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Downregulation of miR-542-3p Contributes to Apoptosis Resistance in Dermal Fibroblasts from Systemic Sclerosis Patients via Survivin Overexpression

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ABSTRACT

Systemic sclerosis (SSc) is characterized by excessive production of collagens by fibroblasts that leads to vast fibrosis. Resistance to apoptosis is one of the possible underlying mechanisms of fibrosis in these patients. Survivinis involved in inhibition of apoptosis and aberrantly functions in SSc. Since dysregulation of *survivin*-targeting microRNAs (miRNAs) has frequently been observed in cancer and some autoimmune disorders, this study aimed to investigate their expression status in dermal fibroblasts from SSc patients.

DiffuseSSc patients were selected according to American College of Rheumatology criteria. Isolated fibroblasts from 10 SSc and 10 healthy skin biopsies were cultured. After examining purity of the cells, mRNA and miRNAs extraction was performed followed by complementary DNA (cDNA) synthesis. Relative expressions of*survivin* mRNA, miR-16-5p, miR-320a, miR-218-5p, miR-708-5p and miR-542-3p were analyzed using real time PCR.

Survivin mRNA expression was significantly 1.85-fold upregulated in fibroblasts from SSc patients compared with healthy controls (p=0.046). Among the studied miRNAs, miR-542-3p expression was significantly decreased (p=0.033), while enhanced expression of miR-708-5p was observed in SSc fibroblasts (p=0.05) in comparison to healthy subjects. Downregulation of miR-542-3p significantly correlated with *survivin* overexpression (r=-0.45, p=0.049).

Downregulation of miR-542-3p that is correlated with higher *surviving* expression levels might be a possible cause of apoptosis resistance in SSc fibroblasts, hence providing a new understanding of the disease pathogenesis.

Keywords: Apoptosis; miR-542-3p; Survivin; Systemic sclerosis

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INTRODUCTION

Systemic sclerosis (scleroderma, SSc) is an autoimmune fibrotic disorder that mostly affects the skin and internal organs with a progressive course and fatal outcome.[1,2] Although the exact etiology of SSc has not been characterized, it has been proposed that environmental factors through epigenetic bridge cause disease in genetically predisposed individuals.[3,4] Clinical manifestations of SSc include various vascular dysfunctions, immune abnormalities, and fibrosis, mediated by various molecules and pathways.[5,6] After tissue injury, fibroblasts tilt homeostasis towards producing collagens and differentiating into myofibroblasts.[7]In opposition to normal wound healing, which is terminated by tissue contraction and cell apoptosis, unknown stimuli keep fibroblasts activated with decreased susceptibility to apoptosis. Afterwards, excessive collagen production by these long-lived cells causes fibrosis in the affected tissues.[8-11]

One of the specific mediators that is responsible for apoptosis resistance is the inhibitor of apoptosis (IAP) protein family. IAP proteins are essential for cell survival by targeting caspase enzymes as central mediators in apoptosis pathways. Survivin (also known as BIRC5) is an IAP member and plays a dual role in promoting cell cycle and inhibiting apoptosis.[12,13] The critical role of survivin in cell division is inferred from its sharp expression at Gap2/Mitosis (G2/M) phases due to inactivation of a cell cycle regulator called cyclin-dependent kinase (Cdk) 4.[14] Although the underlying mechanisms are unclear, studies have shown the apoptosis-regulating role of survivin.[15]Survivinhas gained much attention in SSc research[16,17]and previously unpublished data by our lab along with another findings pointed out to the higher *survivin* expression in these patients.[18].

