IFI44L Gene Promoter is Differentially Methylated in Iranian Patients with Systemic Lupus Erythematosus and Rheumatoid Arthritis.

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Research Article

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Abstract

Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are multisystemic autoimmune diseases with multifactorial nature. Considering limitations of the current conventional serological tests for diagnosis of these diseases, researchers strive to find new and more valid biomarkers. The methylation level of interferon-induced protein 44-like (*IFI44L*) promoter was evaluated in 69 patients with SLE, 61 patients with RA, and 71 healthy subjects. Quantitative methylation of the promoter region of *IFI44L* gene was measured in DNA extracted from peripheral blood mononuclear cells (PBMCs) with methylation-quantification endonuclease-resistant DNA (MethyQESD) method. Our findings revealed a substantial hypomethylation of *IFI44L* promoter in SLE and RA patients compared with healthy volunteers (mean: 60.36%±64.54%, 47.59%±30.34%, and 89.17%±76.96%, respectively; P_{SLE}= 0.018, P_{RA}<0.001). In comparison between SLE and RA patients with control group, *IFI44L* promoter methylation had a sensitivity of 84/06% and 93/65% and specificity of 32/39% and 29/58, respectively. The promoter methylation level was not meaningfully different between SLE and RA patients (P> 0.05). Moreover, our analysis revealed that the methylation level of *IFI44L* promoter was not statistically significantly different between SLE disease activity and renal involvements (P> 0.05). While RA patients with a higher concentration of CRP had a lower DNA methylation level (P= 0.012). The methylation level of *IFI44L* promoter was lower in PBMCs of Iranian patients with SLE and RA than that control group. Furthermore, DNA methylation level of *IFI44L* promoter had a negative correlation with RA disease activity. However, there was not a significant association between clinical characteristics of SLE.

Introduction:

Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are chronic, progressive, and multisystemic autoimmune diseases with multifactorial nature and polygenic etiology [1–3]. In SLE the joints are affected and internal organs and skin are more likely to be involved than that RA [4, 5]. SLE can also lead to life-threatening complications because of some symptoms such as renal involvement, neurological symptoms (e.g., seizures), and hematological manifestations (e.g., clotting problems) [6]. Concerning RA, the first attack occurs in the joints which leads to painful swelling in and around the joints particularly the small joints that consequently results in stiffness, cartilage erosion, and joint deformity [5]. However, in some cases, RA could also be associated with inflammation in the lungs and the occurrence of cardiovascular problems [5, 7]. The female/male ratio of SLE and RA ranges from 9:1 to 3:1, respectively [8]. Based on twin studies, the heritability of SLE is approximately 66% and around 50–60% for RA [9, 10]. The diagnosis and disease activity of SLE and RA is mostly based on clinical symptoms and conventional serological markers such as antinuclear antibodies (ANAs), anti-Smith (anti-Sm) antibodies, anti-double-stranded DNA (dsDNA) antibodies, rheumatoid factor (RF), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and complement proteins (C3 and C4) [11–14]. Although based on previous reports and available guidelines, a combination of these serological parameters is proposed for the diagnosis of SLE and RA; meanwhile, some other studies, based on low specificity and sensitivity, demonstrated that these factors have substantial limitations in the diagnosis of diseases [12,

15–18]. Hence, considering the very wide spectrum of discovered serological proteins, researchers strive to find new and more valid biomarkers for a better diagnosis and management of these diseases.

Numerous studies have revealed that the disrupted gene expression profiles in immune cells are involved in the pathogenesis and represent pro-inflammatory phenotype in RA and SLE diseases. Specifically, dysregulated DNA methylation patterns were well-known as a central contributor to autoinflammatory diseases including RA and SLE [19, 20]. In this way, several studies with work on alteration of DNA methylation in these autoimmune diseases hope to reach a biomarker set for diagnosis and monitoring.

One of these genes is Interferon-induced protein 44-like (*IFI44L*). Although the exact function of this gene is unknown but studies demonstrated that expression of this gene is upregulated in SLE and RA patients which this upregulation was attributed to hypomethylation of *IFI44L* [21–25]. For the first time, in two different studies, Chen and Zhao et al proposed that DNA methylation alteration of *IFI44L* is a promising biomarker for the diagnosis of SLE and RA with high specificity and sensitivity [24, 26]. Therefore, we intended to investigate the utility of *IFI44L* methylation level in peripheral blood mononuclear cells (PBMCs) as a biomarker for the diagnosis of these diseases. Furthermore, in this study, we compared the difference between methylation levels of this gene in RA and SLE patients to evaluate the ability of this factor in distinguishing between two different diseases in the Iranian population.

