Concise report

Aberrant T cell expression of Ca\textsuperscript{2+} influx-regulated miRNAs in patients with systemic lupus erythematosus promotes lupus pathogenesis

Ming-Chi Lu\textsuperscript{1,2}, Chia-Li Yu\textsuperscript{3}, Hua-Chien Chen\textsuperscript{4}, Hui-Chun Yu\textsuperscript{1}, Hsien-Bin Huang\textsuperscript{5} and Ning-Sheng Lai\textsuperscript{1,2}

Abstract

Objective. The aim of this study was to investigate the pathogenic role of calcium (Ca\textsuperscript{2+}) influx-regulated microRNAs (miRNAs) in T cells from patients with SLE.

Methods. Expression profiles of 270 human miRNAs in Jurkat cells co-cultured with or without ionomycin were analysed by real-time PCR. Differential expression of miRNAs in T cell samples from 28 patients with SLE (SLE T cells) and 20 healthy controls were investigated using western blot analysis of proteins expressed by respective miRNA target transcripts. Transfection studies were conducted to investigate miRNA-specific biological functions.

Results. Initial analysis revealed differential expression of nine miRNAs in Jurkat cells after co-culture with ionomycin. Of these, miR-524-5p and miR-449b were overexpressed in SLE T cells. Levels of expressed miR-524-5p showed a significant direct correlation with the SLEDAI. Transfection of Jurkat cells with miR-524-5p mimic suppressed Jagged-1 and Hes-1 protein expression. Likewise, expression of both Jagged-1 and Hes-1 proteins were diminished in SLE T cells. Upon activation of Jurkat cells transfected with miR-524-5p mimic, production of IFN-\gamma increased but the apoptotic rate was unaffected.

Conclusion. In SLE T cells, miR-524-5p and miR-449b (both regulated by Ca\textsuperscript{2+} influx) were overexpressed. Moreover, increased miR-524-5p expression, as shown by patients with SLE, directly paralleled disease activity (SLEDAI). Transfection of miR-524-5p also enhanced IFN-\gamma production in activated Jurkat cells.

Key words: systemic lupus erythematosus, T cells, Ca\textsuperscript{2+} influx, miR-524-5p, miR-449b, Notch.

Introduction

Calcium (Ca\textsuperscript{2+}) influx plays a critical role in the activation of T cells. SLE is an autoimmune disease that stems from complex aberrancies in cellular and humoral immunity. Heightened and prolonged Ca\textsuperscript{2+} influx are recognized features of T cells in patients with SLE (SLE T cells), culminating in overexpression of calmodulin kinases, nuclear factor of activated T cells (NFAT), cyclic adenosine monophosphate (cAMP) response element modulator (CREM) and calcineurin [1–3].

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate the expression of multiple genes by targeting mRNA. Our previous study showed that over-expressed miR-224 in SLE T cells facilitated activation-induced cell death [4]. Furthermore, several miRNAs have been demonstrated to be differentially expressed in T cells from patients with SLE compared with controls. These miRNAs also participated in the immunopathogenesis of SLE [5]. Given the complex pathogenesis of SLE, we believe that aberrantly expressed miRNA may abound in SLE T cells. However, Ca\textsuperscript{2+} influx-regulated miRNA expression in T cells and its ramifications...
for the pathogenesis of SLE have not yet been investigated.

We hypothesized that Ca²⁺ influx-regulated miRNAs could contribute to the immunopathogenesis of T cell dysfunction in patients with SLE. Hence we used a novel strategy to search for aberrantly expressed miRNA in SLE T cells. First, Ca²⁺ influx-regulated miRNAs were identified in Jurkat cells and their corresponding levels of expression in SLE and normal T cells were documented. Then, functional aspects of the aberrantly expressed miRNAs were explored and correlated with clinical parameters of SLE. Finally, we investigated the biological functions of specific miRNAs via a transfection study.

