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Impact of alterations in X-linked *IRAK1*gene and *miR-146a* on susceptibility and clinical manifestations in patients with systemic sclerosis

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Highlights

- *IRAK1* gene expression could be a marker for better stratification of SSc patients
- Carriers CC genotype of miR-146a rs2910164 C>G have 10 times more chances to develop lung fibrosis and active form of SSc
- Reduced miR-146a expression is a risk factor for development and progression of SSc

Abstract

Systemic sclerosis (SSc) is a heterogeneous multisystem autoimmune disease with unknown etiology. Numerous studies have indicated that the disease heterogeneity implies various genetic abnormalities. Considering that SSc is characterized by a strong sex bias and that the position of *IRAK1* gene is on the X chromosome, we assume that variations in *IRAK1* gene could

explain female predominance of SSc. It was previously described that miR-146a has a role in 'fine-tuning' regulation of the TLR/NF-kB signaling pathway through down-regulation of *IRAK1* gene. The aim of the present study was to analyze both variants and expression level of *IRAK1 and miR-146a* genes in terms of susceptibility to SSc and clinical presentation of SSc patients.

We analyzed variants *IRAK1* rs3027898 C>A and *miR-146a* rs2910164 C>G in 102 SSc patients and 66 healthy subjects. Genotyping was performed by Sanger sequencing. Expression level of *IRAK1* mRNA and miR-146a in PBMCs was performed in subset of 50 patients and 13 healthy controls by RT-qPCR.

Our results showed that there was no association between *IRAK1* rs3027898 and the risk of SSc in women. However, the analysis of genotype distribution of the *mir-146a* rs2910164 C>G variant indicated that CC genotype shows strong association with lung fibrosis and active form of the disease. When expression level of *IRAK1* gene was analyzed, we detected significant downregulation of *IRAK1* mRNA in SSc patients compared to controls, as well as in male compared to female patients, in patients with ACAs autoantibodies and in patients with severe skin involvement. Regarding the expression level of miR-146a, we have found significantly reduced expression in SSc patients, in patients with skin involvement and in male SSc patients.

The results from this study indicate that expression of *IRAK1* gene could explain phenotypic heterogeneity of SSc and may be involved in the pathogenesis of SSc due to its differential expression in certain subgroups. Our results also suggested that *miR-146a* rs2910164 CC genotype may be predisposing factor for development lung fibrosis and more progressive form of SSc. Results from relative expression analysis of miR-146a demonstrated that changes in the level of this miRNA may have an impact on development and clinical course of SSc.

Key words: Systemic Sclerosis; Biomarker; IRAK1; miR-146a; gene expression; SNV[1]

IRAK1- Interleukin 1 Receptor Associated Kinase 1

TIR- Toll-like/ IL-1 receptor

NF-κB- Nuclear Factor kappa- B

3'UTRs- 3'-untranslated regions

TRAF6- Tumor Necrosis Factor (TNF) Receptor-associated Factor 6

SNV- Single base Alteration

ESR test- Erythrocyte Sedimentation Rate test

VC- Vital Capacity,

DLCO- Diffusing Capacity of the Lung for Carbon Monoxide

HRCT- High Resolution Computed Tomography

MRSS- Modified Rodnan Skin Score

ACAs- anti-centromere antibody

ATAs- anti-topoisomerase antibody

ANAs- more than one type antinuclear antibody

non-RP- non-Raynaud's phenomenon

PBMCs- Peripheral Blood Mononuclear Cells

1. Introduction

Systemic sclerosis (SSc) is a heterogeneous multisystem autoimmune disease characterized by immune abnormalities, inflammation, microangiopathy, excessive extracellular matrix deposition and progressive interstitial and perivascular fibrosis in the skin and many other organ systems [1, 2]. Due to relative rarity of the disease and the large variability in the clinical manifestations, the diagnosis of the SSc can be difficult. Moreover, the etiology of the disease is largely unknown and therefore the effective medical treatment for SSc is still missing [3]. As is the case with the most of autoimmune disorders (ADs), SSc represents a clear example of a sex biased immune disorder, with women being affected more frequently than men [4], reaching almost 90% of total affected individuals [5]. To date, very little is known about the possible association between the genes located on the sex chromosomes with their role in the immune system homeostasis and the susceptibility of the female sex to ADs [6, 7]. Interestingly, recent reports have indicated that *IRAK1* (*Interleukin 1 Receptor Associated Kinase 1*) gene, was the first X chromosome-linked gene associated with susceptibility and female predominance to systemic lupus erythematosus (SLE) [8] and rheumatoid arthritis (RA) [9]. IRAK1 is a serine/threonine protein kinase with significant role in TLR/IL-1 receptor (TIR) activation of NF-κB transcription factor, which subsequently increases the expression of many genes related to immunological functions [10-12]. Altered activation of NF-κB represents the critical event in pathogenesis of some ADs, acting through aberrant transcriptional regulation of many proinflammatory mediators [13]. Since IRAK1 is considered to be the “on-switch” of TIR signaling complex, it is recognized as a risk gene in ADs [8, 14].

