Overexpression of Smac is associated with the development of primary Sjogren’s syndrome

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Abstract

Background: Primary Sjögren’s syndrome (pSS) is a systemic autoimmune disease characterized by keratoconjunctivitis sicca, xerostomia, and extraglandular abnormalities. The precise etiology remains unclear. Previous studies have revealed that the apoptosis played an important role in the pSS. Herein, we investigated the expression and effect of second mitochondria-derived activator of caspase (Smac) in patients with pSS and associated the expression with clinicopathological parameters.

Methods: Smac expression was checked in labial salivary glands of surgical specimens from cases of pSS using immunohistochemistry, reverse transcription-quantitative PCR, and Western blot. The results of immunohistochemistry were analyzed for clinicopathological parameters. In addition, the content of Smac in cytoplasm and mitochondria were examined.

Results: The mRNA of Smac, the content of Smac in cytoplasm, the Smac protein in the pSS patients increased significantly compared with the healthy controls (p < 0.05). The content of Smac in mitochondria decreased significantly compared with the healthy controls (p < 0.05). The integral optical density of Smac protein levels were positively correlated with IgG (r = 0.7435, p < 0.05) and erythrocyte sedimentation rate (ESR) (r = 0.7925, p < 0.05).

Conclusion: Smac plays an important role in the development of pSS.

Keywords: Cell apoptosis; Primary Sjogren’s syndrome; Smac

1. INTRODUCTION

Sjögren’s syndrome (SS) is a common, systemic autoimmune disease, with a prevalence of approximately 0.5% in the general population.1 SS can be primary Sjögren’s syndrome (pSS) or secondary Sjögren’s syndrome (sSS), the latter being associated with other autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus. The prevalence of pSS reported in different studies ranges from 0.1% to 4.8%.2 pSS is characterized by a progressive lymphocytic infiltration and destruction of the exocrine glands, mainly the lachrymal and salivary glands, but extraglandular manifestations are also reported (e.g., arthralgia, pulmonary involvement, renal tubular acidosis, etc).3 Since the pathophysiology of pSS is multifactorial, different environmental factors have been implicated such as viral infection, hormonal balance, and genetic background.4 The role of apoptosis in the pSS has been studied extensively. Apoptosis was involved in a variety of physiological situations, including immunity, embryogenesis, and carcinogenesis.5 It was generally considered that cell apoptosis was implemented through two different pathways. The endogenous pathway of apoptosis occurred by Fas/FasL interactions.4 Kong et al6 and Treviño-Talavera et al8 have already reported the expression of Fas/Fasl in the salivary glands of patients with pSS, suggested that the endogenous death receptor pathway was involved in pSS. However, the role of exogenous pathway in the patients with pSS is not clear.

Second mitochondria-derived activator of caspase (Smac) is a 29 kDa mitochondrial precursor protein, which is proteolytically processed in mitochondria into a 23 kDa mature protein. It is released from the mitochondrial intermembrane space to cytosol after an apoptotic trigger. Smac acts as a dimer and it contributes to caspase activation by sequestering the inhibitor of apoptosis proteins (IAPs).9 However, the changes in Smac expression in pSS patients remain unclear. In the present study, the expression of Smac in the labial salivary glands of pSS patients and healthy controls were examined to investigate the possible role of Smac in the pathogenesis and development of pSS.

2. METHODS

2.1. Ethics statement

Experiments using human tissues were approved by the ethics committee of the School of Stomatology, China Medical University, and written informed consent was obtained from patients providing tissue specimens.

2.2. Cases and group

Forty cases (27 females and 13 males) with a mean age of 43.67 ± 1.38 years suffering from pSS were eligible for enrollment into
the study. All patients were recruited from the Department of Emergency and oral medicine, school of Stomatology, China Medical University between December 2015 and December 2016 and diagnosed with pSS fulfilled the American-European Consensus Group Criteria for this diagnosis,10 and individuals with other rheumatic diseases, infections, or malignant tumors were excluded from the study. The control group included 30 cases (23 females and seven males) with a mean age of 42.14 ± 0.92 years. Healthy controls specimens come from the lip trauma who were confirmed by serologic tests to exclude the possible autoimmune. The characteristics of the individuals included in the study are depicted in Table 1.

2.3. Experimental specimen collection
Each case was cut down 0.6 cm × 0.6 cm labial gland tissue of the lower lip under the local anesthesia. One part of fresh specimen was immersed in 4% formaldehyde fixed, conventional paraffin embedding. The other part was put into the liquid nitrogen immediately and preserved in −70°C condition.

