Circular RNA circLOC101928570 Suppress Systemic Lupus Erythematosus Progression Via Targeting the miR-150/c-myb Axis

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Abstract

Background: Accumulating evidence supports the implication of circRNAs in systemic lupus erythematosus (SLE). however, little is known about their the detailed mechanisms and the roles of circRNAs in the pathogenesis of SLE.

Methods: Quantitative real time-PCR (qRT-PCR) was used to determine the levels of circLOC101928570 and miR-150 in peripheral blood mononuclear cells (PBMCs) of SLE. Overexpression and knockdown experiments were conducted to assess the effects of circLOC101928570. Fluorescence in situ hybridization (FISH), RNA immunoprecipitation (RIP), luciferase reporter assays, western blot, flow cytometry analysis and enzyme-linked immunosorbent assay (ELISA) were used to investigate the molecular mechanisms underlying the function of circLOC101928570.

Results: The results showed that the level of circLOC101928570 was significantly down-regulated in SLE and correlated with systemic lupus erythematosus disease activity index (SLEDAI). Functionally, circLOC101928570 acted as a miR-150 sponge to relieve the repressive effect on its target c-myb, which modulates the activation of immune inflammatory responses. CircLOC101928570 knockdown enhanced apoptosis. Moreover, circLOC101928570 promote the transcriptional level of IL2RA through directly regulate miR-150/c-myb axis.

Conclusion: Overall, our findings demonstrated that circLOC101928570 played a critical role in SLE. The down-expression of circLOC101928570 suppressed SLE progression through miR-150/c-myb/IL2RA axis. Our findings identified that circLOC101928570 serve as a potential biomarker for the diagnosis and therapy of SLE.

Introduction

Systemic lupus erythematosus (SLE) is a typical autoimmune disease with the production of autoantibodies, the deposition of immune complexes and the impairment of multi-organ functions. The pathogenesis of SLE is not very clear, previous studies have demonstrated that SLE results from the complex interaction between genetic and environmental exposures (Kiriakidou and Ching 2020; Tsokos et al. 2016). The study of SLE biomarkers is critical for early diagnosis, disease activity monitoring, assessment of the likelihood and extent of organ damage and discovery of new therapeutic targets (Cortes and Forner 2019; Yu and Kuo 2019; Sole et al. 2019; Chen et al. 2019).

Circular RNAs (circRNAs) belong to a new type of non-coding RNAs which widely distributed in mammals. CircRNAs are single-stranded transcripts generated by back-splicing and characterized by covalently linked head-to-tail closed loop structures with neither 5’-3’polarity nor a polyadenylated tail in eukaryotic cells (Memczak et al. 2013; Zhang et al. 2013). CircRNAs show highly stability compared with linear RNAs and exhibit a cell-type or developmental-stage-specific expression pattern (Qu et al. 2015). Due to their high abundance, stability, and unique expression pattern, circRNAs are of potential utility for clinical diagnosis and prognosis. Along with the development of the RNA sequencing and bioinformatics, large

numbers of circRNAs have been successfully identified. Increasing evidence suggest that circRNAs are involved in the pathogenesis of a variety of diseases, including autoimmune diseases (Kristensen et al. 2019). Many studies have demonstrated that circRNAs control gene expression at the transcriptional, post-transcriptional, and translational levels through distinct mechanisms, including acting as miRNA sponges, interacting with proteins or DNAs and encoding small peptides (Hansen et al. 2013; Taulli et al. 2013; Li et al. 2017; Zhang et al. 2019; Zhu et al. 2019; Legnini et al. 2017). However, our current understanding of circRNAs in SLE is limited and needs further investigation.

In this study, we aimed to analyze circRNA profiles expressed in PBMCs of SLE patients, investigate whether circLOC101928570 differentially expressed and closely related to the disease activities of SLE patients. We focused on the effect of circLOC101928570 competitively bind to miR-150 and regulate the expression of c-myb, which might regulate the transcription of IL2RA and eventually protected against diseasees progression. This study may provide a promising biomarker for the prevention, diagnose and treatment of the SLE.

Methods

Patients samples

There are 62 SLE patients and healthy volunteers were recruited from the First Affiliated Hospital of Army Medical University between 2017 and 2020. The SLE patients included fulfilled at least four of the American College of Rheumatology (ACR) 1997 revised criteria of SLE (Hochberg, 1997). Demographic, clinical, and laboratory characteristics of each subject were recorded, and disease activity was evaluated with SLEDAI (Bombardier et al. 1992). The information of SLE patients can be found in (Table 1, Additional file 1: Table 1). All participants were Han Chinese.

Table 1 Clinical features of SLE patients and demographic data of HCs

characteristics	SLE patents(n=62)	HCs(n=62)
Age, years, median (IQR)	37.0(20.8-54)	36.0(21.5-53.4)
Female, n (%)	55(89)	55(89)
SLEDAI score, mean (IQR)	9.05(2.73-15)	N/A
Complement C3, median (IQR)	0.58(0.25-0.92)	N/A
Complement C4, median (IQR)	0.14(0.04-0.27)	N/A

IQR: interquartile range.

RNA extraction and RNA quantitative real-time polymerase chain reaction

Total RNA was harvested and separated from PBMCs in samples via TRIzol reagent (Invitrogen, United States) and sequentially complementary DNA (cDNA) was synthesized. Two micrograms of total RNAs was used to synthesize cDNA, a portion of which (1 μl, equal to 0.2 μg cDNA) were used in a PCR. The reverse transcription polymerase chain reaction (PCR) was operated with the SYBR Green Super Mix (Roche, Switzerland). Analysis of experimental results was analyzed through the $2^{-\Delta\Delta Ct}$ method. β-actin was an internal control. The sequences of the primers used for qRT-PCR in this study are shown in (Additional file 2: Table 2).

Ribonuclease R treatment

Total RNA (2 μg) of PBMCs were mixed with 3 U/μg of ribonuclease R (EpicentreTechnologies, United States) at 37°C for 20 minutes. The stability of circLOC101928570 and linear LOC101928570 were determined. Relative expression levels were evaluated by qRT-PCR.

Fluorescence in situ hybridization

Hybridization was performed overnight with circLOC101928570 probes. Specimens were analyzed using a Nikon inverted fluorescence microscope. The circLOC101928570 probe for fluorescence in situ hybridization (FISH) is 5'-TGGCTTGAATAGATTGGGACTAATA-3'. The cell suspension was pipetted onto autoclaved glass slides, followed by dehydration with 70%, 80% and 100% ethanol. Then hybridization was performed at 37 °C overnight in a dark moist chamber. After being washed twice in 50% formamide/2×SSC for 5 min, the slices were incubated with the regents in Alexa FluorTM 488 Tyramide SuperBoost™ Kits by Riobio (Guangzhou, China) for 30 min and sealed with parafilm containing DAPI. This assay was repeated three times.

Cell isolation and culture

Whole blood (10 ml) was collected in EDTA collection tubes from each subject, and human PBMCs were isolated by density-gradient centrifugation using Ficoll-Paque Plus (GE Healthcare Biosciences, United States) and cultured in RPMI Media 1640 (Gibco, United States) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/mL Penicillin, 100 U/mL streptomycin (Gibco, United States) at 37 °C with 5% CO2 for 24 h before transfection.

Cell transfection

The circLOC101928570 overexpression plasmids and empty vector were constructed and designed by Genechem (Shanghai, China). The miR-150-3p mimics, miR-150-5p mimics, miR-150-3p inhibitor, miR-150-5p inhibitor, miRNA mimics NC, miRNA inhibitor NC were chemically synthesized by Riobio (Guangzhou, China). pCDH-CMV-MCSEF1-GFP+Puro_(CD513B-1) vector as a negative control plasmid, pCDH-MYB plasmid was fragment inserted into pCDH-CMV-MCSEF1-GFP+Puro (CD513B-1) vector with BamHI and NotI restriction sites. The result of vector construction was verified by direct sequencing.

