Expression Level of TLR9, but not Hypomethylation, Is Correlated With SLE Disease Activity

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Summary

This study aims to investigate the associations of hypomethylation and over expression of the *TLR9* gene with systemic lupus erythematosus (SLE). Fifteen SLE patients who were diagnosed and not treated, were selected as cases, and 32 healthy subjects were enrolled as controls. DNA and total RNA of peripheral blood mononuclear cells (PBMCs) were extracted. The methylation status of the promoter region CpG motifs of the *TLR9* gene was quantitatively measured using bisulfite sequencing PCR, and the mRNA expression of the *TLR9* gene was determined using real-time fluorescent quantitative PCR. The methylation level of the 10 *TLR9* CpG motifs of gene did not show difference between cases and controls (P>0.05). By contrast, we observed an abnormal increase of *TLT9* mRNA expression in patients (P=9.379×10^{-8}), which was significantly correlated with SLEDAI (Systemic Lupus Erythematosus Disease Activity Index) (P=9.018×10^{-7}). The change of *TLR9* gene expression may play an important role in the pathogenesis of SLE.

Key words

Systemic lupus erythematosus • TLR9 gene • Hypomethylation • Expression level • Disease activity of SLE

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Introduction

In the past 10 years, approximately 30 susceptibility genes were suggested playing a crucial role in the pathogenesis of systemic lupus erythematosus (SLE). Most of these genes were involved in three biological processes, the removal of immune complexes, Toll-like receptor (TLR) function and production of interferon-1 (type-I interferon, IFN-1), and immune signal transduction of lymphocytes. The DNA methylation status induced by environmental and other factors, in particular, abnormal low DNA methylation in immune disorder, may cause autoimmune response. These changes may contribute to the occurrence and development of SLE (Ray *et al.* 2009).

Toll-like receptor 9 (TLR9) is a member of the TLR family. Through the identification of conserved pathogen-associated molecular patterns (PAMPs), TLR9 is involved in specific immune responses, bridging the gap between natural immunity and specific immunity (Latz *et al.* 2004). TLR9 is specifically identifying CpG motifs, which has a core nucleotide sequence containing unmethylated cytosine-guanine dinucleotide, the combination activates the myeloid differentiation protein 88 (MyD88)-dependent signaling pathway, finally activates nuclear factor NF-κB. It leads to the expression of a series of pro-inflammatory cytokine genes and the activation of immune cells (Latz *et al.* 2004, Akira *et al.* 2004), and subsequently, the abnormal survival, differentiation and proliferation of B cells and the production of antibodies (Bernasconi *et al.* 2003). The process results in the abnormal activation of plasmacytoid dendritic cells (pDC) and release of a large amount of IFN-1. Hence, TLR9 plays an important role in autoimmune and inflammatory processes (Shirota *et al.* 2001). A recent study also revealed that the mRNA

expression level of the TLR9 gene was closely related to SLE conditions (Komatsuda *et al.* 2008). To further dissect the role of *TLR9* in SLE pathogenesis, in the present study, we investigated the gene methylation status of *TLR9* in SLE patients, and its correlation between TLR9 expression level and SLE disease activity.

Methods

Subjects

The experimental case group comprised of 15 SLE patients (Han Chinese) from the Department of Dermatology of the Second Affiliated Hospital of Kunming Medical University and Department of Rheumatology, the First People's Hospital of Yunnan Province. These patients were primarily diagnosed and had not been medically treated, including14 female and one male patients. The patients were 15 to 50 years old, with an average age of 28.60±11.95 years. All patients were evaluated strictly in accordance with ≥4 of the 11 items of the 1997 SLE classification standard of the American College of Rheumatism (ACR). In addition, we also collected blood samples of 32 healthy Han Chinese who received physical examination at the Second Affiliated Hospital of Kunming Medical University. The controls comprised of three males and 29 females. The age of these samples ranged from 26 to 52 years old, with an average age of 33.20±7.97 years old.