Although the etiology and pathological mechanisms of SSc have not been completely elucidated, there are growing evidence pointing out to the possible role of miRNAs in the pathogenesis of SSc [15]. MicroRNAs are short non-coding RNA sequences (20–23 nucleotides) which function as posttranscriptional regulators by binding to complementary sequences in the 3'-untranslated regions (3'UTRs) of target mRNA, leading to the translational suppression or to mRNA destabilization and degradation in order to maintain homeostasis [16-18]. In the human genome, miRNA-coding genes comprise 5% of all human genes, making them the most abundant class of regulators that can control the expression of 30% of protein-coding genes [19].

MiRNAs are involved in a broad spectrum of cellular functions, like in developmental timing [19], cell differentiation, proliferation and apoptosis, signal transduction and organ development [20, 21]. These findings point out that the proper miRNA regulation is necessary for the prevention of autoimmunity and maintenance of normal functioning of the immune system [22-24]. Among them, recently described miR-146a, which is expressed predominantly in immune tissues, plays the pivotal role in "fine-tuning" negative feedback regulation of TIR signaling pathway through down-regulation of TRAF6, and IRAK1 [25]. This leads to reduced activation of NF-κB and production of pro-inflammatory mediators such as IL-6, TNF-α, IL-1b, and IL-17 [25, 26]. Recent studies have suggested that alteration in the expression of miR-146a could be associated with pathogenesis of ADs, such as SLE, RA, and Sjögren's syndrome (SS) [24].

Single nucleotide variation (SNV) in the DNA sequences of miRNA-coding genes, even outside of mature miRNA sequence, may alter the expression and/or maturation of miRNA [27]. Also, the existence of the SNVs in the mRNAs target sites can result in the prevention of their recognition by miRNAs that could consequently lead to the lack of inhibition or degradation of the target mRNAs [28]. This could have complex functional consequences and may contribute to some pathological conditions [29]. Recently, a common rs2910164 C>G variant within the pre-miR-146a sequence was reported to be involved in the regulation of mature miR-146a expression and its function indicating the potential role of this variant in the pathogenesis of several diseases, including various cancers [30, 31] and ADs, like RA [32], multiple sclerosis [33], and ulcerative colitis [34]. Similarly, variant rs3027898 C>A in the *IRAK1* gene was reported to be associated with RA, ankylosing spondylitis, and psoriatic arthritis (PsA) susceptibility [32, 35]. This variant, which is located in 3'-UTR of *IRAK1* gene, doesn't reside into a miR-146a target site, but may

affect the correct folding of the 3'-UTR of *IRAK1* mRNA and disrupt the posttranscriptional IRAK1 regulation [36].

In an effort to better understand the pathogenesis of SSc, we have investigated the possible association of *miR-146a* rs2910164 and *IRAK1* rs3027898 variants with susceptibility to this disease. Also, our aim was to determine whether these variants have an effect on miR-146a and *IRAK1* expression levels, and to explore possible correlation between *IRAK1* mRNA and miR-146a in the SSc patients.

2. Material and Methods

2.1 Patients and control subjects

The current case-control study was performed with 102 patients (88 females, 14 males) with SSc with an average age of 57.7 ± 11.0 years. The control group was consisted of 66 healthy subjects (56 females, 10 males) matched to patient's group by ethnicity, gender and age, and unrelated to the SSc patients (mean age of 55.2±10.2 years). Relative expression analyses were performed on a subgroups of 50 SSc patients (45 women and 5 men, mean age 59.6 ± 9.3 years,), and 13 healthy individuals, (10 women and 3 men, 57.2 ± 11.5 years). Demographic, clinical and serological characteristics of SSc and healthy individuals participated in this study are shown in Table 1.

