The role of miR-125b in T lymphocytes in the pathogenesis of systemic lupus erythematosus

X. Luo[1], L. Zhang[2], M. Li[1], W. Zhang[1], X. Leng[1], F. Zhang[1], Y. Zhao[1], X. Zeng[1]

[1]Department of Rheumatology, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing; [2]Department of Rheumatology, Provincal Hospital Affiliated to Shandong University, Jinan, China.

Abstract

Objectives

This study aimed to explore miRNA expression profiles in peripheral blood mononuclear cells (PBMCs) from patients with systemic lupus erythematosus (SLE) and the potential biological functions of the associated miRNA in the pathogenesis of SLE.

Methods

Fifty patients with active SLE and 26 healthy controls were enrolled. Four patients and four controls were used for miRNA microarray analysis to detect the levels of 847 miRNAs in PBMCs. The others were used for qRT-PCR confirmation and miRNA functional studies. A reporter gene assay was used to determine the biological function of miR-125b.

Results

Eleven miRNAs were found to be up-regulated and 26 miRNAs were down-regulated in SLE patients. Further analysis showed that the down-regulation of miR-125b, mainly in T cells, was negatively correlated with lupus nephritis. We also confirmed that ETS1 and STAT3 are target genes of miR-125b using a dual-luciferase reporter transfection assay.

Conclusion

These data identified this miRNA expression profile as a possible new biomarker of SLE. Moreover, the down-regulation of miR-125b mainly in T cells may contribute to the pathogenesis of SLE by regulating ETS1 and STAT3 gene expression.

Key words

systemic lupus erythematosus, microRNA, miR-125b, target gene

*Xiaofang Luo, PhD**
*Limin Zhang, MD**
Mengtao Li, MD
Wen Zhang, MD
Xiaomei Leng, MD
Fengchun Zhang, MD
Yan Zhao, MD
Xiaofeng Zeng, MD

**These authors made an equal contribution to this study.*

Please address correspondence to:
Dr Xiaofeng Zeng,
Department of Rheumatology,
Peking Union Medical College Hospital,
Peking Union Medical College
and Chinese Academy of Medical Sciences,
1 Shuaifuyuan, Dongcheng District,
100730, Beijing, China.
E-mail: zengxfpumc@yahoo.cn

Received on May 25, 2012; accepted in revised form on September 3, 2012.

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Funding: this study was supported by the Chinese National Key Technology R&D Program, Ministry of Science and Technology (2008BAI59B02), Chinese National High Technology Research and Development Program, Ministry of Science and Technology (SS2012AA02A513) and the National Natural Sience Foundation of China (81202824).

Competing interests: none declared.

Introduction

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease with a diverse array of clinical manifestations that is characterised by the production of antibodies to components of the cell nucleus. Although the life expectancy of SLE patients has improved, the 15-year overall survival rate is only 80%. It has been widely reported that T cells and B cells participate in the pathogenesis of SLE, but the specific mechanism remains unclear (1-3).

MicroRNAs (miRNAs) are small, singal-stranded noncoding RNAs, many of which have been highly conserved throughout evolution. Currently, miRNA is known to regulate several cellular processes such as differentiation, cell cycle, apoptosis, and immune functions. MiRNAs are known to regulate post-transcriptional events of certain subsets of messenger RNAs (mRNAs) by binding to their 3´untranslated region (UTR), which targets them for degradation or translational repression. To date, the miRNA sequence database, miRbase, includes over 9.000 predicted miRNAs in numerous species of plants, animals, and viruses. In humans alone, the miRBase lists over 1.000 predicted miRNAs, and other bioinformatics predictions indicate that as much as one-third of all mRNAs may be regulated by miRNA (4-6).

More than 100 different miRNAs are expressed by cells of the immune system. They have the potential to broadly influence the molecular pathways that control the development and function of innate and adaptive immune responses by regulating target mRNA expression (7-11). Altered miRNA expression has been reported in human autoimmune diseases such as SLE, rheumatoid arthritis, and multiple sclerosis (12-18). However, the role of miRNA in autoimmunity is just beginning to be explored, and the specific role remains unclear.

In this study, we used a miRNA-specific microarray to screen a set of SLE-related miRNAs and further predict their targets using bioinformatics and verification with a dual-luciferase reporter transfection assay. In functional studies, we show that miR-125b, which is a miRNA that correlates with lupus nephritis, targets the *ETS1* and *STAT3* genes.