Materials and methods

Jurkat cells pretreated with ionomycin

Purchased Jurkat cells (5 × 10⁶; ATCC, Manassas, VA, USA) were incubated overnight with or without ionomycin (1 μg/ml; Sigma-Aldrich, St Louis, MO, USA) in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing heat-inactivated fetal bovine serum (10%), L-glutamine (2 mmol/l), penicillin (100 U/ml) and streptomycin (100 mg/ml). Each treatment was repeated three times.

Assessment of miRNA expression by real-time PCR

Total RNA (including miRNAs) was extracted from purified T cells or Jurkat cells and the expression levels of miRNAs were quantified as previously described [6].

Isolation of T cells from SLE patients and controls

Twenty-eight patients who met the 1997 ACR revised criteria for the classification of SLE [7] were recruited from outpatients of the study hospital and 20 healthy volunteers were recruited to serve as controls. Written consent was obtained from all participants according to the Declaration of Helsinki. The study was approved by the institutional review board of Buddhist Dalin Tzu Chi Hospital, Taiwan (no. B10004011).

The age and sex ratio were not significantly different between patients with SLE [37.1 years (s.d. 12.5), 8.3:1] and controls [33.4 years (s.d. 6.3), 5:1]. All patients with SLE were treated with methylprednisolone [6.9 mg/day (s.d. 1.8) and HCQ]. The SLEDAI score was 3.0 (s.d. 2.4) and 14 patients with SLE had LN. Serological data showed that the mean serum level of anti-dsDNA was 51.5 IU/ml (s.d. 68.5) and C3 was 96.8 mg/dl (s.d. 23.8). Blood samples were collected at least 12 h after the last dose of immunosuppressant to minimize the effects in the in vitro studies. T cells were subsequently purified with anti-human CD3-coated magnetic beads (IMag Cell Separation System, BD Bioscience, Franklin Lakes, NJ, USA).

Western blot analysis of cell lysates

Western blot analysis was performed as previously described [6]. Primary antibodies were mouse monoclonal anti-Hes-1 and anti-Jagged-1 (Abcam, Cambridge, UK) with anti-β-actin used as an internal control (Sigma-Aldrich) and goat-anti-mouse IgGs (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used as secondary antibodies.

Effects of miRNA-524-5p on IFN-γ secretion in Jurkat cells

Jurkat cells were transfected with miR-524-5p mimic or scramble oligonucleotides using the conditions previously described [6] and then cultured in 25 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) with 500 ng/ml ionomycin for 24 h at 37°C. Cultured cells were pelleted by centrifugation (300 g) and supernatants were collected for the determination of IFN-γ levels using ELISA kits (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s protocol.

Detection of apoptosis by flow cytometry

After transfection with miR-524-5p mimic or scramble oligonucleotides, Jurkat cells were cultured with 250 ng/ml ionomycin plus 10 ng/ml PMA for 72 h at 37°C. Apoptotic rates were subsequently determined by subjecting doubly stained [FITC-annexin V and propidium iodide (PI) kit; BD Biosciences] Jurkat cells to flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA) using Lysis II software.

Statistical analysis

All data were expressed as mean (s.d.). Univariate and multivariate linear regression analyses were performed to obtain correlation coefficients and statistical significance using Stata version 8.0 (StataCorp, College Station, TX, USA). Statistical significance, assessed as appropriate by either paired or unpaired Mann-Whitney U-test, was set at P < 0.05.

Results

Expression profiles of Ca²⁺ influx-regulated miRNAs in T cells from patients with SLE and healthy controls

Expression profiles of 270 miRNAs in Jurkat cells co-cultured with or without ionomycin (1 μg/ml) are displayed in Fig. 1A, with each scatter spot representing the average of three normalized miRNA determinations from each group. Expression of nine miRNAs, including miR-432, miR-497, miR-383, miR-505*, miR-483-3p, miR-25*, miR-449b and miR-524-5p, were significantly higher, whereas miR-96 expression was significantly lower in Jurkat cells after co-culture with ionomycin (fold change >4, P < 0.05; Fig. 1B). Expression levels of miR-524-5p and miR-449b from the T cells of SLE patients were significantly elevated (miR-524-5p, 3.4-fold; miR-449b, 1.9-fold; P < 0.05) compared with those in controls (Fig. 1C). However, further analysis of miR-449b was not pursued due to the inherently low levels of expression in T cells in both patients with SLE and controls. Patients with SLE displayed a significant direct correlation between miR-524-5p expression level and SLEDAI score (P = 0.026, r = 0.420; Fig. 1D) and...
Fig. 1 Altered expression of Ca\(^{2+}\) influx-regulated miRNAs in T cells from patients with SLE and normal volunteers