Patients with SSc were recruited from the Institute of Rheumatology, Clinical Center of Serbia in Belgrade. Written informed consent from all participants and approval from the local ethical committees were obtained in accordance with the tenets of the Declaration of Helsinki. In all cases, SSc patients which fulfilled EULAR 2013 classification criteria for SSc, and had at least one visceral organ involvement were included in the study [37]. For each patient the following data were collected and reported: age, gender, duration of disease, subtype of SSc by pattern of skin involvement, laboratory findings (*erythrocyte sedimentation rate* (*ESR*) *test*, creatinine clearance, C3 and C4), parameters of lung function (VC-vital capacity, DLCO-diffusing capacity of the lung for carbon monoxide), chest X ray findings and/or high resolution computed tomography (HRCT) of lungs and heart ultrasound findings. To perform the subphenotype analyses, SSc patients were divided into subgroups based on relevant clinical parameters. Relying

on modified Rodnan skin score (MRSS) which is a standard outcome measure for skin disease in SSc and is calculated by summation of skin thickness in 17 different body sites, SSc patients were divided into two groups, one with mild (MRSS≤10) and another with severe (MRSS>10) skin involvement. Based on pattern of skin involvement, SSc patients were separated into limited cutaneous SSc (lcSSc) or diffuse cutaneous SSc (dcSSc) group according to the criteria by Leroy *et al.* [38]. Disease activity was assessed by using the criteria proposed by Valentini *et al.* [39], in which evaluation of clinical and laboratory factors results in a score ranging from 0 to 10 (0 represents no disease activity, and 10 represents maximal activity). SSc patients with a score ≥3 were classified as patients with active form of SSc, while patients with score <3 were classified as patients with stable form of SSc. All SSc patients were separated into groups based on the presence of scleroderma-associated autoantibodies including ACAs (anti-centromere), ATAs (anti-topoisomerase), ANAs (more than one antinuclear antibodies) or the absence of these three antibodies (Ab-Neg), detected as previously described [40]. Presence of pulmonary fibrosis was defined radiologically by positive chest X ray findings or ground-glass opacification on HRCT. Disease duration was the time between the onset of the first non-*Raynaud's phenomenon* (non-RP) manifestations and time of the inclusion into the study. SSc patients were grouped according to disease duration: < 3 years, and ≥ 3 years. The most of the patients analyzed received some kind of therapy: endoxan, imuran, prednisone and methotrexate, or combination (Table 1).

2.2 Blood sampling, DNA extractions and genotyping

After informed consent was obtained, blood samples from the 102 patients with SSc and 66 healthy individuals were collected into two 4.5ml sodium citrate anticoagulant tubes (Vacutainer, Becton-Dickinson, Plymouth, UK). Genomic DNA was extracted from whole peripheral blood with QIAampDNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany), and stored at -20°C until analysis. All subjects were analyzed for genetic variants in *miR-146a* (rs2910164 C>G) and *IRAK1* (rs3027898 C>A) genes by direct sequencing of PCR product using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on 3130 Genetic Analyzer (Applied Biosystems, USA). Information about primer sequences and PCR product size are specified in Table 1. in Supplementary material.