MicroRNAs (miRNAs) are small (about 22 nucleotides) noncoding RNAs that regulate their target mRNAs post-transcriptionally through degradation or translational repression. They exert their effects by binding to complementary sequences in 3′-untranslated regions (3′-UTR) of mRNAs.[19]miRNAs have vastly been surveyed in autoimmunity.[4,20,21] Among the transcription factors, signaling molecules and ligands as well as other regulatory mechanisms of *surviving* expression,[22]the role of miRNAs in survivin modulation has received increasing attention in the field of cancer[23] and several critical *survivin*-targeting miRNAs have been identified to date.[23]

The role of miR-218 in *survivin* regulation has been reported in lung, breast, and nasopharyngeal cancers.[24-26]Moreover, miR-320a is involved in the profibrotic signaling pathways in pathogenesis of SSc and cancers through targeting the β-Catenin,[27] a downstream cellular protein in Wingless-type (Wnt) pathway.[28] Data has shown that miR-320a promotes apoptosis by targeting survivin and downregulated miR-320a is a possible mechanism of tumorigenesis in colorectal cancer.[27]miR-16-5p induces leukemic cell apoptosis through the negative regulation of B-cell lymphoma 2 (Bcl-2), an anti-apoptotic protein.[29] miR-16-5p targets survivin and p53, a nuclear protein which controls cell cycle and apoptosis. Data from renal cancer investigations suggest that the tumor-suppressive role of miR-708-5p is mediated through *survivin* downregulation.[30] It was also noted that this miRNA induces its proapoptotic effect by caspase induction.[31]It was observed that survivin was decreased in miR-542-3p-transfected cells, implying to the interaction of miR-542-3p with target *survivin*mRNA.[32]

Considering the higher expression of the apoptosis inhibitory molecule of survivin in SSc patients, and relentless characteristics of SSc fibroblasts in apoptosis, the current study examined expression levels of five predicted and confirmed *survivin*-targeting miRNAs. This could be beneficial in discovering the regulatory mechanisms of *survivin* expression and hopefully lead to devising new therapeutics in SSc medications.

MATERIALS AND METHODS

Patients and Control Subjects

Ten patients (females, 42.2±19.91 years) referring to Rheumatology Research Center, Shariati Hospital were selected as having diffuse SSc according to the American College of Rheumatology (ACR) criteria.[33] Ten age- and gender-matched (9 females and 1 male, 37±10.31 years) individuals with no history of rheumatologic or any other autoimmune disease were selected as healthy controls. Skin involvement was assessed for each patient according to the Modified Rodnan Skin Score (MRSS). Prior to initiation, this study received approval from the Human Research Ethics Committees of Tehran University of Medical Sciences (ethics approval No. IR.TUMS.REC.1394.777) and conducted based on the

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principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all participants based on the approved guidelines.

Isolation and Culture of Fibroblasts

Three-millimeter skin punch biopsy specimens were obtained from the affected forearms of the participants under local anesthesia. Tissues were then transferred in Hank's balanced salt solution (HBSS) containing antibiotic-antimycotic (Sigma, USA) vials to the laboratory. By enzymatic action of Dispase II and Collagenase type I, fibroblasts were isolated from skin biopsy samples. Then the isolated cells were propagated in T-75 cell culture flasks (SPL, Korea) containing Dulbecco's modified Eagle medium (DMEM) (Gibco, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, UK) and 100 U/ml penicillin-streptomycin. Confluent detached fibroblasts with trypsin (Sigma, USA) were used after three passages.

Immunocytochemical Analysis of Skin Biopsies

The purity of fibroblast cultures was confirmed using immunocytochemical staining. To this end, cultured cells at a density of 5×10^4 cells in 24-well tissue culture plates were fixed with pre-cold methanol, rendered permeable using Triton-X100 treatment and blocked with 1% bovine serum albumin (BSA) (Sigma, USA). Then, cells were probed with primary antibodies (anti-α smooth muscle actin (α-SMA, Abcam) and anti-fibroblast surface protein (ab11333, Abcam). Afterward, fibroblasts were washed three times with PBS and subsequently incubated in secondary antibody (Sheep Anti-Mouse Ig (Human Ig Adsorbed) FITC conjugated, Avicenna Research Institute, ACECR, Iran).Ultimately, labeled cells were mounted in DAPI-containing medium (sc-24941, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Fluorescence was visualized using a digital inverted microscope (Nikon, Japan).

