

Investigation of Genes Associated With Atherosclerosis in Patients With Systemic Lupus Erythematosus

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ABSTRACT

Objectives: In this study, we aimed to identify patients with systemic lupus erythematosus (SLE) who are genetically at risk for developing atherosclerosis.

Patients and methods: Between November 2014 and May 2016, a total of 38 patients with SLE (36 females, 2 males; mean age: 37.6 years; range, 18 to 71 years) and 32 healthy females (mean age: 31.5 years; range, 19 to 54 years) were included in the study. Carotid intima-media thickness (CIMT) was measured using high-resolution B-mode ultrasonography. SurePrint G3 Human Gene Expression 8x60K Microarray kit was used in our study. Genes showing differences in expression between the groups were identified by using GeneSpring GX 10.0 program. Pathway analyses of gene expressions were performed using Ingenuity Pathways Analysis (IPA). Gene ontology analyses were performed using the Protein Analysis Through Evolutionary Relationships (PANTHER).

Results: Clinical findings of SLE patients were mainly photosensitivity (71.1%), arthritis (63.2%), lupus nephritis (55.3%), thrombocytopenia (26.3%), and autoimmune hemolytic anemia (21.1%). A total of 155 genes showing expression level difference were detected between SLE patients and healthy controls. In molecular network analysis, 28.2% of all genes were found to be directly or indirectly associated with atherosclerosis and cardiovascular disease.

Conclusion: In SLE patients, many genes are expressed differently from healthy individuals. Expression of these genes is important in the pathogenesis of SLE. Genes identified differently in gene expression analysis can help us to identify SLE patients at risk for atherosclerosis in the Turkish population.

Keywords: Atherosclerosis, clinical parameters, genetic variance, systemic lupus erythematosus, Systemic Lupus Erythematosus Disease Activity Index.

Systemic lupus erythematosus (SLE) is a chronic, autoimmune disease affecting multiple heterogeneous organs and systems. It is associated with an increased risk of atherosclerosis and increased cardiovascular complications. The estimated incidence of new cardiovascular events in patients with SLE is about 1.2 to 1.5% per year, which poses a five to six-fold increased risk compared to

women without SLE.¹ This is more important, as it occurs during the young premenopausal period.¹

Inflammation plays an important role in plaque rupture and thrombus formation, as well as the onset and progression of atherosclerosis. Atherosclerosis begins and progresses as a result of the interaction of immune mechanisms with metabolic risk factors.^{2,3} Detection of patients at

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genetic risk would allow treatment modifications to be made earlier and more effectively.

In the literature, as a result of molecular network analyzes, expression differences of 20-30% of total genes have been reported in SLE and APS pathologies that are directly or indirectly associated with atherosclerosis, inflammation and cardiovascular diseases.⁴ In the present study, we aimed to identify patients with SLE who are genetically at risk for developing atherosclerosis.

PATIENTS AND METHODS

A total of 38 SLE patients (36 females, 2 males; mean age: 37.6 years; range, 18 to 71 years) who admitted to the rheumatology clinic of Trakya University, Faculty of Medicine and 32 healthy female controls (mean age: 31.5 years; range, 19 to 54 years) November 2014 and May 2016 were included in this prospective cohort study. Patients with other rheumatological diseases were excluded from the study. A written informed consent was obtained from each participant. The study protocol was approved by the Trakya University, Faculty of Medicine, Ethics Committee (Date: 28/05/2014, No: 11/12). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Clinical features of SLE patients were recorded. Laboratory tests were taken from the hospital records. All patients met the 1997 American College of Rheumatology (ACR) revised criteria and the 2012 Systemic Lupus International Collaborating Clinics (SLICC) classification criteria for SLE.^{5,6} The SLE Disease Activity Index (SLEDAI) and SLICC/ACR Damage Index scores showing the disease activity of the patients were recorded.

The carotid intima-media thickness (CIMT) was measured twice in both the near and distal walls of both internal carotid arteries and common carotid arteries using high-resolution B-mode ultrasonography.

For the whole-genome gene expression analysis, one-color microarray-based gene expression analysis kit (Agilent Technologies, CA, USA) was used in this study. These microarray slides carry

60-base probes specific to the gene regions. Each array consists of 60,000 different spots to obtain the whole-genome expression view.