Patients and methods

Patients and healthy blood donors

Fifty patients with SLE based on the American College of Rheumatology classification criteria (19) were recruited from the Department of Rheumatology, Peking Union Medical College Hospital (China). Active disease was defined as a Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score of ≥4, and inactive disease was defined as a SLEDAI score of <4. Twenty-six healthy control subjects were recruited from age- and sex-matched healthy blood donors. All subjects gave written informed consent prior to enrollment in the study, which was approved by the ethics committee of our hospital.

Cell isolation

Peripheral blood mononuclear cells (PBMCs) were freshly isolated by Ficoll-Histopaque (Sinopharm Chemical Reagent Co., LtD, China) density gradient centrifugation of heparinised venous blood. The $CD3^+$ T and $CD19^+$ B lymphocytes were obtained by FACSAria (Becton Dickinson, USA) based on the expression of CD3 or CD19 (eBioscience, USA).

Microarray

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The expression of 847 human miRNAs was analysed by miRCURY LNA™ microRNA Arrays (Exiqon, Denmark), using 30 ng total RNA. Changes in microRNA expression in PBMCs from patients with SLE compared to that in PBMCs from healthy control subjects were assessed on a GenePix 4000B Array Scanner and GenePix Pro6.0 software (MDC, USA). MiRNA products with at least a 1.5-fold difference in expression between the 2 groups and a *p*-value less than 0.05 were considered significant.

Real-time PCR

To validate the microarray results, the TaqMan kits specified to quantify miR-125b, miR-326, and miR-26a expression levels normalised to the U6 small nuclear RNA (U6 snRNA). To quantify the mRNA, 400 ng of RNA from each sample was reverse transcribed into comple-

Table I. Sequences of primer used in the qRT-PCR.

MicroRNA	Forward primer	Reverse primer
miR-125b	5'GCTCCCTGAGACCCTAAC3'	5'CAGTGCGTGTCGTGGAGT3'
miR-326	5'CGCCTCTGGGCCCTTCC3'	5'GTGCAGGGTCCGAGGT 3'
miR-26a	5'GCCGCTTCAAGTAATCCAGGAT 3'	5' GTGCAGGGTCCGAGGT 3'

Table II. Sequences of primer of target gene 3'-UTR used in the PCR.

Gene	Forward primer	Reverse primer
ETS1	5'CTCGAGACATCCCTTTACTTACTTG 3'	5'GGTTCTACTCTTACCCATTA 3'
STAT3	5'CTCGAGATGAGTGAATGTGGGTGA 3'	5'TGTTGCTGGAGAAGTAAGAG 3'

mentary DNA (cDNA), using a PrimeScript RT Reagent kit (Takara). The cDNA was amplified by real-time RT-PCR with SYBR Green (SYBR Premix Ex Taq RT-PCR kit; Takara). The relative expression levels were analysed using the comparative threshold cycle (Ct) method according to the manufacturer's instructions. The sequences of primers used for PCR are listed in Table I.

miRNA target prediction analysis

Potential miRNA target genes were identified using the miRanda (http://www.microrna.org), PicTar (http://pictar.mdc-berlin.de/), and TargetScan (http://www.targetscan.org) search engines. To optimise the accuracy of prediction, a potential target gene should be predicted by all three programs and correlate with inflammation or apoptosis.

Plasmid construction

A sequence from the target gene 3'-UTR containing the putative binding sites for miR-125b was amplified by PCR using the primers listed in Table II. The target gene 3'-UTR sequences were inserted into the pMIR-REPORT luciferase microRNA Expression Reporter Vector (Promega). The inserts were confirmed by DNA sequencing.

Luciferase activity assay

One day before transfection, 293T cells were seeded at 2×10^4 cells/well in a 96-well plate. Cells were co-transfected with firefly luciferase reporter vector (150 ng) containing the target gene 3´UTR, 3 pmol of pre-miR-125b, or a negative control using Lipofectamine 2000 (Invitrogen) on the following day. After 24 h incubation, the cells were harvested and luciferase activity was assessed using a Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase was used to normalise the Renilla luciferase and the experiments were performed in triplicate.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). Data were analysed by analysis of variance (ANOVA) followed by a Student's unpaired *t*-test for multiple comparisons. Spearman's rank test was used for correlation studies. All analyses were performed with SPSS v13.0 software (IBM, USA). *p*-values less than 0.05 were considered statistically significant.

