miR-99a-3p Targeting EIF4EBP1 Affects B Lymphocytes Function Through Autophagy and Aggravates SLE Disease Progression

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Abstract

Background: Overactivation of immune cells plays a key role in the pathogenesis of systemic lupus erythematosus (SLE). The regulation of immune cells by miRNA is a research hotspot. In this study, the second-generation high-throughput sequencing found that the expression of miR-99a-3p in SLE decreased, but the specific mechanism is still unclear. The purpose of this study is to explore the potential target genes, target cells of miR-99a-3p and their potential mechanisms affecting the progression of SLE.

Methods: Isolate PBMC from healthy individuals and SLE patients, transfect Ball-1, Jurkat, THP-1 and K562 cells with miR-99a-3p agomir and antagomir, detect miR-99a-3p, predict target genes and autophagy pathway mRNA and protein expression by RT-qPCR and Western blotting. CCK-8 method detects cell proliferation, PI method detects cell cycle, flow cytometry detects cell apoptosis, and luciferase reporter gene experiment determines miR-99a-3p target genes. With C57BL/6J mice as control, construct miR-99a-3p overexpression and interference model based on MRL/lpr mice, ELISA detects plasma ANA, dsDNA, IgE, IgM, IL-6, IL-10, BlyS. Pathological analysis of HE staining and C3 immunofluorescence (IF) deposition in mouse kidney tissue, Immunohistochemistry (IHC) detects changes in target genes and pathway proteins in kidney tissue, isolate B cells to verify the differential expression of miR-99a-3p, target genes, pathway mRNA and protein.

Results: Compared with healthy individuals, miR-99a-3p in SLE was down-regulated, while EIF4EBP1, LC3II, LAMP-2A mRNA and protein expression were up-regulated. After Ball-1 was transfected with miR-99a-3p agomir, cell proliferation decreased and apoptosis increased. After transfected with miR-99a-3p antagomir, the effect was opposite; Luciferase reporter gene detection proved that miR-99a-3p directly targets EIF4EBP1. Rescue experiments confirmed the interaction model between miR-99a-3p and EIF4EBP1. Clinical, in vitro, and in vivo experiments further confirmed that miR-99a-3p agomir can reduce the expression of EIF4EBP1, LC3-II, and LAMP-2A, while miR-99a-3p antagomir had the opposite effect. In vivo experiment antagomir group mice serum ANA, dsDNA, IgE, IgM, IL-6, IL-10, BlyS were higher than those in the MRL/lpr group, EIF4EBP1, LC3-II, LAMP-2A mRNA, protein and IHC levels also increased, and the urinary protein and C3 IF deposition of mice in the antagomir group were increased, and the related indexes of mice in the agomir group were lower than those in the MRL/lpr group.

Conclusion: The expression of miR-99a-3p in SLE PBMC was down-regulated. Up-regulation of miR-99a-3p by transfection can protect B cells from autophagy mediated by EIF4EBP1. The down-regulation of miR-99a-3p induces autophagy by regulating the autophagy signaling pathway mediated by EIF4EBP1 in SLE B cells. These results indicate that miR-99a-3p and EIF4EBP1 may be potential targets of SLE.

Introduction

Systemic lupus erythematosus (SLE) is a type of autoimmune-mediated diffuse connective tissue disease that involves multiple systems throughout the body, characterized by pathological inflammation [1]. Studies had shown that genetic factors influence the clinical phenotype and

progression of SLE, while environmental factors trigger the occurrence of SLE[2], and ultraviolet rays play an important role in stimulating SLE[3]. The age of onset of SLE in high altitude areas is lower, the course of disease is shorter. The proportion of patients with Anti-Sm positive, anemia, thrombocytopenia and elevated serum creatinine is significantly higher than that in low altitude areas [4].

The main ethnic group in the Diqing Tibetan Autonomous Prefecture in Yunnan Province is the Tibetans, they live in a high-altitude, low-oxygen, high-cold, dry and strong ultraviolet environment, relatively isolated, lack of gene exchange with the outside world. The epidemiological and transcriptomics characteristics of Tibetan SLE patients deserve in-depth and systematic research.

MicroRNAs (miRNAs) are a class of highly conserved endogenous non-coding single-stranded small RNAs with a length of about 21-25 nucleotides. More and more miRNAs had been found to play an important role in the pathogenesis of SLE[5]. Our research group found that UVB may be involved in the pathogenesis of SLE by decreasing miR-125b-5p of SLE and increasing the expression of target gene UVRAG and cell autophagy[6].

We collected venous blood from 10 Tibetan SLE patients and 10 healthy Tibetans. The miRNA was differentiated by high throughput sequencing of the second generation after RNA extraction (Fig. 1a). The sequencing results were verified by RT-qPCR, and it was found that miR-99a-3p in Tibetan SLE patients decreased significantly (Fig 1B).

As a member of the miR-99 family, miR-99a-3p is transcribed from the 21 region of the long arm of chromosome 21. miR-99 is expressed at low levels in a variety of human malignant tumors. It participates in the occurrence and development of a variety of urinary tumors[7], head and neck squamous cell carcinoma[8], liver cancer[9] and ovarian cancer[10], and has certain significance in the early diagnosis and staging of tumors[11].

Pradhan and Tomankova were the first to find that the expression of miR-99 in SLE decreased [12]. Jin et al found that the expression of miR-99a in South Korean SLE patients was down-regulated by miRNA PCR chip detection [13]. Frangou et al used cDNA microarrays to compare the gene expression in the effector cells and target tissues of SLE patients and control individuals, and found that miR-99a expression in SLE PBMC decreased, and it was related to the regulation of type I IFN pathway[14]. There is no report on the expression of miR-99a-3p in SLE in the Chinese population.

Therefore, this study explores the role of miR-99a-3p in the pathogenesis of SLE. This study provides help to clarify the complex mechanisms involved in the pathogenesis of SLE and new targets for SLE.