Materials And Method:

Study Populations:

In this case-control study, we enrolled 63 RA patients and 69 SLE patients according to diagnostic criteria created by the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR) (2019). Besides, 71 age and gender-matched apparently healthy voluntaries were included as a control group. SLE and RA patients were recruited from rheumatology clinics and inpatient wards at Al-Zahra hospital, the biggest affiliated hospital of Isfahan University of Medical Sciences. All healthy subjects in the control group had no symptoms or personal and family history of RA and SLE, or other autoimmune and immune-mediated conditions. This study was approved by the Isfahan University of Medical Sciences research ethics committee and all the voluntaries provided written informed consent. Demographic and clinical presentation data of all subjects were collected. These data were sex, age, blood pressure (systolic blood pressure (SBP) and diastolic blood pressure (DBP)), height and weight to calculate body mass index (BMI, calculated as weight [kg] divided by height [m] squared), family history of RA, SLE and other autoimmune disorders and clinical manifestations such as the presence of cutaneous manifestations, neurological disorders, hematological abnormalities, oral ulcer, arthritis, and renal involvement. Likewise, laboratory characteristics such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), anti-dsDNA antibodies, complement component 3 (C3), complement component 4 (C4), white blood cell (WBC) count, hemoglobin, creatinine, platelet count test (PLT), blood urea nitrogen (BUN), fasting blood sugar (FBS), triglyceride (TG), high-density lipoprotein (HDL), and low-

density lipoprotein (LDL) were documented. Ultimately, about 5 ml of venous blood was collected into EDTA anticoagulant tubes from each individual and stored at − 20°C for further processing.

Isolation of peripheral blood mononuclear cells (PBMCs):

PBMCs were isolated via Ficol gradient density (Ficoll-Hypaque, Sigma) based on the standard protocol [27]. Genomic DNA was isolated from PBMCs by PrimePrep Genomic DNA Isolation Kit (GeNetBio, Korea). The yield, purity, and suitability of DNA for the MethyQESD (methylation-quantification of endonuclease-resistant DNA) method was evaluated by spectrophotometry and agarose gel electrophoresis.

DNA methylation assessment:

Methylation of the *IFI44L* gene in both healthy and case groups was assessed by the MethyQESD method. This technique is a combination of methylation-sensitive digestion and real-time polymerase chain reaction (RT-PCR). The amount of methylated DNA that resists digestion by the methylation-sensitive endonuclease Hin6I is determined by RT-PCR and is calibrated using a reference DNA that remains uncut. In detail, this method is a combination of two processes: the first is restriction digestion of template DNA by two sets of methyl sensitive (Hin6I) enzymes, which is called “Methylation Quantification Digestion (MQD)”. Hin6I does not affect methylated GCGC but was used to cut unmethylated GCGC regions. The methyl insensitive enzymes (XbaI & DraI), referred to as “Methylation-Independent Calibrator Digestion (CalD)”, digest separately before RT-PCR amplification and their recognition sites are not present within the amplicon and were used to digest total DNA. The second process is the quantitation of the Hin6I digested template using real-time PCR in which XbaI and DraI restricted templates hold as internal normalizing controls. CpG methyltransferase (M.SssI) enzyme (Thermofisher, USA) was used for the preparation of methylated positive control, according to the manufacturer’s instruction, and a PCR amplified fragment included as an unmethylated negative control in each real-time PCR reaction. Negative and positive controls were run in parallel with our sample. The percentage of promoter methylation was calculated by differences of the Ct values between MQD and CalD according to the formula:

Methylation % = EΔCt × 100. Where ΔCt = Ct Calibrator − Ct methylation quantification. E = PCR efficiency = 2

Statistical analysis:

The MedCalc statistical software version 10.2.0 (MedCalc Software bv, Ostend, Belgium) was used for statistical analyses to assess the percentage of methylation of the *IFI44L* gene in the cases and control groups. Because of the abnormal distribution of *IFI44L* in both groups, Mann–Whitney U-test was used to compare this variable between cases and controls. A receiver operating characteristic (ROC) curve analysis was used to evaluate the areas under the ROC curve (AUC). This established the best cutoff values for % DNA methylation for diagnosing SLE and RA and then sensitivity and specificity were calculated. For demographic, clinical, and laboratory characteristics, P values were calculated by Student’s t-test, Chi-square, or Mann–Whitney test. The significance level was set at $P < 0.05$.

