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# Association Study of CD*226* and CD*247* Genes Single Nucleotide Polymorphisms in Iranian Patients with Systemic Sclerosis

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## ABSTRACT

CD*247* and CD*226* play important roles in signaling of lymphocytes. Single nucleotide polymorphisms (SNPs) of genes encoding CD*247* and CD*226* have been associated with the risk of several autoimmune disorders. This study aimed to evaluate the possible association between *CD226* and *CD247* genes SNPs and risk of systemic sclerosis (SSc) in Iranian population.

Study participants were 455 SSc patients and 455 age, sex, and ethnic -matched healthy individuals. Genotyping of rs2056626 and rs763361 at *CD247* and *CD226* genes, respectively, was carried out using TaqMan MGB-based allelic discrimination real-time PCR. Neither alleles nor genotypes of both SNPs showed significant association with the risk of SSc.

Furthermore, association analysis of the genotypes with clinical manifestations of the disease revealed that rs763361 variants were associated with the forced vital capacity (FVC) in SSc patients.

Our results suggest that genetic variants of *CD226* and *CD247* genes may not be a contributing factor in pathogenesis of SSc in Iranian population.

**Keywords:** CD*226*; CD*247*; Single nucleotide polymorphism; Systemic sclerosis

## INTRODUCTION

Systemic sclerosis (SSc) is a common autoimmune disease characterized by essential vasomotor disturbances, fibrosis, subsequent atrophy of the skin, immunologic abnormalities and autoantibody production.[1,2]

Impaired apoptosis mechanisms of fibroblasts cause prolonged activation of these cells and, therefore, production of cytokines and mediators involved in clinical outcomes of SSc.[3] Many genetic and environmental factors contribute to the pathogenesis of SSc. A huge number of genetic loci has been identified through independent genetic association studies and genome-wide association studies (GWASs) including hundreds of thousands of SNPs located throughout the genome.[4-7]

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Although the precise pathogenesis of SSc is still undetermined, it is commonly considered as an autoimmune disorder. During SSc development, autoimmune responses and vasculopathy initiate further events like fibroblast activation and fibrosis.[8,9] As the central culprits of the SSc immunopathogenesis, B cells produce autoantibodies to several autoantigens like those found on endothelial cells. Additionally, ischemia-reperfusion injury due to Raynaud's phenomenon, production of reactive oxygen species (ROS) along with recruitment of inflammatory cell, and subsequent release of inflammatory cytokines induce myofibroblastic transformation and fibroblasts overactivation, eventuating in immoderate collagen synthesis as well as other extracellular compounds.[10,11] As a subset of $CD4^+$ T cells, helper 2 T (Th) cells have been postulated to be involved in aberrant production of profibrotic mediators, like interleukin (IL)-4, IL-13, IL-33, and transforming growth factor β (TGFβ); hence T cells are involved in pathogenesis of SSc through contributing to fibrosis by fibrotic cytokine production, in addition to their role in helping B cells to produce autoantibodies.[8,12,13]

CD*247* (cluster of differentiation *247*), also known as T-cell surface CD3 zeta chain, is part of T cell receptor (TCR)/CD3 complex. The molecule is involved in the assembly as well as transport of the TCR/CD3 complex toward the cell surface. CD*247* plays an essential role in associating the antigen recognition to several intracellular signaling pathways.[14,15] Mutations in *CD247* gene have been demonstrated to be involved in impaired immune function.[16] GWASs demonstrated that an intronic rs2056626 SNP of the *CD247* gene was associated with SSc risk in European and US Caucasians.[4]

CD*226*, known as the DNAX-accessory molecule-1 (DNAM-1), is expressed on immune cells such as natural killer (NK) cells, T cells, NK-T cells, and B cells. The molecule is involved in various biological mechanisms and function of immune cells, particularly as a co-stimulatory molecule in cell signaling.[17,18] *CD226* gene rs763361 SNP is a nonsynonymous variation, which has been frequently occurred in several autoimmune diseases.[19] This polymorphism is attributed to substitution of glycine instead of serine at position 307 (Ser307Gly) in exon-splicing silencer (ESS) region, which may impress expression of CD*226*.[20] The 307Ser variant modifies the splicing of the CD*226* transcript, leading to stimulation of signaling transduction and, therefore, over-activations of T and NK cells.[19]