2.3 RNA extraction, cDNA preparation and relative quantification analyses (RT-qPCR)

For the expression analyses, samples of the subgroup of 50/102 SSc patients and 13/66 control were available for isolation of peripheral blood mononuclear cells (PBMCs) by Ficoll density-gradient centrifugation (GE Healthcare, Sweden). Total RNA was extracted using TRI Reagent Solution (Ambion, USA) according to the manufacturer's protocol. All RNAs were quantified spectrophotometrically according to 260/280 ratios. For detection of expression level of *IRAK1* gene, complementary DNA (cDNA) samples were synthesized from 1µg of total RNA by using RevertAid M-MuLV Reverse Transcriptase (ThermoFisher Scientific, USA) in an ultimate volume of 20µl. *IRAK1* mRNA expression was determined using TaqMan gene expression assay (Hs01018347_m1, ThermoFisher Scientific, USA) and KAPA PROBE Universal qPCR kit (KAPA Biosystems, USA). Real-time PCR was performed on an on an ABI 7500 Real-Time PCR System (Applied Biosystems, USA). The Glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) was used as an endogenous control to normalize transcript levels of total *IRAK1* mRNA of each sample. The quantity of miR-146a was determined by reverse transcription of 20 ng of RNA from each sample using a TaqMan miR-146a Gene Expression Assays (ThermoFisher Scientific, USA) based on commercial miRNA-specific stem-loop primers. In subsequent studies, TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal PCR Master Mix, No AmpErase UNG were obtained from Applied Biosystems, UK. The expression level of each sample was normalized to that of RNU6B, a reference small nuclear RNA (TaqMan microRNA Control Assay RNU6B, ThermoFisher Scientific, USA). ABI 7900HT Real-Time PCR System (Applied Biosystems, USA) were employed for all real-time PCR analysis. Relative quantification analysis was performed by a comparative ddCT method, using median expression level of the healthy control group as the calibrator (SDS 1.3.1.21, Applied Biosystems, USA). All experiments were performed in duplicates.

2.4 Statistical analysis

Genotype frequencies in patient and control groups were checked for the Hardy–Weinberg equilibrium (HWE) by using goodness-of-fit $\chi 2$-test. The differences in the allele and genotype distributions between patients and controls were performed by Chi-square test with Yates's

correction or Fisher exact test wherever appropriate. Recessive model was used for comparisons between genotypes, where homozygous for minor allele was compared to group for homozygous major allele plus heterozygous genotype. Considering multiple testing, Benjamini-Hochberg correction has been introduced [41]. The false discovery rate was set at the level of 0.1. Odds ratio (OR) and 95% confidence interval (CI) were calculated as a measure of the association between variants in *miR-146a* and *IRAK1* genes and SSc risk. For the relative expression analyses data are presented as medians with range, means ± SD, or as absolute numbers with percentages. Distributions of continuous data were tested with the Shapiro-Wilk and Kolmogorov-Smirnov tests. Differences in this variables were analyzed using nonparametric Mann-Whitney *U* test for distribution between 2 groups. Correlations between variables within the group of SSc patients were analyzed using Spearman's rank-order correlation coefficient (r). The statistical analyses were performed using the SPSS computer software 20.0 ((SPSS, Inc, Chicago, IL, USA)). For all analyses, the *p* values were 2-tailed, and $p < 0.05$ was considered statistically significant.

3. Results

3.1 Association between IRAK1 rs3027898 C>A and miR-146a rs2910164 C>G variants and susceptibility to SSc

Comparison of variants in *IRAK1* and *miR-146a* genes in SSc patients and healthy controls was performed through recessive model.

Considering positioning of *IRAK1* gene on X chromosome, male individuals were not included in associative study for this gene. The distribution of *IRAK1* rs3027898 genotypes was in HWE for female control subjects (χ^2=1.493, p= 0.22) while genotypes of female SSc patients were not consistent with HWE (χ^2=6.024, p<0.014). The genotype frequencies and the results of statistical analysis of the *IRAK1* rs3027898 variant are presented in Table 2. The comparison of genotype frequencies of rs3027898 C>A variant showed no association neither between female SSc patients and controls nor between phenotypic subgroups of patients.

When *miR-146a* rs2910164 C>G was tesed, both patient's and control group were in HWE (χ^2=0.364, p=0.546 and χ^2=0.022, p=0.883, respectively). There were no statistically significant difference between SSc patients and control group regarding genotype and allele distribution. However, statisticaly significant difference was found between patients with lung fibrosis and active form of the disease compared to group of patients without lung fibrosis and non-active disease, respectively (OR=0.082, 95% CI=0.009-0.772, p=0.019; OR=0.101, 95% CI= 0.011-0.948, p=0.033) (Table 3). This results were significant also after correction for fals discovery rate .

3.2 Expression analyses of IRAK1 gene and miR-146a

Analyzing the expression level of *IRAK1* gene, we have found that the expression level among SSc patients was 42% lower than the expression level found among healthy individuals, reaching statistical significance (median 0.517 (0.090 – 1.861) vs. median 1.000 (0.449 - 1.93) p=0.003) (Fig. 1a). Downregulation of *IRAK1* gene was more prominent among male compared to female patients (p=0.042) (Fig. 1b). We have also detected that the group of SSc patients associated with the presence of ACAs had significantly reduced *IRAK1* gene expression compared to patients with ATAs (p=0.032, Fig. 1c). Reduction was also significant in group of patients with severe skin involvement compared to the group of patients with mild skin involvement (p=0.021) (Fig. 1d). More detailed description of the results of *IRAK1* gene expression analysis are reviewed in Supplementary Table 2.