For the ribonucleic acid (RNA) isolation and quality control, total RNA isolation was performed from 2 mL whole blood samples according to the manufacturer's instructions using the InnuPrep RNA miniprep kit (Analytik Jena AG, Jena, Thüringen, Germany). Total RNA quality was determined using the 2100 Bioanalyzer instrument (Agilent Technologies, CA, USA) and RNA6000 Nano kit (Agilent Technologies, CA, USA). As a result of the analysis, RNAs whose RNA integrity number (RIN) value was higher than 7 were selected for use in the microarray study.

For the microarray protocol, complementary deoxyribonucleic acid (cDNA) was obtained from total RNA samples with a concentration of 200 ng and a RIN value greater than 7. The cRNA was amplified and labelled using the Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies, CA, USA) and hybridized to the SurePrint G3 Human GE Microarray 8×60 K version 2.0 (Agilent Technologies, CA, USA) according to the manufacturer's instructions. Purification process was performed using labeled amplified PCR products in accordance with the manufacturer's instructions for the Absolutely RNA Nanoprep kit (Agilent Technologies, CA, USA). The purified cRNA samples were hybridized with probes on microarray slides using the gene expression hybridization kit and incubated at 65°C for 17 h in a hybridization oven. Following incubation, the microarray slides were washed using the Gene Expression Wash Buffers (Agilent Technologies, CA, USA). It was scanned using the Agilent SureScan microarray scanner (Agilent Technologies, CA, USA). The data obtained were analyzed using the GeneSpring GX 10.0 program (Agilent Technologies, CA, USA).

Statistical analysis

Statistical analysis was performed using the SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed in mean \pm standard deviation and percentage of numbers. The Chi-square and Fisher's exact tests were used for the analysis of disease and related genes.

A two-tailed p value of <0.05 was considered statistically significant.

Bioinformatic analysis of gene expression

After extracting the data with Feature Extraction program, the differences in gene expressions between the groups were identified by using the GeneSpring GX 10.0 program (Agilent Technologies, CA, USA). Using this program, gene expression significance levels were considered as the Bonferroni correction, p values less than 0.05, and a fold change (FC) value ≥ 2 were considered statistically significant.

Pathway analyses of gene expressions were performed using the Ingenuity Pathways Analysis (IPA). Gene ontology analyses were performed using the Protein Analysis Through Evolutionary Relationships (PANTHER).

RESULTS

Clinical findings and laboratory data of SLE patients are shown in Table 1. Clinical findings of SLE patients were mainly photosensitivity (71.1%), arthritis (63.2%), lupus nephritis

Table 1. Clinical findings and laboratory data of SLE patients

Finding	n	%	Mean \pm SD		n	%	Mean \pm SD
Age (year)			37.6 \pm 12.1	ANA	38	100	
Sex				Anti-dsDNA	18	47.4	
Female	36						
Male	2						
Disease duration (months)			74.8 \pm 60.4	Anti-Sm	8	21.1	
Arthritis	24	63.2		Anti-nucleosome	7	18.4	
Photosensitivity	27	71.1		Anti-RNP	5	13.2	
Alopecia	3	7.9		Anti-ribosomal-P	2	5.3	
Oral ulcer	11	28.9		Anti-Ro	15	39.5	
Pleural effusion	8	21.1		Hypocomplementemia	19	50	
Pericardial effusion	3	7.9		Total cholesterol (mg/dL)			175.2 \pm 51.3
Lupus nephritis	21	55.3		Fasting blood sugar (mg/dL)			89.8 \pm 19.9
Neurological involvement	2	5.3		HDL (mg/dL)			45.1 \pm 15.4
Leukopenia	17	44.7		LDL (mg/dL)			102.9 \pm 32.5
Thrombocytopenia	10	26.3		Triglycerides (mg/dL)			117.2 \pm 57.7
Hemolytic anemia	8	21.1		CRP (mg/dL)			1.7 \pm 1.9
Thrombosis	5	13.2		Erythrocyte sedimentation rate (mm/h)			61 \pm 30
Active SLE (SLEDAI >4)	22	57.9		Corticosteroids	35	92.1	
SLEDAI			7.6 \pm 6.3	Pulse corticosteroids	12	31.6	
SLICC/ACR-DI			0.9 \pm 0.9	Hydroxychloroquine	38	100	
Obesity	3	7.9		Azathioprine	16	42.1	
Smoking	10	26.3		Cyclosporine	1	2.6	
Hypertension	6	15.8		Methotrexate	1	2.6	
Systolic blood pressure (mmHg)			117.1 \pm 12.7	Mycophenolate mofetil	6	15.8	
Diastolic blood pressure (mmHg)			76.8 \pm 8.1	Cyclophosphamide	5	13.2	
Diabetes	1	2.6		Rituximab	4	10.5	
Hyperlipidemia	7	18.4		Use of statin	0	0	
Increased CIMT	4	10.5		Use of anti-hypertensive	7	18.4	
CIMT (mm)			0.4 \pm 0.1	Anti-aggregant/Anti-coagulant	7	18.4	

