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Reduced gene expression of S*urvivin* in PBMCs from patients with limited systemic sclerosis

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Systemic sclerosis (SSc) is a rheumatologic disease, and fibroblasts are the main cells responsible for SSc pathogenesis. The *BIRC5* gene encodes survivin, an inhibitor of apoptosis protein. Studies have suggested a role for survivin overexpression in leading to decreased apoptosis of fibroblasts in SSc patients. This study explored the frequencies of two single nucleotide polymorphisms (SNPs) in the *BIRC5* gene (rs9904341 [G>C] and rs17878467 [C>T]) in SSc patients and evaluated survivin gene expression in the peripheral blood mononuclear cells (PBMC) of patients and compared it with that of healthy individuals. The allelic and genotypic frequencies of rs9904341 in 459 SSc patients and 487 healthy controls were assessed. For the rs17878467 SNP, the survivin gene in 214 SSc patients and 246 controls was analyzed. Genomic analyses were carried out on DNA samples isolated from whole blood by the phenol-chloroform method. TaqMan rt-PCR was used to investigate the survivin gene alleles. Survivin gene expression was also investigated in 53 patients (lSSc = 25, dSSc = 28) and 55 controls by specific primers for the survivin gene (SYBR Green Real-time PCR method). The allelic and genotypic frequencies of both SNPs showed no significant difference in patients and controls; however, survivin expression level was significantly lower in limited SSc (lSSc) and total SSc patients than in controls. The results suggest that survivin might have a role in the pathogenesis of SSc; however, more research is needed to confirm the relationship.

Keywords: systemic sclerosis, survivin, single nucleotide polymorphism, peripheral blood mononuclear cell

Introduction

Systemic sclerosis (SSc) mainly affects connective tissue in the body with immune system-related pathogenesis [1]. Many factors are suggested to have an impact on the increased risk of SSc in patients, such as genetic factors [2] sexuality, ethnicity, and geographical factors [3]. Environmental exposure to toxins like silica, vinyl chloride, organic solvents, and pesticides can also increase the risk of SSc [4]. Two types of SSc are described according to the location and size of the affected skin. The limited type (lSSc) affects the head, neck, hands, forearms, feet, and calves. The diffuse form (dSSc), however, has a broader distribution extending to the trunk and limbs [5]. The heart, lungs, kidneys, bones, and gastrointestinal tract are more influenced in dSSc, leading to a lower survival rate. The autoantibodies produced by the immune systems of SSc patients reveal the autoimmune nature of the disease. Moreover, there is a correlation between SSc types and the types of autoantibodies found in the patients' blood, as anti-topoisomerase I, anti-fibrillarin, and anti-RNA polymerase III antibodies appear more in dSSc patients. Indeed, anti-centromere and anti-Th/To antibodies are observed more often in lSSc [5].

The extracellular matrix (ECM) is a component of connective tissue, and fibroblasts are the primary producers of ECM. Collagen types I and III, fibronectin, elastin, and hyaluronic acid are synthesized in fibroblasts and then secreted into the ECM [6]. Transforming growth factor β (TGF-β) is secreted by tissue macrophages and

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injured epithelial cells, and it activates fibroblasts and initiates fibrosis and scar tissue formation [7]. TNF-α restricts TGF-β-enhanced collagen production by interfering with TGF-β signaling pathways and inhibiting collagen gene transcription. Other cytokines secreted by different subsets of helper T cells can influence fibrosis. Th_2 secretes IL-4 and IL-13, thus increases collagen synthesis, while INF-γ secreted by Th_1 cells reduces it [8].

Fibroblasts can differentiate into myofibroblasts that have cytoplasmic contractile actin filaments. Furthermore, myofibroblasts can originate from pericytes, bone marrow-derived fibroblasts [9], tissue mesenchymal cells, and hepatic stellate cells [10]. The contraction of myofibroblasts moves the edges of the wound toward each other and helps in wound healing. Myofibroblasts also help in the fibrosis process by secreting collagen and fibronectin as well as vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF) that increase angiogenesis and collagen production, respectively. Indeed, myofibroblasts help in the crosslinking of collagen fibrils by producing extracellular enzymes such as lysyl-oxidase (LOX) and transglutaminase-2 [11]. In healthy conditions, after the tissue repair process is completed, myofibroblasts and fibroblasts undergo apoptosis. In contrast, fibroblasts of SSc patients exhibit resistance to Fas receptor-mediated apoptosis, which is not related to Fas expression on the cell membrane [12].

Baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5), known as survivin, is an inhibitor of apoptosis protein (IAP) that has only one baculovirus IAP repeat (BIR). Dimerization of survivin and the X-linked inhibitor of apoptosis proteins (XIAP) decreases its degradation and inhibits the intrinsic pathway of apoptosis by binding to caspase 9. Moreover, by binding to the second mitochondria-derived activator of caspases (SMAC) or Diablo, it interferes with the induction of apoptosis [13, 14]. Some previous studies have shown that survivin expression is higher on the fibroblasts of SSc patients than healthy controls [15]. This difference in the expression level of survivin could be a result of mutations in the promoter of the gene which controls DNA transcription. The current study evaluated the frequencies of genotypes and alleles of two SNPs in survivin gene promoters (rs9904341 and rs17878467) in SSc patients and analyzed the relationships of these SNPs and the risk of SSc development. Moreover, the survivin expression levels of peripheral blood mononuclear cells (PBMCs) from SSc patients were also measured.

Materials and Methods

Participants

Four hundred fifty-nine patients of the Rheumatology Research Center (RRC) with confirmed SSc diagnoses based on the ACR criteria (American College of Rheumatology) for systemic sclerosis as well as 488 healthy controls were selected for this case-control study [16]. Inclusion criteria for participants were age over 16 years, Iranian ethnicity, and no previous history of autoimmune diseases. Controls and patients were matched for gender, ethnicity, and age. Five milliliters of peripheral blood was obtained from each participant. Analysis of survivin gene polymorphism rs9904341 was carried out for all patients and 487 control participants. Gene analysis of rs17878467 was carried out for 214 patients and 246 controls. Furthermore, 53 patients (12 males, 41 females) and 55 controls (7 males, 48 females) were chosen randomly for observation of survivin gene expression. Erythrocyte sedimentation rates (ESR) were assessed for all participants (Table 1). All participants provided written informed consent before the blood draw, and the study was performed based on the Declaration of Helsinki guidelines. The Ethics Committee of Tehran University of Medical Sciences approved this study (Approval No: IR.TUMS.VCR.REC.1396.42.15).

Genotyping

DNA was isolated from whole blood samples using the Phenol-Chloroform method [17]. The purity of the DNA samples was assessed with the ratio of absorbance (OD) at 260 nm (DNA) versus 280 nm (proteins) by NanoDrop spectrophotometry (NanoDrop ND-2000C Spectrophotometer, Thermo Fisher Scientific, USA).The DNA samples were stored at -20 °C. SNPs were investigated by the TaqMan real-time polymerase chain reaction (PCR) method using ABI Real-time PCR (Applied Biosystems, Foster City, CA, USA).

Gene expression

PBMCs were isolated from peripheral blood samples using Ficoll (Lymphodex, Inno-Train) density gradient centrifugation. A High Pure RNA Isolation kit (Roche, Germany) was used for total RNA extraction from PBMCs. NanoDrop spectrophotometry (NanoDrop ND-2000C Spectrophotometer, Thermo Fisher Scientific, USA) was used to assess the purity of extracted RNA samples.

Complementary cDNA was synthesized from RNA templates using the PrimeScript *RT Reagent kit* (Takara Bio, Japan). Specific primers for *BIRC5* (survivin) and *GAPDH* were used as follows. The *BIRC5* primers were Forward primer: 5'-CCACCGCATCTCTACATTCA-3'; Reverse primer: 5'-GTCTGGCTCGTTCTCAGTGG -3', and the *GAPDH* primers were Forward primer: 5'-AAGGTCGGAGTCAACGGATTT-3'; Reverse primer: 5'-ACCAGAGTTAAAAGCAGCCCTG-3'. The SYBR® green real-time PCR method was used to assess survivin gene expression. The expression level of *GAPDH*, a housekeeping gene, was chosen as the control in the comparative C_T method, and the expression levels of each sample were calculated using the ($2^{-\Delta Ct}$) formula.