The level of miR-146a expression in SSc patients was significantly lower compared to non-SSc subjects (median 0.215 (0.000 – 8.730) *vs*. median 1.000 (0.113 – 4.138), p<0.001) (Fig. 2a). Interestingly enough, we have detected that the male patients had higher miR-146a expression level compared to female subjects (p=0.005, Fig. 2b). After dividing SSc patients into different groups based on specific clinical features, we have found that SSc patients with mild skin involvement (MRSS score ≤10) have expressed considerably less amount of miR-146a than SSc patients with severe skin involvement (less by 36% compared to severe, p=0.045, Fig. 2c). Detailed description of miR-146a relative quantification analysis are summarized in Supplementary Table 3.

Correlation analysis between *IRAK1* and *mir-146a* expression levels within the group of SSc patients revealed strong negative correlation (Spearman's rho= -0.517, p<0.001). However,

determination coefficient for these two variables was r^2 =0.267, and therefore the expression level of miR-146a could explain only 27% of variance of *IRAK1* gene expression.

3.3 Association of the IRAK1 and miR-146a variants with their expression level

To gain insight into the mechanism underlying the observed association between *IRAK1* and miR-146a expression levels, we investigated the influence of the *miR-146a* rs2910164 variant on the production of mature form of miR-146a. Quantification of miR-146a expression showed that carriers of the C allele had decreased miRNA levels compared to the carriers of the GG genotype but without statistical significance (CC carriers had 1.307-fold and GC carriers 2.260 fold decreased miR-146a level compared to GG carriers). These data suggested that this C>G variant might affect the production of mature form of miR-146a. When we analyzed the influence of *IRAK1* gene rs3027898 variant on its relative expression level, we hadn't found any correlation between carriers of A or C alleles and detected level of *IRAK1* mRNA.

4. Discussion

Several ADs, including SSc, are characterized by a strong sex bias, with females being affected more frequently than males [4, 8]. Very little is known about whether genes on the sex chromosomes can directly influence on susceptibility to SSc. In the present study, we focused on *IRAK1* gene, in regard to its position on Xq28 genomic region, and its regulator, miR-146a, in order to elucidate their joint role in susceptibility to SSc and predominant occurrence of SSc in female. Therefore we performed associative study on *IRAK1* rs3027898 C>A variant, as well as *miR-146a* rs2910164 C>G variant.

IRAK1 rs3027898 C>A variant is located in 3'-flanking region of the gene, 68 bp from transcription end site, close to the binding site of the posttranscriptional regulator miR-146a. The role of this variant have been connected to some AD so far. Song *et al*. [42] revealed an association between inflammatory arthritis and the rs3027898 CC genotype, while the study of Yang *et al.* showed that the C allele was associated with an increased risk for RA in patients from China [43]. In a cohort of SLE patients, Changzheng *et al.* have found an increased risk for carriers of *IRAK1*

rs3027898 C allele izmeniti reference [44]. Yet, in the present study we found no association between *IRAK1* rs3027898 C>A variant and the risk of SSc.

Considering *IRAK1* expression analysis, only a small number of studies dealing with the *IRAK1* expression patterns in various ADs have been published. The results of these studies are very contradictory. Study on RA patients didn't reveal any difference regarding *IRAK1* expression level between patients and control subjects [45], while the study on psoriatic patients showed overexpression of *IRAK1* gene [46]. Likewise our study, the expression of *IRAK1* gene was significantly decreased in SS patients analyzed by Zilahi *et al.* [47]. Our study revealed 42% decreased *IRAK1* expression level in SSc patients compared to control samples. Based on this finding, we suggest that the *IRAK1* expression level could serve as a potential biomarker for SSc. When we compared female with male patients, we have found lower expression in men, suggesting that expression level of this gene is gender dependent. Reduced expression level of *IRAK1* can be associated with some distinct clinical features of the SSc, like the presence of ATAs and severe skin involvement, suggesting that it can be used as a marker for better stratification of SSc patients based on individual molecular signature.