(A) Expression profiles of 270 miRNAs in Jurkat cells co-cultured with or without ionomycin (1 \(\mu\)g/ml) overnight, as determined by real-time PCR. Each scatter spot represents the average normalized expression level of miRNA in three repeats of each treatment. (B) Nine miRNAs exhibited aberrant expression in Jurkat cells cultured with ionomycin (1 \(\mu\)g/ml). (C) miRNA overexpression in SLE T cells limited to miR-524-5p and miR-449b compared with normal T cells. (\(P\)-values obtained with unpaired Mann-Whitney \(U\)-test). (D) Correlation of SLE disease activity and the relative expression level of miR-524-5p in patients with SLE. miRNAs: microRNAs.
this association remained significant after adjusting for age and sex ($P = 0.043$).

**Transfection of miR-524-5p mimic suppressed Jagged-1 and Hes-1 protein expression**

Chen *et al.* [8] demonstrated that miR-524-5p could suppress Jagged-1 and Hes-1 expression by directly targeting their mRNA transcription. To clarify the role of miR-524-5p in Jagged-1 and Hes-1 protein expression, Jurkat cells were transfected with miR-524-5p mimic or scrambled oligonucleotides (control) by electroporation. Expression levels of miR-524-5p increased dramatically thereafter (see supplementary Fig. S1A, available at *Rheumatology* Online), with significant suppression of Jagged-1 and Hes-1 protein expression in Jurkat cells (see supplementary Fig. S1B and C, available at *Rheumatology* Online). A representative sample is depicted in supplementary Fig. S1D, available at *Rheumatology* Online.

**Functional studies of miR-524-5p in the pathogenesis of SLE**

To confirm the potential biological and pathological impact of increased miR-524-5p in SLE T cells, protein expression of Jagged-1 and Hes-1 in T cell lysates from SLE patients and healthy controls were compared using western blot analysis. Protein levels of Jagged-1 (Fig. 2A) and Hes-1 (Fig. 2B) were significantly lower in SLE T cells than in normal T cells. Representative T cell sampling results (three from patients with SLE and two from controls) are shown in Fig. 2C. Logically, an increase in miR-524-5p expression should contribute to the inflammatory response characteristic of SLE. Our results indeed showed that IFN-$\gamma$ secretion increased significantly in ionomycin- and PMA-activated Jurkat cells after the transfection of miR-524-5p mimic (vs transfection of scrambled oligonucleotides; Fig. 2D). However, the rate of activation-induced cell death under this setting (i.e. transfection of Jurkat cells with miR-524-5p mimic vs scrambled oligonucleotides) was not significantly altered (Fig. 2E).

**Discussion**

Abnormal expression of miRNAs in patients with SLE has previously been investigated [5]. Using a stepwise approach, the expression of eight miRNAs, including miR-432, miR-497, miR-383, miR-505, miR-483-3p, miR-25*, miR-449b and miR-524-5p, was significantly higher, whereas levels of miR-96 were significantly lower in Jurkat cells after Ca$^{2+}$ influx. Two of the nine Ca$^{2+}$ influx-regulated miRNAs, miR-524-5p and miR-449b, were expressed at comparatively high levels in SLE T cells. Fan *et al.* [9] demonstrated that miR-31 expression in normal T cells was increased after co-culture with both ionomycin and PMA. In our study we did not find any differences in the expression level of miR-31 in Jurkat cells cultured with or without ionomycin.