Every SLE patient had completed the "SLE survey questionnaire" (Li *et al.* 2015), and the SLE disease activity index (SLEDAI) (Liang *et al.* 1989, Jorce *et al.* 1989) was evaluated according to the clinical data. All patients and healthy controls signed the informed consent.

According to the clinical information obtained, all 15 SLE patients have the complete data for the 11 ACR clinical subphenotypes, which are presented as Table 1. All SLE patients were divided into two groups according to the SLEDAI points: remission stage group (<10 points) and active stage group (≥10 points) (Table 1).

Methylation status of the promoter region of the TLR9 gene

Fresh peripheral blood (3 ml per sample) was obtained from SLE patients and healthy controls, and underwent anticoagulation using ethylenediamin-etetraacetic acid (EDTA) and stored in RNA Stabilization Solution (RNA *later* Tissue Collection). Separation and collection of peripheral blood mononuclear cells (PBMCs) was completed within 24 hours using the Ficoll-Hypaque method. Genomic DNA was extracted using the TIANamp Genomic DNA Kit. The bisulfite modification of genomic DNA was conducted (EZ DNA Methylation-Gold™ Kit, ZYMO Research, USA). Promoter region sequence of the *TLR9* gene was obtained from Genebank. CpG motifs were searched by software MethPrimer. A segment of the proximal promoter region that has an important impact on the transcription of the *TLR9* gene (Takeshita *et al.* 2004), a sequence containing 10 CpG motifs within the 811 bp fragment, was selected. The primer was designed by MethPrimer software (synthesized by Shanghai Generay Biotech Co. Ltd.). The amplification reaction was conducted by bisulfite sequencing PCR (BSP) (Zymo Taq PreMix™ Kit ZYMO Research, USA). The PCR product was checked by agarose gel electrophoresis and UV light. PCR product was purified by TIANgel Midi Purification Kit. The purified PCR product was connected to a T vector using the pGEM®-T Easy Vector System II (Promege, USA). Based on blue-white selection, the white colonies that contained target sequence, were picked and performed PCR amplification using universal primers M13F and M13R (Dalian TaKaRa Biotech). The PCR amplification product was sent for sequencing. Analysis of sequencing was conducted by BiQ Analyzer software.

In this study, the 10 Cp G motifs in the proximal promoter region of the TLR9 gene was studied (-736, -683, -620, -546, -488, -481, -389, -105, -78 and -35). The test of normality (Shapiro-Wilk test) on the methylation rates and overall methylation rates of the 10 Cp G motifs in the promoter region of TLR9 gene in 15 SLE patients and 32 healthy controls were conducted using SPSS 17.0 software. The data that was not normally distributed ($P<0.05$) were analyzed using nonparametric rank sum test. Comparisons between groups were conducted using Kruskal-Wallis test. Inspection level was set as $\alpha=0.05$.

Detection of TLR9 gene mRNA expression by RT-PCR

Trizol method was used to extract mRNA from the PBMC samples, and mRNA quality was checked by micro UV/VIS spectrophotometer. When score of A260/A280 was in 1.6-2 and mRNA samples were preserved to -80°C. Synthesis of cDNA was conducted *via* reverse transcription reaction using a PrimeScript® RT reagent kit with gDNA Eraser (TaKaRa Biotech). The mRNA sequence of TLR9 gene (NM_017442.3) was searched from Genebank. The primers were designed by PRIMER 3.0 software, and primers spanned introns. The uniqueness of primer was verified by NCBI primer blast. Primers of the reference gene HPRT (NM_000194.2) was

