

Increased MLKL mRNA level in the PBMCs is correlated with autoantibody production, renal involvement and SLE disease activity

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Abstract

Background: Necroptosis is a form of regulated necrosis that is involved in various autoimmune diseases. Mixed lineage kinase domain-like pseudokinase (MLKL) has been identified as a key executor of necroptosis, however, the significance of MLKL in peripheral blood mononuclear cells (PBMCs) of systemic lupus erythematosus (SLE) has not been investigated. In this study, we aimed to determine the mRNA level of MLKL in PBMCs and examine its relationship with clinical features and serological parameters in SLE.

Methods: Real-time transcription-polymerase chain reaction analysis (RT-PCR) was used to determine expression of MLKL mRNA in PBMCs from 59 patients with SLE, 25 patients with rheumatoid arthritis (RA) and 30 age- and sex-matched healthy controls(HC). Spearman's correlation test was performed to assess the correlation of MLKL mRNA with clinical variables. The receiver operating characteristic curve (ROC) was created to evaluate the diagnostic value.

Results: Our results showed MLKL mRNA in PBMCs was upregulated in SLE patients compared to that in RA and HC individuals. SLE patients positive for anti-nuclear antibodies had significantly higher MLKL mRNA than antibody-negative patients. In SLE patients, MLKL mRNA was found to be upregulated in patients with lupus nephritis (LN) as compared with patients without LN, and also higher in active patients than in stable patients. MLKL mRNA level was significantly and positively correlated with c-reaction protein (CRP) ($r=0.3577$, $p=0.0237$), erythrocyte sedimentation rate (ESR) ($r=0.4091$, $p=0.0043$), serum immunoglobulin G (IgG) concentration ($r=0.3546$, $p=0.0289$) and the numbers of positive antinuclear antibodies (ANAs) ($r=0.3945$, $p=0.0432$). ROC analysis showed that MLKL mRNA in PBMCs had an area under the curve of 0.9277 (95% CI 0.8779 - 0.9775, $p<0.001$) to discriminate SLE from controls.

Conclusions: These results suggest that increased MLKL mRNA level in the PBMCs of SLE patients is correlated with renal involvement and disease activity, identifying a subgroup of patients with SLE or LN who may benefit from early diagnosis and therapies targeting MLKL.

Background

SLE is a complex, heterogeneous systemic autoimmune disease that attacks various cells and tissues, resulting in chronic inflammation and persistent tissue damage[1]. A notable characteristic of SLE is the production of pathogenic autoantibodies recognizing nucleic acids or proteins binding to nucleic acids[2]. Dysregulated cell death processes and defective clearance of dying cells have been proposed to contribute to autoantigen generation and induction of autoantibodies, as well as other aberrant immune responses in SLE[3].

Necroptosis is a special form of necrosis that is triggered by multiple pathways[4]. In cells where caspase-8 is inhibited, inflammatory signaling via Tumor necrosis factor (TNF) super family receptors, interferons (IFNs), Toll- like receptor 3 (TLR3), or TLR4 can lead to the phosphorylation of receptor-

interacting serine/ threonine-protein kinase 1 (RIPK1), RIPK3 and MLKL[5-8]. The phosphorylated MLKL inserts itself into the cell membrane, disrupts its integrity and leads to cell death[9]. Various studies have revealed that necroptosis could be implicated in the pathogenesis of many inflammatory and autoimmune diseases, including SLE[6, 10, 11].

The diversity of the SLE might reflect differences in pathogenesis between different subgroups[12]. Approaches are needed to better understand the pathogenesis and to find new targets for various stages of the disease. Considering the role of necroptosis in the pathogenesis and development of SLE[13-16], we aimed to analyze MLKL mRNA of PBMCs and figure out whether it could serve as a biomarker for disease diagnosis and monitoring.

Subjects And Methods

Study cohorts

We enrolled 59 patients with SLE and 25 patients with RA admitted to the Department of Rheumatology and Immunology of The Third Affiliated Hospital, Southern Medical University, China, from July 2019 to December 2019. Thirty age- and sex-matched HC individuals with no history of SLE or other immune disorders were enrolled at the Health Management Center in the same hospital. All the subjects had no infections. The diagnosis of SLE was according to the 1997-revised American College of Rheumatology (ACR) classification criteria[17]. All participants were provided written informed consent for blood draw and MLKL mRNA testing. Serum samples were obtained from all participants during the study.