Based on the results of a literature search, two known target genes of miR-524-5p, namely, Jagged-1 and Hes-1, were selected for further study [8]. As expected, protein expression of these genes was diminished in SLE T cells. Both proteins are linked to the Notch pathway, an evolutionarily well-conserved signalling mechanism. Humans have four Notch receptors (Notch 1–4) and five distinct Notch ligands (Jagged-1, 2 and $\delta$-like 1, 3 and 4). Hes-1 is a critical protein molecule downstream of the Notch pathogenesis of SLE.
pathway [10]. Accordingly, Sodsai et al. [11] showed a decrease in Notch 1 mRNA and protein expression in T cells from patients with active SLE. However, mRNA expression of molecules downstream (Hes-1 and DeltaX-1) was not decreased, contrary to our finding (by western blot analysis) of lower Hes-1 protein expression in SLE T cells. This discrepancy may be due to the effects of miR-524-5p on Hes-1 mRNA translation. Rauen et al. [12] have also shown that dampening of Notch 1 expression was under epigenetic control in patients with SLE. Activation of CREM-α may suppress Notch 1 expression and thereby increase the production of IL-17. Our results point to an alternative pathway in SLE T cells where overexpressed miR-524-5p inhibits Hes-1 protein expression, thus suppressing Notch signalling.

The potential pathogenic role of decreased expression of Jagged-1 in SLE T cells is unclear. Although Jagged-1 is apparently expressed on the surface of T cells [13, 14], few studies have investigated its effects on T cell function [10]. Stallwood et al. [14] found that knock-down of Jagged-1 by small interfering RNA (siRNA) in CD4+ T cells had no effect on cytokine production. In our study, transfection of miR-524-5p actually enhanced IFN-γ secretion in activated T cells. Because miRNA alone is capable of targeting hundreds of proteins, other signalling pathways influenced by miR-524-5p may account for this discrepancy. Nevertheless, IFN-γ is a major pro-inflammatory cytokine and its levels (in terms of gene expression and serum concentration) were not only elevated in patients with SLE, but correlated directly with their clinical status (SLEDAI score) [15–17]. Our results indicate that increased miR-524-5p can boost the production of IFN-γ in SLE T cells and thereby contribute to an increase in disease activity (SLEDAI score).

Notch signalling and Ca2+ influx may be closely intertwined. In keratinocyte differentiation, for example, the Notch signalling pathway serves as an upstream activator of the calcineurin/NFAT pathway and calcineurin signalling, which in turn mediates the cellular transcriptional response to Notch activation [18]. In SLE T cells, once the calcineurin/NFAT pathway is activated via Ca2+ influx, enhanced expression of miR-524-5p may invoke a negative feedback loop to control Notch signalling activity. Notch signalling in T cells is complex and has been implicated in differential responses by various T cell subsets [19], but more work is needed to understand its functional roles in SLE pathogenesis.

We found expression of miR-449b to be relatively low and therefore elected to forego further study of its functional role in SLE T cells. Nevertheless, Buggele et al. [20] recently showed that influenza A virus infection of A549 cells could trigger miR-449b expression, which then served to regulate histone deacetylase 1 protein expression and IFN-β production. Thus it is still premature to exclude miR-449b as a pathogenic factor in SLE.

In conclusion, we identified nine Ca2+ influx-regulated miRNAs in Jurkat cells. Of these, miR-524-5p and miR-449b were overexpressed in SLE T cells. Levels of miR-524-5p expressed showed a significant direct correlation with the disease activity of SLE patients. The two proteins linked with the Notch pathway, Jagged-1 and Hes-1, were targeted by miR-524-5p. Augmented expression of miR-524-5p increased IFN-γ production in activated T cells, corresponding to an increase in SLE disease activity.

### Rheumatology key messages

- Two Ca2+ influx-regulated microRNAs—miR-524-5p and miR-449b—are overexpressed in SLE T cells.
- miR-524-5p expression significantly correlated with SLEDAI.
- Transfection of miR-524-5p enhanced IFN-γ production in activated Jurkat cells.

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### Disclosure statement
The authors have declared no conflicts of interest.

### Supplementary data
Supplementary data are available at Rheumatology Online.

### References