Statistical Analysis

SPSS software, version 22.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis in this study. The chi-square (χ^2) test was used to assess differences in allelic frequencies between patients and controls. Also, the association of SNP genotypes with SSc phenotypes (*p value* < 0.05) was analyzed using the chi-square test, odds

ratio (OD), and 95% confidence interval. The Benjamini-Hochberg method was used to calculate the adjusted *p values* and reduce the rate of false-positive results caused by multiple comparisons [18].The genotypic frequencies of the two SNPs in the controls and Hardy–Weinberg equilibrium (HWE) was also compared by calculating the *p values*. The cut point for *p value* significance was 0.05.

Ethics approval and consent to participate

Written informed consent was obtained from all participants of this study before enrollment in the study. The study was designed based on the Declaration of Helsinki guidelines, and approval was obtained from the Ethics Committee of Tehran University of Medical Sciences (Approval No: IR.TUMS.VCR. REC.1396.42.15).

Results

Tables 2 and 3 illustrate the allelic and genotypic frequencies of the two SNPs. The distribution of SNP genotypes of rs9904341 (*p value* = 0.844) and rs17878467 (*p value* = 0.259) in the control group was not significantly deviated from the HWE. According to the NCBI database (https://www.ncbi.nlm.nih.gov/snp), the reference allele had the highest frequency, and the minor allele frequency (MAF) is reported in Tables 2 and 3. The G allele for rs9904341 is the reference allele as indicated by higher frequencies in healthy people. In this study, the G allele had a higher frequency compared to the C allele in both SSc patients (71.24%) and controls (68.58%). Indeed, the difference in allelic frequencies of rs9904341 SNP in patients and controls was not significant (*p value* = 0.207). The GG genotype had the highest prevalence in both patients (50.32%) and controls (47.22%), and the CC genotype was the least prevalent genotype in both groups (7.84% of patients and 10.06% of controls). Analysis of genotypic frequencies of CC versus GG and GC versus GG showed that the differences between the two groups were not statistically significant (*p values* were 0.189 and 0.537, respectively). The dominancy of the C or G allele was analyzed by comparing frequencies of CC + GC versus GG and CC versus GC + GG in patients and controls; the results showed no statistically significant differences (*p values* were 0.340 and 0.234, respectively).

The frequency of the C allele (reference allele) of rs17878467 SNP was 90.65% in patients and 89.63% in controls, and the difference was not significant (*p value* = 0.605). The CC genotype had the highest frequency in SSc patients (81.77%). Indeed, 17.75% and 0.46% of patients had the CT and TT genotypes, respectively. Moreover, 79.67% of the controls had the CC genotype in rs17878467. Comparison of the rs17878467 genotypic frequencies showed that the differences in TT versus CC and CT versus CC were not significant. These results suggest that these genotypes are not related to the occurrence of SSc in patients (*p values* were 0.936 and 0.556, respectively). The results of the dominancy analysis of rs17878467 by comparing the frequency of TT + CT versus CC and TT versus CT + CC between patients and controls suggest that there is not a dominancy relationship between the two alleles (*p values* were 0.569 and 0.921, respectively).

Survivin expression levels were assessed in 53 patients (mean age 42.89 ± 9.95 years) and 55 healthy controls (mean age 42.80 ± 9.76 years). The erythrocyte sedimentation rates (ESR) of patients (21.33 ± 22.81) and controls (17.0 ± 15.41) were not significantly different in the expression group (*p value* = 0.275); however, patients had a significantly higher ESR level than controls when analyzed in all participants (*p value* < 0.001). Of 53 patients, 25 had lSSc and 28 had dSSc. *Survivin* expression levels were 0.98 ± 0.89 and 0.62 ± 0.31 (mean ± SD) in controls and SSc patients, respectively. These results suggest that survivin expression is significantly lower in SSc patients (*p value* = 0.01, FC = 0.63). Survivin expression level in patients with lSSc patients was 0.58 ± 0.36, which was significantly lower than survivin expression in controls (*p value* = 0.03, FC = 0.59). The dSSc group had a non-significantly lower survivin expression level (0.65 ± 0.30) than the controls (*p value* = 0.07, FC = 0.66) (Table 4, Figure 1).