SLE: Systemic lupus erythematosus; SD: Standard deviation; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; SLICC/ACR-DI: Systemic Lupus International Collaborating Clinics/American College of Rheumatology-Damage Index; CIMT: Carotid intima-media thickness; ANA: Anti-nuclear antibody; dsDNA: Double-stranded deoxyribonucleic acid; Sm: Smith antigen; RNP: Ribonucleoprotein antibodies; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; CRP: C-reactive protein.

(55.3%), thrombocytopenia (26.3%), and autoimmune hemolytic anemia (21.1%). The main laboratory parameters of SLE patients were anti-nuclear antibody (ANA) positivity (100%), hypocomplementemia (50%), and anti-double-stranded DNA (anti-dsDNA) positivity (47.4%).

All data obtained from the whole-genome gene expression microarray were submitted to NCBI's Gene Expression Omnibus and are available through GEO Series accession number GSE154851. (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE154851>).

A total of 155 genes showing expression level difference were detected between SLE patients and healthy controls. When the patient samples were compared with the controls, an increase was found in the expression level in 110 and a decrease in expression level in 45 of these genes.

Genes with the highest expression increase were interferon-induced protein 44-like (IFI44L), epithelial stromal interaction 1 (breast) (EPSTI1), NLR family, apoptosis inhibitory protein (NAIP), interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), interferon-induced protein 44 (IFI44), spermatogenesis associated, serine-rich 2-like (SPATS2L), 2'-5'-oligoadenylate synthetase 1, 40/46kDa (OAS1), receptor (chemosensory) transporter protein 4 (RTP4), guanylate binding protein 1, interferon-inducible (GBP1), 2'-5'-oligoadenylate synthetase 2, 69/71kDa (OAS2), genes with the highest expression reduction are G0/G1 switch 2 (G0S2), uncharacterized FLJ36000 (FLJ36000), long intergenic non-protein coding RNA 1478 (LINC01478), and LNCipedia lincRNA (linc-ZAP70-2). Comparing the SLE patients and control group, all genes expressed differently (see Additional File 1).

In the molecular network analysis, 28.2% of all genes were found to be directly or indirectly associated with atherosclerosis and cardiovascular disease. For the evaluation of functionally categorized subgroups of genes associated with atherosclerosis and cardiovascular diseases, immunological disease and inflammatory response was seen in 41%, hematological disease and cardiovascular disease in 32%, cell signaling in 24%, and lipid metabolism in 3%.

The heat map of atherosclerosis-related genes according to functional categorization is shown in

Figure 1. The heat map of other genes according to functional categorization is shown in Additional File 2.

In canonical pathways analysis using IPA program, a significant activation was first detected in SLE patients in the role of pattern recognition receptors in recognition of bacteria and viruses, death receptor signaling and interferon signaling versus the control group. The canonical pathways analysis of all genes is detailed in the Additional File 3.

In the upstream analysis of the SLE patients, PRL, IFNA2, IFNL1, NKX2-3, MAPK1 genes, and notably interferon alpha signature (AIM2, CEACAM1, DDX58, DYNLT1, EIF2AK2, EPSTI1, FBXO6, GBP1, HELZ2, IFI44, IFI44L, IFIH1, IFIT2, IFIT3, IL6R, LAP3, OAS1, OAS2, PARP12, PARP14, PARP9, PLSCR1, PML, RNASEL, RNF213, RTP4, SAMD9L, SAT1, TDRD7, TMEM140, TNFSF10, TRIM22, ZBP1) genes were expressed significantly more than the