2.4. Immunohistochemistry
Immunohistochemistry was performed following the manufacturer’s protocols. After deparaffinization and rehydration, the sections were submerged in citrate buffer, treated with 3% H2O2, incubated in 5% goat serum, and incubated with primary antibody against Smac (Abcam) at 4°C overnight, which were diluted 1:1000. The sections were then incubated with a biotinylated secondary antibody by using DAKO Real EnVision Kit (DAKO, Hamburg, Germany) at 37°C for 15 minutes. The sections were then stained with 3,3´-diaminobenzidine (DAB; Maixin Biotech, Fuzhou, China) and counterstained with commercial hematoxylin (Maixin Biotech) before microscopic analysis. At higher magnification (×400), five visual fields were selected randomly, the expression positive signal was analyzed by Image-proplus software. Compared the level of Smac protein in pSS group and healthy control group of labial salivary gland, according to the integral optical density (IOD), as a parameter for semiquantitative detection.

2.5. Reverse transcription-quantitative PCR
Total tissue RNA was extracted from tissue using the Trizol kits (Sangon Biotech Corporation, Shanghai, China), according to the manufacturer’s instructions. The quality and yield of the RNA samples were determined by ultraviolet spectrophotometer and the concentration was adjusted to 1 μg/μL for reverse transcription. The RNA was reverse-transcribed to form cDNA using a ReverTra Ace qPCR RT kit (Toyobo Corporation, Osaka, Japan). RT reaction system consisted of 1 μg of RNA, 4 μL of 5x PrimeScript RT Master Mix, and RNase Free dH2O was added to reach a final volume of 20 μL. The reaction conditions were as follows: 37°C for 15 minutes and 85°C for 5 minutes. qPCR was performed using the Light Cycler TaqMan Master kit (Toyobo Corporation), according to the manufacturer’s instructions. The qPCR reaction system consisted of 10 μL of SYBR Premix Ex Taq II, 1 μL of cDNA, 0.5 μL of forward primer, 0.5 μL of reverse primer, and 8 μL of sterile water. The cycling conditions were as follows: 95°C for 30 seconds, 40 cycles of denaturation at 95°C for 5 seconds, 60°C for 30 seconds, and 72°C for 2 minutes. The relative expression of Smac was calculated using the ΔΔCt method. The expression of mRNA was normalized against the expression of the GAPDH gene.

2.6. Western blot
Western blot was carried out. Protein concentrations were determined using the bicinchoninic acid method. The 50 μg of protein samples was subjected to 10% SDS-PAGE and then transferred onto PVDF membranes. After blocking with 5% nonfat milk, the membrane was probed with anti-Smac and anti-GAPDH antibodies (Abcam, Cambridge, MA, USA) and further probed with secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA). Proteins were visualized with an Thermo Pierce ECL (Thermo Fisher Scientific, Waltham, MA, USA).

2.7. Determination of the content of Smac
The tissue homogenate was prepared and the mitochondria was extracted: Reference Zydowo methods.11 Labial gland tissues were weighed after being rinsed with cold saline, and 10% tissue homogenate was made with precooled medium homogenate (0.1 mol/L of Tris-HCl, 1 mol/L of KCl, 0.25 of mol/L sucrose, pH 7.4). At low temperature, centrifugation at 600g for 15 minutes was conducted. The supernatant was collected and centrifugation at 1800g was conducted again. The cold slurry medium was added with the precipitation, and the mitochondria suspension was made and saved at −20°C. Determination of Smac: 1 mL of Smac standard (80 mg/mL) dilution was taken, add sodium sulfate when vibration, the density of Sodium sulfate was measured at 520 nm. The standard curve was drawn with the concentration of the standard as the horizontal co-ordinate and optical density as the longitudinal co-ordinate. The concentration of sample was calculated by the standard curve.

2.8. Statistical analysis
The Mann–Whitney rank sum test was used to compare Smac mRNA and Smac protein between the pSS and the healthy controls. In addition, correlations between the levels of IOD of Smac protein with clinicopathological parameters were assessed using univariate analysis. Statistical analysis was performed using SPSS17.0 software. p < 0.05 was considered to indicate a statistically significant difference.

3. RESULTS

3.1. Immunohistochemistry
Smac expression levels in 70 labial glands of cases (including 40 cases of pSS and 30 cases of health control) were evaluated by immunohistochemistry. All 40 pSS samples exhibited distinct expression of Smac. Smac was primarily expressed in cytoplasm rather than the nucleus, and brown particles were mainly distributed in the cytoplasm of acinar epithelial cells, ductal epithelial cells, and the infiltration of focal lymphocytic. We found that pSS tissues presented with up-regulated Smac than control and the difference was significant (Figs. 1 and 3A).

