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Research Article

Analysis of Hsa_Circ_0006770 and Mir-146/Mir-155 Expression and the Correlation with the Disease Activity in Children with Systemic Lupus Erythematosus

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Abstract

Objective: To find relation among hsa_circ_0006770 and miR-146/miR-155 and inflammation in children with systemic lupus erythematosus.

Methods: A total of 40 children with systemic lupus erythematosus admitted to our hospital from April 2018 to December 2022 were selected, including 10 children with basically no activity, 10 with mild activity, 10 with moderate activity and 10 with severe activity according to the SLEDAI-2000 scores. Twenty healthy children who underwent physical examination in our hospital during the same period were selected as healthy control group, Peripheral Blood Mononuclear Cells (PBMC) and serum were isolated, extracted and reversely transcribed into cDNA. The expression difference of hsa_circ_0006770, miR-146a, miR-155, TLR4 and NF-κB groups was detected by fluorescence quantitative PCR, and the normalized 2-δδT value was calculated. The expression of IL-1, IL-6, IL-10, IL-18 and TNF-α in plasma was detected by ELISA. And correlation analysis.

Results: The expression levels of hsa_circ_0006770, TLR4 and NF-κB in case group were higher than those in control group, while the expression levels of miR-146a and miR-155 were lower than those in control group. The mRNA relative expression level of hsa_circ_0006770 in the case group was positively correlated with TLR4 and negatively correlated with miR-146a and miR-155. The relative mRNA expression of miR-146a and miR-155 was negatively correlated with NF-κB in the case group.

Conclusion: hsa_circ_0006770 and miR-146/miR-155 may be related to the level of inflammation in children with systemic lupus erythematosus.

Keywords: Systemic lupus erythematosus in children; Circrnas; TLR4; NF-κB

Introduction

Systemic Lupus Erythematosus (SLE) is the most common autoimmune systemic vasculitis in childhood, with clinical manifestations involving skin, gastrointestinal tract, joint, kidney and other systems. It has been reported in domestic and foreign literature that the continuous increase of IL-6 and TNF-α is related to the course of SLE, the degree of organ damage and the prognosis. However, the mechanism of inflammatory activation in children with systemic lupus erythematosus is still unknown [1]. The Toll-Like Receptors (TLRs) family plays an important role in the innate immunity of the body and the recognition of pathogens. TLRs induce the body's immune response by recognizing and binding associated signaling molecules. A large number of studies have shown that LPS stimulation of TLR may lead to the expression of some genes involved in innate immune response by activating multiple signal transduction pathways [2]. Studies have found that miR-146a and miR-155a are related to chronic inflammation, and SNP of pre-miR-146a and miR-155a (rs2910164) may be related to the susceptibility of Chinese Han population to systemic lupus erythematosus, and their mutation sites may be molecular markers of susceptibility to sys-

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temic lupus erythematosus [3]. The anti-inflammatory effect of miR-146a and miR-155a in AR may be caused by inhibiting the expression of toll-like receptor 4(TLR 4)/TRAF 6/NF-κB signaling pathway related proteins [4-6].

Then what is the expression of miR-146a and miR-155a in peripheral blood of children with systemic lupus erythematosus? The relative expression of miR-155 in SLE patients was higher than that in the mild and control groups, and was positively correlated with disease activity. Russka Shumnalieva [7] found miR-155 in 40 SLE patients and 32 normal controls. miR-146a and miR-155a were elevated in peripheral blood of SLE and correlated with disease activity. circular RNA (circRNA) is a special type of endogenous noncoding RNA (ncRNA) produced by selective splicing and widely expressed in eukaryotic cells. Junhui Zhang [8] identified a total of 29 DECs, including 2 up-regulated circRNAs and 27 down-regulated circRNAs, by searching SLE circrRNA sequencing GSE84655 from GEO database. These include (hsa_circ_0006770, hsa_circ_0002904, hsa_circ_0034044, hsa_circ_0023685, hsa_circ_0049271, hsa_circ_0074491, hsa_circ_0074559, and hsa_circ_0023461). We further analyzed the above results and found that hsa_circ_0006770 was related to multiple differentially expressed mirnas in the database. By using targetscan, miRDB and other databases to predict the relationship between hsa_circ_0006770 and miR-146a, miR-155, miR-155 binding, then the expression of hsa_circ_0006770 in SLE and its correlation with disease activity will be further discussed.

Materials

Experimental Reagents

ELISA kit, TRIzol Reagent BR DP424(day root), Reverse transcription kit (DBI-2220), quantitative fluorescence PCR kit (AOPR-1200 Genecopies), anhydrous ethanol, isopropyl alcohol, trichloromethane and DEPC were all domestic reagents.1.2 Primer design: hsa_circ_0006770 F:5 '-ACAGAGAGGA ATCTGGGCCT-3, R: 5 '- AAGGCCCTTACCTTTTCCAGG-3; TLR4 F:5 '-GGGAC TCTGATCATGGCATT-3, R: 5-GTCTCCACAGCCACCAGATT -3; NF-κB F:5 '-TTGGATTTCCACAGCCGTAG-3', R:5 '-AGAGTTACCTGGCCTG CAGGA-3'; GAPDH: F:5 'GCACCGTCAAGGCTGAGAAC 3', R:5' -TGGTGAA GACGCCA GTGGA-3'. Hsa_circ_0006770 Prediction and backwards-priming design were carried out through circinteractome database. The primers above were synthesized by BGI.