It appears that genetic variations in *CD226* and *CD247* genes are involved in impaired function of T cells, which are main players in the pathogenesis of SSc. Taking into account the previously reported associations of SNPs in *CD226* and *CD247* genes with a number of autoimmune diseases like systemic sclerosis,[21-24] and that no study has addressed the potential association of polymorphisms in these genes in Iranian population, herein we decided to evaluate the association of rs2056626 and rs763361 at *CD247* and *CD226* genes with the risk of SSc in Iranian population.

## MATERIALS AND METHODS

### Study Participants

Study population comprised of 455 SSc patients (68 males and 387 females) and 455 healthy controls (67 males and 388 females) with the mean age of 41.55±12.05 and 41.38±12.73, respectively. SSc patients enrolled from the Iranian SSc patients referred to Rheumatology Research Center outpatient clinic, Shariati hospital and diagnosed based on American college of rheumatology (ACR) criteria for SSc. Those patients with past medical history of other autoimmune disorders or family history of SSc were excluded. As the healthy control group, 455 age, sex, and ethnic (Iranian Fars, Turk, Kurd, Lur, and Gilak) -matched individuals were included in this study. Matching the study population ethnically, possibility of spurious results due to population stratification was eliminated.[25] In order to investigate the association between the genotypes of SNPs with SSc phenotypes, the clinical manifestations of the patients were recorded (Table 1). Before sampling, written informed consent was signed by each subject. Ethical Committee of Tehran University of Medical Sciences approved the protocol of the study (No. 93-04-41-27689-290398).

### DNA Extraction and Genotyping

Genomic DNA was extracted from 5 mL whole blood samples containing ethylenediaminetetraacetic acid (EDTA) using the standard phenol/chloroform method.[26] The optical density values were used to evaluate the concentration and purity of the extracted DNA (NanoDrop 2000C). All DNA samples were stored at -20°C until further experiments. Study

**Table 1. Baseline and clinical data of the studied patients with systemic sclerosis**

| Characteristic (n=445) | Value |
|---|---|
| Male/ female | 68 (15%)/ 387 (85%) |
| Age* | 41.55±12.05 |
| Disease Duration* | 10.5 ± 6.14 |
| Limited SSc/ Diffuse SSc | 169 (37%)/ 286 (63%) |
| Raynaud's phenomenon** | 33 (7.25%) |
| Digital ulcer | 345 (75.8%) |
| Lung fibrosis disease | 248 (54.5%) |
| FVC (Pos>120, Neg<80, Normal range: 80-120) | 229 (50.3%) |
| PAP (Neg<35%, Pos>35%) | 77 (17%) |
| LVEF (Pos<40, Neg:55-70, Borderline:40-55) | 55 (12%%) |
| FANA (Neg>1/100, Pos<1/100, Borderline =1/100) | 365 (80%) |
| ACA (Pos>18, Neg<12, Borderline:12-18 RU/mL) | 23 (5%) |
| ATA (Neg<20 RU/mL , Pos≥20) | 317 (69.7%) |
| ARA | 12 (2.6%) |
| Creatinine* | 0.97 ± 0.58 |
| Total protein* | 7.55 ± 3.07 |
| ESR* | 19.35 ± 18.19 |

FVC, forced vital capacity; PAP, pulmonary artery pressure; LVEF, left ventricular ejection fraction; FANA, fluorescent anti-nuclear antibodies; ACA, anti-centromere antibodies; ATA, anti-topoisomerase antibody; ARA, Anti-RNA polymerase III; ESR, erythrocyte sedimentation rate.

