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ORIGINAL ARTICLE

Overexpression of apoptosis-related protein, survivin, in fibroblasts from patients with systemic sclerosis

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Abstract

Background/objectives Recent studies suggest that, in addition to activation and hypersecretion of matrix components, fibroblasts from patients with systemic sclerosis (SSc) are resistant to apoptosis. Previous studies have shown that survivin, a member of inhibition of apoptosis (IAP) family, plays an important role in apoptosis resistance. Accordingly, we decided to study the expression of the most important members of IAP family in SSc fibroblasts, which can block apoptosis either by binding and inhibiting caspases or through caspase-independent mechanisms.

Method Skin biopsy samples were obtained from 19 patients with diffuse cutaneous SSc (DcSSc) and 16 healthy controls. Dermal fibroblasts were cultured and the total RNA was isolated from cells followed by cDNA synthesis. Real-time PCR was performed using SYBR Green PCR master mix and specific primers for *cIAP1*, *cIAP2*, *XIAP*, and *Survivin* mRNA quantification.

Results A significantly increased expression level of *Survivin* was observed in fibroblasts from SSc patients compared to controls (2.26-fold, $P = 0.04$). However, mRNA expression of *cIAP1*, *cIAP2*, and *XIAP* did not change significantly between cases and controls.

Conclusions Our results showed that *survivin* is upregulated in SSc skin fibroblast which may lead to resistance to apoptosis. Further studies should be performed to reveal the role of *survivin* in apoptosis pathway of SSc fibroblasts.

Keywords Apoptosis · Fibroblast · IAP · Survivin · Systemic sclerosis

Introduction

Systemic sclerosis (SSc) is a slowly progressing connective tissue disorder, characterized by three major features, including vasculopathy, fibrosis, and inflammation [1]. SSc is a multifactorial disorder and both genetics and environmental risk factors predispose individuals to disease [2–5]. Fibrosis, the hallmark of SSc, is featured as an increased deposition of the extracellular matrix along with the destruction of normal tissue architecture, causing tissue and organ dysfunction [1, 6, 7]. The cultured fibroblasts isolated from sclerotic lesions of SSc patients preserve characteristics such as increased synthesis of collagen and other extracellular matrix components, proliferative behavior, and resistance to apoptosis. Therefore, therapeutic approaches of SSc have concentrated on apoptosis induction of fibroblasts [8–11]. Alternately, preserved characteristics of cultured SSc fibroblasts make them suitable experimental models for studying the mechanisms involved in overproduction of the extracellular matrix and apoptosis resistance in SSc [12].

The inhibitor of apoptosis (IAP) family proteins is distinguished by Baculovirus IAP Repeat (BIR) and able to suppress apoptosis [13–15]. Three major determinants involved in the apoptosis progress are IAPs, IAP antagonists, and caspases [16]. IAPs are endogenous inhibitors of caspases, which are the main players of apoptosis. IAPs can also restrain the caspases via ubiquitination process. In addition to

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apoptosis suppression, they have been demonstrated to be involved in a variety of cellular processes, such as cell cycle and signal transduction [17]. The behavior of fibroblasts in SSc is similar to those in hypertrophic scars and idiopathic pulmonary fibrosis (IPF), suggesting a dysregulation in apoptosis components of these cells. Thus, it is possible to observe a dysregulation in mRNA expression of IAP family members in SSc fibroblasts, as it is observed in IPF and excessive scar formation.

Transforming growth factor-β (TGF-β) is a critical molecule in the pathogenesis of SSc, emerging as a new molecular target for therapy. Autocrine stimulation by endogenously produced TGF-β is a major molecular hallmark in the pathogenesis of SSc. TGF-β has a dual role on apoptosis and provides relevant signals for survival as well as apoptosis [18]. However, it has been strongly implicated in fibrosis through its anti-apoptotic activities [19]. During liver regeneration, increased levels of cIAP-1 could lead to resistance to the apoptosis induced by TGF-β [20]. In addition, XIAP activates all pathways, including NF-κB, c-Jun amino-terminal kinase (JNK), and SMAD-dependent transcription, after TGF-β signaling [21]. However, survivin (also known as baculoviral inhibitor of apoptosis repeat-containing 5 or BIRC5) indicates a cell-type-dependent effect, depending on TGF-β [22–25]. Survivin is a member of IAP family and suppresses apoptosis through inhibition of caspase activation [26].