We have also analyzed the influence of *IRAK1* gene rs3027898 variant on its expression level. We haven't found any significant correlation between certain genotypes and detected level of *IRAK1* mRNA, indicating that this variant is not crucial for aberrant *IRAK1* expression. Based on our findings as well as others, we suggest that in the further studies the impact of other variants, gene-gene, and gene-environment interaction on *IRAK1* expression must be taken into consideration.

MiR-146a was one of the first miRNAs known to be involved in innate immune response, and therefore associated with the occurrence of several types of cancers [31, 48] and autoimmune disorders [49, 50]. A number of associative studies examining *mir-146a* rs2910164 variant in ADs have been performed, mostly showing no significant role of this genotype for susceptibility to RA [32, 51], SLE [52] or PsA [35]. In accordance to these findings, we didn't find that *mir-146a* rs2910164 variant contribute to susceptibility to SSc in our population. Interestingly, we detected significantly higher prevalence of CC genotype in SSc patients with lung fibrosis and active form of the disease, suggesting that carriers of this genotype have 10 times more chances to develop these specific clinical manifestations.

Regarding *mir-146a* gene expression, level of miR-146a was significantly decreased (by 67%) in our cohort of SSc patients, compared to control samples. In addition, we have found significant increase of expression of miR-146a in male SSc patients, which is consistent with finding of decreased *IRAK1* gene expression found in cohort of our male patients, showing direct regulation of expression of *IRAK1* gene by this specific miRNA. Likewise, the aberrant expression of miR-146a has been shown to correlate with mild skin involvement. Numerous studies on ADs have shown the controversial observations regarding the miR-146a expression levels, especially depending on the tissue that have been investigated. Elevated miR-146a expression was observed in synovial tissue, serum and PBMCs of RA patients and was correlated with disease [45, 50, 53]. Similar findings were reported in study of Sjogren's syndrome [47], and psoriasis [19, 54], where overexpression of miR-146a upregulation was observed in skin lesions of these patients. Downregulation of miR-146a expression have been shown in patients with Type 2 diabetes and SLE [55, 56]. Taking these results together, we can speculate that reduced miR-146a expression represents a risk factor for development and progression of SSc.

The analysis of an impact of *miR-146a* rs2910164 variant on production of mature miR-146a in our SSc patients showed a trend towards the reduction of miR-146a expression level among C allele carriers, compared to the GG genotype carriers. Our finding are supported by studies done by Yue *et al*. [48] and Shen *et al.* [57], showing that this variant causes elevated expression of mature miR-146a. In contrast to this, there are reports showing that C>G substitution in pre-miR-146a results in a reduced efficiency of pre-miR processing to mature form, therefore lowering miR-146a expression level [30, 58]. It remains to be elucidated whether this natural genetic variation in pre-miR-146a can affect the amount of miR-146a, and therefore contribute to the genetic predisposition to SSc.

Correlation analysis within the group of our SSc patients revealed the strong negative correlation between *IRAK1* and *miR-146a* expression levels. These results are consistent with the data that, beside TRAF6, IRAK1 can act like a second target for miR-146a [25], altering NF-KB signaling pathway. It has already been shown that the alteration of this signaling pathway mediate skin fibrosis [59].

5. Conclusion

The data presented here provide first insight into the association between *IRAK1* gene variant and its expression level and female predominance of SSc, indicating that gene located on the sex chromosome can directly influence the predisposition and phenotypic heterogeneity of SSc, and may be a gene relevant for the pathogenesis of SSc. Our study also suggests that *miR-146a* rs2910164 variant may be involved in the mechanisms of the lung fibrosis development and the more progressive form of SSc. Results from relative expression analysis of miR-146a demonstrated that changes in the level of a single miRNA may have a significant impact on development of SSc.

However, the main limitation of this work is relatively small sample size. Future studies with larger sample and control sizes are required to confirm these results and to enable better understanding of their precise role in the pathogenesis to SSc. Another limitation of our study relays to the fact that the SSc phenotype can progress during the course of the disease, and thus prospective follow up of the SSc cohort is needed to improve subphenotypic analyses. Nevertheless, this study represents a significant step towards better understanding of how analyzed molecular markers contribute to the genetic predisposition to SSc, how they can be used as potential diagnostic and prognostic biomarkers, and a possible new targets for future therapies of SSc.