Materials And Methods

clinical experiments

SLE patients and healthy individulas included

SLE patients: 10 cases of SLE patients of Han ethnicity who were treated in the outpatient clinic of the Second Affiliated Hospital of Kunming Medical University from January to December 2020, and 10 cases of SLE patients of Tibetan ethnicity in Diqing Tibetan Autonomous Prefecture. Both Tibetan and Han patients need to be diagnosed in accordance with the American Rheumatism Association's 1997 classification of SLE, and had not taken chloroquine or hydroxychloroquine in the past three months. The ethics was approved by the Ethics Committee of the Second Affiliated Hospital of Kunming Medical University (Trial-PJ-2020-135), and 20ml of peripheral venous blood was collected from patients and healthy individuals. There was no statistical difference in age or gender of each group ($P>0.05$). General clinical data of study subjects see Supplementary Materials Table 1.

In vitro experiments

PBMC, primary B cell separation and culture

PBMC was selected by lymphocyte isolation fluid (LTS1077, Haoyang, Tianjin, China) for 20 ml EDTA anticoagulant. RT-qPCR and Western blotting were used to detect PBMC and intervention after culture.

Take 1ml (1×10^8) of separated PBMC in a 5ml test tube, add 200ul of MagniSort™ Enrichment Antibody Cocktail (Thermo, Germany) and mix well, incubate for 10min, centrifuge and discard the supernatant, add 200ul of MagniSort™ Negative Selection Beads (Thermo, Germany), mix and incubate, insert the test tube into the magnetic pole (Thermo, Germany), collect the supernatant, and centrifuge the precipitate to be B lymphocytes (identification of B cells and culture, see Supplementary Materials Fig 1.).

Take the frozen Ball-1, Jurkat, THP-1 and K562 cells (purchased from the Cell Bank of the Chinese Academy of Sciences) from the liquid nitrogen tank and dissolve them in a 37°C water bath, centrifuge to discard the supernatant, and add 1640 complete medium (containing 1% Double antibody +10% FBS, Thermo, Germany) to resuspend and culture.

RT-qPCR to detect the expression of miRNA/mRNA

Add TRIzol™ Reagent (15596-026, Lifetech, USA) lysate to extract total RNA, use SureScript™ First-Strand cDNA Synthesis Kit (Genecopoeia, Guangzhou, China) for mRNA, use All-in-One™ miRNA First-Strand cDNA Synthesis for miRNA Kit 2.0 (Genecopoeia, Guangzhou, China), take 1ug of total RNA and synthesize the first-strand cDNA of mRNA/miRNA according to its instructions, mRNA at 25°C for 5min, 42°C for 45min, 85°C for 5min, miRNA at 37°C for 60min, 85°C under 5min conditions, reverse transcription was performed on a common PCR machine (4359659, Applied Biosystems 2720 Thermal Cycler, USA). BlazeTaq™ SYBR® Green qPCR Mix 2.0 (GeneCopoeia, Guangzhou, China) for mRNA, All-in-One miRNA RT-qPCR Detection System User Manual (GeneCopoeia, Guangzhou, China) for miRNA, using cDNA as a template and GAPDH/ U6 as the internal reference, using Sybrgreen method, detected on the CFX96 real-time quantitative fluorescent PCR instrument (Bio-Rad, USA). PCR amplification reaction conditions were: 95°C 10 min, 95°C 10 s, 60°C 20 sec, 72°C 30 s, for 40 cycles, collect and record the fluorescence, read the CT value, and use the $2^{-\Delta\Delta CT}$ method to calculate the relative expression of the

gene . The relevant primers were synthesized by Beijing Kinco Xinye Biotechnology Co., Ltd., and the sequence was shown in Table 2 of Supplementary Materials.

miR-99a-3p agomir and antagomir transfect Ball-1, Jurkat, THP-1 and K562 cells

miR-99a-3p agomir (Sense-CAAGCUCGCUUCUAUGGGUCUG, Antisense -CAGACCCAUAGAAG CGAGCUUG) and miR-99a-3p antagomir (Sense-CAGACCC AUAGAAGCGAGCUUG) were synthesized by Ribobio Co. (Guangzhou, China). When the cell confluence reached 80%, 2ul lipo2000 transfection reagent (Thermo, Germany) was added for transient transfection, the medium was changed after 6h of transfection, and RT-qPCR was performed on the cells after 48h of culture.

CCK-8 detection method to detect cell proliferation

Inoculate newly cultured Ball-1, Jurkat, THP-1, and K562 cell suspensions (100ul/well) in 96-well plates, and transfect miR-99a-3p agomir and antagomir into four cell lines respectively. Add 10ul CCK8 (Life iLab Biotech Co., Shanghai, China) to each well at 24h, 48h, 72h, 96h, incubate for 2 hours, and measure the absorbance at 450nm with a microplate reader (BioTek, USA).

PI method to detect cell cycle

Take 250ul (1×10^6 cells) cell suspension, add 750ul of pre-cooled absolute ethanol, seal and place at -20°C for fixation overnight; add 500ul PI staining solution (Becton, Dickinson and Company, USA) to a final concentration of 65ug/ml, Incubate at 37°C for 30 minutes, and immediately perform flow cytometric detection (Thermo, Germany). ModFit LT™ 4.1 software was used to analyze cell cycle.

FITC Annexin V method to detect cell apoptosis

According to the apoptosis test kit Annexin V FITC Apop Dtec Kit I (556547, BD, USA) instructions, use 300ul $1 \times$ Binding Buffer cells at a concentration of 1×10^6 /ml, add 5ul Annexin V-FITC and Propidium Iodide Staining Solution staining, add Binding Buffer, light incubate, and analyze on Attune NxT Streaming Cytometer (Invitrogen, USA).