Given the impaired apoptosis pathway in SSc disease as the important pathogenesis mechanism, the present study focuses on the gene expression of IAP family transcripts in SSc fibroblasts compared to normal dermal fibroblasts.

Materials and methods

Patients and controls

Skin punch biopsies were obtained from the affected dorsal forearms of 19 patients with DcSSc (16 females and 3 males, mean age 42.76 ± 11.28 years), who referred to Rheumatology Research Center, Shariati Hospital, Tehran University of Medical Sciences and fulfilled the American College of Rheumatology Criteria (ACR) for classification and diagnosis of SSc [27]. Healthy dermal fibroblasts were obtained from the dorsal forearm skin punches of 16 sex- and age-matched healthy volunteers (14 females and 2 males, mean age 40.67 ± 10 years). Healthy controls had no history of skin, autoimmune, and rheumatologic diseases, cancer, and glucocorticoid medications. Informed consent was obtained from all the study subjects. The study was approved by the Ethic Committee of Tehran University of Medical Sciences.

Fibroblast isolation and culture

Dermal fibroblasts were isolated from each biopsy sample (4 mm) by enzymatic digestion. Briefly, each biopsy specimen was washed with phosphate-buffered saline (PBS), then incubated with Dispase II (overnight) to separate the epidermis and dermis. Incubation of the dermis in collagenase type I for 3 h at 37 °C resulted in single-cell suspension of dermal fibroblasts. Fibroblasts were cultured in Dulbecco Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 2% penicillin streptomycin (pen-strep) at 37 °C in 5% CO_2 cell culture incubator. Finally, fibroblasts yielded from passages 3 to 5 were utilized for further experiments.

Confirmatory test for fibroblast isolation

The morphology of the fibroblasts was studied using an invert microscope after several days and after they had reached 100% confluency. A specific antibody against fibroblast-surface protein and the DAPI dye for the fibroblast nucleus were used to confirm correct fibroblast isolation and check for possible cellular contaminations (Figs. 1 and 2).

RNA isolation and quantitative real-time PCR

The total RNA was isolated using High Pure RNA Isolation Kit (Roche, Nutley, NJ) according to the instructions of the manufacturer. Complementary DNA (cDNA) was synthesized from 1 μg of the total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo scientific, Wilmington, DE, USA). The relative expression levels of mRNA were measured by the SYBR Green Takara gene expression master mix and StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Primers for quantification of XIAP, cIAP1, cIAP2, and survivin (Table 1) were designed using primer3 online tool (http://simgene.com/Primer3) and for spasticity and accuracy were blasted in NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were produced by the custom oligonucleotide synthesis service Metabion (Martinsried, Germany). The values were expressed as the difference in Ct values normalized to β2-microglobulin (β_2M) for each sample, using the following formula: relative RNA expression = $(2^{-\Delta Ct}) \times 10^3$ [28, 29].

Statistical analysis

Statistical analysis was performed using the IBM SPSS software version 22 (Armonk, NY, SUA). The normality of variants was analyzed using the Kolmogorov–Smirnov test. Two-tailed Student's *t* test was used for statistical evaluation. GraphPad PRISM 6 (GraphPad Software, La Jolla, CA, USA, www.graphpad.com) was used to draw graphs. *P*