Results

Microarray analysis reveals a distinct pattern of miRNA expression in PBMCs from patients with SLE

We analysed the expression of 847 mature miRNAs in the PBMCs of 4 patients with SLE and 4 healthy individuals. The microarray analysis identified 11 miRNAs that were significantly up-regulated (1.5–3.0-fold) and 26 miRNAs that were significantly down-regulated (1.5–3.5-fold) in patients with active SLE compared to the controls ($p<0.05$; Table III, Fig. 1A). The microarray results were confirmed by quantitative real-time PCR (Fig. 1B).

Decreased expression of miR-125b in patients with SLE correlates with lupus nephritis

Since miR-125b was found to be significantly down-regulated in patients with active SLE compared to controls, and it has been reported to regulate naive $CD4^+$ T cell differentiation, expression of NF-κB and p53, and apoptosis (20-24), we further explored the role of miR-125b in SLE. We used RT-PCR to subsequently examined miR-125b expression in a larger group of samples, including 50 patients with active SLE, 8 patients with primary Sjögren Syndrome, 8 patients with rheumatoid arthritis, and 26 normal controls. As shown in Figure 2A, the expression of miR-125b was significantly lower in lupus patients compared to the controls ($p<0.01$), and the expression of miR-125b was also significantly lower in patients with primary Sjögren syn-

Table III. Differentially expressed miRNAs in peripheral blood mononuclear cells (PBMCs) from patients with systemic lupus erythematosus (SLE).

Name	Test/control
hsa-miR-7	1.50778
hsa-miR-300	1.6879
hsa-miR-326	3.01035
hsa-miRPlus-E1024	1.62828
hsa-miRPlus-E1030	1.55356
hsa-miR-519e	1.56387
hsa-miR-1246	2.44836
hsa-miRPlus-E1038	2.11636
hsa-miR-1308	1.85795
hsa-miRPlus-F1066	1.58372
hsa-miRPlus-F1086	1.63082
hsa-miR-140-5p	0.61735
hsa-miR-125a-5p	0.57099
hsa-miR-26a	0.61063
hsa-miR-31	0.2862
hsa-miR-365	0.50534
hsa-miR-378	0.63024
hsa-miR-95	0.4306
hsa-miR-29c*	0.51467
hsa-miR-660	0.66783
hsa-miR-590-5p	0.58126
hsa-let-7a	0.64967
hsa-miR-362-3p	0.62499
hsa-miR-28-3p	0.58018
hsa-miR-374a*	0.61089
hsa-miR-125b	0.36401
hsa-miR-769-5p	0.62127
hsa-miR-576-5p	0.65013
hsa-miR-362-5p	0.49626
hsa-miR-505	0.64718
hsa-let-7b*	0.63609
hsa-miR-29a*	0.5151
hsa-miRPlus-E1035	0.43755
hsa-miRPlus-F1037	0.54158
hsa-miR-27a	0.63951
hsa-miR-26b	0.61314
hsa-miR-1297	0.53611