Dual-Luciferase Reporter Assay

The miR-99a-3p target genes were predicted, and EIF4EBP1, NCAPG, IKBKB, PRKCB were selected for luciferase detection. The related target gene 3'UTR Wild/Mutant type luciferase vector pmirGLO Vector was constructed by Wuhan GeneCreate Company. Take miRNA and plasmid 2ug incubated for 5min, and add 2ul lipo2000 transfection reagent to culture medium for incubation. When the cell fusion degree reaches 80%, add 500ul of the above transfection complex, add the extracted protein to 100ul of firefly luciferase substrate, measure its firefly luciferin value, add Renilla luciferase substrate, and measure the sea level. Kidney fluorescence value. The renilla fluorescence value of each well divided by the firefly fluorescein value was the fluorescence value of the reporter plasmid in each well.

Western-blotting

Add 250ul RIPA lysis buffer (containing protease inhibitor, Thermo, Germany) to each group of cells to be tested, and BCA protein quantification kit (P0010, Beyotime, China) to determine the protein concentration. Take 30ug of total protein for SDS-PAGE electrophoresis, and block after transfer to membrane. Add the primary antibodies EIF4EBP1 (1:1000, GTX133182, GeneTex, USA), LAMP-2A (1:1000, ab24170, Abcam, USA), LC3B (1:1000, ABS1513, Millipore, USA), β -Actin (LMAI Bio, Shanghai, China), shake overnight at 4°C, rewarm for one hour the next day, add secondary antibody (Peroxidase labeled goat anti-rabbit IgG, 1:5000, Sigma, USA), incubate for 30min, exposure instrument (Monad, Suzhou, China), add ECL color developing solution (Thermo, Germany) to take pictures, use Image-Pro Plus 6.0 software to analyze the optical density of the stripe, the optical density ratio of the target protein and the light density ratio of the endoprotein β -actin represents the relative content of the target protein to compare the difference in protein expression.

SiRNA and antagomir jointly intervene in Ball-1 cells for Rescue experiment

siRNA EIF4EBP1 was synthesized by Guangzhou Ruibo Biological Company, and transfected Ball-1 cells with siEIF4EBP1-1, siEIF4EBP1-2, and siEIF4EBP1-3. After 48 hours, RT-qPCR and Western-blotting were detected. It was found the expression of EIF4EBP1 in the siEIF4EBP1-1 group was lower than siNC, the difference was statistically significant (sequence and screening see supplementary material Table3, Fig 2). Collect Ball-1 cell count and plate, add siRNA EIF4EBP1 and NC respectively, and then add lipo2000 transfection reagent. After 24h, half of the sample was separated from the siRNA tube and added miR-99a-3p antagomir for further culture. After incubation at 37°C for 48 hours, proliferation, cycle, apoptosis and Western blotting were detected.

In vivo experiment

miR-99a-3p over-expression and interference with MRL/lpr mouse model building

Take 18 MRL/lpr mice (20-30g, female, 6-8 weeks old, purchased from SPF Biotechnology Co., Ltd.), and group them into a single high-dose tail vein injection of miR-99a-3p agomir, antagomir and NC, injection volume 200 μ l, miR-99a-3p agomir/antagomir injection doses were 20nmol/200nmol respectively [15, 16]. 6 C57BL/6J (C57) mice (20-25g, female, 6-8 weeks old) purchased from Hunan Slack Jingda Experimental Animal Co., Ltd.

Eye ball venous blood was collected, and B lymphocytes were separated by immunomagnetic bead method, followed by Western-blotting, RT-qPCR, and plasma retention for ELISA. Mice were sacrificed by neck breaking method, and the kidneys were removed in layers and placed in 4% paraformaldehyde (P0099, Beyotime, China) for fixation. The experimental protocol was approved by the Animal Research Committee of Kunming Medical University (kmmu2021724).

Coomassie brilliant blue method for quantitation of total protein in the urine

Take appropriate amount of standard dilution, and take appropriate amount of urine to be measured, PBS equal multiple dilution, add 5 ml dilution of the Coomassie brilliant blue solution (Xinfan Biological Biological Technology Co., Shanghai, China), the color changes from red to blue , the absorbance was determined at 595 nm.

ELISA detects ANA, dsDNA, IgE, IgM, IL-6, IL-10, BLyS

Take out the ELISA kit (JL12477-96T, Jiang Lai Bio, China) slats, add 100μL of HRP-labeled antibody, incubate at 37°C for 60min, wash the plate; add substrate A and B 50μl to each well , then incubate at 37°C in dark incubation and add the stop solution. Measure the OD value of each well at 450nm wavelength, and calculate the sample concentration by the absorbance value of the sample and the standard curve.

HE staining

Take the longitudinally sectioned kidney tissues of each group and fix them in paraformaldehyde for 24 hours, and then put them in a low-to-high concentration ethanol solution for dehydration, transparency, wax immersion, embedding, and cut into slices with a thickness of about 4μm, and bake at 64°C for 30 minutes. After dewaxing with xylene, put the slides in the ethanol solution of high concentration to low concentration to gradually hydrate, counterstain with hematoxylin (C0105-1, Beyotime, China) for 4 minutes, rinse with distilled water, and put it into alcohol-hydrochloric acid solution for differentiation, return to blue in tap water for 20 minutes, stain with eosin (C0109, Beyotime, China) for 10 seconds, and wash with distilled water. Finally, use ethanol dehydration, transparent xylene and neutral gum to mount the slides, take pictures with a microscope (Lab.A1, ZEISS, Germany) and analyze the staining results.