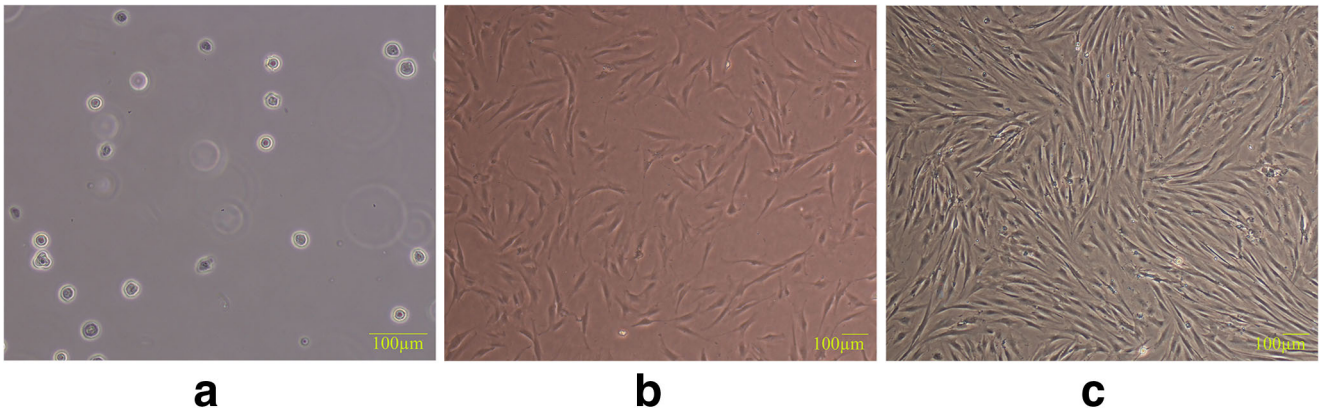

Fig. 1 Fibroblasts under invert microscope. **a** Fibroblasts right after extraction. **b** Fibroblasts after several days of extraction. **c** Fibroblasts with 100% confluency on culture plates. Magnification for **a**, **b**, and **c** is × 10 (scale 100 μm)

values < 0.05 were considered significant. All data were represented as the mean ± standard error of mean (SEM).

Results

Our results indicated a significant upregulation of *survivin* mRNA expression level ($P = 0.04$) in fibroblasts from SSc patients compared with healthy controls. However, mRNA expression levels of *XIAP* ($P = 0.48$), *cIAP1* ($P = 0.82$), and *cIAP2* ($P = 0.60$) in fibroblasts demonstrated no significant change between patients and controls (Fig. 3 and Table 2).

Discussion

The IAP gene was initially detected in insect SF-21 cells infected by the baculovirus [13, 16]. The BIR domain is the determining feature of IAP family members in the N-terminal of IAPs [30]. XIAP, cIAP1, and cIAP2 are the most