Analyzing subgroups of SLE is increasingly important to better understand the pathogenesis of disease and provide more tailored medic protocols. Then we sorted SLE patients into different groups based on serological features, renal involvement and disease activity. Firstly, SLE patients were divided into two groups: positive ANAs group (n=48) and negative ANAs group (n=11). Another variable was renal involvement, defined as fulfilling the ACR classification criteria for renal manifestation of SLE (≥ 0.5 grams of proteinuria per day or 3+ protein on urine dipstick analysis) or having evidence of LN on kidney biopsy. SLE patients were divided into two groups: LN patients (n=23) and non-LN (n=36) patients. Lastly, SLE patients were evaluated using the SLE Disease Activity Index (SLEDAI) [18] and divided into 2 groups: stable patients (SLEDAI score <5 , n=32) and active patients (SLEDAI score ≥ 5 , n=27), according to the physicians' evaluation.

Isolation of PBMCs and RNA extraction

Considering that autoreactive PBMCs, mainly lymphocytes, may participate the autoimmune inflammatory process, we chose PBMCs as a source for determining MLKL mRNA level in SLE patients. The venous blood samples (4–5 ml) was collected in an EDTA-K2 tube from all the participants before breakfast, and PBMCs separated within 2 h by Ficoll (TBD science, Tianjin, China) gradient centrifugation for 30 min at 1700r/min. PBMCs were then transferred into 1 ml TRIzol Reagent in 1.5 ml centrifuge tubes and stored at -80°C until RNA extraction.

Total RNA was extracted from PBMCs by using TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer's protocol and quantified with the NanoDrop ND-1000 (Thermo Scientific, USA). Approximately 200-800 ng of RNA obtained from 1 ml of venous. Samples were used only if the ratio of the absorbance at 260 nm to that at 280 nm (A_{260} / A_{280}) was between 1.8 and 2.1. RNA samples with concentrations $>0.2\mu\text{g}/\mu\text{l}$ were used for following reverse transcription reaction.

Real-time polymerase chain reaction (RT-PCR) validations

According to the manufacturer's recommendations, 20 μl of final reaction mixture was used containing 10 μl of SYBR Green PCR Master Mix (Takara, Dalian, China), 0.8 μl of sense primer, 0.8 μl of antisense primer, 0.4 μl ROX Reference Dye (50 \times), 6 μl of sterile deionized water and 2.0 μl of the synthesized cDNA. Primers were designed by Primer Primer 5.0 and synthesized by Sangon Biotech (Sangon, Shanghai, China). Primers, targeting MLKL and human 18S-rRNA were used. MLKL, forward: 5'-GCCACTGGAAAGATCCCGTT-3', reverse: 5'-CAACAACCTC

GGGGCAATCCT-3'; human 18S-rRNA, forward: 5'-TGGAAATCCCATCACCATC

TTCC-3', reverse: 5'-GGTTCACACCCATGACG-3'. The relative expression level of MLKL was normalized to the internal control 18s-rRNA expression and calculated by the comparative C_T ($\Delta\Delta C_T$) method.

Amplification was performed in 40 cycles (30 s at 95°C, 5 s at 95°C, 34 s at 60°C) by ABI Step One Plus Real-Time PCR system (Applied Biosystem, CA, USA). A melt curve analysis was used to confirm the specificity of amplification.

Serological assays

The serum total ANA was measured by an indirect immunofluorescence assay (Euroimmun, AG) with a titre of $>1:80$ scored as positive. The antibodies to 15 antigens including double-stranded DNA (ds-DNA), smith antigen (Sm), and nucleosome (Nuc), SSA/60, SSA/52, SSB/La, ribonucleoprotein (rRNP), centromere protein B (CENPB), ribosome P protein (Rib-p), histone (His), proliferating cell nuclear antigen (PCNA), Scl-70, Jo-1 and mitochondria (M2) were detected by chemiluminescent immunoassay (CLIA) (HOB, Suzhou, China). Serum complement 1q (C1q), complement 3 (C3), complement 4 (C4), immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA) were detected by immunoturbidimetric assay (Roche, Shanghai, China), D-Dimer concentration was determined with immunoturbidimetric assay (Sysmex, Guangzhou, Japan) according to the manufacturer's instructions.