RNA Extraction and cDNA Synthesis

The seeded cells in T-75 flask culture plates were collected using trypsinization. Then, the enzymatic reaction in the flasks was neutralized through adding 10% FBS supplemented DMEM solution. To isolate the total RNA content, including large coding and small non-coding RNAs (such as microRNAs) from the collected cells, miRNeasy Mini kit (Qiagen, Valencia, CA, USA) was used following the manufacturer's manual. The quantity and purity of total RNA were evaluated using a NanoDrop-2000c Spectrophotometer (ThermoScientific, Wilmington, Delaware, USA), and the criterion for the RNA inclusion was 260/280.

Using miScript II RT kit (Qiagen, Valencia, CA, USA), cDNA was generated from 1 µg of RNA. In summary, reactions were carried out in the final volume of 20 µl using a Peqlab thermal cycler (Peqlab Biotechnology GmbH, Erlangen, Germany). Briefly, each reaction consisted of 6 µL5x miScript HiSpec buffer, 2 µL 10x nucleics mix, 2 µL miScript Reverse Transcriptase, and remaining RNase-free water. Samples were incubated for 60 min at 37 °C and then for 5 min at 95°C to inactivate the miScript Reverse Transcriptase Mix.

Survivin Is A miRNA Target gene

To identify putative miRNAs which target *survivin*, the miRNA target sites in 3′-UTR region of the *survivin* mRNA were screened using miRBase (http://www.mirbase.org) and microRNA databases (http://www.microrna.org). miR-16-5p, miR-218-5p, miR-320a, miR-542-3p, and miR-708-5p were selected to be evaluated in experimental phase (Table 1).

Real-Time Quantitative Polymerase Chain Reaction

This study investigated the expression of *survivin* mRNA and five s*urvivin*-targeting miRNAs in the dermal fibroblasts of SSc patients. Quantifications were performed using the StepOnePlus real-time PCR System (Applied Biosystems, USA). The expression data of *survivin* mRNA and miRNAs were normalized using *B2M* mRNA and *U6* snRNA as endogenous controls, respectively. The comparative $C_T$method was used to calculate the relative expressions in samples.[34]

Relative quantification of miRNAs (miR-16-5p, miR-218-5p, miR-542-3p, miR-708-5p, and miR-320a) was achieved according to protocol by the miScript SYBR Green PCR Kit. Each reaction mixture contained 12.5 µL2x QuantiTect SYBR Green PCR Master Mix,1.8 µL10x miScript Universal Primer, 1.8µL10x miScript Primer Assay, diluted cDNA, and a corresponding amount of RNase-free water to the final volume of 25 µL. Reactions were initiated with 15 min incubation at 95°C for Hot Start Taq DNA Polymerase activation followed by 40 cycles of 15 s denaturing at 94°C, 30 s annealing at 55°C, and 30 s at 70°C for extension.

Table 1. Computational analysis of the regions in *Survivin* gene that are targeted by each miRNA and their specifications