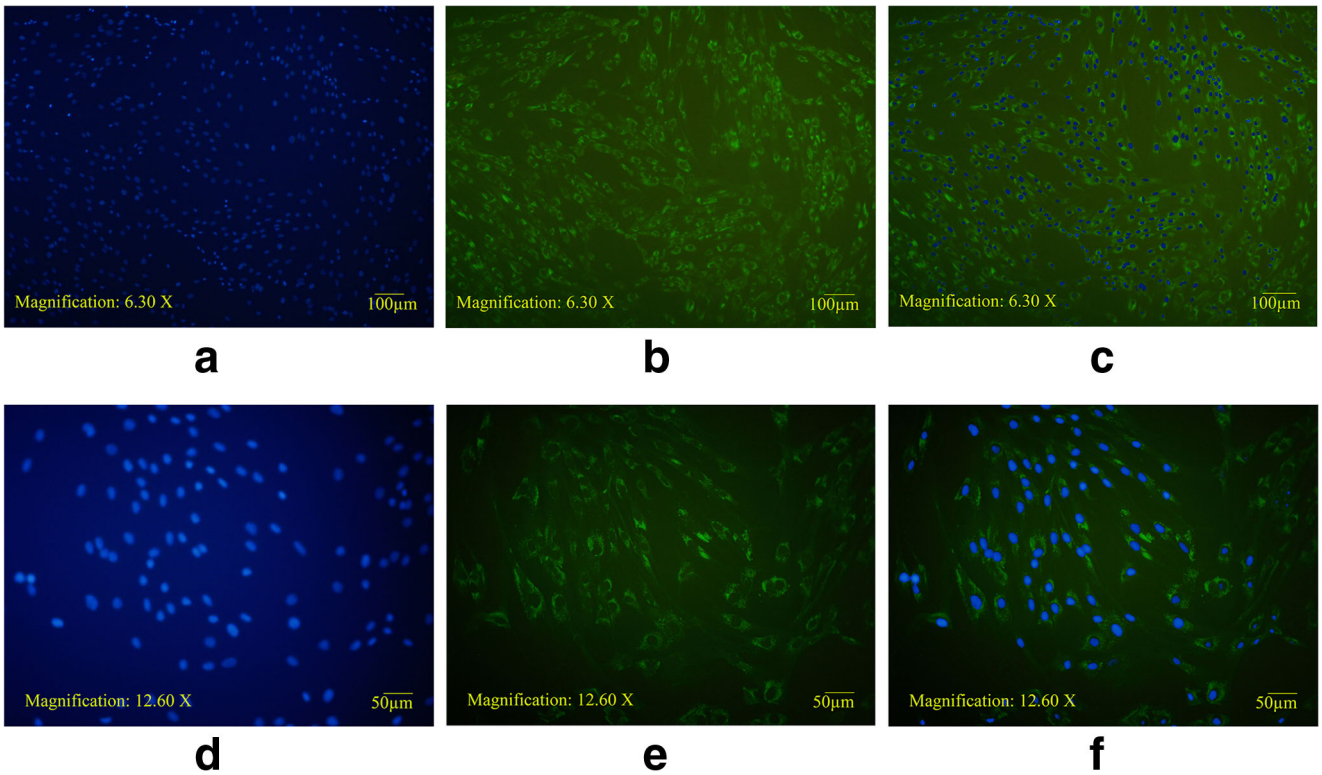

Fig. 2 Stained extracted and cultured fibroblasts with specific antibody against cytoplasmic membrane proteins, and DAPI dye. **a** Invert microscopy of fibroblasts and DAPI staining of nucleus, displayed simultaneously. **b** Stained fibroblasts using specific antibodies against their cytoplasmic membrane protein. **c** Invert microscopy of fibroblasts and DAPI staining of nucleus, displayed simultaneously. **d** Stained fibroblasts using specific antibodies against their cytoplasmic membrane protein. Magnification for a, b and c is × 10 (scale 100 μm), and for **d**, **e**, and **f** is × 20 (scale 50 μm)

Table 1 Sequences of primers for real-time QPCR (*XIAP*, *cIAP1*, *cIAP2*, *survivin*)

Primer name	Accession number	Primer sequence	Amplicon size	Tm (°C)
XIAP-F	NM_001167.3	5′-GACAGTATGCAAGATGAGTCAAGTCA-3′	93	61
XIAP-R	NM_001167.3	5′-GCAAAGCTTCTCCTCTTGCAG-3′		60
cIAP1-F	NM_001166.4	5′-TACTGGCCATCTAGTGTTCC-3′	124	56
cIAP1-R	NM_001166.4	5′-CAGATTCCCAACACCTCAAG-3′		56
cIAP2-F	NM_001165.4	5′-ATGCCAAGTGGTTTCCAAGG-3′	128	58.5
cIAP2-R	NM_001165.4	5′-ATCTCCTGGGCTGTCTGATGTG-3′		61
Survivin-F	NM_001168.2	5′-CCAGATGACGACCCCATAGAG-3′	152	59.4
Survivin-R	NM_001168.2	5′-TTGTTGGTTTCCTTTGCAATTTT-3′		56.8
B2M-F	NM_004048.2	5′-CCTGAATTGCTATGTGTCTGGG-3′	244	59.05
B2M-R	NM_004048.2	5′-TGATGCTGCTTACATGTCTCGA-3′		59.83

extensively studied mammalian IAPs [31–33]. The ability of IAPs to inhibit apoptosis via blocking the caspases activity is guaranteed by BIR. Unlike other IAPs, survivin not only inhibits apoptosis but also regulates cell division [34].