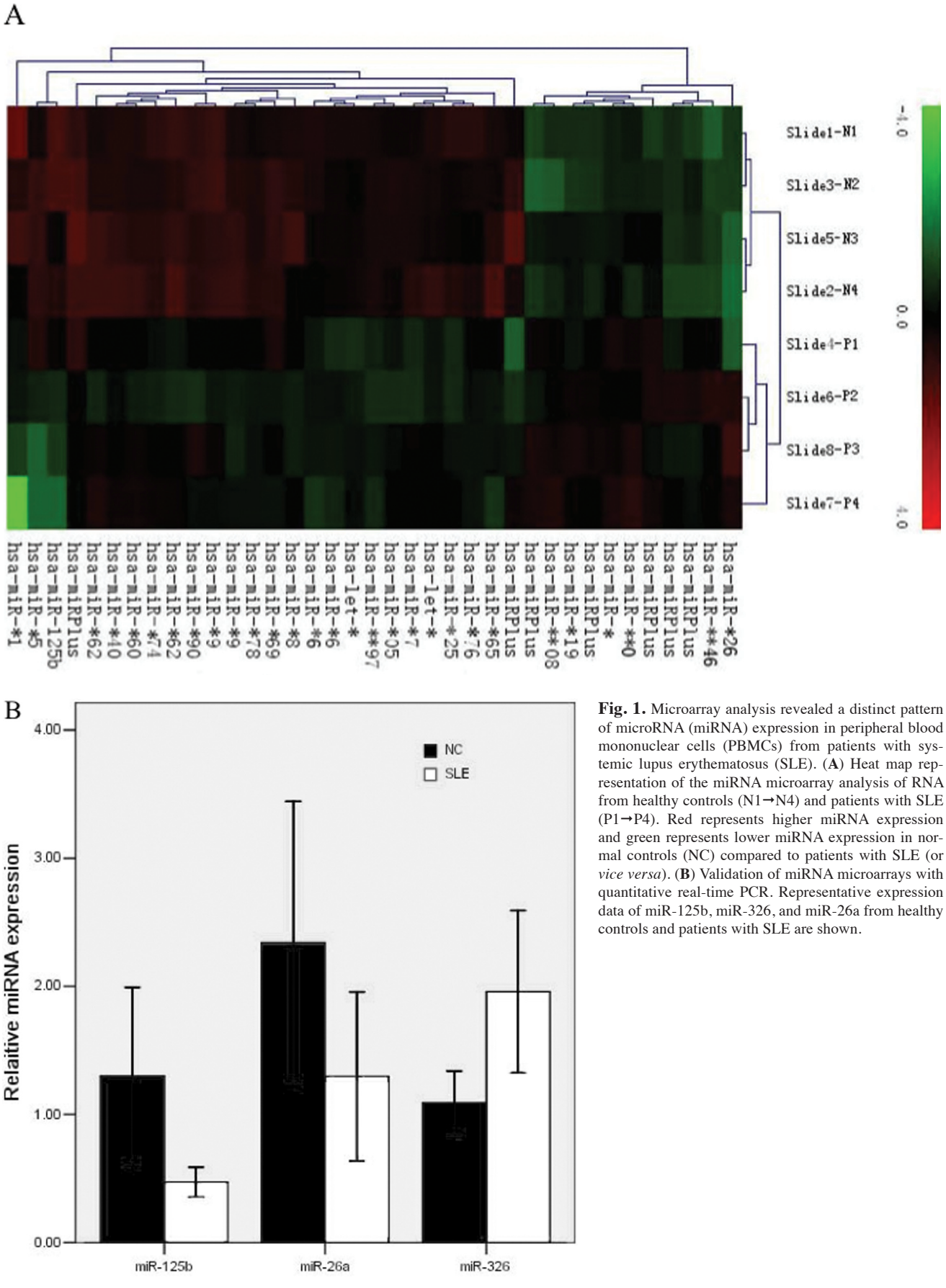

Fig. 1. Microarray analysis revealed a distinct pattern of microRNA (miRNA) expression in peripheral blood mononuclear cells (PBMCs) from patients with systemic lupus erythematosus (SLE). (**A**) Heat map representation of the miRNA microarray analysis of RNA from healthy controls (N1→N4) and patients with SLE (P1→P4). Red represents higher miRNA expression and green represents lower miRNA expression in normal controls (NC) compared to patients with SLE (or *vice versa*). (**B**) Validation of miRNA microarrays with quantitative real-time PCR. Representative expression data of miR-125b, miR-326, and miR-26a from healthy controls and patients with SLE are shown.

drome or rheumatoid arthritis ($p<0.01$) than the controls. However, there was no difference in the level of expression of miR-125b among patients with SLE, primary Sjögren's syndrome, or rheumatoid arthritis.

We next performed an analysis to determine if there was any correlation between miR-125b levels and clinical features. We divided the patients into the following 2 groups: no-lupus nephritis group and lupus nephritis group. Patients with lupus nephritis had significantly lower levels of miR-125b expression than those without lupus nephritis ($p<0.05$; Fig. 2B). However, we found that there was no correlation between miR-125b levels and SLEDAI ($r = -0.209$; $p=0.144$; Fig. 2C). Moreover, there was no correlation between miR-125b levels and the value of C3, anti-dsDNA, or erythrocyte sedimentation rate (ESR) (data not shown).

Differential expression of miR-125b in immune cells

We analysed the expression of miR-125b in different immune cells from 5 patients with untreated SLE and 5 healthy individuals. We used FACS to purify $CD3^+$ T and $CD19^+$ B lymphocytes on the basis of expression of the respective antigens and found that the differential expression of miR-125b in T lymphocytes was significantly higher than B lymphocytes as well as non-T and non-B cells ($p<0.01$; Fig. 3).