ProbeSetID	Accession number	Predicted consequential pairing of miRNA (top) and Survivin (bottom)	Seed match
hsa-miR-218-5p	MIMAT0000275	3' uguaccaaucuaguUCGUGUu 5' 5'…uuaauucacagaauAGCACAa 3'	7mer-A1
hsa-miR-320a	MIMAT0000510	3' aagcggGAgaguugg-GUCGAAAa 5' 5' …uuuugCUagagcuga CAGCUUUg…3'	7mer-m8
hsa-miR-16-5p	MIMAT0000069	3' gcgguuauaaa ugcACGACGAU 5' 5'…ugccugugcagcgggUGCUGCUG… 3'	7mer-m8
hsa-miR-708-5p	MIMAT0004926	**Site 1 : (556-579)** 3' gggUCgaUCU- AacauUCGAGGAA 5' 5'...uggAGacAGAgUcccuGGCUCCUC 3' **Site 2 : (794-816)** 3' gggucgaucuaacauuCGAGGAa 5' 5'.. ggggcacaugcuggccGCUCCUc… 3' **Site 3 : (1304-1329)** 3' gggucgauc-UA—AcaUUCGAGGAa 5' 5'..accuguuuguAUcaUccGGGCUCCUu…3'	7mer-m8
hsa-miR-542-3p	MIMAT0003389	**Site 1 : (394-401)** 3' aaagucaauaguuaGACAGUGu 5' 5' ...cucauguuguugaggCUGUCACa... 3'	8mer
		Site 2 : (457-464) 3' aaagucAAUAguuaGACAGUGu 5' 5'...cugcagguuccUUAU-----CUGUCACa... 3'	8mer
		Site 3 : (926-932) 3' aaagucaauaguuaGACAGUGu 5' 5' ...gcccaaccuucacauCUGUCACg... 3'	7mer-m8

miRNA: microRNA

Table 2. Relative expression of *Survivin* and each miRNA, the corresponding fold change, and the measured *p* values for the comparisons by the Mann–Whitney U test in fibroblasts from systemic sclerosis patients and healthy controls

Gene / miRNA name	SSc samples	Control samples	Fold change	*p*-value
Survivin	72.19±43.56	38.88 ± 22.76	1.85	0.046[a]
miR-16-5p	22.73±8.73	17.30 ± 8	1.31	0.165
miR-218-5p	7.86 ± 6.51	7.78 ± 4.09	1.01	0.973
miR-320a	0.0051 ± 0.0061	0.0039 ± 0.0042	1.30	0.971
miR-542-3p	0.40 ±0.21	0.77 ± 0.43	−2	0.033[a]
miR-708-5p	0.15 ± 0.13	0.06 ± 0.02	2.53	0.05[a]

miRNA: microRNA, SSc: systemic sclerosis

[a] Values showed statistically significant amounts ($p < 0.05$)

Table 3. Spearman's Rank-Order results to demonstrate the correlation between *Survivin* and miRNAs expressions in dermal fibroblasts of the systemic sclerosis patients

	miR-16-5p	miR-218-5p	miR-542-3p	miR-708-5p	miR-320a
Survivin	$r=-0.205$, $p=0.387$	$r=-0.139$ $p=0.571$	$r=-0.457$ $p=0.049$[a]	$r=0.269$ $p=0.251$	$r=0.195$ $p=0.409$

miRNA: microRNA, SSc: systemic sclerosis

[a] Significant correlation at 0.05 level

Based on the necessary components provided by SYBR green Master Mix, *survivin* gene expression was evaluated. Primers (*survivin* forward CCAGATGACGACCCCATAGAG, and reverse: TTGTTGGTTTCCTTTGCAATTTT; *B2M*forward: CCTGAATTGCTATGTGTCTGGG, and reverse: TGATGCTGCTTACATGTCTCGA) were designed using the BioEdit Sequence Alignment Editor and blasted using the Basic Local Alignment Search Tool on the USA National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/tools/primer-blast). At this step, PCR conditions were as follows: predenaturation step for 30 s at 95°C, and 40 cycles with 15 s at 95°C and 1 min at 60°C. Then, the relative expression for each sample was calculated using the following equation: Relative mRNA expression=$(2^{-\Delta Ct}) \times 10^3$.

Statistical Analysis

All statistical analyses were carried out using SPSS version 21.0 (SPSS Inc, IL, USA) and GraphPad Prism version 5 (La Jolla, CA, USA) softwares. Comparisons between two groups was conducted using the Mann–Whitney U test. The correlations between *survivin* mRNA and miRNAs expression profiles or each miRNA was analyzed using Spearman's Rank-Order. $p<0.05$ was considered as statistically significant.