In this study, we observed 2.26-fold upregulation of the mRNA expression level of *survivin* ($P = 0.04$) in fibroblasts from SSc patients, while no significant alteration was observed in cIAP1, cIAP2, and XIAP. Increased expression of *survivin* has been reported in many studies in cancer since 1997 and it has also been validated as a therapeutic target in cancer [35–38]. In 2012, Sisson et al. reported increased mRNA expression of *survivin* in fibroblasts from idiopathic pulmonary fibrosis (IPF) patients, which is a fibrotic lung disorder with the presence of apoptosis-resistant fibroblasts, as seen in SSc. Their study revealed that increased expression levels of TGF-β and Endothelin-1 leads to upregulation of survivin, which plays an important role in resistance to apoptosis [22]. TGF-β and Endothelin-1 are also upregulated in SSc fibroblasts [39]. Excessive scars also show overproduction of extracellular matrix and resistance to apoptosis. Horowitz et al. indicated that *Endothelin-1* can increase *survivin* expression in excessive scar fibroblasts, which in turn

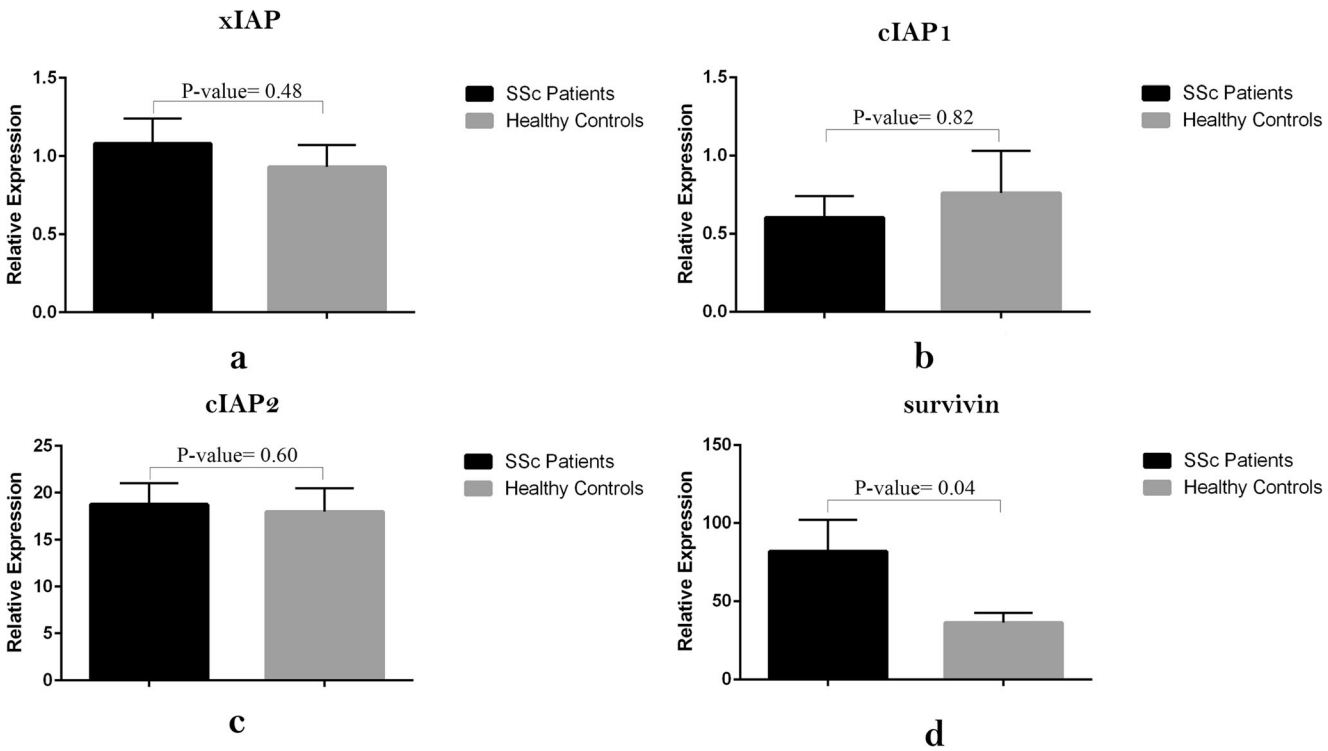

Fig. 3 **a**–**c** Expression of *xIAP*, *cIAP1*, and *cIAP2* and survivin in SSc patients ($n = 19$) and normal fibroblasts ($n = 16$). No significant differences were observed in mRNA expression levels of *xIAP*, *cIAP1*, and *cIAP2* in fibroblasts from SSc patients in comparison to control group. **d** SYBR green based real-time PCR for survivin in SSc fibroblasts indicated significantly increased expression of *survivin* mRNA compared to normal fibroblasts (fold change = 1.18; $P = 0.017$). Data are indicated as mean ± SEM

Table 2 Gene expression fold change of *XIAP*, *cIAP1*, *cIAP2*, and *survivin* mRNA in fibroblasts from SSc patients in comparison to healthy subjects

Target gene	Fold change (SSc vs. HS)	*P* value
XIAP	1.16	$P = 0.48$
cIAP1	1.04	$P = 0.82$
cIAP2	0.79	$P = 0.60$
Survivin	2.26	$P = 0.04$