MiR-125b targets ETS1 *and* STAT3 *genes*

We next used miRanda, PicTarand, and TargetScan search engines to predict the target genes of miR-125b. According to the principles for optimising the accuracy of prediction of these databases, we focused on genes *ETS1* and *STAT3*. To determine whether these genes were direct targets of miR-125b, we generated three reporter plasmids with firefly luciferase fused downstream to a segment of the *ETS1* or *STAT3* 3'-UTR, respectively, which each contained the putative miR-125b-binding sequence (Fig. 4A-4B). The constructs were then co-transfected into 293T cells and luciferase activity was measured 24 h later. As shown in Figure 4C, miR-125b

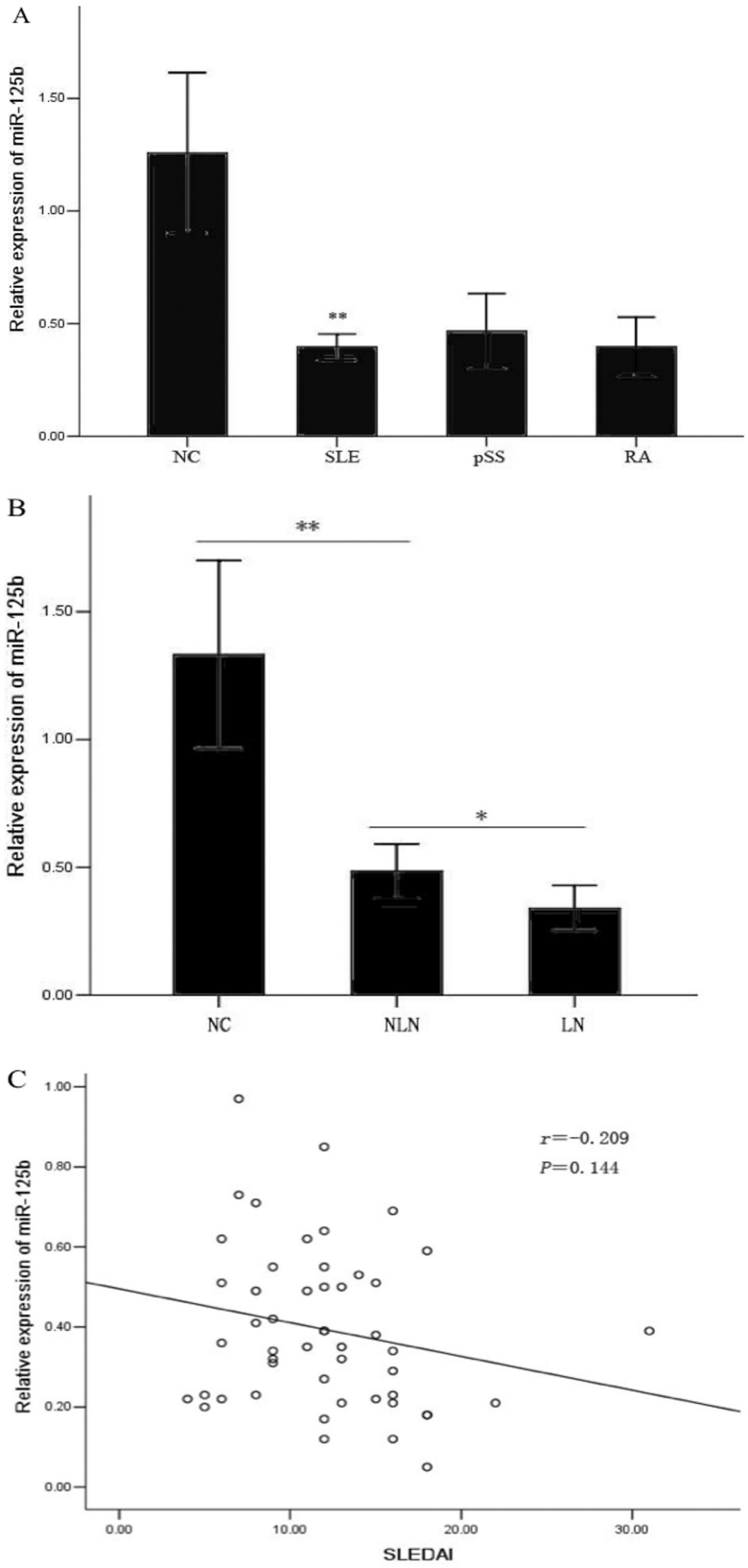

Fig. 2. Down-regulation of miR-125b in patients with SLE and the correlation between miR-125b levels and lupus nephritis and disease activity. (**A**) Expression of miR-125b in 50 patients with SLE, 8 patients with primary Sjögren syndrome (pSS), 8 patients with rheumatoid arthritis (RA), and 26 normal controls (NC). Values are the mean and standard deviation (SD). (**B**) Expression of miR-125b in 21 patients with lupus nephritis (LN) and 29 patients without lupus nephritis. NLN: no lupus nephritis. (**C**) Correlation between miR-125b levels and SLEDAI scores.