Table 1. Information of clinical sub-types and group dividing for SLE patients

Case number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Total	ratio
Clinical sub-types																	
malar rash	+	+	-	+	+	+	-	-	+	+	+	-	+	+	+	11	73.3
discoid rash	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	2	13.3
photo sensitivity	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	3	20.0
oral ulcers	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	2	13.3
Arthritis	+	-	+	+	-	+	+	+	-	+	+	+	-	+	+	11	73.3
Serositis	-	+	-	-	-	+	-	-	+	+	-	-	-	-	+	5	33.3
renal disorder	+	+	-	-	+	+	+	-	+	+	-	-	+	-	+	9	60.0
neurologic disorder	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	1	6.6
hematologic disorder	+	+	+	+	-	+	+	-	+	+	-	+	+	+	+	12	80.0
immunologic disorder	+	+	+	-	+	+	+	-	+	+	-	-	+	-	+	11	73.3
positive anti-nuclear	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	14	93.3
Antibody Vasculitis	-	-	-	-	-	+	-	-	+	+	-	-	+	+	+	6	40.0
SLEDAI score	13	13	9	7	8	31	11	8	15	21	6	5	17	15	22		
active stage / remission stage (A/R)	A	A	R	R	R	A	A	R	A	A	R	R	A	A	A	A=9 R=6	

designed. The mRNA expression of the TLR9 gene was detected by RT-PCR using SYBR® Premix Ex Taq™ II reagent (TaKaRa Biotech) on a DNA Engine Opticon TM2PCR system.

With the expression of HPRT gene as reference, the relative expression level of TLR9 was estimated: Gene expression level $\Delta Ct = Ct_{target\ gene}$ value - $Ct_{reference\ gene}$, $\Delta\Delta Ct$ = expression level of detected gene - expression level of reference gene, and the relative expression was expressed as $2^{-\Delta\Delta Ct}$. The reference sample was H42.

On the DNA Engine Opticon TM2 PCR machine, TLR9 gene mRNA expression in PBMC samples was analyzed using relative fluorescence quantitative analysis. The PCR machine automatically recorded data and drew the amplification curve and dissolution curve during the experiment. After the end of the PCR test, the efficiency and Ct value of the PCR test were automatically analyzed using the MJ Opticon Monitor software.

Statistical Analysis

Data were analyzed using statistical software SPSS 17.0. Figures were drawn using the Microsoft Excel 2010. Data with a normal distribution were analyzed using *t*-test. Measurement data with non-normal distribution were expressed as median ± interquartile range. Comparisons between two groups were conducted using the Wilcoxon rank-sum test in a random design. $P \leq 0.05$ was considered statistically significant.

Shapiro-Wilk test was conducted on the methylation rates of the 10 CpG motifs in 15 cases and 32 controls by SPSS 17.0 software. If results revealed that only part of the data was normally distributed, or all data was not normally distributed, these data were compared using nonparametric rank sum test by Kruskal-Wallis test.

The relative mRNA expression levels of TLR9 in the active stage group, the remission stage group, and control group were compared using Shapiro-Wilk test by SPSS17.0 software. Results showed that the experimental data were normally distributed ($P>0.05$). The relative mRNA expression levels of TLR9 as mean ± standard deviation were compared using independent samples *t*-test among three groups. The relative m RNA expression levels of TLR9 in cases and controls were compared using Shapiro-Wilk test by SPSS 17.0 software. And the relative m RNA expression levels of TLR9 in patients in the active stage group, the remission stage group, and control group were also compared using Shapiro-Wilk test on the SPSS17.0 software. Results revealed that the experimental data were normally distributed ($P>0.05$). Therefore, the relative mRNA expression levels of TLR9 as mean ± standard deviation were compared using independent sample *t*-test between case with control, and different stage groups.

Results

Methylation status of the promoter region of the TLR9 gene in SLE cases and controls

The 10 CpG motifs (-736, -683, -620, -546, -488, -481, -389, -105, -78 and -35) in the promoter region of the TLR9 gene was evaluated using bisulfite sequencing PCR. It turned out that only part of the data was normally distributed. Therefore, these data were compared using nonparametric rank sum test (the Kruskal-Wallis test). We did not detect the differences of methylation and the overal methylation levels between cases and controls among the 10 CpG motifs ($P>0.05$, Table 2, Fig. 1).