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Table 1. Demographic, clinical and serological characteristics of SSc patients and healthy controls

		case-control study		relative expression study	
		SSc patients	Healthy individuals	SSc patients	Healthy individuals
Demographic data					
Number		102	66	50	13
Age	mean ± SD	57.7 ± 11.0 yr	55.2±10.2 yr	58.9 ± 9.2 yr	57.2±11.5 yr
	Median	59.0 yr	55.5 yr	61.0 yr	54 yr
Sex distribution	Women	88 (86%)	56 (83%)	45 (90%)	10 (77%)
	Men	14 (14%)	10 (17%)	5 (10%)	3 (23%)
Clinical characteristics					
Disease duration*	mean ± S.D	8.9 ± 8.2 yr		8.0 ± 6.4 yr	
	Median	6.5 yr		6.0 yr	
	Early form	28 (27%)		13 (26%)	
	Late form	74 (73%)		37 (74%)	
Clinical subtypes**	Diffuse	22 (22%)		8 (16%)	
	Limited	79 (77%)		42 (84%)	
Disease activity***	Active	32 (31%)		17 (34%)	
	Stable	70 (69%)		33 (66%)	
Renal impairment		22 (22%)		12 (24%)	
Lung impairment****		65 (64%)		27 (54%)	
Digital ulcers		9 (18%)		9 (18%)	
Lung fibrosis		28 (27%)		18 (36%)	
MRSS score	≤10	63 (62%)		25 (50%)	
	>10	39 (38%)		25 (50%)	
Serological characteristics					
ACA-positive		35 (34%)		19 (38%)	
ATA-positive		28 (27%)		15 (30%)	
ANA-positive		25 (25%)		12 (24%)	
Ab-Neg		14 (14%)		4 (8%)	
Therapy					
	endoxan	69 (67%)		35 (70%)	
	Imuran	19 (18%)		11 (22%)	
	prednisone	40 (39%)		22 (44%)	
	methotrexate	46 (45%)		20 (40%)	

* Disease duration from 1st non-Raynaud's symptom; Early form-duration less than 3 years and late form- duration of 3 or more years;
** Two distinct subtypes on the basis of the pattern of skin involvement and the timing and type of internal organ involvement
*** Disease activity was assessed by using the criteria proposed by Valentini *et al.*[39] to active form with score ≥3 and stable form of disease with score <3;
**** Patients were considered to have lung impairment if measured DLCO value was less than 80%.

Table 2. Genotype distributions and summarized results of association study of *IRAK1* rs3027898 C>A among female SS patients and female controls

	type	AA	CA	CC	OR 95%CI	P value*
Patients vs controls	patients (88)	58	22	8	0.566 0.280-1.102	0.130
	controls (56)	29	25	2		
Skin involvement	Limited (73)	50	17	6	1.902 0.616-5.878	0.407
	Diffuse (15)	8	5	2		
Lung fibrosis	Yes (22)	14	6	2	1.143 0.417-3.132	1.000
	No (66)	44	16	6		
Renal impairment	Yes (19)	14	4	1	0.629 0.202-1.952	0.586
	No (69)	44	18	7		
Disease activity	Stable (62)	39	17	6	0.625 0.228-1.712	0.501
	Active (26)	19	5	2		

***CA+CC vs AA**

GeneBank accession number for referent sequence used for the analysis: NM_001569.3 for *IRAK1* rs027898 gene variant

Table 3. Genotype distributions and summarized results of association study of *miR-146a* rs2910164 C>G among SS patients and controls

	types	GG	GC	CC	OR 95%CI	P value*
Patients vs controls	Patients (102)	57	40	5	0.606 0.114-3.220	0.705
	Controls (66)	46	18	2		
Skin involvement	Diffuse (22)	13	8	1	1.105 0.117-10.423	1.000
	Limited (80)	44	32	4		
Lung fibrosis	Yes (28)	17	7	4	0.082 0.009-0.772	**0.019**
	No (74)	40	33	1		
Renal impairment	Yes (22)	14	7	1	1.105 0.117-10.423	1.000
	No (80)	43	33	4		
Disease activity	Stable (70)	40	29	1	0.101 0.011-0.948	**0.033**
	Active (32)	17	11	4		

***GG+GC vs CC**

GeneBank accession number for referent sequence used for the analysis NR_029701.1 for *miR-146a* rs2910164 variant

Fig. 1. Analysis of relative expression of *IRAK1* gene in samples of SSc patients and healthy controls and different subgroups of SSc patients.