Table 1
Characteristics of the patients and controls enrolled in the study

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>pSS (n = 40)</th>
<th>Control (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>13/27</td>
<td>7/23</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43.67 ± 1.38</td>
<td>42.14 ± 0.92</td>
</tr>
<tr>
<td>Serological activity (n, %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-SSA</td>
<td>27 (67.5)</td>
<td></td>
</tr>
<tr>
<td>A-SSB</td>
<td>25 (62.5)</td>
<td></td>
</tr>
<tr>
<td>ANA</td>
<td>17 (42.5)</td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>8 (20.0)</td>
<td></td>
</tr>
<tr>
<td>RF</td>
<td>15 (37.5)</td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td>20 (50.0)</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>9 (22.5)</td>
<td></td>
</tr>
</tbody>
</table>

ANA = antinuclear antibody; A-SSB = anti-La/SSB; CRP = C-reactive protein; RF = rheumatoid factor; pSS = primary Sjögren’s syndrome; SSA = anti-Ro/SSA antibody.
3.2. RT-qPCR and Western blot

To determine the mRNA expression level of Smac, the total mRNA was isolated and the mRNA expression level of Smac was investigated with qPCR. As shown in Fig. 2A, we found that there was a 2.11-fold increase in the relative mRNA expression of Smac in the pSS patients than the healthy controls ($p < 0.05$; Fig. 2A). We also used Western blot to explore the protein expression level of Smac. The protein expression level of Smac was increased in the pSS cases compared to the healthy controls ($p < 0.05$; Fig. 2B).

3.3. The content of Smac

There was great difference between the two groups ($p < 0.05$). The cytoplasm content of Smac in pSS was $7.21 \pm 0.22$, while the control group was $1.62 \pm 0.85$. The mitochondrial content of Smac in pSS was $2.36 \pm 1.32$, while the control group was $6.67 \pm 1.79$ (Fig. 3B).

3.4. The correlation between the expression of Smac protein and clinicopathological parameters

The association between Smac expression and clinicopathological parameters (SSA, SSB, ANA, erythrocyte sedimentation rate [ESR], CRP, RF, and IgG levels.) of pSS patients was also analyzed in this study. The univariate analysis was used for evaluation.
(Table 2). It was found that the IOD of Smac protein levels was positively correlated with ESR ($r = 0.7435$, $p < 0.05$) (Fig. 4A) and IgG ($r = 0.7925$, $p < 0.05$) (Fig. 4B). Other factors were not found to be associated with Smac expression. Interestingly, when patients were grouped according to test results as normal or abnormal, elevated IOD of Smac protein levels being exhibited were found in the groups with high titers of ANA ($1.13 \pm 0.45$ vs $6.94 \pm 0.43$, $p = 0.0025$) or high concentration of RF ($0.97 \pm 1.16$ vs $6.31 \pm 2.54$, $p = 0.0012$), as shown in Table 2.

### Table 2
Comparison of the IOD levels of Smac protein between pSS patients with normal or abnormal laboratory values

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal mean ± SD (n)</th>
<th>Abnormal mean ± SD (n)</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-SSA</td>
<td>3.72 ± 1.17 (10)</td>
<td>6.23 ± 3.12 (30)</td>
<td>1.38</td>
<td>0.2474</td>
</tr>
<tr>
<td>A-SSB</td>
<td>7.12 ± 1.34 (19)</td>
<td>4.62 ± 1.96 (21)</td>
<td>5.70</td>
<td>0.0220*</td>
</tr>
<tr>
<td>ANA</td>
<td>1.13 ± 0.45 (5)</td>
<td>6.94 ± 0.43 (35)</td>
<td>10.45</td>
<td>0.0025**</td>
</tr>
<tr>
<td>CRP</td>
<td>5.67 ± 3.15 (27)</td>
<td>9.32 ± 0.97 (13)</td>
<td>2.46</td>
<td>0.1251</td>
</tr>
<tr>
<td>RF</td>
<td>0.97 ± 1.16 (15)</td>
<td>6.31 ± 2.54 (25)</td>
<td>12.30</td>
<td>0.0012**</td>
</tr>
<tr>
<td>IgM</td>
<td>7.11 ± 2.15 (16)</td>
<td>9.32 ± 1.04 (24)</td>
<td>0.88</td>
<td>0.3541</td>
</tr>
<tr>
<td>IgA</td>
<td>4.26 ± 2.22 (30)</td>
<td>8.19 ± 0.76 (10)</td>
<td>6.50</td>
<td>0.0149*</td>
</tr>
</tbody>
</table>

$p < 0.05$ means significant difference ($*p < 0.05$, **$p < 0.01$).