Results:

Demographic and laboratory characteristics:

In this case-control study, we investigated 69 SLE patients (15 males and 54 females with mean age of 43.72 ± 13.41) and 63 subjects as RA patients (23 males and 40 females with mean age of 42.54 ± 11.80). The healthy control group consisted of 71 subjects (18 males and 53 females with mean age of 45.08 ± 12.32). The mean age of onset in the SLE and RA patient groups was 24.81 ± 10.80 and 40.31 ± 11.10, respectively. Table 1 shows the characteristics of the patients and healthy controls. Clearly, SLE and RA patients had higher BMI compared with the control group ($P > 0.001$). Likewise, there was a significant difference between the SLE patients and the control group in terms of systolic blood pressure (SBP) and diastolic blood pressure (DBP) ($P < 0.05$). However, there was not any difference between the RA patients and the control group in terms of SBP and DBP ($P > 0.05$).

Table 1
Baseline characteristics of patients (SLE and RA) and control subjects who participated in the study

Characteristics	Controls	SLE	$P_{\text{Controls vs SLE}}$	RA	$P_{\text{Controls vs RA}}$
Total number	71	69		63	
Age (mean ± SD)	45.08 ± 12.32	43.72 ± 13.41	0.416	42.54 ± 11.80	0.225
Gender n (%)					
Male	18(25.4)	15(21.7)	0.692	23(36.5)	0.191
Female	53(74.6)	54(78.3)		40(63.5)	
Age of onset (mean ± SD)	–	24.81 ± 10.80	–	40.31 ± 11.10	–
BMI (mean ± SD)	23.77 ± 3.51	25.80 ± 2.40	< 0.001*	26.22 ± 2.41	< 0.001*
SBP (mean ± SD)	120.56 ± 9.39	125.80 ± 16.31	0.023*	121.90 ± 13.20	0.503
DBP (mean ± SD)	79.56 ± 8.34	82.70 ± 6.01	0.014*	79.52 ± 7.40	0.928
Positive family history n (%)	0	12(17.4)	–	10(15.9)	–
Neurological symptoms n (%)	0	19(27.5)	–	0	–
Skin manifestations n (%)	0	47(68.1)	–	0	–
Hematological manifestations n (%)	0	34(49.3)	–	0	–
Oral ulcers n (%)	0	52(75.4)	–	0	–
Arthritis n (%)	0	49(71.0)	–	63(100)	–
Renal involvement n (%)	0	28(40.6)	–	0	–

* *P* value < 0.05. SLE: Systemic lupus erythematosus; RA: Rheumatoid arthritis; BMI: Body mass index; SD: Standard deviation; SBP: Systolic blood pressure; DBP: Diastolic blood pressure.

In the SLE group, twelve patients (17.4%) had a family history of SLE or other autoimmune disorders, most of the patients presented oral ulcers (52 patients, 75.4%), skin manifestations (47 patients, 68.1%), and arthritis (49 patients, 71.0%). Furthermore, 34 (49.3%), 19 (27.5%), and 28 (40.6%) of patients with hematological, neurological, and renal symptoms were documented, respectively. Based on laboratory tests, ESR, CRP, and creatinine were significantly higher in case groups than healthy controls ($P < 0.05$). While serum concentration of hemoglobin was obviously higher in the control group than in cases ($P >$

0.001). Furthermore, in SLE patients, the concentration of anti-dsDNA antibodies was significantly higher than in controls, while C3 and C4 levels were expressively higher in controls than in patients ($P > 0.001$). On the other hand, white blood cell count, FBS, HDL, LDL, and TG were not markedly different between two groups of patients and healthy individuals ($P > 0.05$). The laboratory characteristics of patients (SLE and RA) and healthy controls are presented in Table 2.