Relative expression analysis of a) *IRAK1*gene in PBMCs samples of SSc and healthy controls. Comparison of *IRAK1* expression level between different subgroups of SSc patients based on demographical, specific clinical or serological parameters: b) male and female SSc patients, c) ACAs and ATAs positive SSc patients, d) SSc with mild and severe skin involvement patients. The line inside the box represents the median. Each box represents the 25th to 75th percentiles of data.

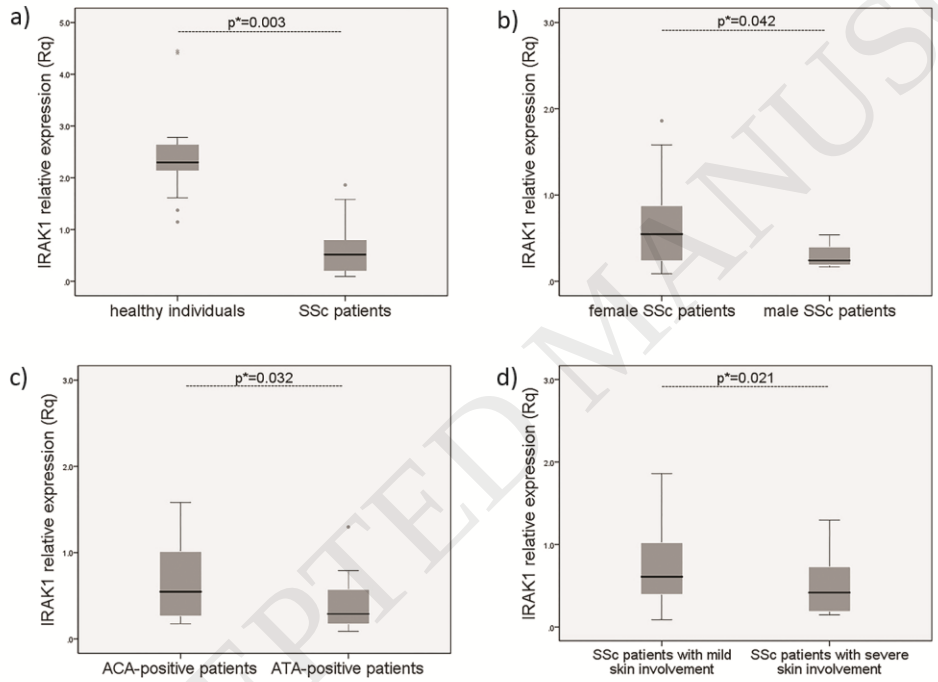
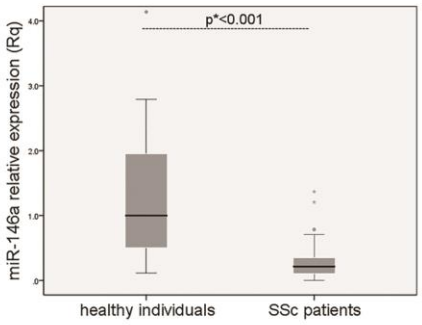


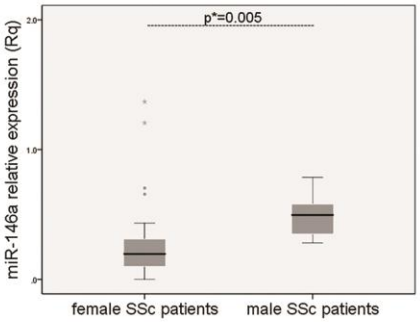
Fig. 2. Analysis of relative expression of miR-146a in samples of SSc patients and healthy controls and different subgroups of SSc patients.

Relative expression analysis of a) miR-146a in PBMCs samples of SSc and healthy controls. Comparison of miR-146a level between: b) male and female SSc patients and c) SSc patients with mild and severe skin involvement. The line inside the box represents the median. Each box represents the 25th to 75th percentiles of data. ATAs: anti-topoisomerase antibodies; ACAs: anti-centromere autoantibodies.

a)



b)



c)