Statistical analysis

All data were statistically analyzed using Graph Pad Prism5 (version 5.0) software. Quantitative data were expressed as the mean \pm SD. Data with a Gaussian distribution was analyzed using an unpaired t test or one-way analysis of variance (ANOVA), Spearman's rank was used to analyze the correlation of the numbers of leucocyte, lymphocyte, monocyte, with the numbers of positive ANA, CRP, ESR and D-Dimer (D-D) levels. The area under the curve (AUC) was used to assess the specificity and sensitivity of using

MLKL mRNA as a novel diagnostic tool for the detection of SLE. *P* values less than 0.05 were considered statistically significant.

Results

Characteristics and subgroups of SLE patients.

Characteristics of the 59 SLE patients, 25 RA patients, and 30 matched HC individuals are shown in Table 1. The median age of SLE patients was 33.68±13.55 years, with 96.6% females(57/59). The majority of SLE patients (48/59, 81.4%) were ANA-positive, less than half of the patients diagnosed with LN (23/59, 40.4%), 32 patients classified as stable patients (low disease activity) and 27 patients as active patients (high disease activity).

Table1. Baseline characteristics of study groups

Variable	Groups		
	HC	RA	SLE
Patients/individuals(n)	30	25	59
Age (y), median (range)	32 (19-80)	30 (21-72)	34 (11-87)
Sex(F/M)	29/1	24/1	57/2
ANA test			
ANA+ (Patients with positive ANA), n(%)			48 (81.4%)
ANA- (Patients with negative ANA), n(%)			11 (18.6%)
Diagnosis based on Renal involvement			
LN patients			23 (40.4%)
Non-LN patients			36 (59.6%)
Disease status			
Active patients (SLEDAI≥5) , n (%)			27 (45.8%)
Stable patients (SLEDAI<5) , n (%)			32 (54.2)

MLKL mRNA was upregulated in the PBMCs of SLE patients.

We detected MLKL mRNA levels in the PBMCs of SLE, RA patients as well as an HC individuals. The levels of MLKL mRNA were significantly higher in SLE patients than in RA patients and HC individuals ($p<0.0001$, respectively, Fig. 1).

We further analyzed the expression of MLKL mRNA in the subgroups of SLE patients and found that SLE patients positive for ANAs exhibited significantly higher levels of MLKL mRNA than those with negative ANAs ($p<0.05$, Fig. 2a). MLKL mRNA was also found to be obviously upregulated in LN patients when compared with patients without LN ($p<0.005$, Fig. 2b), and higher in active patients than in stable patients ($p<0.05$, Fig. 2c).

MLKL mRNA was positively correlated with clinical and pathological parameters of SLE

The relationships between the levels of MLKL mRNA in PBMCs and the clinical or pathological characteristics of SLE were assessed and detailed in Table 2. Interestingly, MLKL mRNA level was significantly and positively correlated with ESR ($r=0.4091$, $p=0.0043$), CRP ($r = 0.3571$, $p = 0.0237$), serum IgG concentration ($r = 0.3546$, $p = 0.0289$) and the numbers of positive ANAs ($r = 0.3597$, $p=0.0432$) (Fig. 3), but not associated with C3, C4 and other serologic indicators. Taken together, we found that increased MLKL mRNA was correlated with the activity in SLE patients.

Table 2. Association of MLKL mRNA with clinical pathological parameters of SLE

Clinical parameters	Data (Mean±SD)	Relative MLKL mRNA expression	
		<i>r</i>	<i>p</i>
Age (years)	33.68±13.55	-0.0132	0.346
ESR (mm/h)	36.10±28.22	0.4091	0.0043
CRP (mg/L)	8.64±24.03	0.3577	0.0237
IgG total (g/L)	13.80±4.55	0.3546	0.0289
IgM total (g/L)	1.07±0.49	-0.1166	0.4919
IgA total (g/L)	2.59±1.27	0.148	0.3821
C1q (ng/L)	177.80±46.61	-0.0421	0.8047
C3 (g/L)	4.92±24.99	-0.0439	0.7936
C4 (g/L)	0.18±0.10	-0.1836	0.2766
D-Dimer (g/L)	397.4±771.30	-0.0569	0.7488
Leucocyte count (*10 ⁹ /L)	6.62±2.68	-0.0317	0.8382
Lymphocyte count (*10 ⁹ /L)	1.81±0.72	0.1263	0.3819
Positive ANA numbers	2.78±2.56	0.3945	0.0432
anti-dsDNA antibody (IU/mL)	59.73±113.3	0.1265	0.5214
anti-Nuc antibody (RU/mL)	84.39±139.60	0.0328	0.8656
anti-sm antibody (RU/mL)	41.75±98.59	0.2085	0.3282