causes fibroblasts resistance to apoptosis [40]. In 2015 of Mokuda et al. reported the overexpression of survivin in SSc skin lesions, while they observed no difference in expression patterns of both XIAP and cIAP between SSc and non-SSc skin lesions using Immunohistochemistry (IHC) staining. They also have reported an elevated mRNA expression of wild type survivin in SSc PBMC compared to non-SSc [41]. Our observation complies with previous results from SSc skin lesions, IPF, and excessive scar formation settings, suggesting a partially same role of survivin in pathogenesis of SSc with regard to molecular mechanism of apoptosis resistance. It seems that apoptosis regulation of Bcl2 and survivin are independent but interestingly a study on hepatocarcinoma cells showed that *survivin* inhibition is critical for Bcl-2 inhibitor-induced apoptosis, which suggests a related path for these two anti-apoptotic proteins. Our previous study showed that there is a decrease in *Bax: Bcl2* ratio in SSc skin fibroblasts compared to non-SSc fibroblasts [10, 42, 43].

The only modified BIR domain of survivin mediates its homo-dimerization and its interaction with other chromosome passenger proteins [44]. The C-terminus of survivin also differs from that of other IAPs. It is substituted by a coiled-coil α-helix domain, which is responsible for regulation of cell division [34]. Survivin can regulate apoptosis and cell division in several significant pathways (Fig. 4).

TGF-β demonstrates a cell-type-dependent effect on survivin expression. In most non-metastatic cancer cells, TGF-β strongly suppresses survivin and acts as a tumor suppressor gene. Nonetheless, TGF-β upregulates survivin expression and triggers in normal lung fibroblasts and sometimes in metastatic cancer cells [22–25]. The cell-type-dependent role of TGF-β in suppressing or activating of survivin might partly explain the dual role of TGF-β in cancers.