Sequence used was (5'-CCGGAATTCCGGGAAAGCGTCACTTGGGGAAAA-3'). PLKO.1-puro (Addgene plasmid # 8453) was used to design short hairpin RNA , they were transfected by Lipofectamine 3000 (Invitrogen, United States) into cells. The transfection process lasted 48 hours.

Luciferase reporter assay

293T cells in 24-well plates were cotransfected with miR-150-3p/5p mimic, inhibitor, and negative control, luciferase reporter vectors (SV40-Luc-MCS) with wild type or mutant circLOC101928570 were designed and constructed by Genechem (Shanghai, China). The IL2RA 3' UTR sequences containing two wild-type c-myb predicted binding sites were inserted into the region directly downstream of a T7 promoter-driven firefly luciferase cassette in a psiCHECKTM-2 vector (Promega, United States). All constructs were verified by sequencing. 293T cells were seeded into 24-well plates and were co-transfected with a mixture of 1ug of luciferase reporter plasmid and PCDH, plasmid pCDH-MYB, shNC, shMYB. After 48h, luciferase activity was measured using Dual-Luciferase® Reporter Assay System (Promega, United States). The relative firefly luciferase activity was normalized to Renilla luciferase activity. All experiments were performed in triplicate.

Short hairpin RNA

To stably knock down circLOC101928570 expression, Jurkat cells were cultured and infected with lentivirus carrying shRNA targeting circLOC101928570 and a negative control vector, PLKO.1-puro was used to design short hairpin RNA, the restriction site were AgeI (R3552S), EcoRI (R3101T), after 1300bp has a single restriction site KpnI (R0142M). For the lentivirus package, HEK-293T cells were transfected with the core plasmid PLKO.1-shRNA, with the psAX2 packaging plasmid and pMD2G envelope plasmid for 48 h to obtain the lentivirus supernatant. The shRNA sequences used was showed in (Additional file 3: Table 3). All constructs were verified by sequence analysis. with no homology to any other human genes.

Apoptosis

Double staining of Annexin V and 7-aminoactinomycin-D (7-AAD) was carried out using a PE Annexin V Apoptosis Detection Kit (BD Pharmingen™, United States) and APC Annexin V Kit (BD Pharmingen™, United States) according to the manufacturer's recommendations. Briefly, cells were washed with cold phosphate-buffered saline and then resuspended in Binding Buffer at a concentration of 1 x106 cell/ml. Then, 100 µl solution (1 x 10^5 cells) was transferred to a tube, and 5 µl PE Annexin V and 5 µl 7-AAD were added. After incubation for 15 min at room temperature in the dark and addition of 400 µl of binding buffer, flow cytometric analysis was done by flow cytometer (FACScan , BD Biosciences, San Jose, CA, United States) within 1 hour, and the data were analysed with FlowJo software (Treestar, Ashland, OR). PE Annexin V (+) or APC Annexin V (+), 7-AAD (-) cells represent early stage of apoptosis, whereas PE Annexin V (+) or APC Annexin V (+), 7-AAD (+) cells were in-end stage apoptosis or already dead.

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of IL-4 and IFN-γ in Peripheral blood serum supernatants were analyzed by Human IL-4 instant ELISA Kit (eBioScience, United States) and Human IFN gamma Platinum ELISA Kit (eBioScience, United States) following the manufacturer's instructions, respectively. The concentrations were calculated according to their corresponding stand curves.

Prediction of ceRNAs for circLOC101928570

Mutually targeted method was applied to predict putative ceRNAs for circRNA. To predict ceRNAs for circLOC101928570, we use software circMir1.0 identified circLOC101928570 targeting miRNAs.

Pull-down assay

Biotinylated circLOC101928570 probe was specifically designed and synthesized for binding to the junction site of circLOC101928570. The biotin-coupled RNA complex was pulled down by incubating the cell lysates with Pierce™Streptavidin Magnetic Beads (Thermo Fisher Scientific, United States) following the manufacturer's instructions. The enrichment of miRNAs in the capture fractions was evaluated by qRT-PCR analysis. Probe sequences used were listed in (Additional file 4: Table 4).

RNA-binding protein immunoprecipitation (RIP)

RIP assay was performed by using a Magna RIP RNA Binding Protein Immunoprecipitation Kit (Millipore, United States), according to the manufacturer's instructions. Briefly, PBMCs were harvested and lysed in RIP lysis buffer on ice for 30 min. After centrifugation, the supernatant was incubated with 30μl of Protein G agarose beads (Roche, United States) and antibodies. After overnight incubation, the immune complexes were centrifuged then washed six times with washing buffer. The beads bound proteins were further analyzed using western blotting. The immunoprecipitated RNA was applied to qRT-PCR analysis.

Western blot analysis

Complete proteome from PBMCs was extracted after lysing in RIPA lysis buffer (Beyotime, China) supplemented with protease inhibitors (Sigma-Aldrich, United States) and then separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis. The samples were electro transferred into polyvinylidene fluoride membrane (Millipore, United States). Post blocking with 5% fat free milk, the membranes were treated with prepared primary antibodies: anti-c-myb (Abcam, England), rabbit IL2Rα antibody (CST, United States), rabbit GAPDH antibody (CST, United States) was used as control. Membranes were washed, followed by treating with secondary antibody anti-rabbit antibody (CST, United States). The signal of the blot was examined by Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, United States) with ChemiDocTM Touch Imaging System (Bio-Rad, United States). The integrated density values were calculated using Quantity One software (Bio-Rad, United States).

Flow cytometry analysis

The flow cytometric analysis was performed on freshly isolated PBMCs as a previous study showed that CD25 expression is affected by freezing–thaw procedures. PBMCs were stained with fluorochrome-conjugated antibodies to identify blood CD4+ and CD8+ T cell subsets by flow cytometry. The staining procedure was conducted blinded to genotype and performed simultaneously for each pair. Prior to staining, FcR Blocking Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) was added to the PBMCs to prevent non-specific binding. We used the following monoclonal antibodies specific for: CD3 (Biolegend, clone, UCHT1), CD4 (Biolegend, United States), CD8 (Biolegend, United States), CD25 (Biolegend, United States), IFN-γ (Biolegend, United States), IL-4 (Biolegend, United States) FOXP3 (Biolegend, United States), IL17A (Biolegend, United States). All antibodies were purchased from BioLegend (San Diego, CA, United States). The stained PBMCs were washed 3 times in cold PBS/2%FCS/0.1% NaN3 and data were acquired on a FACS Canto II 8 color flow cytometer (BD Biosciences, San Jose, CA, United States) aiming for 30,000 acquisitions.

Statistical analysis

Data were analyzed and visualized with GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, United States). Quantitative values are expressed as means±standard deviation (SD) of at least three independent repetitions. Statistical differences among groups were tested with unpaired two-tailed student's t test. A P-value less than 0.05 was chosen to indicate statistical significance.

Results

Identification and characterization of circLOC101928570, a circRNA that specifically showed a lower expression level in SLE

To identify the essential circRNAs that contribute to the progression of SLE, we analyzed the circRNA RNA-seq data to identify circRNAs (Additional file 5: Table 5) with significant differences (|fold change|>2, FDR<0.05) between healthy controls and SLE patients (Miao et al. 2019). We selected and identified 4 circRNAs significantly related with SLE (Fig. 1A). We applied 62 patient samples and 62 healthy samples for further analysis. We then focused on circLOC101928570, which was expressed at low level in PBMCs of SLE patients, for further study (Fig. 1B). Patients expressed low level of circLOC101928570 and had a significantly higher the SLEDAI score (Fig. 1C), higher complement C3 level (Fig. 1D). To assess the diagnostic value of circLOC101928570 for SLE, receiver operating characteristic (ROC) curve analysis was performed to determine the relative circLOC101928570 expression between the 62 patients and 62 healthy controls (Fig. 1E). The area under the curve (AUC) was 0.8985 and the 95% confidence interval (95%CI) was 0.8441–0.9530. We next evaluated the circular structure of circLOC101928570, which was aroused from exon 3 of the LOC101928570 gene (chr6: 77271340-77273307), sanger sequencing validated the backspliced junction of circLOC101928570, as showing in (Fig. 1F). There are two CPG islands existed in the transcriptional core region of circLOC101928570 by using the software (http://www.ebi.ac.uk/emboss/cpgplot/), as showing in (Fig. 1G). Then we used the exonuclease RNase R to examine the stability of the circLOC101928570, circLOC101928570 showed a strong resistance to

digestion by RNase R, whereas the linear RNA of LOC101928570 and β-Actin were highly degraded (Fig. 1H). FISH result showed that circLOC101928570 is predominantly localized in the cytoplasm (Fig. 1I). Together, these data suggest that circLOC101928570 was significantly low expressed in SLE patients and correlated with SLE.

