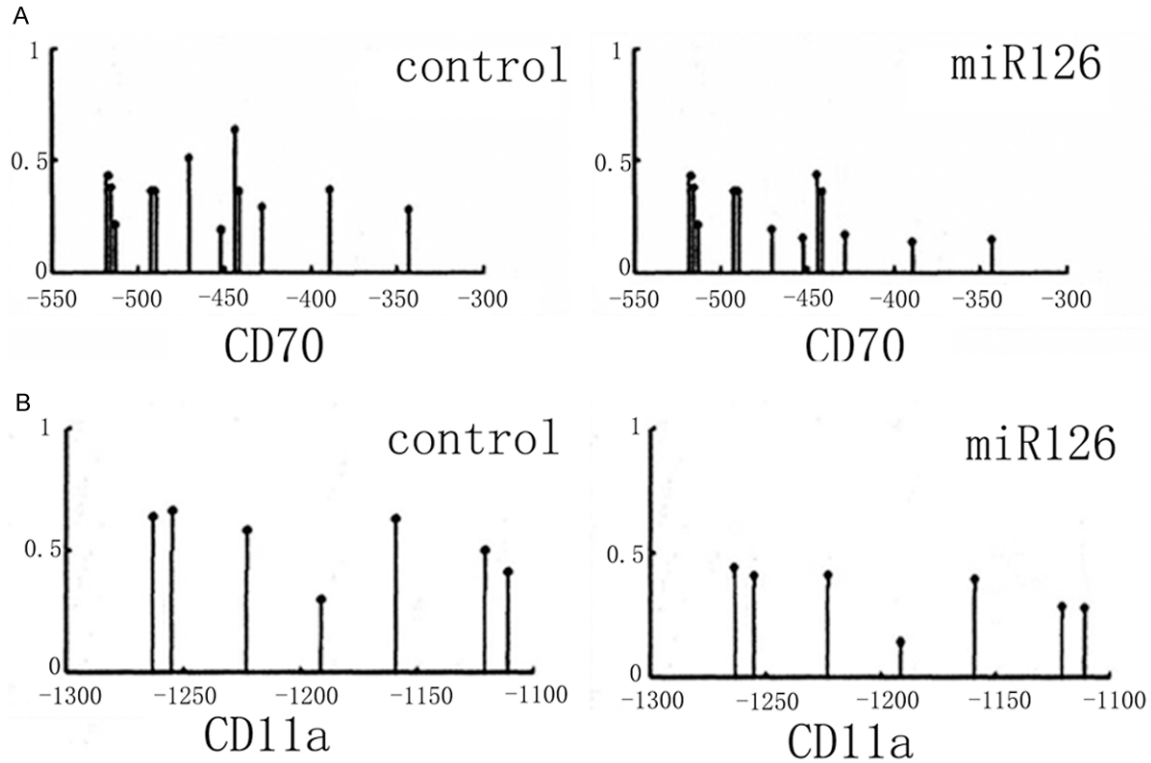


Figure 3. Gene promoter region methylation level. A. CD70 gene; B. CD11a gene.

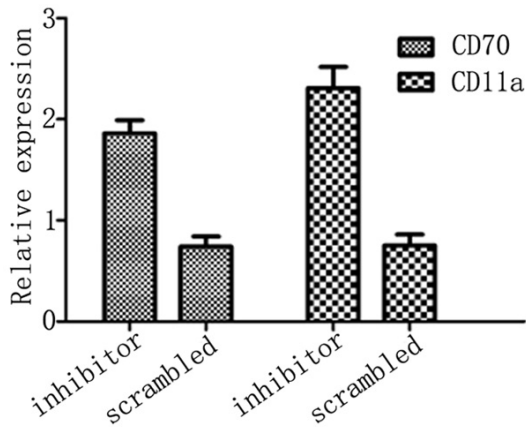


Figure 4. CD70 and CD11a mRNA level after inhibitor transfection.

significantly elevated to $52.32\% \pm 3.58\%$ (Figure 6A, $P < 0.05$). CD11a gene methylation level showed similar patterns, as the increase from $48.16\% \pm 3.96\%$ to $55.98\% \pm 4.23\%$ (Figure 6B, $P < 0.05$).

Discussion

With the advancement of epigenetics study, DNA methylation has been found to be closely

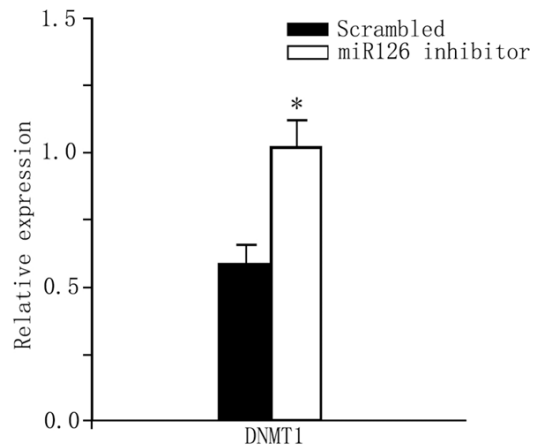


Figure 5. DNMT1 protein expression level. * $P < 0.05$ compared to the scrambled oligonucleotide control group.

correlated with autoimmune diseases. Currently more than 100 DNA methylation sensitive genes have been identified by oligonucleotide sequencing analysis, including CD11a, CD70, CD10L/IgEFCRY1 and perforin [15-18]. Those abovementioned methylation sensitive genes were found to be over-expressed in CD4+ T cells from SLE patients, with suppressed DNA methylation status at the gene

miR-126 and DNA methylation

Table 3. CD70 and CD11a expression in transfected CD4+ T cells

	Cell percentage		Mean fluorescent intensity (MFI)	
	Scrambled control	MiR126 inhibitor transfection	Scrambled control	MiR126 inhibitor transfection
CD11a+	53.33±0.57	48.23±1.15*	17.6±2.1	14.34±2.5
CD70+	14.97±2.65	6.06±2.20*	10.77±2.46	7.44±2.75

Note: *P<0.05 compared to the scrambled control group.