Table 2
Laboratory characteristics of patients and controls groups

	Controls (71)	SLE (69)	$P_{\text{Controls vs SLE}}$	RA (63)	$P_{\text{Controls vs RA}}$
ESR (mm/h)	15.44 ± 6.94	38.80 ± 18.64	< 0.001*	39.14 ± 25.80	< 0.001*
CRP (mg/l)	4.00 ± 2.19	18.54 ± 9.94	< 0.001*	17.15 ± 13.14	< 0.001*
White blood cell (10^9/1)	6576.62 ± 1423.22	6915.94 ± 1633.01	0.192	7073.02 ± 2103.03	0.117
Hemoglobin	14.20 ± 1.45	11.80 ± 1.51	< 0.001*	12.51 ± 1.15	< 0.001*
PLT(10^9/1)	250.21 ± 69.47	216.36 ± 59.95	0.002*	255.22 ± 70.80	0.680
Creatinine (mg/dL)	0.90 ± 0.19	1.01 ± 0.25	0.024*	1.06 ± 0.20	< 0.001*
BUN	16.16 ± 4.63	19.16 ± 10.08	< 0.001*	17.50 ± 4.70	0.112
FBS	92.32 ± 21.35	87.1 ± 9.42	0.063	92.24 ± 15.36	0.979
HDL	49.59 ± 11.70	50.64 ± 8.24	0.662	49.10 ± 15.36	0.568
LDL	108.51 ± 37.03	103.26 ± 26.43	0.338	111.81 ± 29.15	0.571
TG	153.97 ± 62.48	159.46 ± 47.64	0.560	162.79 ± 45.10	0.356
Anti-dsDNA (IU/ml)	10.60 ± 4.60	207.1 ± 187.81	< 0.001*	–	–
C3 level (mg/dl)	145.93 ± 35.74	49.23 ± 37.51	< 0.001*	–	–
C4 level (mg/dl)	19.83 ± 5.53	10.93 ± 7.92	< 0.001*	–	–

* P value < 0.05. Data are mean ± SD, or n (%). SD: Standard deviation; SLE: Systemic lupus erythematosus; RA: Rheumatoid arthritis; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; BUN: Blood urea nitrogen; PLT: Platelet; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; TG: Triglyceride; FBS: Fasting blood sugar; C3: Complement component 3; C4: Complement component 4; dsDNA: Double-stranded DNA.

IFI44L methylation analysis:

Based on our findings, the mean *IFI44L* promoter methylation level in the SLE group was 60.36%±64.54% and in RA patients was 47.59%±30.34%, while in the control group was 89.17%±76.96%. Differences

between the average percentages of methylation between RA and control groups were obviously significant ($P > 0.001$). Similarly, the difference between SLE and control groups was noticeably significant ($P = 0.018$). Although the average percentage of methylation in SLE groups was higher compared with RA subjects, this difference was not statistically significant (P = 0.144). Figure 1 demonstrates the comparison of *IFI44L* promoter methylation level between cases (RA and SLE patients) and the healthy control group. Additionally, the ROC analyses showed that the diagnostic power of the *IFI44L* promoter methylation level for RA was 0.725 (95%CI [0/641–0/798]) and for SLE was 0.687 (95%CI [0/603 to 0/763]). The appropriate cutoff point for *IFI44L* promoter methylation level to distinguish between RA cases and controls was ≤ 93/487 with a sensitivity of 93/65% (95%CI [84/5–98/2]) and specificity of 29/58 (95%CI [19/3–41/6]). Similarly, the proper optional cutoff point for *IFI44L* promoter methylation level to distinguish between SLE cases and controls was ≤ 88/433, therefore the sensitivity and specificity was 84/06% (95%CI [73/3–91/8]) and 32/39% (95%CI [21/8–44/5]), respectively (Fig. 2).

Our analysis about the association between *IFI44L* promoter methylation level and clinical characteristics revealed that there was no statistically significant difference between laboratory parameters associated with SLE disease activity ($P > 0.05$). For instance, the DNA methylation level of *IFI44L* promoter in SLE subjects with renal involvement (54.25%±36.41) was lower compared to patients without renal involvement (69.31% ±91.58), however, it was not statistically significant (Table 3). Furthermore, the results of this study demonstrated that RA patients in whose serum concentration of CRP was ≥ 6 (mg/l) had a lower methylation level in the *IFI44L* promoter (38.42%±28.29) than those patients with CRP < 6 (mg/l) (39.42%±28.29) ($P = 0.012$).