Table 2. Compare of methylation status of 10 Cp G motifs in promoter region of TLR9 between SLE cases and controls

Cp G site	SLE patients n	SLE patients M±Q	Health controls n	Health controls M±Q	Wilcox on W	*P* value
-736	15	0.250±0.250	32	0.250±0.250	323.500	0.391
-683	15	0.000±0.250	32	0.125±0.250	337.500	0.582
-620	15	0.000±0.125	32	0.125±0.250	302.000	0.161
-546	15	0.125±0.250	32	0.125±0.125	321.500	0.360
-488	15	0.125±0.375	32	0.125±0.125	328.000	0.451
-481	15	0.125±0.250	32	0.250±0.250	308.500	0.228
-389	15	0.750±0.250	32	0.750±0.250	359.000	0.981
-105	15	0.125±0.250	32	0.125±0.250	765.000	0.942
-78	15	0.000±0.125	32	0.000±0.000	717.500	0.140
-35	15	0.250±0.250	32	0.313±0.125	306.000	0.207

Statistical analysis of TLR9 gene expression in cases and controls

The results showed that the mRNA expression level of TLR9 was significantly higher in case group (167.685±63.895) than in control group (2.816±1.723), ($P=9.379\times10^{-8}$; Fig. 2).

When the SLE patients were divided into two groups according to the SLEDAI: the remission stage group, 6 patients had a SLEDAI of <10 points; and the active stage group, 9 patients had a SLEDAI of ≥10 points, we observed a higher TLR9 expression in the active stage (207.370±25.227) than in the remission stage

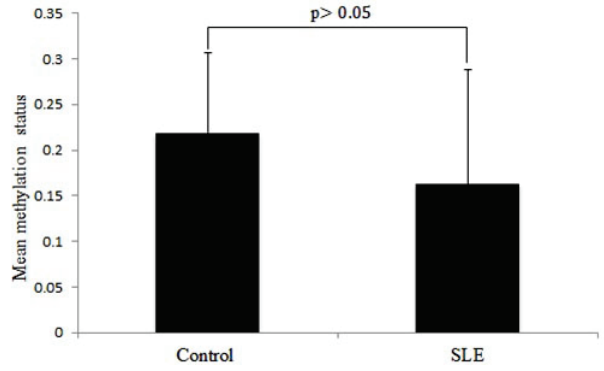

Fig. 1. Comparison of methylation rate of the 10 CpG motifs in the promoter region of the TLR9 gene in SLE patients

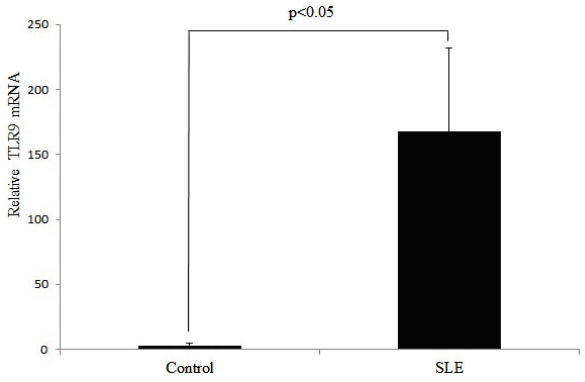

Fig. 2. Comparison of the relative mRNA expression levels of TLR9 in SLE patients and controls

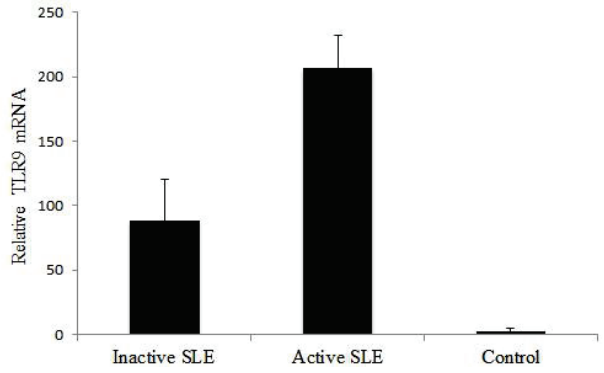

Fig. 3. Comparison of the relative mRNA expression levels of TLR9 in SLE patients of active stage group, remission stage group, and healthy control group

(88.316±32.334) (P=2.664×10^{-6}). Also, the mRNA expression level of TLR9 was significantly higher in the active stage than the control group (2.816±1.723) (P=9.674×10^{-10}). Similarly, the TLR9 mRNA expression level in the remission stage was significantly higher than the control group (P=0.04, Fig. 3).