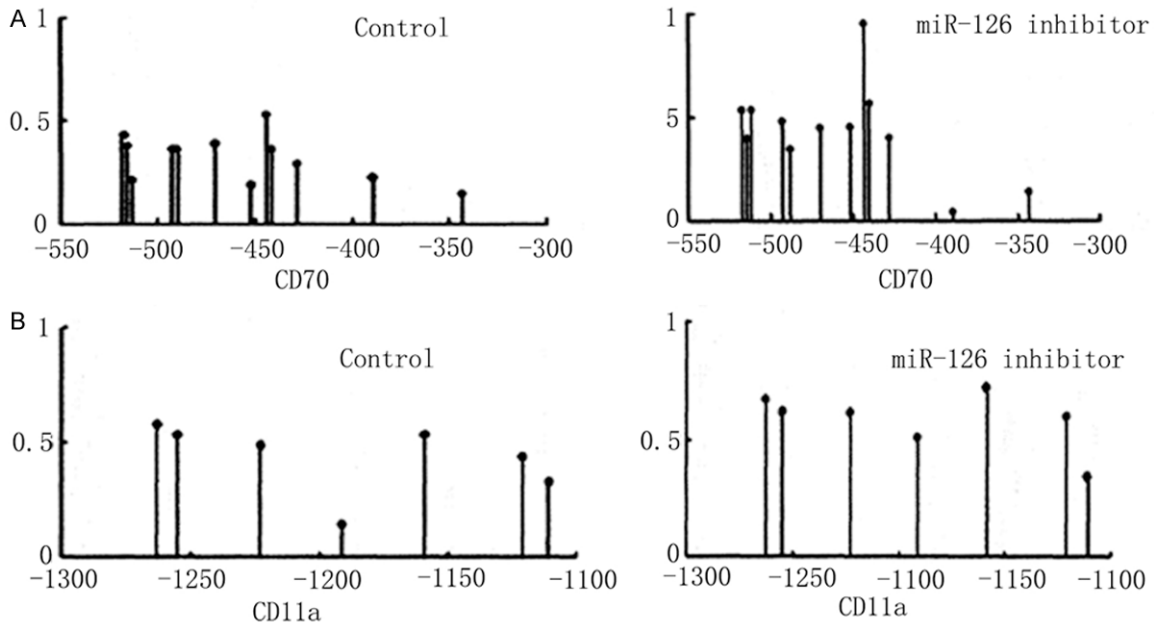


Figure 6. DNA methylation level of CD70 (A) and CD11a (B) genes.

promoter region [13-15, 26]. MiRNA is one important factor regulating DNA methylation level, and has been found to be correlated with the occurrence of RA [20]. This study thus detected both gene expression and DNA methylation levels of CD11a and CD70 genes by over-expression and silencing of miRNA-126. Our results showed significantly elevated mRNA and protein expression levels of both CD11a and CD70 in RA patients derived CD4+ T cells transfected with miR-126 plasmids, along with depressed DNMT1 protein but not mRNA. The introduction of miR-126 inhibitor reversed such effects, suggesting the potency of elevated miR-126 expression in inducing hypomethylation of CD11a and CD70 genes, possibly via depressing DNMT1 protein levels, thus causing over-expression of CD11a and CD70, leading to the occurrence and progression of RA.

CD11a is one subunit of lymphocyte related antigen-1 and is expressed on the surface of lymphocytes, macrophage and mononuclear

cells. It is known to play a critical role in the activation and proliferation of T cells, as well as in the inflammation and body immune response. Studies have reported the induction of autoimmune disease by over-expression of CD11a in animal models [27]. DNA methylation is one typical epigenetic mechanism regulating gene expression. It has been reported that CD11a hypomethylation was correlated with over-expression of CD4+ T cells in SLE patients [14]. The over-expression of cytokines may stimulate B cells to produce large amounts of auto antibodies, further causing the occurrence of autoimmune diseases. CD70, as one B cell co-stimulator molecule, can facilitate the production of immunoglobulin (Ig) by the synergistic effects with CD27. The over-expression of CD70 also leads to over-production of auto antibodies, further causing tissue damage [28]. The hypomethylation level of CD70 gene has been correlated with over-expression of CD4+ T cells in SLE patients [15]. Based on all those previous

studies and our results, the over-expression of miR-126 may suppress DNMT1 expression, causing depressed DNA methylation level of CD11a and CD70 genes, thus elevating the gene expression level and leading to RA occurrence.

In summary, this study for the first time found the role of miR-126 in depressing DNA methylation level of CD4+ T cell-related autoimmune genes, thus activating gene expression and causing RA pathogenesis. This study provides new insights for the etiology of RA and indicate novel treatment strategy.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zihua Rao, Department of Clinical Laboratory, Affiliated Second People's Hospital of Luzhou Medical College, 244 New River Road, Dongxing District, Neijiang 641000, Sichuan, China. Tel: +86-832-2383108; Fax: +86-832-2383108; E-mail: hgteyt889@yeah.net

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miR-126 and DNA methylation

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