Experimental Procedure

Research Objects

A total of 40 children with systemic lupus erythematosus admitted to the Department of Allergy and rheumatology in our hospital from April 2020 to December 2020 were selected, including 10 children with basically no activity, 10 children with mild activity, 10 children with moderate activity and 10 children with severe activity. The diagnosis was consistent with the 1986 diagnostic criteria for systemic lupus erythematosus. The SLEDAI-2000 score was used to determine the severity of SLE. Twenty healthy children who underwent physical examination in the Department of Pediatrics of our hospital during the same period were selected as the control group, excluding chronic diseases and acute infectious diseases in the past one month.

RNA Extraction from Peripheral Blood Mononuclear Cells and qPCR Detection

lml Trizol reagent was set at room temperature for 5min and centrifuged at 4℃ for 12000r /min for 5min. Take the supernatant, add 0.2ml chloroform, violently shake and mix, centrifuge at 4℃, 12 000dmin for 15rain. The supernatant was transferred and 0.5ml isopropyl alcohol was added, which was mixed and left at room temperature for 10min. After centrifugation for 10rain at 12000r /min, the supernatant was abandoned. The supernatant was precipitated and added with 1ml diethyl pyrocarbonate, DEPC water for 75% ethanol and washed and dried at room temperature. Total RNA extract was obtained by dissolving 40m DEPC water.

The absorbance (A) of total RNA at 260nm and 280nm was measured by ultraviolet spectrophotometer to determine the purity and concentration of RNA. Total RNA was analyzed by electrophoresis with 1.5% agarose gel to detect its integrity. qPCR reaction: predenaturation at 94℃ for 2 minutes, denaturation at 94℃ for 30 seconds, annealing at 59℃ for 30 seconds, extension at 72℃ for 30 seconds, a total of 35 cycles, extension at 72℃ for 5 minutes. Stop the reaction at 4℃. The amplified products were subjected to 1% agarose-gel electrophoresis and analyzed by Quantity One software. The results were expressed by the ratio of absorbance of target gene band to GAPDH band.

ELISA Experiment

ELISA was used to measure the concentration of IL-1, IL-6, IL-10, IL-18, and TNF-α: (according to the steps of the kit) after sample addition, enzyme-labeled antibody was added, incubation and washing was performed, substrate solution was added for color development, and reaction was terminated with termination solution. OD value was measured by enzyme-labeled instrument and the concentration was calculated.

Statistical Analysis

SPSS13.0 was used for statistical analysis. The relative mRNA expression levels of hsa_circ_0006770, miR-146a, miR-155, TLR4 and NF-κB were expressed by 2-δδCT. Non-parametric test (Kruskal-Wallis test) was used for comparison between groups. T test was used to compare baseline data and IL-2, IL-6 and IL-10 concentrations between case group and control group, and $P<0.05$ was statistically significant.

Result

Combining Targets can and miRDB, Starbase prediction database and literature search, location of miR-146a, miR-155 for combining the NF-kappa B micrornas

In addition, targeted miR-146a and miR-155 circrnas hsa_circ_0006770 were found through literature search and database prediction. Forward and backward primers were designed, and the ring formation of hsa_circ_0006770 was verified by qPCR.

Expression of hsa_circ_0006770 and miR-146a/miR-155 in PBMC of children with systemic Lupus erythematosus and healthy control group

The expression level of hsa_circ_0006770 (Table 1) in the range of activity (mild, moderate and severe) in the case group was higher than that in the control group, and the difference was statistically significant (P=0.01). There was no significant difference in the expression of hsa_circ_0006770 between the disease-free group and the control group. The expression levels of miR-146a and miR-155 (Table 1) in the range of activity (mild, medium and severe) in the case group were lower than those in the control group, and the difference was statistically significant

(*P*=0.01). There was no significant difference in the expression of miR-146a and miR-155 between disease-free group and control group.

Expression of TLR4/NF-κB in PBMC of children with systemic lupus erythematosus and healthy control group

The expression level of TLR4 in case group was higher than that in control group, the difference was statistically significant P=0.01, and the expression level of NF-κB in case group was higher than that in healthy control group, the difference was statistically significant P=0.01. The expression level of NF-κB (Table 1) in case group was higher than that in control group, and the expression level of NF-κB in case group was higher than that in healthy control group, the difference was statistically significant P=0.01.