The TLR9 mRNA expression levels in SLE patients had a significant and positive linear correlation with SLEDAI (r=0.924, P=9.018×10^{-7}; Fig. 4).

However, there was no correlation between the total methylation rate of the 10 CpG motifs in the promoter region of the TLR9 gene with the mRNA expression level of TLR9 in SLE patients (r=0.097, P=0.730; Fig. 5).

Discussion

A large number of studies had revealed that gender, drugs, ultraviolet, bacterial or viral infections, and even diet can lead to changes in DNA methylation status (Ray *et al.* 2009) and subsequently induce or aggravate the condition of SLE. The overall methylation level of the genome in PBMCs from SLE patients significantly decreased (Liu *et al.* 2011). It was confirmed that a number of genes could regulate this pathway (Harley *et al.* 2009). The excessive production of IFN-1 can accelerate the maturation of dendritic cells and promote the expression of cytokines and chemokines before inflammation, bringing about multiple effects to the function of the immune system, including the activation of autoreactive B and T cells, auto-antibody production, and loss of auto-tolerance (Kalliolias *et al.* 2010). TLR9 is one of the important genes in the IFN-1 signaling pathway, and its abnormal activation may be associated to the pathogenesis of SLE through the induction of the production of a large amount of IF-1 (Shirta *et al.* 2001).

Whole-genome DNA methylation analysis of CD4$^+$ T cells, CD19$^+$ B cells, and CD14$^+$ monocytes in patients with SLE, the severe hypomethylation status of multiple genes involved in the IFN-1 signaling pathway was detected. It revealed that the methylation levels of 1,013 Cp Gs in T cells, 166 Cp Gs in B cells and 97 Cp Gs in monocytes in SLE patients significantly changed (P<1×10^{-8}). At present, no study has reported the

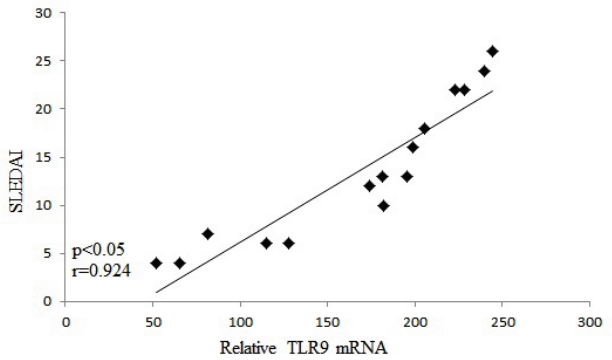

Fig. 4. The linear correlation diagram about m RNA expression levels of TLR9 in SLEDAI scores of SLE patients.

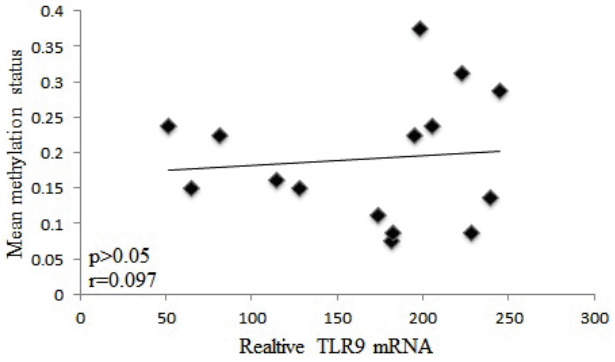

Fig. 5. The linear correlation diagram about methylation rate of the 10 CpG motifs in the promoter region of TLR9 gene and the relative mRNA expression levels of TLR9 in SLE patients