* Data represented as mean ± SD; ** Positive count reported

**Table 2. CD*226* and CD*247* genetic variants analyzed in systemic sclerosis (SSc) patients and healthy controls**

| SNP | Chromosome | Position | Alleles | Amino acid change |
|---|---|---|---|---|
| rs763361 | 18 | 69864406 | C/T | Ser307Gly |
| rs2056626 | 1 | 167451188 | G/T | Intron |

subjects were genotyped for *CD247* gene rs2056626 and *CD226* gene rs763361 SNPs (Table 2) using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and allelic discrimination TaqMan MGB-based assays (Applied Biosystems, Foster City, USA). All PCR reactions mixture contained approximately 25-75 ng of DNA, 5 µL Taq-Man Master Mix containing Taq DNA polymerase and dNTPs (Applied Biosystems, Foster City, USA), 0.25 µL Taq-Man Genotyping Assay mix containing primers and FAM or VIC labeled probes (Applied Biosystems, Foster City, USA), and distilled water for a final volume of 10 µL. Thermocyclic conditions of PCR were: initially 60°C for 30 seconds and then 95°C for 10 mins, and subsequently 40 cycles of amplification (95°C for 15 seconds and 60°C for 1 min), and finally 60°C for 30 seconds. Allele calling was performed by analyzing allelic discrimination plots using ABI SDS V 2.3 software (Applied Biosystems, Foster City, USA).

**Statistical Analysis**

Demographic and clinical characteristics of the study population were assessed by descriptive statistical analysis. The associations between SSc and *CD226* and *CD247* genes SNPs were analyzed by Logistic Regression and $\chi 2$ test or two-tailed Fisher's exact test. Odds ratios (OR) and confidence intervals (95% CI) were employed for risk estimation. *p* values were adjusted by Benjamini-Hochberg Method (BHM) and considered statistically significant if they were less than 0.05. Adherence to the Hardy–Weinberg Equilibrium (HWE) was evaluated using $\chi 2$ test in Package 'genetics' of R-Software (R Core Team, Austria).

## RESULTS

Table 1 shows the clinical specifications of the studied SSc population with more details.

Genotype distribution of rs2056626 ($p$=0.66) and rs763361 ($p$=0.44) in control subjects did not disclose significant deviation from HWE (Table 3). For both SNPs, the allele with the highest frequency was considered as the reference allele and minor allele frequency (MAF) was reported, according to NCBI database (https://www.ncbi.nlm.nih.gov/snp). The reference genotype was also selected according to the monozygotic genotype with alleles of highest frequency. The C allele of the rs763361 SNP was less represented in SSc patients than controls (39.12% *vs*. 41.75%). However, the frequency difference was not significant (OR= 0.81, CI: 0.55-1.20; $p$=0.30). The CT genotype of rs763361 SNP had lower frequency in SSc patients compared with healthy controls (47.13% *vs*. 51.15%) and the difference was not significant (OR= 0.80, CI: 0.54-1.21; $p$=0.30). Alternately, the CC genotype was distributed almost equally between patient and control groups (15.57% *vs*. 16.13%); hence the frequency distribution difference was not statistically significant (OR= 0.84, CI: 0.48-1.47; $p$=0.55). As the dominant model, the CT+TT genotype had no significant distribution difference between SSc and healthy control groups (62.64% *vs*. 67.25%; OR=0.81, CI: 62-1.07; $p$=0.15).

For rs2056626 (Table 3), the G allele was found to be highly represented in SSc patients in comparison to controls (38.89% *vs*. 36.87%); however, no significant difference was observed in the allele distribution between patients and controls (OR=1.14, CI: 0.68-1.92; $p$=0.61). Among the genotypes of rs2056626, both GT and GG genotypes did not show statistically significant differences between study groups. The GG+GT model was assigned as the dominant genotype and its distribution difference was not statistically significant between patients and controls (OR=1.10, CI: 0.84-1.44; $p$=0.45).