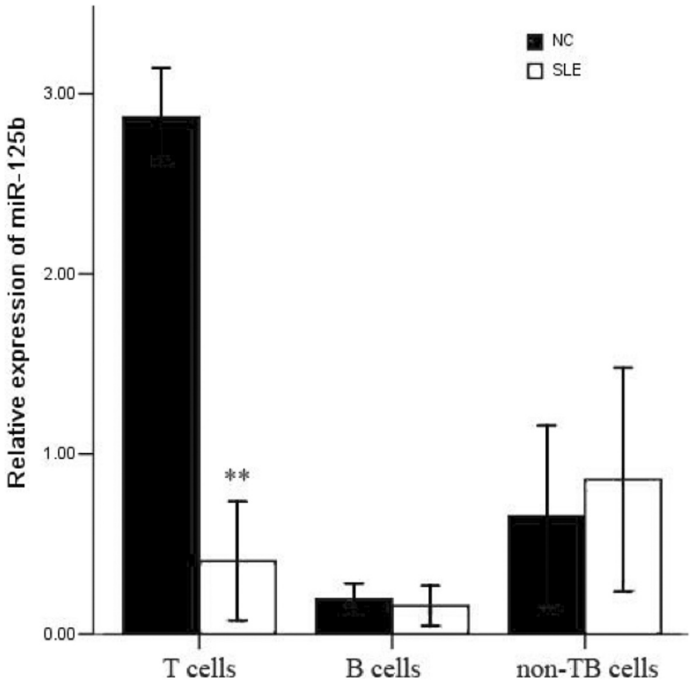

Fig. 3. Differential expression of miR-125b in different types of immune cells.

significantly reduced *ETS1* and *STAT3* luciferase activity ($p<0.05$), with suppression ratios of 50% and 23%, respectively.

Discussion

It has recently become evident that miRNA plays an important role in immune homeostasis and aberrant expression of certain miRNAs, which is thought to contribute to the initiation and progression of autoimmunity. Altered miRNA expression has been reported in the pathology of systemic lupus erythematosus (SLE). For example, Dai *et al*. identified 16 dysregulated miRNAs in the PBMCs from lupus patients (14). Tang *et al*. showed that down-regulation of miR-146a disrupts normal type I interferon pathway signalling in the PBMCs from lupus patients (15), and Zhao *et al*. found that the underexpression of miR-125a contributes to elevated expression of Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) by targeting Kruppel-like factor 13 (KLF13) in lupus T cells (16). Moreover, Zhao *et al*. showed that the Dnmt1-targeting miRNA gene miR-126 is up-regulated in SLE $CD4^+$ T cells and contributes to SLE (17). Stagakis *et al*. found that the up-regulated miR-21 affects programmed cell death 4 (PDCD4) expression and regulates aberrant T cell responses in human SLE (18). Despite the abundance of studies showing the involvement of miRNAs in SLE, the precise role of dysregulated miRNAs in SLE remains to be determined.

By using the miRCURY LNA™ microarray containing 847 human miRNAs, we identified 11 miRNAs that were up-regulated and 26 miRNAs that were down-regulated in patients with SLE compared to healthy controls. Importantly, the microarray results were confirmed by quantitative real-time PCR, and we chose miR-125b for further assessment of the role in SLE.

MiR-125b is a homologous gene of *lin-4* that regulates the spatial and temporal development of C. elegans and can regulate proliferation and differentiation of several types of cells, including neurons (25). It has also been shown to suppress the proliferation of cancer cells (26). MiR-125b was reported to negatively regulate expression of p53 in human lung cells, neurons, and zebrafish embryos (22). In immune cells, miR-125b has been shown to regulate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling by influencing tumour necrosis factor (TNF-α) (21). It has also been reported to regulate naive $CD4^+$ T cell differentiation (20) and is involved in TNF-α-mediated cutaneous inflammation of psoriasis (27). To the best of our knowledge, the functions of miR-125b in the immune system are still unclear, and therefore additional studies are needed to determine the function of miR-125b in immune cells as well as its contribution to the pathogenesis of SLE.