Laboratory values such as A-SSA, A-SSB, ANA, and RF positive were defined as abnormal, while laboratory parameters such as CRP, IgM, and IgA above the limit values were defined as abnormal. ANA = antinuclear antibody; A-SSA = anti-Ro/SSA antibody; A-SSB = anti-La/SSB antibody; CRP = C-reactive protein; IgA = immunoglobulin A; IgM = immunoglobulin M; IOD = integral optical density; pSS = primary Sjögren’s syndrome; RF = rheumatoid factor; Smac = second mitochondria-derived activator of caspase.

### 4. DISCUSSION

pSS is a complex and heterogeneous chronic autoimmune disease primarily characterized by a focal lymphocytic chronic inflammation of exocrine glands leading to progressive loss of secretory function. Estimates of prevalence range from 0.2% to 2.7% depending on study protocols, with a strong female propensity from 9:1 to as high as 20:1. Mean age of onset is usually in the 4th to 5th decade, with juvenile forms being a very rare occurrence. At present, however, the detailed molecular mechanism of pSS was still unclear. Recently, research interest is shifted to the apoptosis. Katsiougiannis et al. discovered that endoplasmic reticulum stress is activated in minor salivary gland epithelial cells from pSS patients and controls. Endoplasmic reticulum stress-induced apoptosis in human salivary gland cells leads to cell surface and apoptotic blebs relocation of Ro/SSA and La/SSB autoantigens. Manoussakis et al. found that efferocytosis is frequently impaired in pSS and is primarily due to the presence of inhibitory IgG anti-ApoCell antibodies and secondarily due to phagocytes’ dysfunction. Horai et al. observed that poly I:C induced apoptosis of salivary gland epithelial cells in vitro compared with a relatively low prevalence of apoptosis found in the ducts and alveoli of labial salivary glands in vivo.

Smac is also known as direct IAP-binding protein. It functions as an endogenous antagonist of IAP family proteins, XIAP, cIAP1, and cIAP2 that inhibit apoptosis by sequestering caspases. Mechanistically, the wild-type Smac protein (residues 1 to 184) forms a dimer in solution and interacts with both BIR2 and BIR3 domains of XIAP and or the linker region of XIAP. Such a dynamic and competitive protein–protein interaction between Smac and XIAP cooperatively neutralizes the inhibition of caspases.

![Fig. 3](image1.png)

Expression levels of Smac were examined by immunohistochemical staining (A). The comparison of the content of Smac between the pSS and the control (B). *$p < 0.05$, vs healthy controls. Smac, second mitochondria-derived activator of caspase.

![Fig. 4](image2.png)

Correlation of the IOD of Smac protein levels with laboratory values. Positive correlation was observed between the IOD of Smac protein levels and ESR (A), IgG (B). ESR, erythrocyte sedimentation rate; IgG, immunoglobulin G; IOD, integral optical density; Smac, second mitochondria-derived activator of caspase.
The present study demonstrated that the expression of Smac mRNA turns enhanced in the labial gland of pSS-patients and it has statistical significance when compared with the group of health patients \((p < 0.05)\). But it is notable that the Smac in mitochondria is lower than control group \((p < 0.05)\). We think that in normal circumstances, the Smac protein is located in the mitochondria, and the content of the protein in the cytoplasm is low. When the apoptotic signal was received, the N-terminal signal peptide of Smac protein was removed from mitochondria into the cytoplasm, which played an important role in promoting the apoptosis of the cells. So the content of Smac in cytoplasm and Smac protein in pSS patients increased significantly than the healthy controls but the content of Smac in mitochondria decreased.

In addition, our investigations have revealed a close correlation of Smac levels with the disease activity and severity in pSS patients. Our data have clearly shown that the levels of Smac protein positively correlate with ESR and IgG. Interestingly, when the patients were grouped according to test results normal or abnormal, we found that the patients group with abnormal ANA, RF, A-SSB, and IgA exhibited higher Smac protein levels significantly. At the same time, we found that there were higher IOD levels of Smac protein in abnormal parameter groups except for anti-SSB group, which can be attributed to the function of inhibiting apoptosis of anti-SSB. In a word, we provide new evidence indicating involvement of Smac overactivation in the disease pathophysiology.

The present study has some certain limitations that must be noted. First, the sample size was relatively small, which might reduce the power of the statistical significance and the generalization of the findings. Second, the relevant mechanisms have not been obtained. Therefore, future studies in larger populations, as well as prospective studies with the use of animal experiments with histopathologic and clinical correlations, will be useful for further probing the effect of Smac in pSS.

In conclusion, our findings provide compelling evidence that the Smac play an important role in the pathogenesis of pSS.

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