CircLOC101928570 acted as a sponge for miR-150

To explore the possible mechanism of functional circLOC101928570, we identified its ceRNAs with circMir1.0 software (http://www.bioinf.com.cn/). The result showed that circLOC101928570 has two targets with miR-150 (Fig. 2A). The miRNAs were extracted after pull-down assay, and the level of 10 candidate miRNAs was detected by real-time PCR. MiR-150-3p/5p were abundantly pulled down by circLOC101928570 in PBMC cells (Fig. 2B). To determine whether circLOC101928570 functions as a miRNAs sponge, We next preformed AGO2 immunoprecipitation to determine whether circLOC101928570 served as a platform for AGO2 and miR-150. The result showed that circLOC101928570 does bind to AGO2, QRT-PCR results further supported this observation (Fig. 2C). To confirm circLOC101928570 could be regulated by miR-150-3p/5p, we constructed luciferase reporters containing wild type and mutated putative binding sites of circLOC101928570 transcripts respectively (Fig. 2D), then co-transfected with miR-150-3p/5p mimcs or inhibitors into 293T cells. Luciferase reporter assays showed that the luciferase activities of circLOC101928570 wild type reporter were significantly reduced when transfected with miR-150-3p/5p mimics compared with control reporter or mutated luciferase reporter (Fig. 2E). Whereas, miR-150-3p/5p inhibitor significantly increased the luciferase signal of wild-type circLOC101928570 reporter (Fig. 2F). QRT-PCR further confirmed that circLOC101928570 knockdown could increase the miR-150 level and circLOC101928570 overexpression had an opposite role in Jurkat cell lines (Fig. 2G). However, miR-150 can't significantly influence circLOC101928570 level (Fig. 2H). Moreover, RNA FISH assay revealed that circLOC101928570 and miR-150 were co-localized in cytoplasm (Fig. 2I). These results showed that circLOC101928570 can bind to miR-150.

CircLOC101928570 decreased the c-myb expression by sponging miR-150

In order to explore the function of miR-150 in SLE, we then detected the expression of miR-150 using qRT-PCR in PBMCs obtained from 36 patients with SLE and 25 healthy controls. The result showed that the expression of miR-150 was upregulated in SLE (Fig. 3A). MiR-150 expression was also contrarily correlated with complement C3 level (Fig. 3B). There was strong positively correlated between miR-150 expression and the SLEDAI score in patients with SLE (Fig. 3C). To assess the diagnostic value of miR-150 in SLE, we also performed ROC curve analysis with the relative miR-150 expression in the 36 patients and 25 healthy controls (Fig. 3D). The AUC was 0.7889 and the 95% CI was 0.6643-0.9135. These results demonstrated that miR-150 high expressed in SLE patients and correlated with SLE.

It has been widely reported that c-myb is the target protein of miR-150. MiR-150 is highly expressed in mature lymphocytes, and c-myb is an important transcription factor for regulating lymphocyte development and participating in the pathogenesis of SLE (Xiao et al. 2019; Lin et al. 2019; Deguchi et al. 1987; Klinman et al. 1986). To further study the role of circLOC101928570 in the progression, we

performed two short hairpin RNAs (shRNA-circLOC101928570#1 and shRNA-circLOC101928570#2). Jurkat cells with stable circLOC101928570 knockdown with lentiviral shRNA and circLOC101928570 overexpression plasmid with lentiviral were constructed. We found that shRNA-circLOC101928570 could successfully knockdown circLOC101928570 expression but had no effect on LOC101928570 mRNA expression in jurkat cells (Fig. 3E). Similarly, circLOC101928570 was successfully overexpressed in jurkat cells, while LOC101928570 mRNA expression had no obvious change (Fig. 3F). To explore whether circLOC101928570 could regulate the c-myb via competitively binding with miR-150, we transfected miR-150 inhibitor and circLOC101928570 overexpression plasmids into jurkat cells. CircLOC101928570 overexpression increased the expression of c-myb, while transfected miR-150 mimics significantly attenuated the circLOC101928570-induced increased expression of c-myb (Fig. 3G). Downregulation of circLOC101928570 resulted in decreased expression of c-myb. Furthermore, transfected miR-150 inhibitor promoted the decreased expression of c-myb (Fig. 3H). These data demonstrated that circLOC101928570 regulates the c-myb by competitively binding of miR-150 to mediate the immune inflammatory response in SLE.

CircLOC101928570 knockdown enhanced apoptosis

As a proto-oncogene, c-myb plays a crucial role in the process of cell development, differentiation and apoptosis. Down-regulated expression of c-myb will cause cell apoptosis (Malaterre et al., 2007; Oh and Reddy, 1999; Liu, 2004). Our study has shown that circLOC101928570 decreased the c-myb expression by sponging miR-150. To further explore the function of circLOC101928570, we examined the cell apoptosis in stable Jurkat cells, and found that the percentage of cells in apoptosis was significantly increased in the shRNA-circLOC101928570#1 group compared with shRNA-NC group (Fig. 3I and 3J). The proportion of cells that were in apoptosis was lower in the circLOC101928570 overexpression group than that of NC group (Fig. 3I and 3K). These findings suggested that circLOC101928570 negatively regulated early apoptosis of Jurkat cells. Identifying the factors contributing to the enhanced apoptosis of SLE T cells will deepen our understanding of SLE pathogenesis.

C-myb transcriptionally regulating IL2RA expression by binding to IL2RA

To further explore the pathogenesis of circLOC101928570 involvement in SLE. Next, through the prediction of transcription factor c-myb target genes by software (http://cistrome.org/db/#/), result showed that IL2RA might bind c-myb in Th1 and Th2 CD4+ T lymphocytes (Fig. 4A and 4B). CD4+T lymphocytes are an important factor in the pathogenesis of SLE, mainly manifested by the immune imbalance of CD4+T lymphocytes, and the differentiation of CD4+T lymphocytes is regulated by IL-2(He et al. 2016). However, the specific mechanism remains to be clarified. Normal serum IL-2 level is an important condition to maintain the normal function of CD4+T lymphocytes, B cells and NK cells. As a transcription factor, c-myb may regulate target gene expression in transcriptional level, thereby exerting biological functions. We analyzed the potential binding DNA sequence loop of c-myb and found two theoretical binding sites in the top 2000nt of the promoter domain of IL2RA gene (http://jaspar.genereg.net) (Fig. 4C). Hence, we speculated that c-myb may regulate IL2RA expression at

the transcriptional level. To confirm the supposition, a luciferase plasmid with the top 2000nt of the promoter domain of IL2RA gene (psicheck2-WT) and a luciferase plasmid with mutant sequences in both two binding sites of the top 2000nt of the promoter domain (psicheck2-Mutant) were generated (Fig. 4D). In addition, we performed two short hairpin RNAs (shRNA-MYB#1 and shRNA-MYB#2), MYB overexpression plasmid was constructed with PCDH vector. We found that shRNA-MYB could successfully knockdown MYB expression and MYB was successfully overexpressed in 293T cells (Fig. 4E and 4F). Luciferase reporter assays demonstrated that c-myb enhanced the luciferase activity of psicheck2-WT in a dose dependent manner, but not that of psicheck2-Mutant (Fig. 4G and 4H), suggesting that c-myb enhanced IL2RA expression by directly binding to the promoter domain of IL2RA. These data demonstrated that c-myb may suppress SLE progression by positively regulating IL2RA expression in the transcriptional level. To explore whether circLOC101928570 could regulate the expression of IL2RA, we transfected circLOC101928570 overexpression plasmid, circLOC101928570-NC, shRNA-circLOC101928570#1, shRNA-NC into jurkat cells. Upregulated circLOC101928570 increased the expression of IL2RA (Fig. 4I). Downregulation of circLOC101928570 resulted in decreased expression of IL2RA (Fig. 4J). These data indicated function of circLOC101928570 participating in regulating IL-2RA expression.