In this study, we observed increased expression of survivin in SSc fibroblasts, which might imply that TGF-β autocrine activation increased survivin gene expression at least partly through NF-κB activation, leading to apoptosis resistance through survivin anti-apoptotic pathways and TGF-β

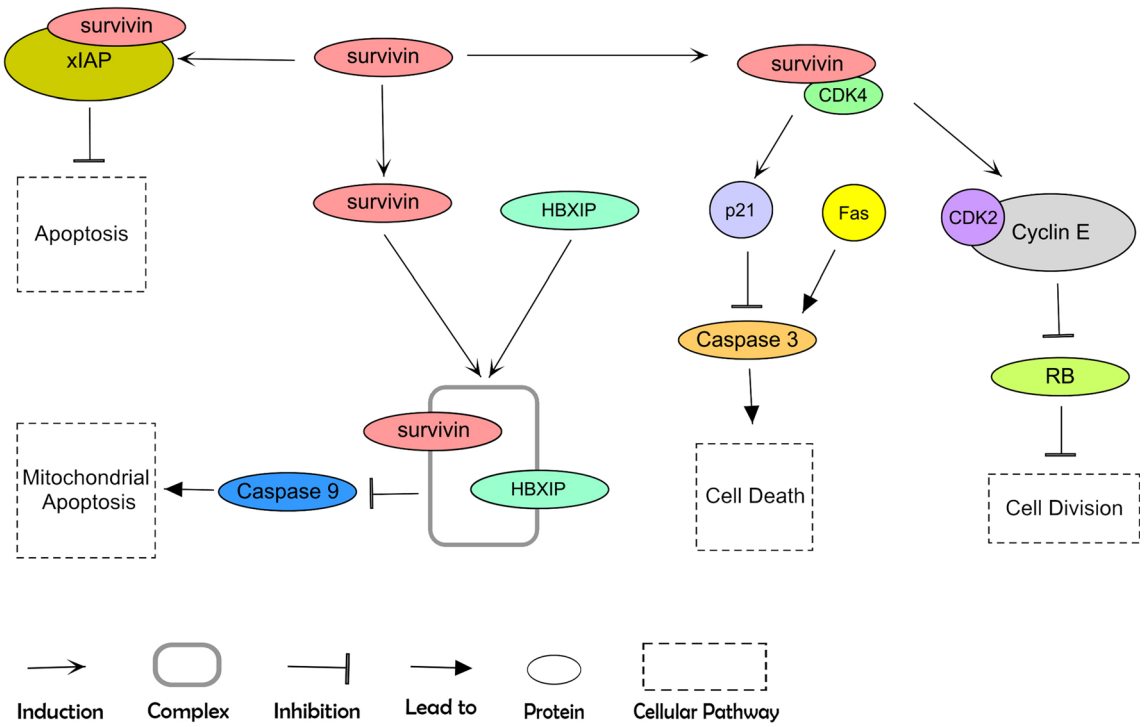

Fig. 4 Survivin has an important component in inhibiting apoptosis called hepatitis B X-interacting protein (HBXIP). Survivin and HBXIP assemble into a complex, which is able to bind to pro-caspase-9, which in turn prevents the activation of mitochondrial apoptosis. In the cytosol, mitochondrial-derived survivin assembles in a complex with XIAP, enhancing the stability of XIAP and its apoptosis inhibitory activity against caspases. Survivin interacts with CDk4 resulting in the CDK2/cyclin E activation and retinoblastoma (RB) phosphorylation. The interaction of survivin with CDK4 releases P21. On the other side, P21 interacts with procaspase 3 and suppresses apoptosis. The illustration was designed by Pathvisio version 3.2.4

signaling. Increased SSc fibroblasts resistance to apoptosis has been associated with excessive fibrosis [18, 21, 45].

All in all, survivin upregulation observed in this study may partly influence resistance to apoptosis and improved growth abilities of SSc fibroblasts, which might be accompanied by increased fibrosis. This is the first report, to our best knowledge, of the upregulation of *survivin* in SSc fibroblasts. This finding introduces *survivin* as a new potential molecule for better understanding of the molecular pathophysiology of SSc, which requires further studies to shed light on its precise role and understand its mechanisms of action in SSc pathogenesis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethic Committee of Tehran University of Medical Sciences and with the 1964 Helsinki declaration.

Informed consent Informed consent was obtained from all individual participants included in the study.

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