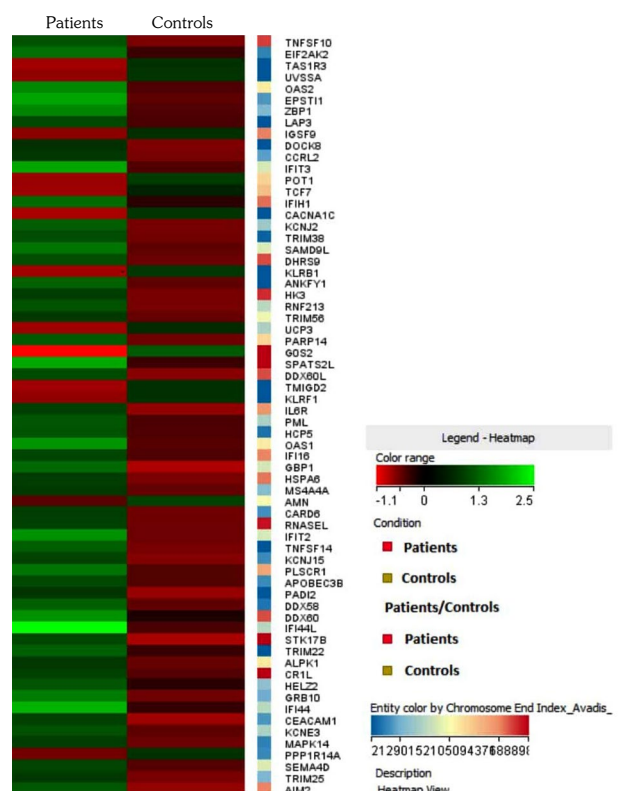


Figure 1. Heat map of atherosclerosis related genes according to functional categorization.

Table 2. Biological processes differently expressed between SLE and control groups

Biological process	Genes in PANTHER database	Observed	Expected	χ^2 p value
Defense response to virus	186	20	1.06	2.88E-19
Immune response	1783	38	10.17	5.53E-13
Immune system process	2630	44	15.00	1.52E-11
Immune effector process	1059	27	6.04	5.90E-11
Innate immune response	708	22	4.04	1.30E-10
Interferon-gamma-mediated signaling pathway	71	7	0.40	2.99E-07
Cellular response to cytokine stimulus	931	20	5.31	3.84E-07
Cytokine-mediated signaling pathway	619	16	3.53	6.16E-07
Regulation of type I interferon production	110	7	0.63	4.68E-06
Response to type I interferon	71	6	0.40	5.00E-06

SLE: Systemic lupus erythematosus; PANTHER: Protein Analysis Through Evolutionary Relationships.

control group. The detailed upstream analysis data are shown in Additional File 4.

The Ingenuity Pathways Analysis software was used to analyze the network between genes showing the relation between genes. The detailed data can be found in the Additional File 5.

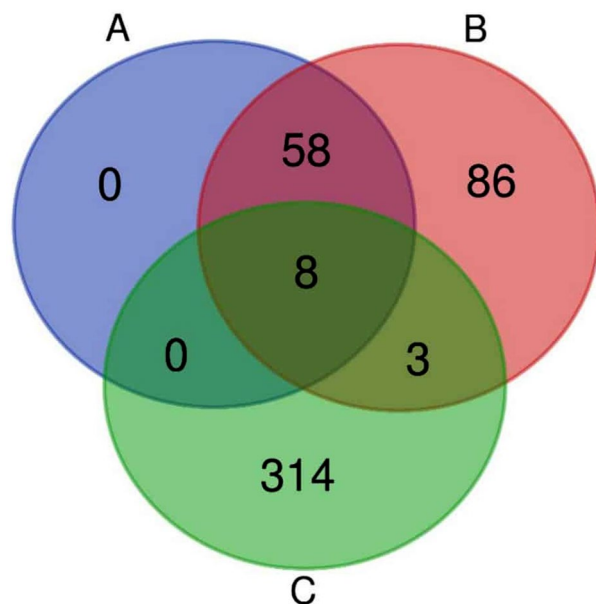


Figure 2. Comparative Venn diagram of the genes we detected related to atherosclerosis. **(A)** Genes that we detect associated with atherosclerosis in our study. **(B)** All genes that we detected expression difference compared to the control group in our study. **(C)** Genes associated with atherosclerosis by Huang et al.⁶

According to the results of the diseases or functions annotation analysis using the IPA program of the genes whose expression differences were detected, the most significant results were found in antimicrobial response, inflammatory response, and infectious diseases (see Additional File 6).