CircLOC101928570 suppresses IL2RA expressions in CD4+T cells subsets of SLE

We analyzed the cell cytokine expressions in the supernatant of PBMCs from different 55 SLE and 33 healthy groups by ELISA. IL-4 expression was increased in SLE groups compared with healthy groups (Fig. 5A). There was no significant difference in the IFN-γ expression in SLE patients compared with healthy groups (Fig. 5B). IL-4/IFN-γ level in SLE was higher than healthy groups (Fig. 5C). The IL-4/IFN-γ ratio of SLE was negatively correlation with the expression of circLOC101928570 (Fig. 5D). Meanwhile, the Treg/Th17 ratios negatively correlated with the expression of circLOC101928570 (Fig. 5E). We analyzed the Treg and Th17 percentages in PBMCs from different SLE and healthy groups by flow cytometry (Fig. 5F and 5G). By a linear regression analysis, the ratio of Th1/Th2 and Treg/Th17 ratios were correlated with the expression of circLOC101928570. Next, We analyzed the expression of IL2RA on Th1, Th2, Tc1 and Tc2 in the CD4+ T cell populations between SLE patients and healthy controls by flow cytometry. The results demonstrated that the expression of IL2RA on Th1, Th2, Tc1 and Tc2 in SLE patients was lower than healthy groups (Fig. 5H and 5I). We analyzed the percentages IL2RA of Th1, Th2, Tc1 and Tc2 cells in PBMCs from different SLE and healthy groups by flow cytometry (Fig. 5J and 5K). These data indicated circLOC101928570 suppresses IL2RA expressions in CD4+T cells subsets of SLE.

Discussion

CircRNAs are a novel type of non-coding RNAs that have multiple potential biological functions. Recently, an increasing number of studies have reported circRNAs participating in the physiology and pathology of various diseases, circRNAs have been identified as potential biomarkers for diseases diagnosis or prognosis, including autoimmune diseases etc. CircRNAs exert their biological functions through interaction with miRNAs or RNA binding proteins (RBPs) or as protein scaffolds, modulating gene

transcription and protein translation (Xu et al. 2017; Guarnerio et al. 2019; Liu et al. 2020). The recent discovery of thousands of circRNAs and their novel functions in gene expression regulation prompted us to investigate their roles in SLE. A misregulated circRNA-PKR-RNase L axis was found in SLE (Liu et al. 2019). Zhang et al demonstrated that hsa_circ_0012919 was associated with clinical variables and the abnormal DNA methylation present in SLE CD4+ T cells. It acted as a miRNA sponge for miR-125a-3p, regulating the gene expression of targets RANTES and KLF13, which are involved in the physiology and pathophysiology of acute and chronic inflammatory processes (Zhang et al. 2018). Zhao et al suggested that upregulated plasma circRNA_002453 level in LN patients is associated with the severity of renal involvement and may also serve as a potential biomarker for LN patients (Ouyang et al. 2018). circIBTK was downregulated in SLE and it might regulate DNA demethylation and the AKT signaling pathway via binding to miR-29b in SLE (Wang et al. 2018). These findings support the role of circRNAs in the pathophysiology of SLE.

In this study, we screened out a downregulated circRNA named circLOC101928570 according to the results of RNA-seq and qRT-PCR analysis. We confirmed that circLOC101928570 expression was downregulated in SLE patients compare with healthy controls, and the expression of circLOC101928570 was correlated with disease activity of SLE. Furthermore, we investigated the function and mechanism of circLOC101928570. Bioinformatics analysis showed that circLOC101928570 binds to miR-150-3p/5p. Luciferase reporter and RIP assays confirmed the direct interaction between circLOC101928570 and miR-150, suggesting the circLOC101928570 functions as a “miRNA sponge” of miR-150. Previous studies have demonstrated the pathogenic role of miR-150 in SLE. MiR-150 was identified to be with positively correlated with chronicity scores and the expression of profibrotic proteins in lupus nephritis patients. Elevated miR-150 could target antifibrotic protein SOCS1 with upregulated profibrotic proteins (Zhou et al. 2013).

C-myb is an evolutionary conserved miR-150 target, researches have showed the miR-150/c-myb interaction played important role in the differentiation of T cells and B cells, pressure overload-induced cardiac fibrosis, regulating epithelial-mesenchymal transition (EMT) in ovarian cancer cells (Luan et al. 2019). In our study, further molecular experiments demonstrated that circLOC101928570 decreased the c-myb expression by sponging miR-150. Additionally, abnormal apoptosis and cytokine secretion of T cells are involved in the pathogenesis of SLE (Emlen et al. 1994; Frieri et al. 2013). We analyzed the role of circLOC101928570 in the apoptosis of T cells of SLE, knockdown of circLOC101928570 led to elevation of early apoptosis of Jurkat cells. Next, we identified that c-myb is interacted with IL2RA by bioinformatics analysis and Dual luciferase reporter assay, which suggesting that circRNAs is implicated in numerous aspects of post-transcriptional of mRNA. Moreover, we found that circLOC101928570 expression is negative correlation with IL-4/IFN-γand Treg/Th17 ratios in SLE patients. The expression of IL2RA on Th1, Th2, Tc1 and Tc2 of CD4+ T cell populations in SLE patients was also significantly lower than healthy groups. Which may show that circLOC101928570 may affect the cytokine expressions and the differentiation of T cell in SLE.

IL2RA is a subunit of the high affinity receptor for interleukin-2 (IL2). The gene locus of IL2RA has been ascertained as risk factor for a diverse series of autoimmune diseases including SLE. The IL-2 pathway is critical for the maintenance of immune homeostasis. IL-2 signaling plays a role in activation-induced cell death and is vital to regulatory T cell homeostasis (Carr et al. 2009; Huang et al. 2012). In our study, we found that the expression of IL2RA on Th1, Th2, Tc1 and Tc2 of CD4+ T cell populations in SLE patients was also significantly lower than healthy groups. C-myb transcriptionally regulating IL2RA expression by binding to IL2RA. These data indicated circLOC101928570 may influence IL2RA expression in CD4+T cells subsets of SLE.

There are accumulating examples of circRNAs acting as miRNA sponges, thereby influencing the target mRNAs. Moreover, several miRNAs regulating the pathogenesis and involving in apoptotic pathway showed therapeutic potential of SLE (Lieberman and Tsokos 2010; Zhang et al. 2018). Whether and to what extent circRNAs expression contributes to the dysregualted miRNA profile in SLE remain to be solved. Our study demonstrated that the functional dysregulations including apoptosis seen in SLE may be related to the circLOC101928570 regulation on relevant transcripts. While the signaling pathway of apoptosis correlated with circLOC101928570 need further exploration. On the other hand, Our study showed that circLOC101928570 acted as a miR-150-3P/5p sponge to modulate the activation of immune inflammatory responses mediated by the c-myb/IL2RA pathway. Even though we found circLOC101928570 influences IL2RA expression in CD4+T cells subsets of SLE. How the low expression of IL2RA in the CD4+ T cell subsets participate in the pathogenesis of SLE is not clear. We suspect the IL2RA in different CD4+ T cells subsets will influence the binding with IL-2. Defective IL-2 production is one of many factors involved in the immune dysregulation responsible for SLE. Decreased IL-2 production in SLE patients leads to many immune defects such as decreased production, decreased activation-induced cell death (AICD) and decreased cytotoxicity (Su et al, 2018). While a follow-up study to elucidate a deeper understanding of circLOC101928570 on the defective function of IL-2 is still needed.