RESULTS

Survivin mRNA was significantly overexpressed (Fold change=1.85) in fibroblasts from SSc patients as compared with controls (Table 2).

Among the five miRNAs, only miR-542-3p and miR-708-5p demonstrated statistically significant aberration in expression level in fibroblasts from SSc patients compared with healthy subjects (Table 2). Notably, the miR-542-3p expression level in fibroblasts from SSc group was significantly downregulated in comparison to control group (Fold change= -2, p=0.033).Additionally, miR-708-5p was significantly overexpressed in patients than in healthy individuals (Fold change=2.53; p=0.05).

No correlation was found with respect to the expression level of miRNAs and clinical manifestations, especially MRSS. Moreover, the expression levels of all miRNAs, except than miR-542-3p, had no significant correlation with *survivin* mRNA expression level (Table 3). A significant negative correlation ($r=-0.45$, p=0.049) was observed between miR-542-3p level and *survivin* mRNA expression.

DISCUSSION

Skin lesions from SSc patients contain fibroblasts that show uncontrolled proliferation ability and produce higher matrix deposition. For many years, researchers supposed that the presence of some growth factors stimulate these cells to proliferate uncontrollably.[35] Since fibroblast proliferation has been seen in other non-fibrotic conditions, researchers have focused on the elimination of these cells as a mechanism of pathogenesis as well as therapeutic approach.[9] Previous studies have shown a decreased susceptibility to apoptosis in SSc fibroblasts.[36]With this regard, the role of IAP proteins, particularly survivin, has been specified in some autoimmune diseases and cancers.[23,37,38] Chen et al.[37] reported elevated expression levels of *survivin* in untreated rheumatoid arthritis (RA) patients versus normal individuals. Specifically, in the field of SSc, several studies have emphasized on the role of survivin.[18,39] As shown in a study by Mokuda et al.,[18] only *survivin* expression differed in skin lesions and peripheral blood mononuclear cells (PBMCs) of SSc patients. The current study also investigated the expression levels of *survivin* in SSc patients. However, unlike Mokuda et al. study, which covered both SSc disease types, this study only focused on diffuse SSc patients to provide homogenous samples needed for statistically valid

results. The data was significantly similar to prior findings about the modulation of *survivin*m RNA in other fibrotic conditions.[40]

Despite the observed change in *survivin* expression, the mechanism of its regulation in SSc is poorly understood. Recent studies have focused on the role of epigenetics such as miRNAs in skin-involved autoimmune diseases specially SSc.[41,42] Furthermore, dysregulated *survivin*-targeting miRNAs have provided new perspectives in the field of cancer.[23] Therefore, much more needs to be learned about the regulatory role of *survivin*-targeting miRNAs in SSc.

Herein, we hypothesized to evaluate miR-16-5p, miR-218-5p, miR-542-3p, miR-708-5p, and miR-320a, which were previously implicated to be involved in apoptosis through direct binding to *survivin* mRNA.[23]The role of miR-218 in survivin regulation has been reported in lung, breast, and nasopharyngeal cancers.[24-26] In nasopharyngeal carcinoma, downregulated miR-218 due to epigenetic silencing of its promoters was associated with *survivin* overexpression. This overexpression is the main culprit of cell survival and poor prognosis in nasopharyngeal carcinoma.[26] Another *survivin*-targeting miRNA is miR-320a, which targets β-Catenin.[27] It has been proposed that β-Catenin, a downstream cellular protein in Wingless-type (Wnt) pathway, is involved in the profibrotic signaling pathways in disease processes like SSc and cancers.[28] Data has shown that miR-320a promotes apoptosis by targeting *survivin* as one of the β-Catenin downstream genes.[27] miR-16-5p induces leukemic cell apoptosis through the negative regulation of B-cell lymphoma 2 (Bcl-2), an anti-apoptotic protein.[29] In this regard, miR-16-5p targets p53, a nuclear protein which controls cell cycle and apoptosis, and survivin. Surprisingly, P53 can affect miR-16-5p maturation and upregulation, which in turn represses *survivin* expression.[43]