Clinical manifestations of the SSc patients including Raynaud's phenomenon, digital ulcer history, lung fibrosis disease, forced vital capacity (FVC), pulmonary artery pressure (PAP), left ventricular ejection fraction (LVEF), fluorescent anti-nuclear antibody (FANA), anti-centromere antibody (ACA), anti-topoisomerase antibody (ATA), anti-RNA polymerase III (ARA), Creatinine, total protein, erythrocyte sedimentation rate (ESR) were evaluated in relation to genotypes of both SNPs (Table 4). Among them, there was significant correlation of FVC with rs763361 genotypes ($p$= 0.036).

**Table 3. Allele and genotype distribution of *CD226* gene rs763361 SNP and *CD247* gene rs2056626 SNP in the systemic sclerosis patients and healthy controls**

| SNP | Allele /Genotype | Case (N=455) N (%) | Control (N=455) N (%) | OR (95% CI) | *p* |
|---|---|---|---|---|---|
| **rs763361** | T (Reference) | 554 (60.88) | 530 (58.24) | - | - |
| | C | 356 (39.12) | 380 (41.75) | 0.81 (0.55-1.20) | 0.30 |
| | TT (Reference) | 170 (37.30) | 149 (32.72) | - | - |
| | CT | 214 (47.13) | 233 (51.15) | 0.80 (0.54-1.21) | 0.30 |
| | CC | 71 (15.57) | 73 (16.13) | 0.84 (0.48-1.47) | 0.55 |
| | CT+TT | 285 (62.64) | 306 (67.25) | 0.81 (0.62-1.07) | 0.15 |
| HWE | | | $p$= 0.44 | | |
| **rs2056626** | T (Reference) | 556 (61.11) | 575 (63.13) | - | - |
| | G | 354 (38.89) | 335 (36.87) | 1.14 (0.68-1.92) | 0.61 |
| | TT (Reference) | 174 (38.22) | 185 (40.55) | - | - |
| | GT | 208 (45.78) | 205 (45.16) | 1.07 (0.72-1.61) | 0.72 |
| | GG | 73 (16.00) | 65 (14.29) | 1.12 (0.68-2.09) | 0.54 |
| | GG+GT | 281 (61.75) | 270 (59.34) | 1.10 (0.84-1.44) | 0.45 |
| HWE | | | $p$= 0.66 | | |

HWE; hardy–weinberg equilibrium

**Table 4. Frequencies of rs763361 and rs2056626 genotypes with various clinical features of patients with systemic sclerosis**