MLKL mRNA in the PBMCs was sensitive for the diagnosis of SLE

ROC analysis was employed to analyze the diagnostic efficiency of the MLKL mRNA for SLE patients. The diagnostic ability of MLKL mRNA achieved very high diagnostic accuracy 0.9277 (95% CI: 0.878-0.978) with high sensitivity (81.36%) and specificity (93.3%), implying that MLKL mRNA of PBMCs may be a potential diagnosis biomarker for SLE (Fig. 4).

Discussion

In this study, we showed that MLKL mRNA level in the PBMCs of SLE patients were significantly upregulated, especially in patients with positive serum ANAs. MLKL mRNA level in the PBMCs was also

significantly and positively correlated with ESR ($r=0.4091$, $p=0.0043$), CRP ($r = 0.3571$, $p = 0.0237$), serum IgG concentration ($r = 0.3546$, $p = 0.0289$) and the numbers of positive ANAs ($r = 0.3597$, $p=0.0432$). So far as we know, this is the first report that MLKL mRNA level in the PBMCs is increased in SLE patients.

Once phosphorylated, MLKL translocates from cytosol to the plasma membrane to execute necroptosis. Defective clearance of necroptotic cells have been proposed to initiate inflammatory responses by the release of danger-associated molecular patterns (DAMPs). DNA acts as a major DAMP and is sensed in endolysosomes by Toll- like receptor 9 (TLR9) and in the cytoplasm by cyclic GMP–AMP (cGAMP) synthase (cGAS), inducing the production of type I and type III IFNs and eliciting strong inflammatory responses[19, 20]. Several studies have demonstrated that patients with SLE have elevated circulating IFNs[21-23], whose signaling contributes to the steady-state expression of MLKL and the initiation of necroptosis, which not only causes tissue damage[6], but may also form a dynamic feedback loop in SLE pathogenesis.

Although SLE is a chronic inflammatory disease that can affect many organs, the kidneys are the mostly attacked[24]. LN is one of the most frequent and serious complications in SLE, and a real challenge for SLE treatment[25]. We surprisingly found that MLKL mRNA was obviously upregulated in the PBMCs of LN patients when compared with patients without LN ($p<0.005$). To date, only one paper reported the correlation of necroptosis with LN, showing that PIPK3 and MLKL were activated in podocytes in renal biopsies from patients with LN[11]. Whether there is a crosstalk between the renal parenchymal cells and peripheral blood cells in necroptosis process still needs to be analyzed.

The current understanding of SLE implies autoimmunity to nuclear and cytoplasmic antigens, leading to generation large amount of ANAs[26]. Our findings that SLE patients positive for ANAs exhibited higher MLKL mRNA levels than serum negative patients, and very significantly positive correlations between MLKL mRNA in the PBMCs and the numbers of positive ANAs or serum IgG concentrations, suggesting that necroptosis may play a potential role in the production of ANAs.

Conventional serologic ANAs are of limited sensitivity and/or specificity for diagnosis and monitoring in SLE [27]. Here we reported that MLKL mRNA in the PBMCs could differentiate between SLE patients and HC individuals, the AUC was as high as 0.9277 (95% CI 0.878-0.978) with high sensitivity (81.36%) and specificity (93.3%). As PBMCs are easy to obtain, suggesting that MLKL mRNA of PBMCs may be a novel biomarker for the diagnosis and monitoring of disease activity of SLE.

There were also several limitations. Firstly, as the patients in this study are from one hospital, whether there is a difference between patients from different areas is not known. Therefore, a multi-center cohort might be necessary for future implementation of techniques. Secondly, the molecular mechanism that how MLKL is involved in the progression of SLE remains unclear. Lastly, which specific cell of PBMCs expressed high MLKL mRNA level needs to be future explored.