Immunohistochemical staining

Prepare slices, bake slices, and dewax. After hydration with gradient alcohol, add 0.01M citric acid buffer and boil for 15 minutes to restore the antigen, block with 5% BSA (LMAI Bio, Shanghai, China) at room temperature for 30 minutes, add primary antibody EIF4EBP1 (1:100, GTX133182, GeneTex, USA), LAMP-2A (1:100, ab24170, Abcam, USA), LC3B (1:100, ABS1513, Millipore, USA) overnight at 4°C, dropwise reaction. Incubate with the enhancement solution, add the secondary antibody (Sheep anti-mouse, A21235, Invitrogen) and incubate at 37°C for 30 minutes, add the DAB (ZLI-9019, Zhongshan Golden Bridge, China) dye solution dropwise to the tissue block for 5 minutes, and place the slide on the Stained in hematoxylin for 4 minutes, and the tap water turned blue for 20 minutes; dehydrated, transparent, and photographed after mounting, scanning the positive area and calculating the positive rate. First score according to the staining intensity: 0 is divided into colorless, 1 is divided into light yellow, 2 is divided into brown and 3 is divided into brown, and the staining intensity needs to be compared with the background coloring; Then score according to the percentage of positive cells: 0 is negative, 1 is positive cells ≤ 25%, 2 is 25%-50%, and 3 is > 50%.

Tissue and cell immunofluorescence staining

Prepare slices, bake slices, dewax, and put them in the repair solution after hydration with gradient alcohol; draw circles with oily brushes, add diluted serum, Anti-C3 antibody (1:100, ab11887, Abcam, USA) incubate overnight and then add secondary antibody Goat Anti-Rabbit IgG H&L (AmyJet Scientific, Wuhan, China), drop DIPA (Weifang Bincheng Chemical Industry) dyeing solution. Put it under a fluorescence microscope (Mshot, Guangzhou, China), and take photos of a total of 5 fields of view in the center and surrounding areas of each film, and calculate the positive rate.

Collect the cells and fix them with 4% paraformaldehyde. After blocking, add the 1:200 diluted primary antibody LC3B (1:100, ABS1513, Millipore, USA) and incubate overnight. Then add the secondary antibody (Sheep anti-mouse, A21235, Invitrogen), and then add DAPI (ID0080, Solarbio, China) to incubate, mount, take pictures, and calculate the positive rate.

Statistical methods

The data were expressed as Mean $\pm$ SD, analyzed by ANOVA and LSD-t test, and statistically analyzed by SPSS 23.0 software. Two tailed $P < 0.05$ was statistically significant. The correlation analysis used Spearman's rank correlation analysis, the count data used the χ^2 test. GraphPad Prism 6.0 is used for statistical analysis of histograms.

Results

Differential verification of miR-99a-3p in SLE

The venous blood of Tibetan SLE patients and healthy individuals was collected, and RNA was extracted and then subjected to second-generation high-throughput sequencing. RT-qPCR verified the sequencing results which shows miR-99a-3p in Tibetan SLE patients had decreased significantly (Fig 1A, B).

RT-qPCR detected miR-99a-3p in PBMCs of 10 Tibetan&Han SLE patients and 10 Tibetan&Han healthy individuals. The miR-99a-3p in both Tibetan and Han SLE patients was decreased compared with the healthy controls, with statistical significance (Fig 1C and D).

Functional difference of miR-99a-3p on Ball-1, Jurkat, THP-1, K562 cells

The synthesized miR-99a-3p agomir and antagomir were transfected into Ball-1, Jurkat, THP-1, K562 (see Supplementary Material Fig 1 for cell morphology), and the expression of miR-99a-3p was detected by RT-qPCR 48h after transfection. Compared with the NC group, miR-99a-3p increased after miR-99a-3p agomir transfected with the four cell lines, and the difference was statistically significant. miR-99a-3p decreased after miR-99a-3p antagomir transfected the four cell lines, the difference was statistically significant (Fig 2A).

After Ball-1 and THP-1 were transfected with miR-99a-3p agomir, cell proliferation decreased on 1, 2, 3, and 4 days compared with NC group; Ball-1 and THP-1 were transfected with miR-99a-3p antagomir, cell proliferation increased on 1, 2, 3, and 4 days compared with NC group, and the difference was statistically

significant; Jurkat and K562 were transfected with agomir and antagomir, cell proliferation had no significant changes (Fig 2B).

After Ball-1, Jurkat, THP-1 were transfected with miR-99a-3p agomir, the apoptosis rate was significantly higher than that of NC group; Ball-1, Jurkat were transfected with miR-99a-3p antagomir, the apoptosis rate was significantly lower than that of NC group, and the difference was statistically significant. After K562 transfection, the apoptosis rate of agomir and antagomir groups did not change significantly compared with NC group (Fig 2C).

After Ball-1 and THP-1 transfected miR-99a-3p agomir, the number of cells in G0/G1 phase was higher than that of NC group; Ball-1 and THP-1 transfected miR-99a-3p antagomir, the number of cells in G0/G1 phase was lower than that of NC group, and the difference was statistically significant.

After Ball-1 transfected miR-99a-3p agomir, the number of cells in G2/M and S phase decreased compared with NC group; after transfected miR-99a-3p antagomir, the number of cells in G2/M and S phase was higher. After Jurkat transfected miR-99a-3p antagomir, the number of cells in G2/M phase was lower than that in NC group ($P < 0.05$). After K562 transfected, the agomir and antagomir groups had no significant changes in each cycle compared with the NC group (Fig 2D). The cell proliferation, apoptosis and cycle changes of the Ball-1 line were relatively stable after transfected miR-99a-3p agomir, antagomir and NC.

Target gene confirmation

The target genes of miR-99a-3p were predicted by database TarBase, <http://mirtarbase.mbc.nctu.edu.tw/php/index.php>, miRDB, <http://www.mirdb.org>, Targetscan, http://www.targetscan.org/vert_71, miRBase, <http://www.mirbase.org>. According to the literature search, EIF4EBP1, NCAPG, IKBKB, PRKCB were selected as the research objects.

RT-qPCR detected the expression of EIF4EBP1, NCAPG, IKBKB, and PRKCB in the PBMC of Han SLE patients and Han healthy individuals. The expression of related genes was up-regulated in the PBMC of SLE patients, and the difference was statistically significant (Fig 3A).