In the gene ontology analysis using the PANTHER database between SLE and control patients, defense response to virus, immune response, immune system process, interferon-gamma-mediated signaling pathway, and response to type I interferon biological processes were found to significantly increase ($p < 0.001$) (Table 2).

In terms of CIMT value, gene expression differences in SLE patients within themselves and between the patient and control groups were examined and no significant difference was found in terms of gene expressions. When we compared the genes that we detected significantly in our study with 344 genes associated with atherosclerosis, CEACAM1, FFAR2, GBP1, HSPA6, IL6R, KCNJ15, KCNJ2, LHFPL2, MAPK14, PARP9, and TNFSF14 genes were also found to be associated with atherosclerosis (Figure 2).⁷ The 344 genes were previously identified by Huang et al.⁷

DISCUSSION

In our study, IFI44L was the gene found to have the highest expression increase (seven folds)

in SLE patients, compared to healthy controls. The IFI44L gene plays a role in type I interferon-inducible genes and in the immune response pathway. In a study conducted by Zhao et al.,⁸ renal involvement was found in patients with SLE with IFI44L gene hypomethylation. Based on these data, the authors proposed that hypomethylation of the IFI44L gene in SLE patients might be a biomarker for patients with SLE renal involvement. In addition, there was no significant difference in comparison of IFI44L expression between SLE patients with lupus nephritis and SLE patients without lupus nephritis. In the meta-analysis by Bae and Lee,⁹ they found the most significant enrichment in the 317 GO category on the type I interferon (IFN) -mediated signaling pathway. In our study, we also found the enrichment in the type I IFN-mediated signaling pathway with $P: 4,03 E-04$.

The EPSTI1 gene is one of the genes known to be upregulated in invasive breast carcinomas.¹⁰ In the study conducted by Ishii et al.,¹¹ for the first time in the literature, they found a significant increase in expression of ESPTI1 gene in SLE patients, compared to the control group. Kennedy et al.¹² reported in their study that CMPK2, HERC5, and EPSTI1 genes can be used as IFN biomarkers in patients with SLE. In our study, we found an increased expression of EPSTI1 and CMPK2 genes in SLE patients. Our study results are consistent with the literature.

We found a significant increase in the expression level of IFIT3 gene in SLE patients, compared to healthy control group. In recent studies using the IFIT3 gene, it was reported that this gene could act as an important regulator in the signaling pathway.¹³ Although our finding is consistent with the literature, it should be supported by further studies that the IFIT3 gene may be a therapeutic target.

The GOS2 gene encodes a protein that acts as a cell cycle inhibitor in the transition of cells to the G1 phase. In studies by Ishii et al.¹¹ and Kobayashi et al.,¹⁴ GOS2 gene expression increased in SLE patients, compared to controls; however, we found a significant decrease in expression of GOS2 gene expression in our study. A decrease in the level of GOS2 gene expression in SLE patients was first detected in our study. Further studies are needed to reveal

the relationship between changes in the increase or decrease in GOS2 gene expression and autoimmune diseases.

In the current study, we also found a significant decrease in the expression level of the HIF3A gene and a significant increase in the expression level of the IFI44 gene in SLE patients. Based on the literature finding and our results together, it is predicted that changes in expression levels of HIF-related genes may cause nephritis-related organ damage in patients with lupus nephritis or SLE. However, there is no evidence that the HIF3A gene is directly related to SLE. Although the role of the IFI44 gene has not been fully elucidated yet, some evidence suggests that this IFN-inducible gene acts as a suppressor of viral replication. It has been reported that hypomethylation of the IFI44L gene, which is a paralog of the IFI44 gene, is associated with SLE and may be a biomarker for SLE, compared to healthy individuals with other autoimmune diseases.¹⁵ Consistent with the literature, a significant increase in expression levels of IFI44 and IFI44L genes was found in our study.

In a study conducted by Zhou et al.,¹⁶ the expression level of the POT1 gene was found to decrease in SLE patients, compared to the control group; however, this decrease was not found in the clinical features of the patients. Similarly, we found a decrease in the expression level of the POT1 gene, compared to the control group in our study, while we could not detect a feature reflected in the clinic of the patients.

In a study conducted by Perez-Sanchez et al.,⁴ 20 to 30% of all genes were found to be directly or indirectly associated with atherosclerosis and cardiovascular diseases in molecular network analysis. Similarly, this rate was found to be 28.2% in our study.