When we examined miR-125b expression in a larger group of samples, the expression of miR-125b was significantly lower in patients with lupus, primary Sjögren Syndrome, and rheumatoid arthritis compared to controls. These three diseases are all autoimmune disorders, and it is hypothesised that miR-125b is involved in the pathogenesis of autoimmune diseases by affecting different target genes. No correlation between miR-125b levels and SLEDAI score, C3 values, anti-dsDNA, and erythrocyte sedimentation rate (ESR) were identified. These results suggest that the down-regulation of miR-125b is not a consequence, but rather a potential cause of SLE disease activity.

Pathogenesis of SLE involves dysfunction of innate and adaptive immunity, including the dysfunction of T and B lymphocytes. We analysed the expression of miR-125b in different types of immune cells and found that miR-125b had a significantly higher expression level in T lymphocytes than B lymphocytes or non-T and non-B cells. The dysfunction of T cells in SLE contributes to the initiation and perpetuation of the autoimmunity, which promote inflammation by secreting cytokines and activating dendritic cells and B cells (2, 28-31). Furthermore, SLE T cells seem to be directly involved in the development of related organ pathology (28). We analysed the correlation between miR-125b levels and lupus nephritis and found that patients with lupus nephritis had markedly lower levels of miR-125b expression than those without lupus nephritis. These results suggested that the down-regulation of miR-125b in SLE T cells contribute to lupus nephritis.

We also used miRanda, PicTarand, and TargetScan search engines to predict target genes of miR-125b. Based on the principles for optimising the accuracy of prediction, we focused on v-ets erythroblastosis virus E26 oncogene