This is the first study addressing the expression profile of *survivin*-targeting miRNAs in SSc pathogenesis. Results revealed that the expression of miR-542-3p was significantly downregulated in dermal fibroblasts of SSc group in comparison with the control group. The findings of the current study support the previously published data about the association between miR-542-3p and *survivin.*[44] Yoon et al.[44] validated *survivin* as a direct target of miR-542-3p. As revealed in their luciferase activity assay, co-transfection of miR-542-3p with *survivin* resulted in decreased activity of the reporter. They found that, by utilizing survivin siRNA, similar levels of inhibition could be achieved. The analysis of neuroblastoma cell lines in the study by Althoff et al.[45] indicated a negative correlation between *survivin* expression and miR-542-3p, which is similar to the current results. They also synthesized miR-542-3p-loaded nanoparticles in order to test the therapeutic potential of this miRNA. These nanoparticles induced apoptosis and decreased cell proliferation through survivin reduction.[45] Another finding established the association of survivin with miR-542-3p in osteoblast differentiation. Bone morphogenetic protein (BMP)-7, one of the important mediators of osteogenesis, is another direct target of miR-542-3p. It should be noted that miR-542-3p suppresses osteogenic differentiation by blocking BMP-7 and its downstream signaling molecules like survivin.[46]

According to the current web-based analysis, another miRNA that significantly changes in fibroblasts is miR-708-5p. As an almost recently identified miRNA,[47] little has been studied with regard to the implication of miR-708-5p in human disorders. Studies of the function of miR-708-5p in renal cell carcinomas demonstrated that this miRNA was involved in apoptosis and modulate the tumorigenesis of renal cells.[30,48] miR-708-5p has been evaluated in cancers mainly and was observed to be upregulated in childhood acute lymphoblastic leukemia[49] and lung carcinoma.[50]In renal cell carcinoma,[30] an enhanced apoptosis was seen after *survivin* knockdown which was also observed when miR-708-5p was transfected. Contrary to those results, no correlation between miR-708-5p and survivin was found in this study. A hypothesis to explain this observation is that miR-708-5p might be upregulated as a compensatory response to suppress *survivin* expression after downregulation of miR-542-3p. However, it is highly complicated to analyze the disease in a certain time point to validate this contemplation, in which miR-708-5p is upregulated later to cover the absence of miR-542-3p in repressing the *survivin* mRNA.

As expected, decreased miR-542-3p led to higher *survivin* expression levels in SSc fibroblasts. This regulation can be considered as one possible cause of apoptosis resistance in this disease. Since miR-542-3p has three binding sites on the *survivin* gene, this miRNA could act more specifically than others as a therapeutic target.[23] The study team recommends

further research to be conducted to gain bright insight into the function and plausible therapeutic application of miR-542-3p in SSc.

This investigation may be subject to some limitations and caveats. The protein level of survivin was not evaluated in our study that could be functionally important in evaluating consequence of miRNA dysregulation. Furthermore, the validation study of miRNA expression dysregulation was not conducted that could be helpful in gaining a better understanding of the role of miRNAs in determining the pathogenesis of SSc.

In conclusion, the data in the current study indicates that miR-542-3p has a pro-apoptotic role which is mediated by the regulation of *surviving* mRNA expression. As this miRNA is downregulated in SSc fibroblasts, overexpression of *survivin* may contribute to apoptosis resistance in these cells. Considering the current concentrations in the application of miRNAs as therapeutic tools in treatment of a variety of human disorders, it appears miR-542-3p is of promising potential to be further investigated and hopefully led to a medication in SSc treatment.

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