| Clinical Features | rs763361 genotype distribution | | | | | rs2056626 genotype distribution | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Frequency N (%) | CC N (%) | CT N (%) | TT N (%) | *p** | Frequency N (%) | GG N (%) | GT N (%) | TT N (%) | *p** |
| Raynaud's phenomenon | 350 (76.92) | 52 (14.86) | 158 (45.14) | 140 (40.00) | 0.313 | 349 (76.70) | 55 (15.76) | 159 (45.56) | 135 (38.68) | 0.339 |
| Digital ulcers | 250 (54.95) | 39 (15.60) | 116 (46.40) | 95 (38.00) | 0.434 | 220 (48.35) | 37 (16.82) | 96 (43.64) | 87 (39.54) | 0.613 |
| Lung fibrosis disease | 179 (39.34) | 19 (10.61) | 86 (48.04) | 74 (41.34) | 0.536 | 162 (35.60) | 30 (18.52) | 57 (35.18) | 75 (46.30) | 0.771 |
| FVC | 140 (30.77) | 19 (13.58) | 52 (37.14) | 69 (49.28) | **0.036** | 258 (56.70) | 142 (55.04) | 55 (21.32) | 61 (23.64) | 0.746 |
| PAP | 50 (10.98) | 5 (10.00) | 30 (60.00) | 15 (30.00) | 0.071 | 58 (12.74) | 8 (13.79) | 22 (37.93) | 28 (48.28) | 0.127 |
| LVEF | 35b(77.78) | 4 (11.43) | 13 (37.14) | 18 (51.43) | 0.261 | 26 (5.71) | 2 (7.69) | 18 (69.23) | 6 (23.08) | 0.631 |
| FANA | 358 (79.56) | 52 (14.52) | 175 (48.89) | 131 (36.59) | 0.211 | 356 (78.24) | 59 (16.57) | 155 (43.54) | 142 (39.89) | 0.351 |
| ACA | 22 (4.83) | 4 (18.18) | 13 (59.09) | 5 (22.73) | 0.869 | 16 (3.51) | 4 (25.00) | 8 (50.00) | 4 (25.00) | 0.108 |
| ATA | 310 (68.13) | 47 (15.16) | 142 (45.81) | 121 (39.03) | 0.480 | 295 (64.83) | 39 (13.22) | 132 (44.75) | 124 (42.03) | 0.365 |
| ARA | 41 (9.01) | 7 (17.07) | 21 (51.22) | 13 (31.71) | 0.359 | 12 (2.64) | 2 (16.67) | 4 (33.33) | 6 (50.00) | 0.493 |
| Creatinine | 26 (5.71) | 2 (7.69) | 15 (57.69) | 9 (34.62) | 0.598 | 34 (7.47) | 10 (29.41) | 12 (35.29) | 12 (35.29) | 0.284 |
| Total protein | 47 (10.33) | 9 (19.15) | 21 (44.68) | 17 (36.17) | 0.799 | 46 (10.11) | 6 (13.04) | 18 (39.13) | 22 (47.83) | 0.609 |
| ESR | 63 (13.85) | 11 (17.46) | 30 (47.62) | 22 (34.92) | 0.818 | 98 (21.54) | 16 (16.33) | 46 (46.94) | 36 (36.73) | 0.695 |

* Benjamini-Hochberg was applied to control the false discovery rate (FDR).

FVC, forced vital capacity; PAP, pulmonary artery pressure; LVEF, left ventricular ejection fraction; FANA, fluorescent anti-nuclear antibodies; ACA, anti-centromere antibodies; ATA, anti-topoisomerase antibody; ARA, Anti-RNA polymerase III; ESR, erythrocyte sedimentation rate

## DISCUSSION

SSc is a complex heterogenic disorder of connective tissue and small arteries, defined by the hallmarks of triad of fibrosis, inflammation and vascular injury.[27,28] Fibrosis caused by dermal fibroblast accounts for a wide range of disease outcomes.[29-31] Previous studies classified the genetic variants of SSc in two distinct groups as follows: first, the genetic factors involved in immune system dysfunctions, which many of them have been detected in GWASs. Second, the genetic variants which promote the cellular and molecular mechanisms involved in the progression of inflammation, autoantibody formation and fibrosis development.[32] Based on the pathogenesis of SSc, great attention has been dedicated to the genetics, which affect the immune regulation mechanisms and autoimmunity pathways. Hence, the SNPs in each of these genes might predispose individuals to SSc disease or supply a susceptibility condition for the effect of genetic variants.[33,34]

Accumulating evidence has implicated to a number of immune system perturbations in pathogenesis of SSc. Immune cells, particularly lymphocytes, demonstrate aberrant activation trends in the initial course of SSc pathogenesis. Immune cells, including T cells, macrophages, mast cells, and B cells, infiltrate to skin before any histologic signs of skin fibrosis.[35,36] T cells accumulate in the skin lesions of the SSc patients and demonstrates the signs of activation such as increased expression of IL-2, CD69, and HLA-DR. Additionally, increased serum levels of T-cell associated cytokines like IL-4, IL-13, and IL-17 have been identified in SSc patients.[37] Studies show that both αβ and γδ T cells infiltrate in skin lesions of SSc patients, which is specified only as a consequence of antigen-driven proliferation of T cells.[37] The role of T cells in induction of fibrosis is mediated through production of cytokines or direct cell-cell contact with B cells and fibroblasts. SSc skin is characterized by infiltrating T cells as well as peripheral blood T cells with a predominantly Th2 profile, which mediates the production of profibrotic cytokines like IL-4, IL-13 and TGFβ.[38,39]