Conclusions

In conclusion, we provided the first evidence that the expression of circLOC101928570 is significantly decreased in SLE patients, further experiments demonstrated the circLOC101928570 acted as a miR-150-3P/5p sponge to modulate the activation of immune inflammatory responses mediated by the c-myb/IL2RA pathway. CircLOC101928570 negatively regulates apoptosis and influences the regulating IL2RA expression in CD4+T cells subsets of SLE. Our results suggested that circLOC101928570 might be as a biomarker for SLE. The regulatory network involving the circLOC101928570/miR-150/c-myb/IL2RA pathway might provide new insight into the potential mechanism of the pathogenesis and development to SLE (**Fig. 6A**).

Abbreviations

SLE: Systemic lupus erythaematosus；circRNAs: Circular RNAs; PBMCs：Peripheral blood mononuclear cells；MiR-150: MicroRNA-1150; miRNAs：MicroRNAs；SLEDAI: Systemic Lupus Erythaematosus Disease Activity Index; ROC：Receiver operating characteristic；AUC : Area under curve; MYB: MYB Proto-Oncogene, Transcription Factor; IL2RA：Interleukin 2 Receptor Subunit Alpha；IgG: Immunoglobulin G; cDNA: Complementary DNA; WT: Wild-type; EMT: Epithelial-mesenchymal transition; RBPs：RNA binding proteins；ELISA: Enzyme-linked immunosorbent assay; FISH：Fluorescence in situ hybridization；qRT-PCR: Quantitative real-time polymer chain reaction; RIP：RNA-binding protein immunoprecipitation；AGO2： Argonaut 2. WB:Western blotting;

Declarations

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Authors' contributions

XWZ, YY, BN were responsible for study conception and design. XWZ performed the experiments, analysed the data and wrote the manuscript. LLZ for technical support and assistance with data analysis. JW, ZQS, YY performed case selection and clinical assessment of diseases. BN provided specialist advice. All authors read, participated in improving and approved the final version of the manuscript.

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Availability of data and materials

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

Ethics approval and consent to participate

This study was approved by the ethical committee of the First Affiliated Hospital of Army Medical University and conducted according to the declaration of Helsinki principles. All patients signed written informed consent for participation.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

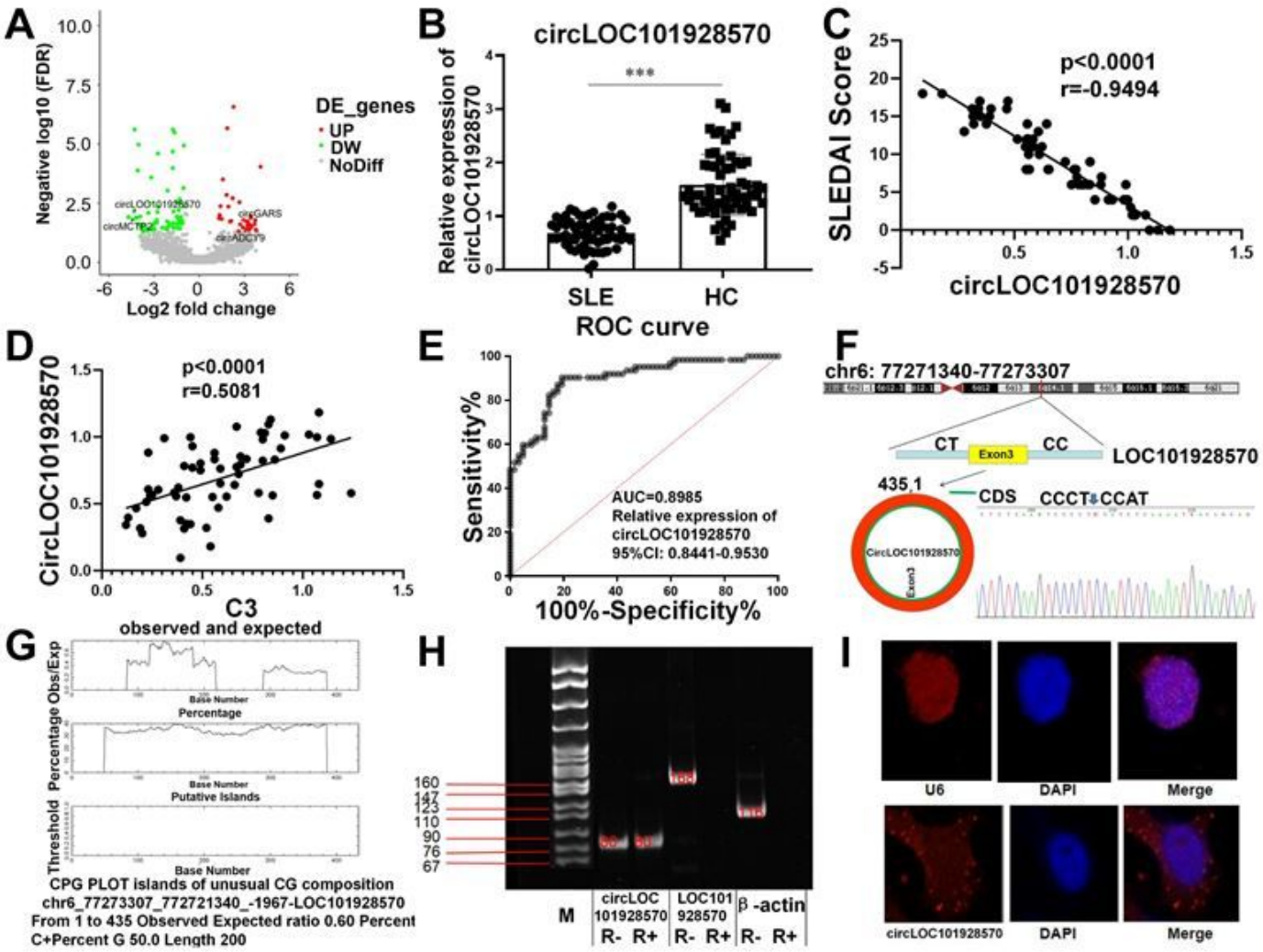

Figure 1

Identification and characterization of circLOC101928570, a circRNA that is significantly downregulated in SLE. A Volcano plot describing the profile of differentially expressed circRNAs between control and SLE. Slected and identified 4 significantly changed circRNAs are marked with green and red dots (FDR < 0.05, |fold change| > 2.0). B CircLOC101928570 expression was significant lower in SLE patients. Results are represented as mean ± SD (n=3). ***p < 0.001. C, D Correlation between the expression of circLOC101928570 and the SLEDAI score, complement C3 level were analyzed. E ROC curve of relative circLOC101928570 expressions for differentiating the 62 patients with SLE from 62 healthy controls. F Structure of the circLOC101928570 genome and transcript. CircLOC101928570 is produced by exons 3 and also identificated of the junction point of circLOC101928570 by qRT-PCR followed by sanger sequencing. G Two CPG islands existed in the transcriptional core region of circLOC101928570. H Relative expression of circLOC101928570, linear-LOC101928570 and actin were tested by PCR upon RNase R treatment. I FISH showed circLOC101928570 localization, U6 was mainly localized in nucleus, used as negative control. circLOC101928570, U6 probes were labeled with Cy3, nuclei were stained with DAPI, scale bar: 10 μm.