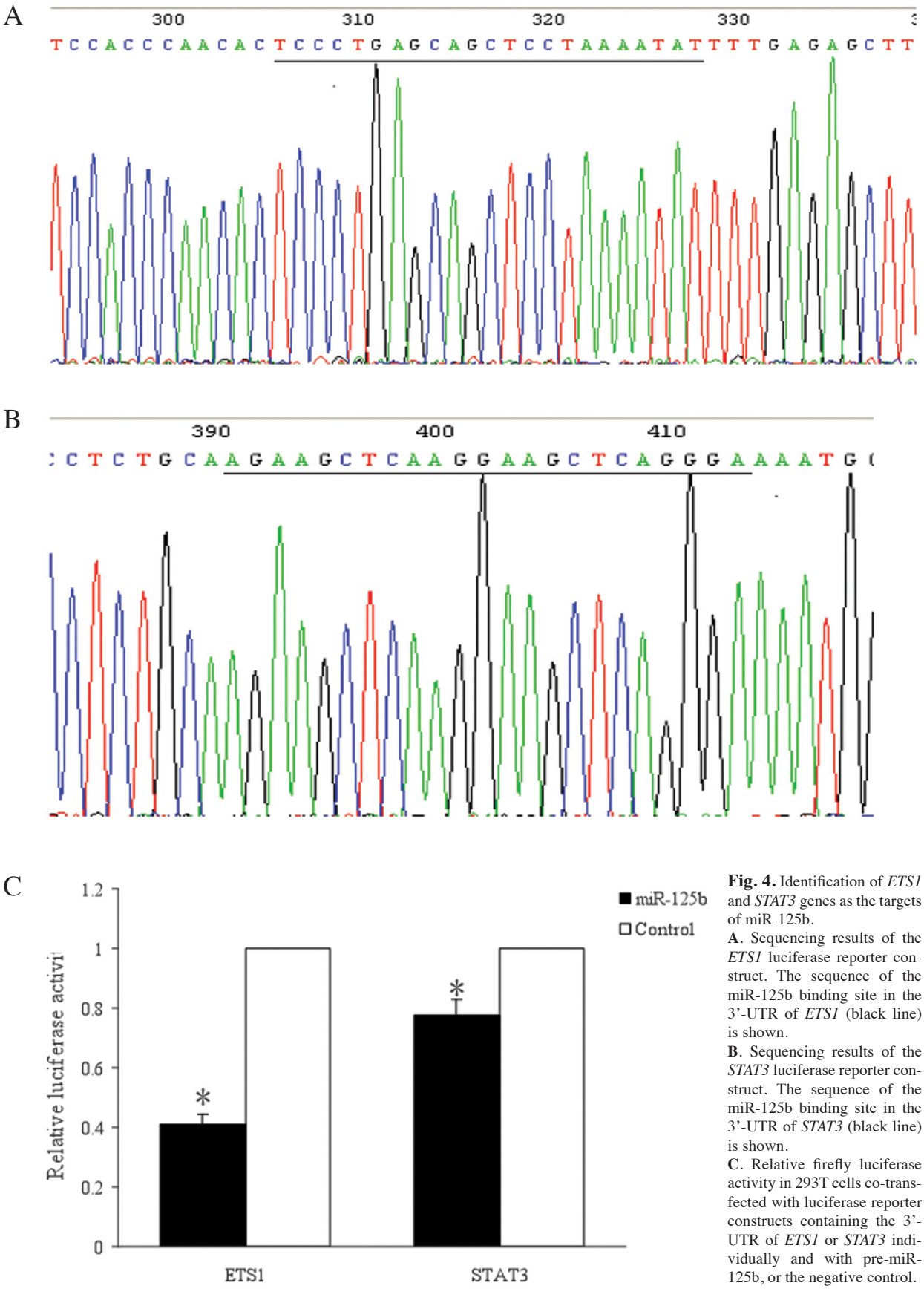

Fig. 4. Identification of *ETS1* and *STAT3* genes as the targets of miR-125b.

A. Sequencing results of the *ETS1* luciferase reporter construct. The sequence of the miR-125b binding site in the 3'-UTR of *ETS1* (black line) is shown.

B. Sequencing results of the *STAT3* luciferase reporter construct. The sequence of the miR-125b binding site in the 3'-UTR of *STAT3* (black line) is shown.

C. Relative firefly luciferase activity in 293T cells co-transfected with luciferase reporter constructs containing the 3'-UTR of *ETS1* or *STAT3* individually and with pre-miR-125b, or the negative control.

homolog 1 (*ETS1)* and signal transducer and activator of transcription 3 (*STAT3)* genes. *ETS1* is a transcription factor that is an important member of the *ETS* family and regulates cell proliferation, apoptosis, angiogenesis, and migration of cancer (32-34). *ETS1* also plays a vital role in immune homeostasis. It was reported that *ETS1* is involved in the differentiation of T cells by regulating the expression of interleukin 2 (IL-2), IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (35-37). Grenningloh *et al*. showed that *ETS1* regulates the Th1 cell-mediated immune response (38). An analysis of polymorphisms of *ETS1* found that the gene correlated with SLE (39-40). *STAT3*, which is the core element of the Janus kinase-Signal Transducer and Activator of Transcription (JAK-STAT) signal transduction pathway, is involved in cell growth, differentiation, proliferation, apoptosis, and other processes with the Mitogen-activated protein kinases (MAPK), Transforming growth factor beta (TGF-β), nuclear receptor, and integrin signalling pathways (41-42). Pramanik *et al*. reported that activation of *STAT3* by IL-6 plays an important role in the pathogenesis of lupus-susceptible mice (43). Harada *et al*. showed that increased expression of *STAT3* in SLE T cells contributed to enhanced chemokine-mediated cell migration (44). Therefore, *ETS1* and *STAT3* seem to play an important role in the pathogenesis of SLE by regulating development, differentiation, proliferation, and apoptosis of immune cells, especially T cells.

In this study, we used bioinformatics to show that miR-125b binds the three prime untranslated region (3'UTR region) of *ETS1* and *STAT3* to regulate the expression of these three genes. When dual-luciferase expression vectors containing the binding sites of the 3'UTR region of *ETS1* or *STAT3* were individually co-transfected with miR-125b into 293T cells, we found that miR-125b reduced *ETS1* and *STAT3* luciferase activity, indicating that these genes are targets of miR-125b. We also found that miR-125b was significantly reduced in SLE T cells, and that the expression of *ETS1* and *STAT3* correlated with regulation of T cell function. Taken together, these results suggested that the down-regulation of miR-125b is involved in the dysfunction of T cells by affecting target genes *ETS1* and *STAT3*. Moreover, miR-125b seems to have an important role in the pathogenesis of SLE.

Conclusion

In conclusion, we have provided evidence for the altered expression of miRNAs in patients with active SLE. MiR-125b expression correlated with lupus nephritis and the dysfunction of T cells by affecting target genes *ETS1* and *STAT3*. Therefore, this miRNA may be exploited as a novel biomarker of SLE. Additional functional studies and studies in knockout and transgenic animal models are needed to explore the role of miR-125b in the pathogenesis of SLE.

Acknowledgements

We would like to thank the patients and healthy volunteers who participated in this study.

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