Ball-1 cells were transfected with miR-99a-3p agomir, antagomir, and NC, and the target gene expression was detected by RT-qPCR 48h after transfection. Compared with NC group, the target genes all decreased after miR-99a-3p agomir transfection. The target genes all increased after miR-99a-3p antagomir transfection, and the difference was statistically significant (Fig 3B).

Hsa-miR-99a-3p+EIF4EBP1-WT was transfected into 293T cells. Compared with NC+EIF4EBP1-WT group, the fluorescence intensity was significantly reduced ($P = 0.0055$); while Hsa-miR-99a-3p+EIF4EBP1-MT was transfected compared with the NC+EIF4EBP1-MT group, the fluorescence intensity did not change significantly after staining 293T cells ($P = 0.5411$), and the predicted position was the target relationship between Hsa-miR-99a-3p and EIF4EBP1 (Fig 3C).

Hsa-miR-99a-3p+NCAPG-WT was transfected into 293T cells. Compared with NC+NCAPG-WT group, the fluorescence intensity was significantly reduced ($P=0.0002$); while Hsa-miR-99a-3p+NCAPG -MT was transfected compared with the NC+NCAPG-MT group, the fluorescence intensity of 293T cells did not change significantly ($P=0.0712$), and the predicted position was the target relationship between Hsa-miR-99a-3p and NCAPG. Hsa-miR-99a-3p had no targeting relationship with IKBKB and PRKCB (Fig 3D).

Western blotting further verified the expression of target genes in SLE and healthy individuals. The expression of EIF4EBP1 and NCAPG in SLE increased, and the difference was statistically significant (Fig 3E).

miR-99a-3p participates in autophagy signaling pathway through target genes

RT-qPCR detected the expression of LC3- β and LAMP-2A in the PBMC of SLE and healthy individuals. SLE was higher than that of healthy control group, and the difference was statistically significant (Fig 4A). Western blotting analyzed SLE and healthy individual PBMC autophagy pathway marker protein LC3- β and LAMP-2A. The ratio of LC3- β and LAMP-2A in SLE was higher than that of healthy individuals ($P=0.031$, Fig 4B).

miR-99a-3p agomir, antagomir, NC transfected Ball-1, the expression of LC3-II and LAMP-2A was detected by RT-qPCR 48h after transfection. After miR-99a-3p agomir was transfected with Ball-1, both LC3- β and LAMP-2A decreased, and the difference was statistically significant. The result was opposite after miR-99a-3p antagomir transfection (Fig 4C).

Compared with NC group, miR-99a-3p increased after miR-99a-3p agomir transfected with B cells ($P=0.0153$), while EIF4EBP1, LC3- β , LAMP-2A decreased. After transfection of miR-99a-3p antagomir into B cells, miR-99a-3p decreased ($P<0.0001$), while EIF4EBP1, LC3- β , and LAMP-2A all increased, and the difference was statistically significant (Fig 4D).

48h after miR-99a-3p agomir, antagomir, and NC transfected Ball-1 and B cells, compared with the NC group, the EIF4EBP1, LC3- β and LAMP-2A proteins in the miR-99a-3p agomir group were decreased, and the difference was statistically significant. The transfection result of miR-99a-3p antagomir group was opposite (Fig 4E).

Fluorescence microscope was used to observe the IF of LC3- β after transfection. The IF of LC3- β in the miR-99a-3p agomir group in Ball-1 and B cells was lower than that in NC group. The LC3-II IF of the miR-99a-3p antagomir group was higher than that of NC group (Fig 4F).

Ball-1 function changes after Rescue experiment

Western blotting was used to detect the expression of EIF4EBP1, LC3- β , LAMP-2A after siNC, siEIF4EBP1, siEIF4EBP1+antagomir transfected Ball-1. After transfection with siEIF4EBP1, Ball-1's EIF4EBP1, LC3- β , LAMP-2A protein expressions were all lower than that of the siNC group. After transfection with

siEIF4EBP1+antagomir, the protein expressions of EIF4EBP1, LC3-II, and LAMP-2A in Ball-1 were higher than those in the siEIF4EBP1 group, and the difference was statistically significant (Fig 5A).

Flow cytometry was used to detect the difference in cell apoptosis after transfection of Ball-1. After Ball-1 was transfected with siEIF4EBP1, the apoptotic rate was significantly higher than that of the siNC group; after transfection with siEIF4EBP1+antagomir, the apoptosis rate was significantly lower than that of the siEIF4EBP1 group, and the difference was statistically significant (Fig 5B).

After Ball-1 transfection, the cell proliferation of the siEIF4EBP1 group at 1, 2, 3, and 4 days was lower than that of the siNC group; the cell proliferation of the siEIF4EBP1+antagomir group at 2, 3, and 4 days was higher than that of the siEIF4EBP1 group. The difference was statistically significant (Fig 5C).

After transfection with siEIF4EBP1, the number of cells in the G0/G1, G2/M phase was higher than that in siNC group, and the number of cells in the S phase was less than that in siNC group. After transfection with siEIF4EBP1+antagomir, the number of cells in the G0/G1 phase decreased compared with the siEIF4EBP1 group, and the number of cells in the G2/M and S phase increased and the cell proliferation recovered (Fig 5D). Fluorescence microscope was used to observe the LC3-II IF of siNC, siEIF4EBP1, siEIF4EBP1+antagomir transfected with Ball-1. It was found that the LC3-II IF of siEIF4EBP1 group was lower than that of siNC group, and the LC3-II IF of siEIF4EBP1+antagomir group was higher than that of siEIF4EBP1 group. The difference was statistically significant (Fig 5E).