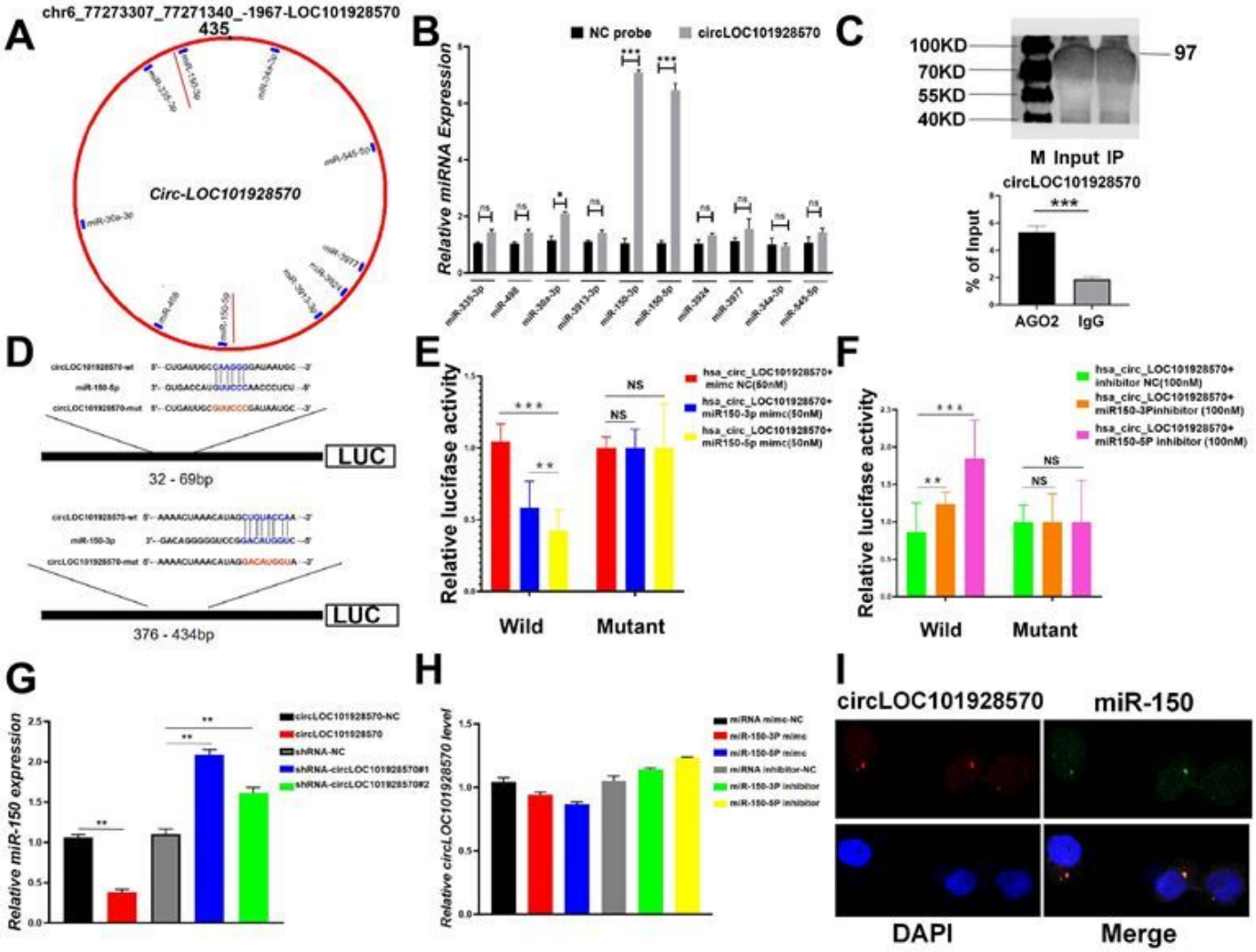

Figure 2

CircLOC101928570 acted as a sponge for miR-150. A ceRNAs that could competitively bind to this exonic circRNA. MiR-150 has two targets marked with red line. B The relative level of 10 miRNA candidates in the PBMCs lysates was detected by real-time PCR. miR-150-3p/5p was pulled down by circLOC101928570 in PBMCs. C Interaction of circLOC101928570 with miR-150 was testified by RNA imunoprecipitation (RIP) of AGO2 from HEK293T cells. CircLOC101928570 levels were quantified by RIP-qPCR. D Schematic illustration of wild type or mutant transcripts of circLOC101928570 luciferase reporter. E, F Luciferase reporter, effects of miR-150-3p/5p on the expression of wild type or mutant transcripts of circLOC101928570 luciferase reporter. Luciferase activity was normalized to the value obtained in cells transfected with NC oligonucleotides. G The expressions of miR-150 were analyzed by using qRT-PCR in cells transfected with circLOC101928570 or mock vector or shRNA-circLOC101928570 or shRNA-NC. H The expression levels of circLOC101928570 were determined with qRT-PCR in cells transfected with miR-150-3p/5p mimics or inhibitor. I The co-localization of circLOC101928570 and miR-150 in PBMC cells was detected using a FISH assay. Red: labeled with Cy3. Green: labeled with Dig. Blue: stained with DAPI.

Scale bar: 10μm. IgG: immunoglobulin G. All data are presented as the means±SD (n=3). *P < 0.1, **P < 0.01, ***P < 0.001.