Table 5 shows the survivin expression levels of different genotypes of the two SNPs (rs9904341 and rs17878467) in SSc patients. The survivin expression levels of rs9904341 GG, GC, and CC genotypes were 0.67 ± 0.23, 0.60±0.29, and 0.49 ± 0.19, respectively. Indeed, rs9904341 polymorphisms were significantly associated with reduced survivin expression (*p value* = 0.001). SSc patients with the CC and CT genotype of rs17878467 had 0.68 ± 0.46 and 0.73 ± 0.68 survivin expression levels, respectively. The results showed that there was no association between survivin expression and the rs17878467 genotype (*p value* = 0.782).

Table 1. Statistical comparisons of characteristics between SSc patients and healthy controls.

	Characteristics		SSc	Healthy control	*P value*
Genotyping	**Age (Year)***		42.07 ± 11.45	41.59 ± 12.09	0.546
	ESR*		19.98 ± 17.46	11.88 ± 10.56	**< 0.001**
	Sex+	Male	72/459	64/488	0.267
		Female	387/459	424/488	
	Type of SSc+	Limited	128/338	-	NA

Characteristics			SSc	Healthy control	*P value*
Expression	Ethnicity	Diffuse	210/338	-	0.747
		Persian	139/369	219/475	
		Lur	48/369	26/475	
		Kurd	35/369	29/475	
		Azeri	120/369	162/475	
		Other	27/369	39/475	
	Age (Year)*		42.89 ± 9.95	42.80 ± 9.76	0.964
	ESR*		21.33 ± 22.81	17.0 ± 15.41	0.275
	Sex+	Male	12/53	7/55	0.212
		Female	41/53	48/55	
	Type of SSc+	Limited	25/53	-	NA
		Diffuse	28/53	-	

*indicated as mean ± SD, + indicated as n/N. NA; Not applicable

Table 2. Associations between rs9904341 (G>C) and risk of SSc.

SNPs	Variation	Frequency		Association Test		
		Case (N=459)	Control (N=487)	Odds Ratio	*P value*	(95 % C.I)
rs9904341 (G>C)	G	654 (71.24%)	668 (68.58%)	-	-	-
	C	264 (28.75%)	306 (31.41%)	-	-	-
	GG	231 (50.32%)	230 (47.22%)	-	-	-
	GC	192 (41.83%)	208 (42.71%)	-	-	-
	CC	36 (7.84%)	49 (10.06%)	-	-	-
	C vs. G	-	-	0.88	0.207	(0.72-1.07)
	CC vs. GG	-	-	0.73	0.189	(0.45-1.16)
	GC vs. GG	-	-	0.92	0.537	(0.70-1.20)
	CC+GC vs. GG	-	-	0.88	0.340	(0.68-1.14)
	CC vs. GC+GG	-	-	0.76	0.234	(0.48-1.19)

Table 3. Associations between rs17878467(C>T) and the risk of SSc.

SNPs	Variation	Frequency		Association Test		
		Case (N=214)	Control (N=246)	Odds Ratio	*P value*	(95 % C.I)
rs17878467(C>T)	C	388 (90.65%)	441 (89.63%)	-	-	-
	T	40 (9.34%)	51 (10.36%)	-	-	-
	CC	175 (81.77%)	196 (79.67%)	-	-	-
	CT	38 (17.75%)	49 (19.91%)	-	-	-
	TT	1 (0.46%)	1 (0.40%)	-	-	-
	T vs. C	-	-	0.89	0.605	(0.57-1.37)
	TT vs. CC	-	-	1.12	0.936	(0.07-18.04)
	CT vs. CC	-	-	0.86	0.556	(0.54-1.39)
	TT+CT vs. CC	-	-	0.87	0.569	(0.54-1.39)
	TT vs. CT+CC	-	-	1.15	0.921	(0.07-18.50)

Table 4. Comparison of survivin expression level between limited and diffused SSC and healthy control groups.