Table 3: Association of IFI44L promoter methylation level with

various parameters in SLE and RA patients

Characteristics	Mean of IFI44L promoter methylation	P value
SLE		
CRP (mg/l)		
Negative (< 6)	47.69 ± 25.90	0.652
Positive (≥ 6)	61.34 ± 66.63	
C3 level (mg/dl)		
< 90	63.12 ± 69.67	0.443
≥ 90	47.25 ± 28.52	
C4 level (mg/dl)		
< 10	60.14 ± 55.46	0.975
≥ 10	60.63 ± 75.15	
Anti-dsDNA (IU/mL)		
Negative (< 20)	51.15 ± 18.41	0.629
Positive (> 20)	61.92 ± 69.38	
Renal involvement n (%)		
Positive	54.25 ± 36.41	0.414
Negative	69.31 ± 91.58	
RA		
CRP (mg/l)		
Negative (< 6)	76.77 ± 31.44	0.012*
Positive (≥ 6)	39.42 ± 28.29	
Data are mean ± SD, or n (%). * P value < 0.05. SD: Standard deviation; SLE: Systemic lupus erythematosus; RA: Rheumatoid arthritis; CRP:C-reactive protein; C3: Complement component 3; C4: Complement component 4; dsDNA: Double-stranded DNA.		

Discussion:

Considering the limitations of the current conventional serological test for diagnosing SLE and RA, researches to identify new biomarkers that enable the development of new diagnostic tools with high sensitivity and specificity are imperative. The goal of the current study was to compare the DNA methylation level of the *IFI44L* promoter and its application in the diagnosis of SLE and RA patients as

well as evaluate the association between this genetic factor with clinical characteristics of these disorders. Until 2016, the role of *IFI44L* in the pathogenesis of autoimmune diseases was unclear. Luo et al in 2016 reported that induction of *IFI44L* leads to upregulation of inflammation via activating the TBK1/IRF3 pathway [28]. Newly, some studies demonstrated that this gene also is involved in the negative regulation of innate immune response after induction of viral infections [29, 30]. In this way, a body of works revealed that the expression of this gene is upregulated in peripheral blood and immune cells of RA and SLE patients [21, 22, 31]. For example, Luo et al represented that the *IFI44L* gene is upregulated in PBMCs of SLE patients and this overexpression was associated with disease activity. In their study, further analysis demonstrated that upregulation of *IFI44L* was due to the hypomethylation of the *IFI44L* promoter [28]. Furthermore, Zhu et al reported that in RA patients, the *IFI44L* promoter was hypomethylated and this methylation status was correlated with enhanced gene expression [32].

In this study, we reported that the level of promoter methylation of *IFI44L* in Iranian patients with RA and SLE was meaningfully lower. However, our results specified that DNA methylation level as a diagnostic biomarker had low power in separating SLE and RA patients from healthy controls. Our results indicated that the DNA methylation level of the *IFI44L* promoter had high sensitivity but low specificity as a diagnostic biomarker. In concordance with our findings, Coit et al in two different studies revealed an obvious hypomethylation of *IFI44L* promoter in naïve T cells, neutrophils, and low-density granulocytes (LDGs) of patients with SLE [33, 34]. Chen et al in a genome-wide DNA methylation study showed the hypomethylation of *IFI44L* promoter in immune cells of SLE and RA patients compared with healthy subjects [24]. Zhao and coworkers reported that *IFI44L* promoter is significantly hypomethylated in SLE, RA, and primary Sjögren's syndrome (pSS) than healthy controls and also showed that this biomarker can distinguish SLE from the healthy population, in addition to pSS and RA. They reported a sensitivity of 93.6% and specificity of 96.8% when evaluated methylation level in CpG site of *IFI44L* promoter (site 1) for diagnosis of patients with SLE from controls. Furthermore, their study indicated that DNA methylation level was significantly lower in patients with SLE with renal involvement than in patients without renal involvement [26]. Likewise, Coit et al. represented that DNA demethylation in IFN-regulated genes (*ISG15*, *ISG20*, *IFI44*, *PARP12*, and *BST2*) in SLE participants with renal involvement was expressively lower than subjects without renal symptoms [35]. Contrarily, our results were inconsistent with their findings in terms of differences between SLE patients with and without renal involvements. This difference could be ascribed to the small sample size of our study. On the other hand, we delineated that RA patients with a higher concentration of CRP have meaningfully a lower DNA methylation level of the *IFI44L* promoter, indicating DNA methylation level of the *IFI44L* promoter had a negative correlation with RA disease activity.