Conclusions

This is the first study to point out the upregulation of the MLKL mRNA in the PBMCs of SLE patients. The data presented here may provide certain evidence for the role of necroptosis in the pathogenesis and development of SLE, and also suggest new therapies by blocking signaling of necroptosis pathway in human SLE, especially in LN patients. Importantly, the MLKL mRNA expression levels in PBMCs may be useful in identifying those subgroups of SLE patients that may benefit from necroptotic blocking therapies. Finally, we believe that these findings could be of relevance for understanding the pathogenesis and diversity of SLE.

Abbreviations

MLKL: Mixed lineage kinase domain-like pseudokinase; PBMCs: Peripheral blood mononuclear cells; SLE: Systemic lupus erythematosus; RT-PCR: Real-time transcription-polymerase chain reaction analysis; RA: Rheumatoid arthritis; HC: Healthy controls; ROC: Receiver operating characteristic curve; LN: Lupus nephritis; CRP: C-reaction protein; ESR: Erythrocyte sedimentation rate; ANAs: Antinuclear antibodies; IgG: Immunoglobulin G; IgM: Immunoglobulin M; IgA: Immunoglobulin A; CLIA: Chemiluminescent immunoassay; TNF: Tumor necrosis factor, TLR: Toll-like receptor; IFNs: Interferons; RIPK: Receptor-interacting serine/threonine-protein kinase; ACR: American College of Rheumatology; SLEDAI: SLE Disease Activity Index; DAMP: Damage-associated molecular pattern; cGAS: Cytoplasm by cyclic GMP-AMP (cGAMP) synthase.

Declarations

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Availability of data and materials

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

Authors' contributions

Mingjiao Zhang and Hongyu Jie carried out most of the experiments, participated in the analysis of data, and drafted the manuscript. Yong Wu participated in the design of the study, data analysis and interpretation, and drafted the manuscript. Xinai Han performed the statistical analysis. Xing Li et al participated in the RT-PCR assays and revising the manuscript. E.W. Sun participated in the design and

coordination of the study, and finalized the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study has been approved by the Ethics Committee of The Third Affiliated Hospital, Southern Medical University, and all participants signed informed consent forms.

Consent for publication

All authors consented to the publication of this manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figures