Expression Level	Control (N=55)	SSc (N=53)	Limited SSc (N=25)	Diffused SSc (N=28)	FC, adj.P^{+}	FC, adj.P$^{\&}$	FC, adj.P$^{\$}$
Survivin	0.98 ± 0.89	0.62 ± 0.31	0.58 ± 0.36	0.65 ± 0.30	0.63, 0.01	0.59, 0.03	0.66, 0.07

All of the expression values expressed as Geometric Mean ± SD, +: comparison between SSc and HC groups, &: comparison between limited SSc and HC groups, $: comparison between diffused SSc and HC groups.

Table 5. Association between survivin expression level and SNPs (rs9904341, rs17878467) in the SSc patients.

Expression Level	rs9904341				rs17878467			
	GG	GC	CC	*P value*	CC	CT	TT	*P value*
Survivin	0.67 ± 0.23	0.60 ± 0.29	0.49 ± 0.19	0.001	0.68±0.46	0.73 ± 0.68	NA	0.782

F=fold change, adj.P= *P value* adjusted by Benjamini-Hochberg method for multiple comparisons, all of the expression values expressed as Geometric Mean ± SD, NA: not available

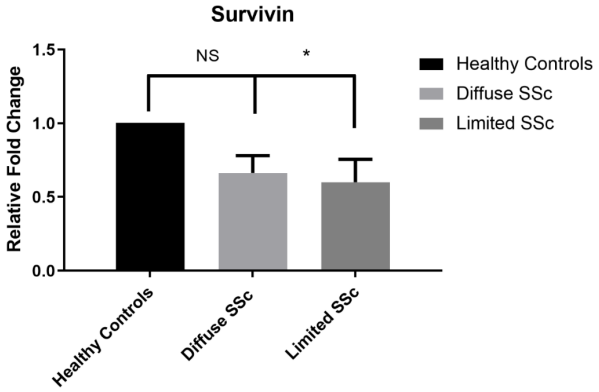

Figure 1: Significant difference in survivin expression levels between limited SSc group and healthy controls (healthy control as a reference group with fold change=1), NS: Not significant; *$p\ value \leq 0.05$

Discussion

As previously mentioned, the main pathogenic features of SSc are the general fibrosis of connective tissues and the production of autoantibodies. The fibrosis affects blood vessels leading to vasculopathy, thereby causing Raynaud's phenomenon and subsequent reperfusion injury. Collagen deposition and fibrosis can affect various parts of the body, like the lungs and kidneys [5, 19]. Fibroblasts are the most significant suspect involved in the SSc pathogenesis that produce proteins like type I collagen when they receive signals such as TGF-β, CTGF, and PDGF [19]. These signaling molecules initiate signaling pathways that affect the gene expression of ECM (extracellular matrix) proteins and even the differentiation of fibroblasts into myofibroblasts [19, 20].

Survivin acts by inhibiting apoptosis and controlling the cellular cycle. Cellular survivin levels change during different stages of the cellular cycle, and peak levels are observed in the G2M phase. Therefore, survivin contributes to the mitosis process by controlling microtubules and centrosomes. Furthermore, survivin affects the signaling pathways of apoptosis by interfering with cyclin-dependent kinase and caspases [21]. Therefore, it is one of the possible proteins that could induce resistance to apoptosis in fibroblasts. However, it has been found that TGF-β can reduce survivin expression. TGF-β binds to a membrane receptor which is composed of TbRI and TbRII and activates Smad2 and Smad3, leading to hypo-phosphorylation of retinoblastoma protein (Rb) and affecting cellular gene expression [22].