Expression of IL-2, IL-6 and IL-10 in peripheral blood of children with systemic lupus erythematosus and healthy control group was detected by ELISA

Case group activity (no, light, medium, heavy), IL-1 were 4.57±1.25, 10.56±2.35, 13.23±3.62, 16.38±4.52 (Table 2); IL-6 were 5.56±3.15, 20.56±4.32, 50.32±10.09, 70.86±11.63 (Table 2); IL-18 were 7.56±2.03,12.63±3.21,16.99±3.68 and 21.45±4.63, respectively, higher than those in healthy control group (*P*=0.01), and the difference was statistically significant. The IL-10 concentration of 1.25±0.62 in the case group (Table 2) was lower than that in the control group (*P*=0.01).

Correlation analysis of hsa_circ_0006770, miR-146a and miR-155 with disease activity and inflammation (Table 3)

hsa_circ_0006770 mRNA expression of relative quantity and disease activity (*P*=0.01, R = 0.84), the NF-kappa B (*P*=0.01, R=0.58), TLR4 (*P*=0.01, R=.68), IL-1 (*P*=0.01, R=0.78), IL-6 (*P*=0.01, R=0.82), IL-18 (*P*=0.03, R = 0.58), TNF-α (*P*=0.01, R=0.74) present positive correlation; The mRNA relative expression of hsa_circ_0006770 in the case group was negatively correlated with IL-10(P=0.04,R=-0.56) miR-146a(*P*=0.01,R=-0.74) and miR-155 (*P*=0.01, R=-0.63). MiR-146a and miR-155 mRNA expression of relative quantity and disease activity (*P*=0.01, R = 0.64), (*P*=0.01, R=0.55).

Discussion

The pathogenesis of systemic lupus erythematosus is related to a variety of factors and the etiology is complex. Most studies believe that the pathophysiological changes of systemic lupus erythematosus are mainly caused by inflammatory mediators caused by dust mites. Mast cells, eosinophils, T/B lymphocytes and other immune cells as well as various cytokines synthesized and secreted by immune cells, such as IL-4, IL-5 and TNF-α, all participate in or lead to the complex pattern of SLE pathogenesis [9], and its pathogenesis has not been fully clarified. In the body's immune system, the toll-like receptor family plays an important role in the body's natural immunity and the recognition of pathogens. After the recognition of ligands by TLR family members, they can activate some signaling pathways and cause inflammation and immune response against microbial infection. Therefore, the study of their activation pathway has become a focus. A large number of studies have shown that pathogens stimulating TLR [10] may lead to the expression of some genes involved in innate immune response by activating multiple signaling pathways, among which the activation pathway of Nuclear factor-kappaB (NF-κB) has attracted more and more attention. Tlr-nuclear factor NF-κB may mediate TH1 (T Helper cell-1, TH1) cytokine expression and thus mediate cellular immunity.

Many studies have found that miR-146a and miR-155 are involved in the occurrence of systemic lupus erythematosus [11]. Studies have found that the abnormal expression of miR-146a and miR-155 is closely related to the severity of lupus erythematosus. miR-146a and miR-155 may regulate the differentiation direction of T helper cells induced by allergens. miR-146a and miR-155 may be important regulatory molecules in the negative feedback regulation of TLR-4-mediated NF-κB activation, contributing to the maintenance of immune balance. Our study also found that miR-146a and miR-155 were negatively correlated with NF-κB downstream of TLR-4 in the peripheral blood of children with systemic lupus erythematosus, suggesting that miR-146a and miR-155 may be involved in the regulation of NF-κB. However, the mechanism of regulating miR-146a and miR-155 is still unclear.

CircRNAs are a class of non-coding Rnas with a closed loop structure, whose closed-loop structure can escape the degradation of RNA enzymes, so they are more stable than linear RNA and may have better biomarker function. This study found that hsa_circ_0006770 expression in peripheral blood of systemic lupus erythematosus increased significantly, which may be a potential biomarker for SLE. At the same time, circRNAs mainly act through sponge miRNA.

We predicted that hsa_circ_0006770 has multiple miR-146a and miR-155 binding sites through the database, and it was reported that hsa_circ_0006770 can sponge miR-146a and miR-155. Through database analysis, we predicted that hsa_circ_0006770 (Table 1) expression level of hsa_circ_0006770 in case group and disease control group was higher than that in control group. Meanwhile, mRNA relative expression level of hsa_circ_0006770 was significantly higher than that of IL-1(*P*=0.02, R=0.85) was positively correlated with miR-146a and miR-155 (*P*=0.01, R=-0.64), suggesting that hsa_circ_0006770 may promote TLR4 expression through sponge miRNA involved in the formation of systemic lupus erythematosus in children. The specific process and mechanism about the hsa_circ_0006770 regulating inflammation remains to be further explored.

Author Statements

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Availability of Data and Material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Patient Consent for Publication

The research proposal for human menstrual blood collection was approved by the Ethics Committee of The Red Cross hospital in guangzhou (approval no.20220514). All volunteers participating in the experiment signed the informed consent.

Author's Contributions

QZ performed multiple experiments, data acquisition and data analysis. HWL collected experimental data and participated in revising the manuscript. SWY contributed to the ex-

perimental design and participated in completing the relevant experiments.

Ethics Approval and Consent to Participate

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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