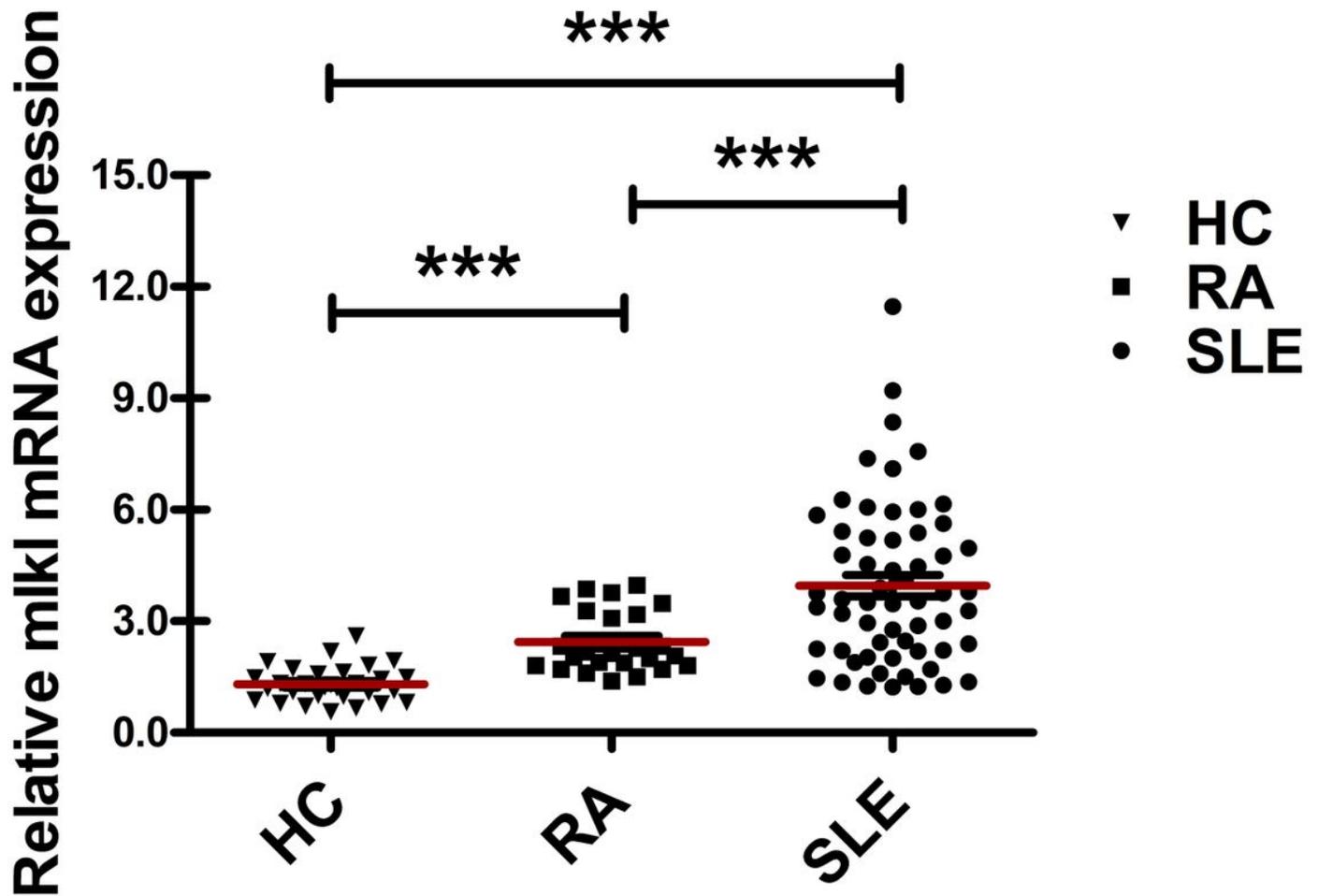


Figure 1

The increased levels of MLKL mRNA in PBMCs of SLE patients. RT-PCR was used to determine relative expression level of MLKL mRNA in the PBMCs of SLE (n=59), RA (n=25) and HC individuals (n=30), *** (p<0.0001).