Survivin expression is suggested to have roles in rheumatoid arthritis (RA). Levitsky *et al*. analyzed survivin levels in the blood samples of 302 rheumatoid arthritis patients. Thirty-eight percent of patients (n = 114) expressed survivin at the first examination. Rheumatoid factor (RF) levels of patients with survivin expression were significantly higher than in patients who did not show survivin expression ($p\ value < 0.001$). Moreover, the survivin expression status at first evaluation was related to clinical outcomes and patient response to different treatments [23]. In another study, serum survivin levels of patients with arthralgia were used to predict RA development [24]. Bokarewa *et al.* chose 131 RA diagnosed patients and 34 controls and analyzed survivin levels in the plasma and knee-joint synovial fluids. The RA patients and erosive-RA patients had significantly higher plasma survivin levels than the controls and non-erosive-RA patients, respectively. Indeed, an evaluation of white blood cell (WBC) counts and C-reactive protein levels

(CRP) of erosive-RA patients showed that both WBC and CRP levels were significantly higher in patients with high survivin levels. Analysis of joint erosion and survivin levels further revealed that joint erosion is more likely in patients with high survivin levels with an odds ratio of 16.02 [25].

Another type of rheumatic disease with a possible association with survivin is systemic lupus erythematosus (SLE). One study analyzed the serum survivin levels of 62 SLE patients (active SLE=25, inactive SLE=37) and 92 controls. The results showed that serum survivin levels were significantly lower in the SLE group than the control group (*p value* = 0.036). However, a comparison of survivin levels between active SLE and inactive SLE patients showed that there was no significant difference [26]. Also, Koike *et al.* found that SLE patients (n = 20) had significantly lower serum survivin levels compared to controls (n = 29) [27].

In SSc patients, the results of survivin expression are controversial. Vahidi Manesh *et al.* reported that dermal fibroblasts of ten dSSc had higher survivin expression levels than controls. Moreover, miR-542 was demonstrated to regulate survivin expression, and it may be responsible for the decreased apoptosis [28]. Also, Mokuda *et al*. reported that the PBMCs of SSc patients had higher survivin expression compared to controls. Furthermore, they observed the presence of $CD1a^{+}$ dendritic cells with a $survivin^{+}$ status in skin lesions of SSc patients [29]. Koike *et al.* analyzed serum survivin and anti-survivin antibodies of lSSc patients, dSSc patients, controls (n = 29), and SLE patients (n = 20). The serum survivin and IgG anti-survivin antibodies were higher in lSSc patients than in SLE patients and controls, and the differences were statistically significant. Moreover, dSSc patients had significantly higher serum survivin and IgG anti-survivin antibodies than SLE patients and controls [27]. In contrast, the current results suggest that SSc patients in total and lSSc patients had significantly lower survivin expression in PBMCs compared to our healthy controls. However, in dSSc patients, the decrease in survivin expression was not significant. The lSSc patients had the lowest survivin expression levels compared to dSSc patients, total SSc patients, and controls. This difference in survivin expression reported in various articles may be explained by environmental factors that affect gene expression, disease stage, age, or ethnicity. Indeed, the role of survivin in tumor cells is to induce the production of VEGF via a signaling pathway including β-catenin and Tcf-Lef [30]. In contrast, as mentioned earlier, abnormal blood vessels and blood supply to tissues are two important features of SSc pathogenesis. Thus, the decreased survivin expression and hence, the decreased angiogenesis caused by lower VEGF levels might lead to SSc. This study also analyzed two SNPs of the survivin gene (rs9904341 and rs17878467) in PBMCs and their association with survivin expression. As the results showed, none of the allelic or genotypic frequencies had a significant difference comparing SSc patients and controls; however, rs9904341 had a significant association with survivin expression in SSc patients. Indeed, the mean survivin expression level of the GG genotype in rs9904341 was higher compared to the CC and GC genotypes.

Conclusion

The current results showed that none of the allelic and genotypical frequencies of the two SNPs were significantly different between patients and controls. This finding suggests that these SNPs might not affect SSc development. The results further showed that SSc patients had lower survivin gene expression compared to the controls. This disagreement among different studies on survivin expression and SSc might be due to the different disease stages and ethnicities of SSc patients in each research. More studies are needed to completely explore this subject. The relationship between survivin expression levels and lSSc or dSSc development and progression also needs more investigation.

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Conflict of interest

The authors declare they have no conflicts of interest.

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