In summary, in this study, we indicated that promoter methylation of *IFI44L* in PBMCs of Iranian SLE and RA patients was obviously lower compared with healthy individuals. However, regarding sensitivity and specificity of the methylation level of *IFI44L* promoter, this biomarker has low power in distinguishing RA and SLE from healthy subjects. In this work, probably, some limitations in the statistical validity of our results such as small population size exist, so further similar studies with larger sample size would help to confirm the suggested correlations. Moreover, we propose that this factor should be evaluated in the

other autoimmune diseases to confirm the specificity of DNA methylation and also clarify the role of *IFI44L* promoter methylation in the pathogenesis of these diseases.

Declarations

Authors' contributions: All authors were involved in whole work

Manuscript has been seen and approved by all the authors.

Conflict of interest: Authors don't have any conflict of interest and we hope that you will find our manuscript acceptable for publication in this Journal**.**

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Figures

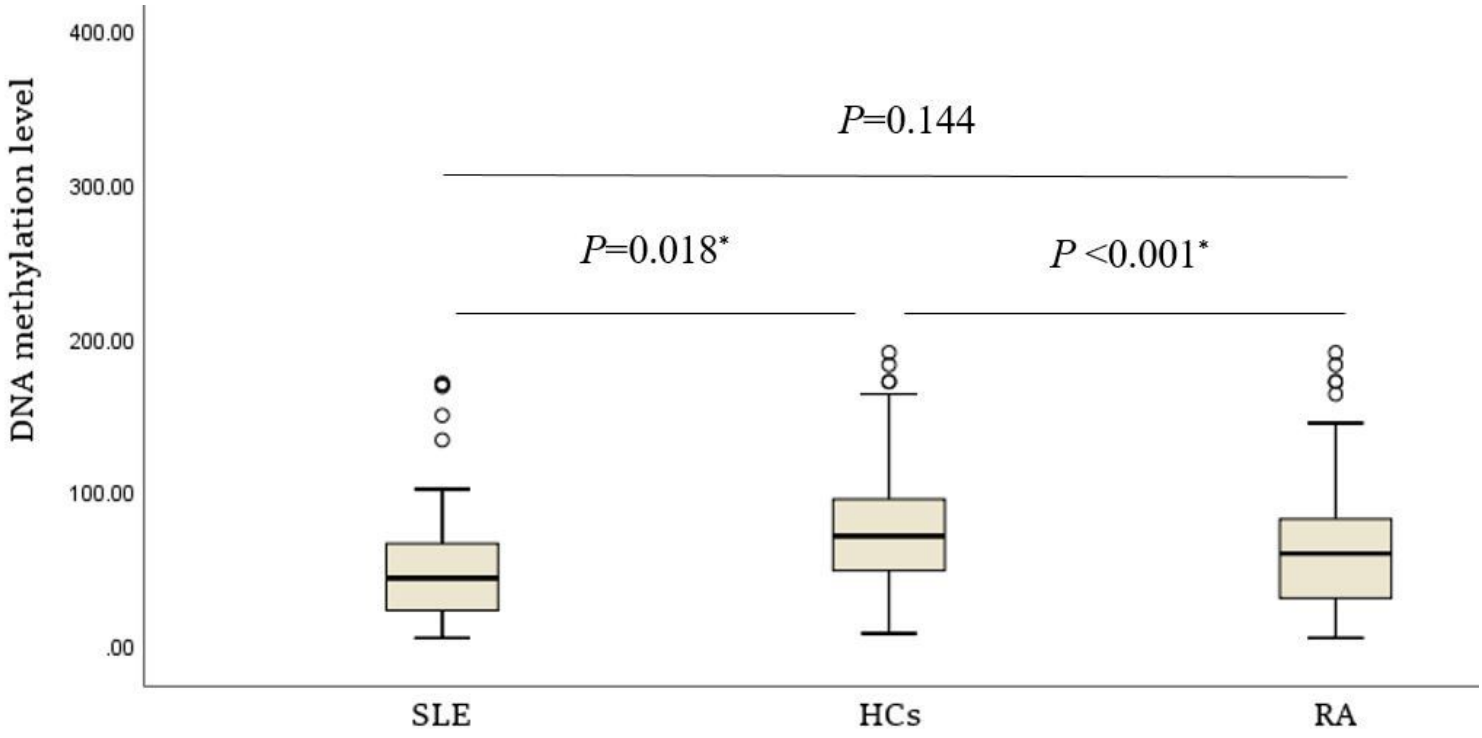

Figure 1

Comparison of interferon-induced protein 44-like (IFI44L) promoter methylation level between patients with Systemic lupus erythematosus and Rheumatoid arthritis and healthy controls. * P value < 0.05

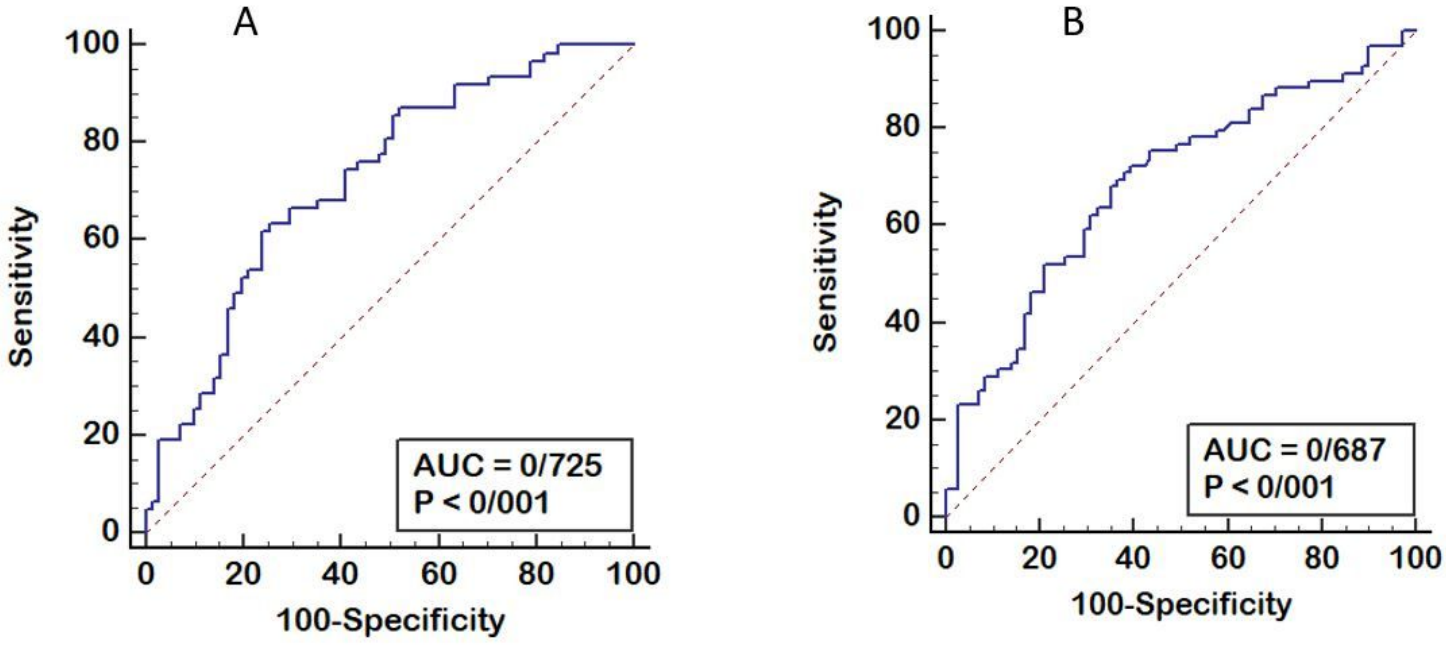

Figure 2

The receiver operating characteristic (ROC) curves of the methylation levels in patients with rheumatoid arthritis (A) and systemic lupus erythematosus (B) compared with healthy controls.