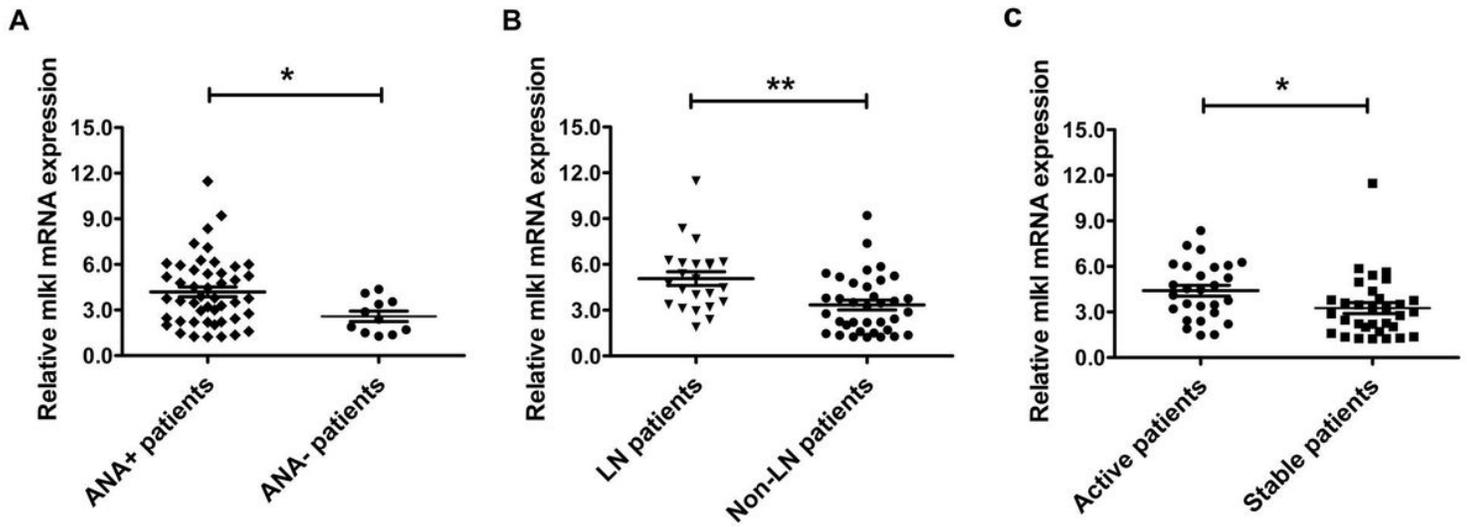


Figure 2

The differential expression of MLKL mRNAs in three subgroups of SLE. A. SLE patients with positive ANA (ANA+, n=48) vs negative ANA (ANA-, n=11); B. LN patients (n=23) vs non-LN patients(n=36); C. Active patients (SLEDAI score ≥ 5 , n=27) vs stable patients (SLEDAI score < 5, n=32), * (p<0.05); ** (p<0.005).

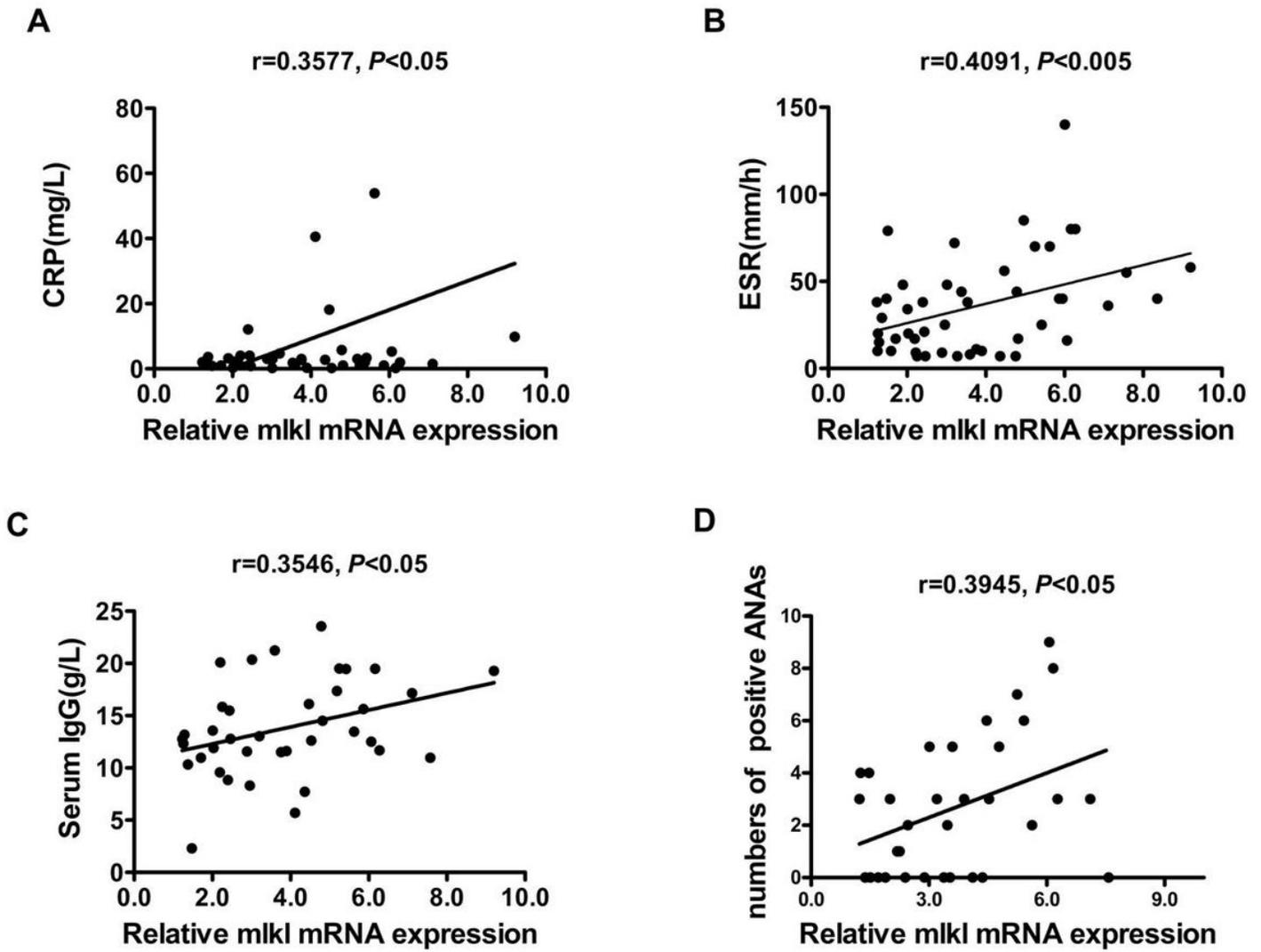


Figure 3

Positive correlations of MLKL mRNA levels with clinical parameters of SLE patients.