Disease progression of MRL/lpr lupus mice after experimental intervention of miR-99a-3p in vivo

The body weight of the four groups of mice increased with the increase in the number of feeding weeks. One mouse in the agomir group died at 12w, and the body weight of the MRL/Lpr group was higher than that of C57 at 10w, 12w, and 13w. However, miR-99a-3p intervention did not significantly change the body weight (Fig 6A). At 13w, the hair around the nose and eyes of the MRL/lpr group decreased, and there was fewer and slower behavioral activities; the antagomir group mice had alopecia area around the nose, eyes and forehead, and the behavioral activity was obviously slow.

There was no difference in urine protein between the four groups at 8 weeks ($P>0.05$). However, at 10w, 12w, and 13w, the urine protein in the MRL/Lpr, agomir, and antagomir group increased significantly. The urine protein of the MRL/Lpr group was higher than that of the C57 group. The urine protein in the antagomir group was higher than that of the C57 group; the urine protein in the agomir group was lower than that of the C57 group, and the difference was statistically significant (Fig 6B).

CaseViewer 3.3 counted the glomerular area, the glomerular area of the MRL/lpr group was higher than that of the C57 group ($P=0.0184$), suggesting that the mice in the MRL/lpr group had some glomerular edema. The glomerular area of the agomir group was lower than that of the MRL/lpr group, but the difference was not statistically significant ($P=0.2098$). The glomerular area of the antagomir group was higher than that of the MRL/lpr group, but the difference was not statistically significant ($P=0.5888$) (Fig 6C, D).

The C3 IF deposition of MRL/lpr mice and C57 mice after intervention was calculated, and it was found that the C3 IF deposition of the MRL/lpr group was higher than that of the C57 group ($P<0.0001$). C3 deposition in the agomir group was lower than that in the MRL/lpr group ($P=0.0002$). C3 deposition in the antagomir group was higher than that in the MRL/lpr group ($P=0.0008$, Fig 6E, F).

ELISA detection found that the levels of ANA, dsDNA, IgE, IgM, IL-6, IL-10, and BlyS in the MRL/lpr group were higher than those in the C57 group. The levels of ANA, dsDNA, IgE, IgM, IL-6, IL-10 and BlyS in the agomir group were lower than those in the MRL/lpr group. The levels of ANA, dsDNA, IgE, IgM, IL-10, and BlyS in the antagomir group were higher than those in the MRL/lpr group, and the difference was statistically significant (Fig 6G).

Changes of target genes and pathway proteins in MRL/lpr lupus mice after experimental intervention of miR-99a-3p in vivo

RT-qPCR detected the expression of miR-99a-3p, EIF4EBP1, LC3- β , and LAMP-2A after tail vein injection. The expression of miR-99a-3p in the MRL/lpr group was lower than that in the C57 group ($P=0.0293$). The expression of miR-99a-3p in agomir group was higher than that in MRL/lpr group ($P=0.0013$). The expression of miR-99a-3p in the antagomir group was lower than that in the MRL/lpr group ($P=0.0272$). The expression of EIF4EBP1, LC3- β and LAMP-2A in the MRL/lpr group was higher than that in the C57 group. The mRNA expression of EIF4EBP1, LC3- β and LAMP-2A in agomir group was lower than that in MRL/lpr group. The expression levels of EIF4EBP1, LC3- β , and LAMP-2A mRNA in the antagomir group were higher than those in the MRL/lpr group, and the difference was statistically significant (Fig 7A).

Western blotting was used to detect the protein expression of EIF4EBP1, LC3- β and LAMP-2A after intervention in MRL/lpr and C57 mice. The expression levels of EIF4EBP1, LC3- β , and LAMP-2A in the MRL/lpr group were higher than those in the C57 group ($P=0.0293$). The expression of EIF4EBP1, LC3- β and LAMP-2A in agomir group was lower than that in MRL/lpr group ($P=0.0013$). The expression of EIF4EBP1, LC3- β and LAMP-2A in the antagomir group was higher than that in the MRL/lpr group ($P=0.0272$) (Fig 7B).

IHC staining showed that the expression of EIF4EBP1, LC3- β , LAMP-2A in the kidney of C57 mice was weak, while the MRL/lpr group increased, the antagomir group increased much more significantly, and in the agomir group significantly weakened (Fig 6C). EIF4EBP1, LC3- β , LAMP-2A in the MRL/lpr group were lower than the C57 and agomir groups at 0 points, and higher than the antagomir group, the difference was statistically significant. As the score increased, the intensity of IHC staining and the percentage of positive cells in the C57 and agomir groups gradually decreased, while the trend in the antagomir group was opposite (Fig 7C).

Discussion

SLE is an immune system disease caused by over-activation of immune cells and massive secretion of autoantibodies. Extremely active B cells are involved in almost all the pathogenesis of SLE. Although the

treatment of SLE has made great progress, there are still patients with ineffective treatment or relapse.

miRNA is a type of endogenous non-coding RNA widely distributed in the human body, which plays an important role in regulating cell proliferation, apoptosis and disease progression. MiRNAs are involved in immune disorders and organ damage in SLE. MiRNA-based biomarkers and treatment methods may become viable options for the treatment of SLE[5].

Zhang et al provide a novel insight into the role of the circRNA-miRNA-mRNA regulation network in the SLE and 29 DECs (2 up and 27 down) of SLE were found[17]. Latini et al found that miR-155, miR-499a and miR-142 are involved in the pathogenesis and clinical phenotype of SLE[18]. Tao et al found that miR-152-3p promotes TLR-mediated CD4+T cell inflammatory response by regulating the DNMT1/MyD88 signaling pathway, which may become a new target for SLE treatment[19].

This study found that miR-99a-3p decreased significantly in Tibetan and Han SLE patients, which was consistent with Pradhan's report on the Indian population[12], Jin's report on the Korean population[13], and Frangou's report on the European population[14]. There was no report on the expression of miR-99a-3p in SLE in Chinese population. This study aimed to explore the functional mechanism of miR-99a-3p. MiR-99a-3p decreased in both Tibetan and Han SLE patients. The Han SLE patients who are more convenient to obtain were selected as further research objects.