relationship between the methylation status of the TLR9 gene with disease activity of SLE. Our results suggested that the overall methylation rate of the 10 CpG motifs in the promoter region of the TLR9 gene had a decreasing tendency in untreated primary SLE patients, but the difference was not statistically significant compared with the healthy control group (P>0.05). This result revealed that no abnormal methylation status of the 10 Cp G motifs in the promoter region of TLR9 was observed. However, this experiment only detected the methylation status of CpG motifs located in the proximal segment of the promoter containing 800 bps in the TLR9 gene, which could not reflect the methylation status of the entire promoter region of TLR9. Further studies are needed to determine the existence of other mechanisms such as histone acetylation and RNA silencing that induce the high expression of the TLR9 gene in SLE patients. In addition, the sample size was small in the present experiment. Studies with large sample sizes should be carried out in the future.

TLR9 mRNA expression levels in SLE patients were positively correlated with SLEDAI (Dong *et al.* 2008, Peng *et al.* 2007). It was reported that the mRNA and protein expression levels of TLR9 in PBMCs were significantly higher in the active stage than in the remission stage and in healthy controls, and the differences between the latter two were also statistically significant (Dong *et al.* 2008, Peng *et al.* 2007). In addition, the mRNA expression of TLR9 in PBMCs and B cells was higher in SLE patients than in controls, but the expression of TLR9 mRNA in pDC cells was not significantly different from that in controls (Komatsuda *et al.* 2008, Migita *et al.* 2007). The expression of TLRs such as TLR7 and TLR9 in SLE patients was elevated, which had racial differences and may become a target of research for new therapeutic drugs (Lyn-Cook *et al.* 2014). It was reported that TLR3- and TLR9-positive cells in PBMCs, B cells ($CD19^+$) and T cells ($CD3^+$, $CD4^+$, $CD8^+$) were significantly higher in SLE patients compared with healthy controls, the TLR7-positive cells in B cells ($CD19^+$) was significantly higher in SLE patients compared with healthy controls. But, no correlation between the disease activity index (DAI) with disease damage index (DDI) was found. In some clinical symptoms such as arthritis, laboratory indicators such as the decrease in lymphocytes and granulocyte, anemia and increase in erythrocyte sedimentation rate (ESR), as well as menopause in female patients, suggested that TLR3, TLR7 and TLR9 play certain roles in organ damage in SLE (Klonowska-Szymczyk *et al.* 2014). Based on RT-PCR method to determine the mRNA levels of TLR9 and interferon regulatory factor 5 (IRF5) in PBMCs in Chinese patients with SLE, revealed that TLR9 expression in SLE patients increased (P=0.011) (Mu *et al.* 2012). It was revealed that the mRNA levels of SLEDAI, ds-DNA, ANA and TLR9 were significantly elevated in SLE patients in the active stage (Ghaly *et al.* 2013). A retrospective study revealed that TLR9 expression was significantly higher in the hormone resistance group than in the hormone sensitivity group before treatment, but there was no difference after treatment. Our results revealed that the mRNA expression level of TLR9 was significantly higher in untreated primary SLE patients compared with controls. Furthermore, for SLE patients, those in the active stage showed a significantly higher expression of TLR9 than those in the remission stage, and both SLE stages showed a higher TLR9 expression than the healthy controls. A recent study from China showed that persistent proteinuria of >0.5 g/day, C-reactive protein levels and high-TLR9 mRNA expression were independent risk factors of poor prognosis during a 2-year follow-up period. Furthermore, the expression of TLR9 mRNA remained high in patients with poor prognosis at the end of a 2-year follow-up period, but in patients with a favorable prognosis, TLR9 mRNA expression was significantly reduced compared with the levels measured at SLE onset (P<0.0001). Therefore, the expression of TLR9 mRNA in whole blood samples at SLE onset is associated with SLE disease activity and its expression may be used as an indicator of poor prognosis in patients with SLE (Yuan *et al.* 2019).

In summary, the TLR9 gene may play an important role in the pathogenesis of SLE by highly expressing in immune cells in SLE patients and activating

the IFN-1 signaling pathway, which was likely related to the disease activity and severity of SLE. TLR9 may become a research target for new therapeutic drugs.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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