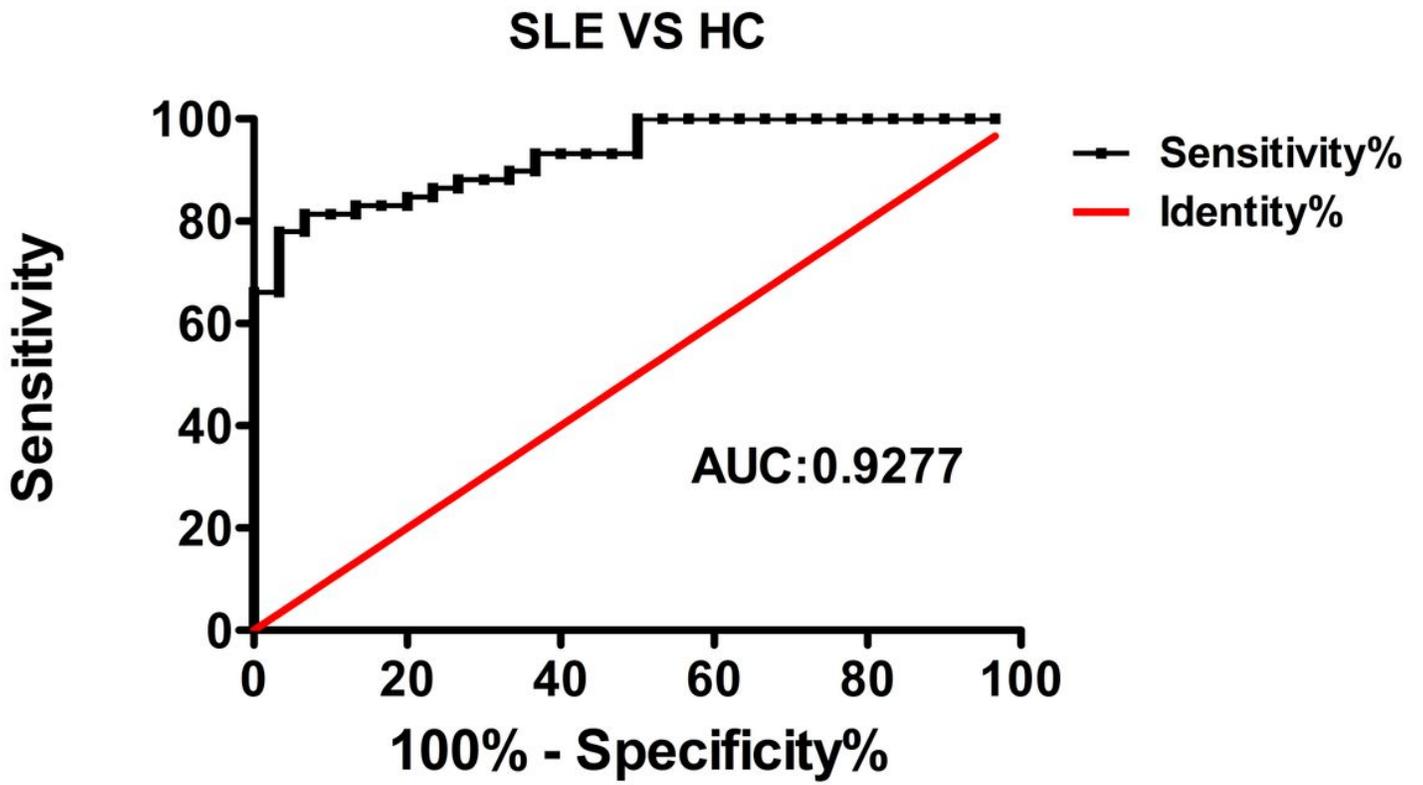


Figure 4

ROC analysis of MLKL mRNA for SLE patients.