In order to clarify the function of miR-99a-3p in different immune cells, this study selected Ball-1, Jurkat, THP-1, K562 cell lines for functional verification. After Ball-1 transfection of miR-99a-3p agomir, antagomir, NC, cell proliferation, apoptosis and cycle changes were relatively stable. In this experiment, Ball-1 was selected as the research object. In the pathogenesis of SLE, B cells not only produce autoantibodies, but also regulate the activation of T cells through various cytokines and antigen presentation processes, which also aggravates the progress of SLE[20]. Therefore, it is particularly important to study how B cells play a role in SLE and how to produce pathogenic auto antibodies through signal transmission between cells.

In order to further study the specific molecular mechanism of miR-99a-3p functioning, it was predicted that EIF4EBP1 was the target gene of miR-99a-3p through bioinformatics methods. In this study, 293T cells were transfected to construct the luciferase reporter gene vector of the 3'UTR wild-type/mutant region of EIF4EBP1, which confirmed that miR-99a-3p can combine with the 3'UTR complementary sequence of EIF4EBP1 and down-regulate EIF4EBP1.

EIF4EBP1 is a translation initiation inhibitor, which regulates its activity by preventing the assembly of eIF4E into the eIF4F complex: hypophosphorylated form competes with EIF4G1/EIF4G3 and strongly binds to EIF4E, leading to repress translation. In contrast, hyperphosphorylated form dissociates from EIF4E, leading to initiation of translation[21]. The pathogenesis of EIF4EBP1 and SLE had not been reported.

Autophagy is a process of engulfing one's own cytoplasmic proteins or organelles, coating them into vesicles, and then fusing with lysosomes to degrade the contents of the package. A large number of studies had confirmed that miRNA can regulate autophagy[22, 23]. Abnormal autophagy function leads to the accumulation of apoptosis and induces the production of autoantibodies, thereby inducing and aggravating the condition of SLE. EIF4EBP1 can regulate mTORC1 to induce autophagy[24], microtubule-associated protein 1 light chain 3 (LC3) is a key protein involved in autophagy[25]. The lysosomal associated protein 2a (LAMP-2A) is a key regulatory protein in the chaperon-mediated autophagy (CMA) pathway. Inhibiting the LAMP-2A protein can specifically block the CMA pathway[26].

This study further confirmed that transfection of miR-99a-3p agomir reduced the expression of autophagy-related genes LC3 and LAMP-2A in clinical, in vitro cells (Ba1.1, B cells), and in vivo experiments (MRL/lpr mouse B cells). Transfection of miR-99a-3p antagomir had the opposite effect, suggesting that miR-99a-3p had a negative regulatory effect on autophagy, and the change trend of EIF4EBP1 is consistent with the change trend of autophagy level, suggesting that EIF4EBP1 had a positive regulatory effect on autophagy.

In this study, rescue experiment was carried out to confirm the interaction model of miR-99a-3p and EIF4EBP1, miR-99a-3p affects cell proliferation and apoptosis through target genes.

B lymphocyte stimulator (BLyS) is a new member of the tumor necrosis factor family, involved in the regulation of B cell proliferation, differentiation and antibody production. Transgenic mice overexpressing BLyS increased the number of B cells, increased serum ANA and dsDNA, and deposited immunoglobulin in the kidney[27]. Benlysta was the first inhibitor to act on BLyS, which binds to soluble BLyS with high affinity and inhibits its activity to achieve disease control[28].

In this study, an in vivo experimental model of MRL/lpr was constructed by injecting miR-99a-3p agomir, antagomir, and NC into the tail vein. The ANA, dsDNA, IgE, IgM, IL-6, IL-10, and BLyS of mice in the antagomir group were significantly increased, SLE disease activity was stronger, and B cell proliferation and differentiation actively produced more antibodies. In the antagomir group, the urinary protein and C3 IF deposition in the kidneys were increased, and the kidney damage was more serious, consistent with reports in the literature[29]. They all reflected that miR-99a-3p inhibition increased the progression of SLE disease. However, the IgM level of mice in the antagomir group was higher than that of the MRL/lpr group, and the IgM level was not negatively correlated with the severity of SLE disease[30]. This may be related to the 13-week-old when the mice were sacrificed in this study, and they were still in the early stage of negative feedback.

Conclusions

This study first discovered that miR-99a-3p targeting EIF4EBP1 participates in the autophagy signaling pathway and affects the function of B cells, thereby aggravating the progression of SLE. This study had deepened the understanding of the molecular mechanisms of miRNA regulatory networks related to the

pathogenesis of SLE. The abnormally expressed miR-99a-3p and its target gene EIF4EBP1 found in the study are expected to become potential targets of SLE.

Limitations

There are some limitations in this study. First, most of the experiments in this study were done on Ball-1 and B cells, but there are still some differences between Ball-1, B cells of healthy individuals and B cells of SLE patients. The results of the B cells in this study were not ideal, and the research was not in-depth. Second, the rescue experiment did not use gene knockout mice and lacked relevant functional verification experiments. Third, due to various restrictions, Tibetan patients were not directly involved in the research. The number of clinical cases was small, and the correlation analysis between clinical indicators and laboratory indicators was lacking.

Declarations

Competing interests

The authors have no proprietary interest in any aspect of the study.

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Availability of data and materials

All datasets generated for this study are included in the article/Supplementary Material.

Authors' contributions

Deng Danqi conceived and designed the experiments, Yang Meng performed the experiments and analyzed the results of the experiments.

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Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

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Figures

Figure 1

Differential verification of miR-99a-3p in SLE. (A) Screening of significantly differentially expressed miRNAs by second-generation high-throughput sequencing (B) RT-qPCR verification of significantly differentially expressed miRNAs (C, D) RT-qPCR detected miR-99a-3p in PBMCs of Tibetan&Han SLE patients and Tibetan&Han healthy individuals.