Both CD*226* and CD*247* play crucial roles in the stimulation and activation of T cells. It has been found that the CD*247* expression is changed in chronic autoimmune and inflammatory disorders, as decreased

expression of this molecule was associated with impaired immune response.[40-42] Previous investigations revealed the association of CD*247* genetic polymorphisms with susceptibility to systemic lupus erythematosus (SLE), a systemic autoimmune disorder.[43,44] Furthermore, the 3′ untranslated region (3′UTR) of *CD247* gene harbors a number of genetic variations, which have been attributed to downregulation of CD*247*, manifested through immune dysfunction and systemic autoimmunity.[43] CD*247*, which is T-cell surface CD3 zeta molecule, participates in signal transduction in T cells. A GWAS reported that rs2056626 SNP at *CD247* gene is associated with the risk of SSc.[4] Thereafter, this association was validated by an independent cohort study of French Caucasian population and was shown that minor G allele of rs2056626 SNP conferred susceptibility to SSc in dominant model.[45] On the other hand, SSc patients of Han Chinese did not demonstrate significant association between rs2056626 SNP and SSc or its subtypes such as diffuse (dcSSc) and limited (lcSSc) SSc.[46] In compliance with Chinese population, rs2056626 SNP did not impress the SSc risk in Iranian population. Lundholm et al. described that *CD247* might be a prime candidate gene for *Idd28*, a novel susceptible gene for autoimmune disorders such as autoimmune diabetes, by harboring mediators such as CD25 and FoxP3.[47] Therefore, it could be figured out that CD*247* might induce autoimmunity by affecting the cellular environment and could be considered as a predisposing factor rather than a pathogenic variant which might somewhat justify the lack of relation between rs2056626 variant and the risk of SSc in this survey.

CD*226*, a member of immunoglobulin super family, is the main co-stimulator of NK cells, $CD4^+$ and $CD8^+$ T cells, monocytes, platelets and certain B cells, as these cells play notable role in SSc immunopathogenesis of autoimmune disorders.[48,49] While a bulk of studies has demonstrated that the *CD226* gene rs763361 SNP is associated with the risk of several autoimmune diseases, such as rheumatoid arthritis (RA), SSc, and SLE, there are notable reports showing no associations.[19,48,50-52] Evaluation of the polymorphism in Iranian SSc population did not show significant association of this SNP with the risk of the disease. These discrepancies are probably due to inefficient statistical power, small sample sizes, clinical and ethnical heterogeneity. Nonetheless, meta-analysis could be helpful to resolve the inconsistencies and limitations of the disparate individual investigations. For this reason, Song *et al.* performed a meta-analysis in several autoimmune diseases and observed that the *CD226* gene rs763361 SNP reduced the susceptibility to SLE, SSc, and type 1 diabetes (T1D) in Asians, Europeans, and South Americans.[53]

More than 50% of SSc patients all over the world suffer from reduced FVC, which is considered as the main cause of mortality, despite the recent advances in the treatment of SSc.[54,55] In our study, we detected a significant association of FVC with three distinct rs763361 genotypes which may confirm the hypothesis of CD*226* gene variation involvement in reduced FVC of SSc patients.

Our study did not show significant association of CD*226* and CD*247* genetic polymorphisms with the risk of SSc or the presence of clinical manifestations except than FVC. In other words, CD*226* and CD*247* genetic variants may not contribute to SSc pathogenesis in Iranian population. Further studies with large sample size are encouraged in order to assess the contribution between CD*226*, CD*247* and other profibrotic factors in the blood serum of SSc patients. Alternately, considering other genetic variations discerned by GWAS in association with SSc, such as STAT and IRF genes, further studies in Iranian population aiming to dissect the exact role of immune-related molecules underlying SSc etiopathogenesis will be of great interest.

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