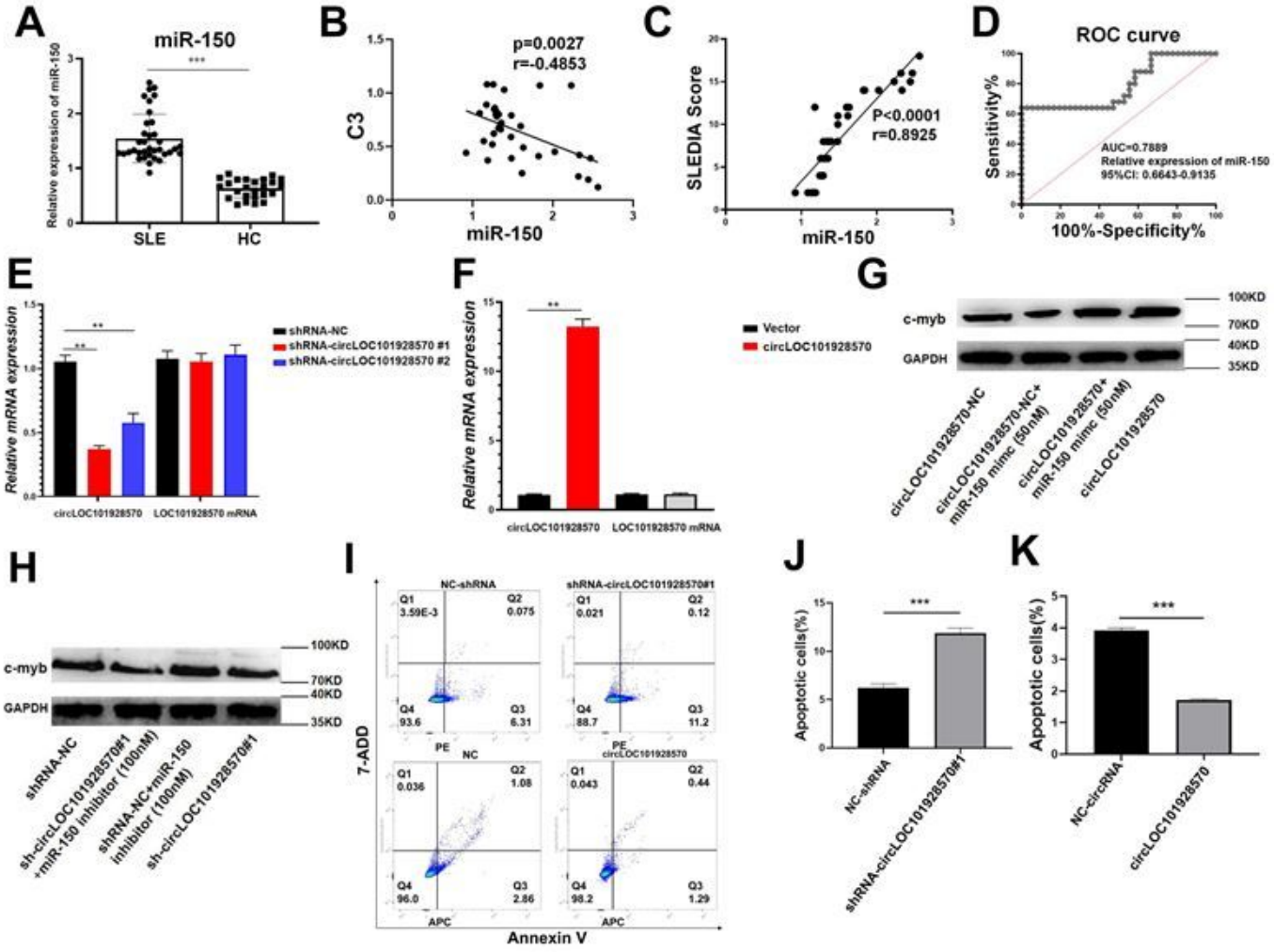

Figure 3

The expression of miR-150 in SLE. CircLOC101928570 decreased the c-myb expressiom, circLOC101928570 knockdown enhanced apoptosis. A Expression of miR-150 in PBMCs from 36 patients with SLE and 25 healthy controls (HC). B, C Correlation between expression of miR-150 and SLEDAI score or complement C3 level analyzed. D The ROC curve of relative miR-150 expressions for the different 36 patients with SLE from 25 healthy controls. E Expression levels of circLOC101928570 and LOC101928570 in jukat cells treated with shRNA-NC, shRNA-circLOC101928570#1, shRNA-circLOC101928570#2 lentivirus. F Expression levels of circLOC101928570 and LOC101928570 in jukat cells after transfection with circLOC101928570 lentivirus. G, H Western blot analysis of c-myb proteins after transfected with miR-150 mimics, circLOC101928570 overexpression plasmid, circLOC101928570-NC and miR-150 inhibitor, shRNA-circLOC101928570#1, shRNA-NC in jurkat cells respectively. I Representative graphs of cell apoptosis determined by APC Annexin V and 7-AAD staining in stably transfected Jurkat cells with circLOC10192857 and negative control circRNA (NC-circRNA). PE Annexin V and 7-AAD staining in stably transfected Jurkat cells with shRNA-circLOC101928570#1 and negative

control shRNA (NC-shRNA). J, K The percentage of cells of apoptosis was significantly increased in the shRNA-circLOC101928570#1 group, significantly decreased in the circLOC101928570 group. Results are represented as mean ± SD (n=3). ***P < 0.001.

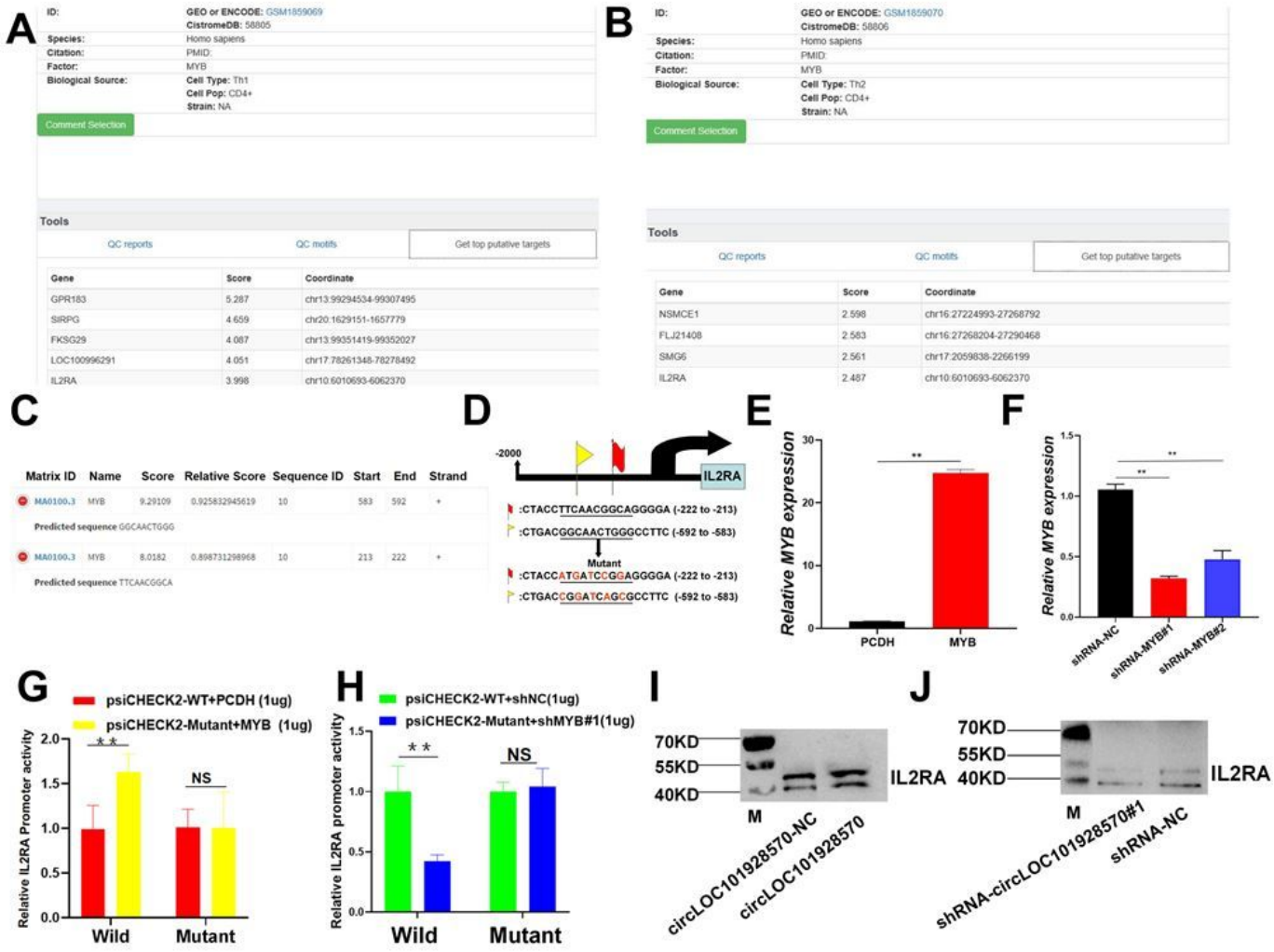

Figure 4

C-myb transcriptionally regulated IL2RA expression by binding to IL2RA. CircLOC101928570 suppresses IL2RA expressions. A, B Prediction of target gene of transcription factor c-myb. C The binding sites of c-myb in the promoter of IL2RA was shown in a model. D Schematic illustration of the sequences of wild type of IL2RA promoter domain and mutant sequences in the binding sites of c-myb on IL2RA promoter domain are shown. E Expression levels of MYB in 293T cells treated with shRNA-NC, shRNA-MYB#1,shRNA-MYB#1. F Expression levels of MYB in jukat cells after transfection with MYB plasmid. G, H Relative luciferase activities were detected in 293T cells after transfecting luciferase reporter plasmids with wild type of IL2RA promoter domain or mutant IL2RA promoter domain with pCDH, MYB , shRNA-NC, shRNA-MYB#1. I, J Western blot analysis of IL2RA proteins in jurkat cells transfected with circLOC101928570 overexpression plasmid, empty vectors, shRNA-NC, shRNA-circLOC101928570#1, respectively. Results are represented as mean ± SD (n=3). NS: no significance, **p < 0.01.