In the study of Huang et al.,⁷ toll-like receptors (TLRs) and interleukin (IL)-10 signaling pathways were detected in the canonical pathway analysis in SLE patients with atherosclerosis. The MAPK14 gene was identified as one of the important key genes in both pathways. The authors found an increase in the expression level of MAPK14 gene in their study. The EIF2AK2 was previously reported to increase expression in SLE patients in the

literature.¹⁷ Therefore, the authors suggested that hypomethylation might play a role in the regulation of expression levels of genes in the TLR pathway in SLE patients. In our study, we found a significant increase in the expression of TLR signaling pathways, EIF2AK2, MAPK14, and IL-10 signaling pathways. We believe that this finding may be used as a biomarker in SLE patients, if supported by further studies.

Death receptor signaling pathway is one of the important apoptotic pathways.¹⁸ In the event of DNA damage, the PARP system in the death signaling pathway initiates the first step in the chain of events leading to DNA repair or apoptosis.¹⁹ The PARP system was previously found to be associated with apoptosis in SLE patients.²⁰ In our study, we found a significant difference in the expression levels of 5 genes (NAIP expression level increase, PARP9, PARP12, PARP14, TNFSF10 decrease in expression levels) in death receptor signaling. The expression levels of PARP9, PARP12, PARP14 genes that we found in our study are consistent with the literature in terms of differences and pathways to which they are related.

Lie et al.²¹ reported that, in patients with SLE, the IFN signaling pathway activation might be associated with atherosclerosis by triggering plaque formation. In our study, we found a significant increase in the expression in the IFN signaling pathway in patients with SLE.

When the genes were classified according to diseases or functions annotation, the most significant result was found in the antimicrobial response and inflammatory response category (APOBEC3B, DDX58, DDX60, EIF2AK2, IFIT2, OAS1, OAS2, PLSCR1, RNASEL, TRIM22). Of these genes, EIF2AK2 and PLSCR1 have previously been reported to increase expression in SLE patients in the literature.¹⁷ Our findings also support the increased inflammatory response in SLE patients.

In the study by Korman et al.,²² a significant expression increase in the immune system process was found in biological process analysis using the PANTHER database between SLE patients and healthy controls. Similarly, we found a significant increase in the expression in defense response to virus, immune response, and immune system

process. Yang et al.²³'s findings seem to be compatible with our study, according to the biological process analysis.

In our study, we compared 344 atherosclerosis-related genes previously described by Huang et al.⁷ Accordingly, 14 genes (10.3%) (CEACAM1, CEP19, CSFR2RA, FFAR2, GBP1, HSPA6, IL6R, KCNJ15, KCNJ2, LHFPL2, MAPK14, PARP9, SEMA4D and TNFSF14) which were mapped by IPA and found to be significant were the same as those found by Huang et al.⁷ In the study conducted by Korman et al.,²² 13.5% of the genes were found to be common with the genes that Huang et al.⁷ found to be associated with atherosclerosis. In the meta-analysis conducted by Yang et al.,²³ the findings seem to be compatible with our results according to the biological process analysis. According to these studies, the 321 common differentially expressed genes are mainly enriched in biological processes for immune responses and inflammatory responses, including natural immune response, defense response, cytokine-mediated signaling pathway, IFN-alpha response, and I-kappaB kinase/NF-kappaB signal. We also observed significant gene expression alterations in the genes involved in similar biological processes, consistent with the study of Yang et al.²³

The major limitation of our study is the small sample size. Increasing the number of samples belonging to the study group will support our results. However, we think that our study is compatible with the literature and contains strong findings in terms of the results we obtained on atherosclerosis genetics. Another limitation of the study is that the findings could not be confirmed by a second method such as Real Time PCR. The reason for this limitation is the large number of genes that are expressed differently.

In conclusion, in SLE patients, many genes are expressed differently from healthy individuals. Expression of these genes is of utmost importance in the pathogenesis of SLE. Genes that we have identified differently in gene expression analysis can help us to identify SLE patients at risk for atherosclerosis in the Turkish population.

Additional files

Additional file 1: All genes expressed differently, comparing the SLE patients and control group

Additional file 2: Heat maps of other genes according to functional categorization

Additional file 3: Canonical pathways analysis performed for all genes (ingenuity canonical pathways)

Additional file 4: Upstream analysis data

Additional file 5: Gene network analysis. Analyses were performed using ingenuity pathways analysis (IPA) program.

Additional file 6: Diseases or functions annotation

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Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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