Figure 2

The functional difference of miR-99a-3p on Ball-1, Jurkat, THP-1 and K562 cells. (A) miR-99a-3p expression after miR-99a-3p agomir, antagomir, NC transfected in different cells (B) CCK-8 method to detect the differences in cell proliferation changes on 1, 2, 3, and 4 days after miR-99a-3p agomir, antagomir, and NC transfected with different cells (C) After miR-99a-3p agomir, antagomir, and NC transfected different cells, flow cytometry was used to analyze the changes in apoptosis of each cell and the histogram to analyze the differences in the proportion of apoptosis in each group (Q1 necrosis, Q2 late apoptosis, Q3 normal, Q4 early apoptosis) (D) miR-99a-3p agomir, antagomir, and NC were transfected with different cells to analyze the cycle changes of each cell by flow cytometry and the histogram to compare differences in the ratio of cells (Note in Fig B: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, comparison between agomir and NC; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, antagomir and NC Comparison; in Fig D: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

Figure 3

Target gene confirmation. (A) RT-qPCR detect the predicted target gene expression between SLE and healthy individuals (B) Changes in target genes after miR-99a-3p agomir, antagomir, and NC transfected Ball-1 cells (C) Prediction of binding site of miR-99a-3p and target gene and mutation construction (D) Transfect 293T cells with mutant and wild-type fluorescein vectors of binding sites and agomir, read the fluorescein report value through a fluorescence spectrophotometer, and analyze the difference in the influence of agomir between mutant and wild-type (E) Western blotting verified the difference in expression of EIF4EBP1, NCAPG in SLE (B1, B2, B3, B4) and healthy individuals (A1, A2, A3, A4).

Figure 4

miR-99a-3p participates in the autophagy signaling pathway through target genes. RT-qPCR detected the expression of LC3 and LAMP-2A in the PBMC of SLE and healthy individuals. Western blotting detected the expression of LC3, LAMP-2A in SLE (B1, B2, B3, B4) and healthy individuals (A1, A2, A3, A4). RT-qPCR detected the expression of LC3 and LAMP-2A after miR-99a-3p agomir, antagomir and NC transfected Ball-1. RT-qPCR detected the expression of miR-99a-3p, EIF4EBP1, LC3 and LAMP-2A after miR-99a-3p agomir, antagomir and NC transfected B cells. Western blotting detected the expression of EIF4EBP1, LC3 and LAMP-2A protein after miR-99a-3p agomir, antagomir, NC transfected Ball-1, B cells. The IF of LC3 after miR-99a-3p agomir, antagomir, and NC transfected Ball-1 and B

cells (×400, scale 50um). The green fluorescence was the positive staining of LC3-β, the blue fluorescence was the nuclear staining of DAPI, and the Merge fluorescence was the image of the fusion of LC3-β and DAPI.

Figure 5

Ball-1 function changes after Rescue experiment. A Western blotting was used to detect the expression of EIF4EBP1, LC3-β, LAMP-2A after siNC, siEIF4EBP1, siEIF4EBP1+antagomir transfected Ball-1. B After siNC, siEIF4EBP1, siEIF4EBP1+antagomir were transfected with Ball-1, flow cytometry was used to analyze the changes in apoptosis of each cell line and the histogram to analyze the differences in the proportion of apoptosis in each group(C1 necrosis, C2 late apoptosis, C3 normal, C4 early apoptosis). C CCK-8 method to detect the difference in cell proliferation of siNC, siEIF4EBP1, siEIF4EBP1+antagomir transfected with Ball-1 at 1, 2, 3, and 4 days. D After siNC, siEIF4EBP1, siEIF4EBP1+antagomir were transfected into Ball-1, flow cytometry was used to analyze the differences in the cycle changes of each cell and the histogram to compare the differences in the proportion of cells in each group. E The IF of

LC3- β after siNC, siEIF4EBP1, siEIF4EBP1+antagomir transfected Ball-1 ($\times 400$, scale 50 μ m). Green fluorescence was the positive staining of LC3- β , blue fluorescence was the nuclear staining of DAPI, and Merge fluorescence was the image of the fusion of LC3- β and DAPI- β Note in Fig C:
 ** $P < 0.01$, *** $P < 0.001$, comparison between siEIF4EBP1 and siNC; ## $P < 0.01$, comparison between siEIF4EBP1+antagomir and siEIF4EBP1; Fig D: ** $P < 0.01$, *** $P < 0.001$, ns, not significant

Figure 6

Disease progression of MRL/lpr lupus mice after experimental intervention of miR-99a-3p in vivo. A Body weight changes after intervention of MRL/lpr and C57 mice. B Coomassie brilliant blue method to detect the changes of 8w, 10w, 12w, 13w urine protein after intervention of MRL/lpr and C57 mice. C HE staining of glomerular area after intervention of MRL/lpr and C57 mice. D HE staining of MRL/lpr and C57 mice after intervention ($\times 2.5, \times 400$). E The C3 IF of MRL/lpr and C57 mice after intervention ($\times 400$, scale 50um). Green fluorescence was C3 positive staining, blue fluorescence was DAPI nuclear staining, and Merge fluorescence was the image after fusion of C3 and DAPI. F C3 IF after intervention of MRL/lpr and C57 mice. G ELISA detected ANA, dsDNA, IgE, IgM, IL-6, IL-10, BLYS expression after intervention in MRL/lpr and C57 mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, not significant.

Figure 7

Changes of target genes and pathway proteins in MRL/lpr lupus mice after experimental intervention of miR-99a-3p in vivo

A RT-qPCR detection of miR-99a-3p, EIF4EBP1, LC3, LAMP-2A changes after intervention

B Western-blotting detection of EIF4EBP1, LC3, LAMP-2A protein expression after intervention

C IHC staining of EIF4EBP1, LC3, and LAMP-2A after intervention($\times 2.5, \times 400$, brown-yellow was positive staining, * $P < 0.05$, ** $P < 0.01$, ns, not significant)

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