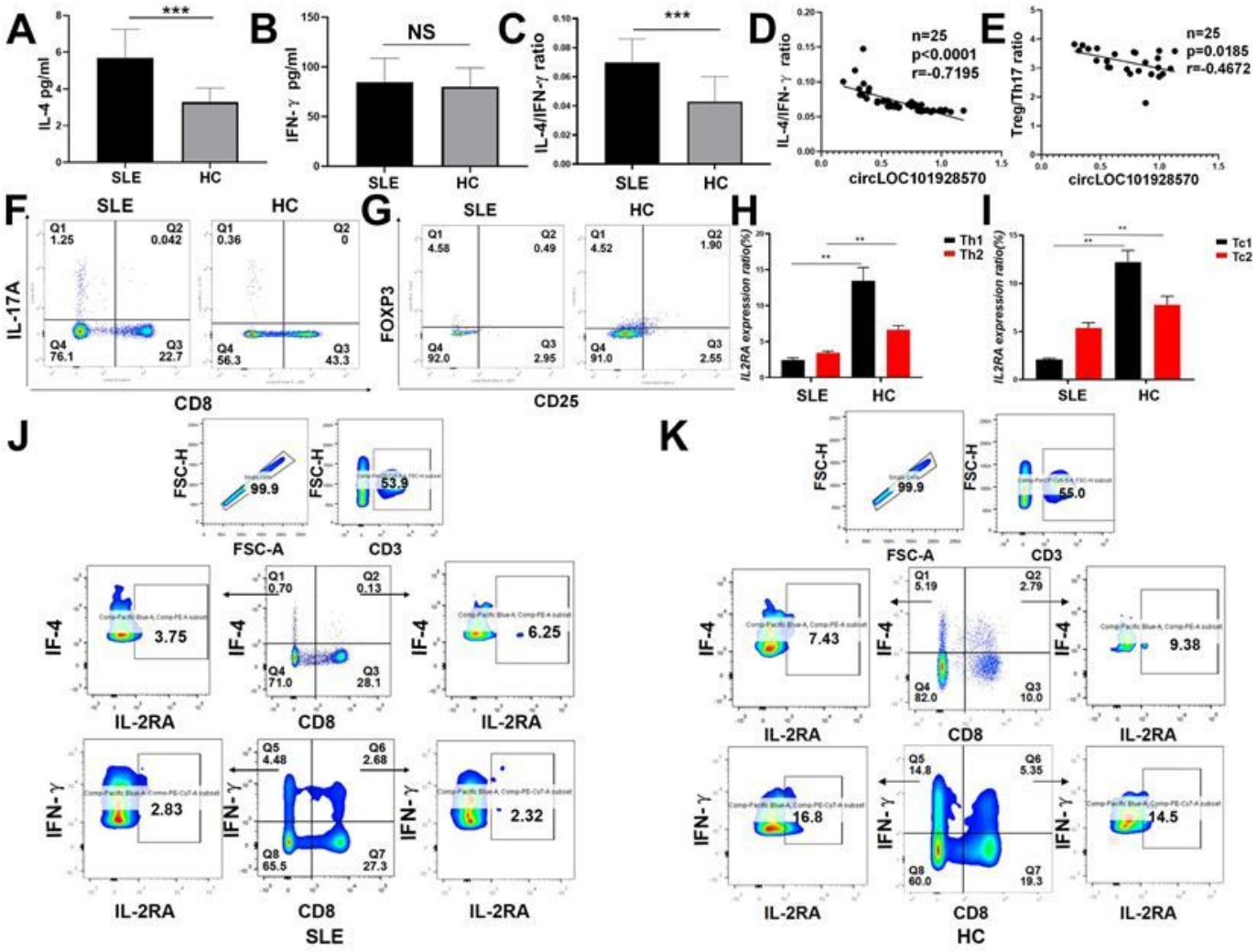

Figure 5

CircLOC101928570 suppresses IL2RA expressions in CD4+T cells subsets of SLE. A-C Quantitative measurements of the IL-4、IFN-γ、IL-4/IFN-γ level in the supernatant of PBMCs from 25 SLE patients and healthy controls by ELISA. D, E Correlation analysis IL-4/IFN-γ、Treg/Th17 ratio with the expression of circLOC101928570 in SLE. F, G Representative flow cytometry results, showing identify the percentages the Treg, Th17 cells in PBMCs from SLE patients compared with the healthy controls. H, I The percentages IL2RA of Th1, Th2, Tc1 and Tc2 cells in PBMCs from SLE patients compared with the healthy controls. J, K Representative flow cytometry results, showing identify the percentages IL2RA of Th1, Th2, Tc1 and Tc2 cells in PBMCs from SLE patients compared with the healthy control group. Results are represented as mean ± SD (n=3). NS: no significance. ***p < 0.001.

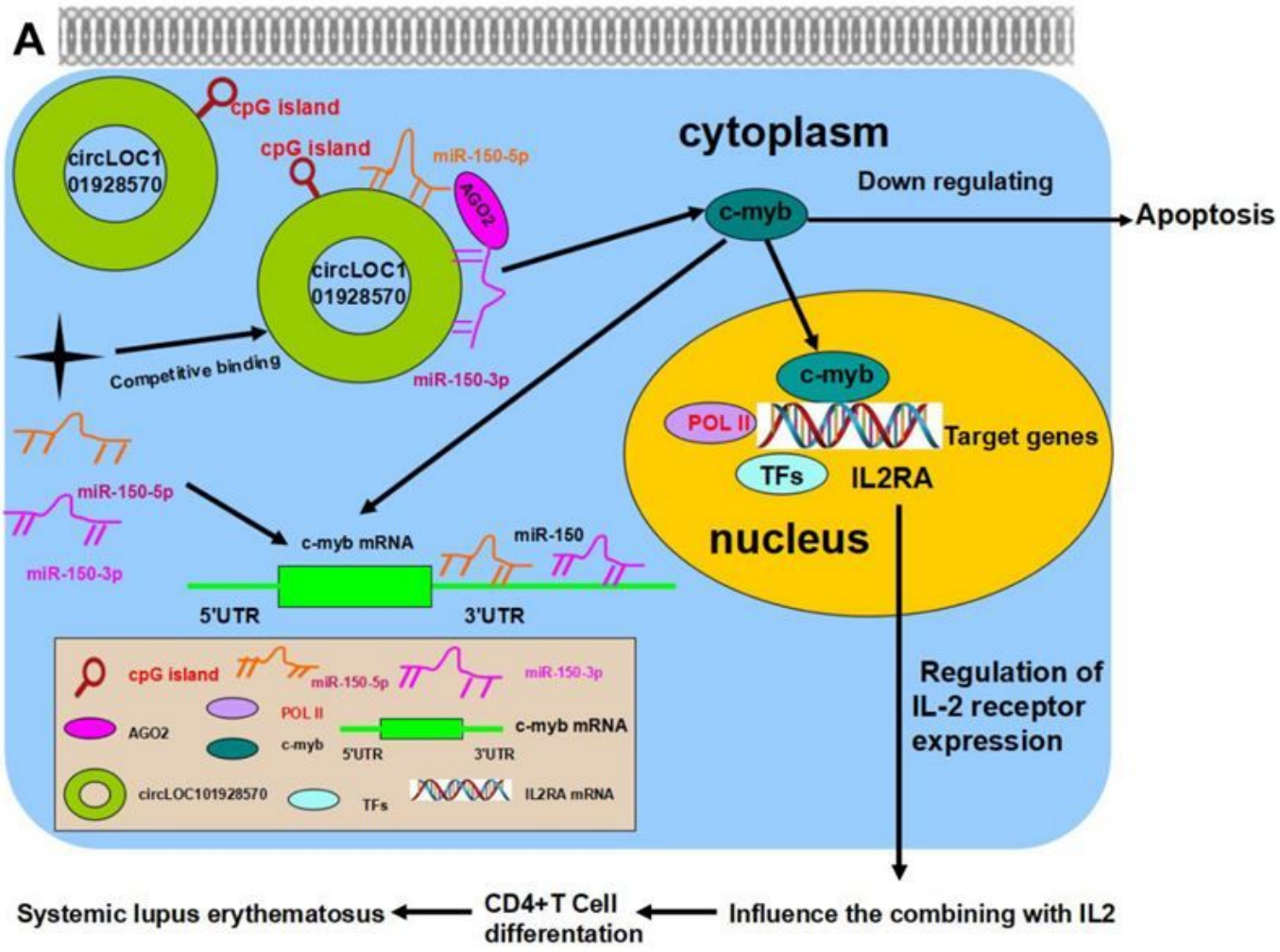

Figure 6

Schematic depicting the dominant function of circLOC101928570. A CircLOC101928570 acted as a miR-150-3P/5p sponge to modulate the activation of immune inflammatory responses mediated by the c-myb/IL2RA pathway. CircLOC101928570 knockdown enhanced apoptosis.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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