Cytochrome *c* is important in apoptosis of labial glands in primary Sjogren's syndrome

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Abstract. The present study aimed to investigate the expression and effect of cytochrome *c* (Cytc) in patients with primary Sjogren's syndrome (pSS). In total, 35 newly diagnosed pSS patients and 35 healthy subjects were enrolled in the present study. The mRNA expression levels of Cytc were detected using reverse transcription-polymerase chain reaction and RT-quantitative PCR. The expression of the Cytc protein in labial salivary glands was detected by immunohistochemistry and was associated with the integral optical density (IOD) of clinical and laboratory variables. In addition, the content of Cytc in the cytoplasm and mitochondria were examined. The mRNA and protein expression levels of Cytc, and the content of Cytc in the cytoplasm of the pSS patients was increased significantly compared with the healthy controls ($P<0.05$). The content of Cytc in the mitochondria was significantly decreased compared with the healthy controls ($P<0.05$). The IOD of Cytc protein levels was positively correlated with immunoglobin G ($r=0.8142$, $P<0.05$) and erythrocyte sedimentation rate ($r=0.7512$, $P<0.05$). Cytc was upregulated in the pSS patients, indicating the potential role of Cytc in the pathogenesis and development of pSS. Further studies may facilitate the development of targeting this molecular pathway for the treatment of pSS.

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Introduction

With a prevalence of 0.2-1.4%, primary Sjogren's syndrome (pSS) is one of the most common chronic, slowly progressing systemic autoimmune diseases. Its main symptoms are kerato-conjunctivitis sicca and xerostomia. The extremely various clinical picture is characterized by multiple extraglandular manifestations (EGMs) and associated diseases. The women accounting for 90% of all cases in pSS. The onset age in 30-60 years. However, both distributions by age and by sex show geographical and ethnic differences (1-3).

The precise etiology remains unclear. Multiple factors, including viral infection, hormonal balance and genetic background were involved in the pathogenesis of pSS (4). The influence of abnormal apoptosis in this disease has attracted considerable attention (5-8). Cell apoptosis was a cell suicide under the control of gene in the development of individual activities. It was a programmed cell death process actively by the gene encoding with multicellular organism to regulate the body growth and maintain the stability of the internal environment. Cell apoptosis was involved in a variety of physiological situations, including immunity, embryogenesis and carcinogenesis. It was generally considered that cell apoptosis was implemented through two different pathway (exogenous and endogenous pathways). Exogenous pathway of apoptosis was regulated by the family of the Bcl-2 protein, promoting material such as the cytochrome *c* (Cytc), which induced apoptosis acts on the mitochondria. The endogenous pathway of apoptosis was occurred by Fas/FasL interactions (9). It is generally believed that the regulatory mechanisms for pSS are mainly through endogenous death receptor pathway. Kong *et al* (10), Treviño-Talavera *et al* (7) 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, it is not clear that whether the exogenous plays an important role in the patients with pSS.

Cytc was a key factor of exogenous pathway. Once released into the cytosol, Cytc binds to apoptotic protease activating facter-1 (Apaf-1) and procaspase-9, apoptosome was formed, apoptosis was initiated (11). However, the changes in Cytc

expression in pSS patients remain unclear. In the present study, the expression of Cytc in the labial salivary glands of pSS patients and healthy controls were examined to investigate the possible role of Cytc in the pathogenesis and development of pSS.

Materials and methods

Patients and group. The group comprised 35 patients (33 women and 2 men) with a mean age of 44.42±2.62 years. All patients were recruited from the Department of Emergency and Oral Medicine, School of Stomatology, China Medical University between September 2014 and September 2016 and diagnosed with pSS fulfilled the American-European Consensus Group Criteria for this diagnosis (12) and individuals with other rheumatic diseases, infections, or malignant tumors were excluded from the study. The control group included 35 cases (32 women and 3 men) with a mean age of 40.11±1.32 years. Healthy controls specimens mainly come from the lip trauma (the 30 cases come from lip trauma, the 5 cases come from the abnormal labial gland surrounding the cyst during the operation of mucous cyst). There were no significant differences in the ages or sex ratios between the two groups. Both the research protocol and the consent forms were approved by the Research Ethics Committee of School of Stomatology, China Medical University.

Experimental specimen collection. Each patient was cutted down 0.6x0.6 cm labial gland tissue of the lower lip under the local anesthesia. One part of fresh specimens was immersed in 4% formaldehyde fixed, conventional paraffin embedding. The other part was put into the liquid nitrogen immediately, preservation in -70˚C condition.

Experimental methods

Reverse transcription-polymerase chain reaction (RT-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 Cytc and GADPH was run in the same reaction. RT reaction system: RNA 2 μl, 10X RT buffer 2 μl, $MgCL_2$ 4 μl, dNTP 2 μl, RNase free H_2O 7.5 μl, RNase inhibitor 0.5 μl, AMV 1 μl, random 9 primer 1 μl, reaction condition: 30˚C for 10 min, 42˚C for 30 min, 99˚C for 5 min, 5˚C for 5 min. PCR reaction system: cDNA 2 μl, forward primer 1 μl, reverse primer 1 μl, dNTP 0.5 μl, 5X RT buffer 5 μl, EsayTaq 0.125 μl. Reaction condition: 95˚C 1 min, 95˚C 15 sec, 60˚C 15 sec, 72˚C 45 sec for 40 cycles. RT-PCR products was separated on 2% agarose gels. After stained with ethidium bromide, gel images were photographed with Chemi Imager™ 4400. The gray value of positive electrophoresis strip were tested by professional digital analysis software. The expression of mRNA was normalized against the expression of the GAPDH gene.

The sequences of the primer pairs are: Cytc, 5'-GGGCGAGAGCTATGTAATGCAAG-3' and 5'-TACAGCCAAAGCAGCAGCTCA-3' (132 bp); GAPDH, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3' (439 bp).

RT-qPCR. Total tissue RNA was extracted from tissue using the TRIzol kits (Sangon Biotech Corporation), 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, Osaka, Japan). RT reaction system: RNA 1 μg, 5X PrimeScript RT Master Mix 4 μl, RNase Free dH_2O was added to reach 20 μl, reaction condition: 37˚C for 15 min, 85˚C for 5 min. qPCR was performed using the Light Cycler TaqMan Master kit (Toyobo), according to the manufacturer's instructions, qPCR reaction system: SYBR Premix Ex Taq II 10 μl, cDNA1 μl, forward primer 0.5 μl, reverse primer 0.5 μl, Sterile water 8 μl. The cycling conditions were as follows: Initial denaturation at 95˚C for 1 min, 30 cyles of denaturation at 94˚C for 30 sec, annealing at 58˚C for 30 sec and extension at 72˚C at 10 sec, followed by 72˚C for 2 min and 16˚C for 5 min. The relative mRNA expression levels of Cytc were determined using the comparative Ct method, using arithmetic formulae from the MxPro software tool. The relative expression of Cytc was calculated using the $\Delta\Delta$Ct method. The expression of mRNA was normalized against the expression of the GAPDH gene.

Immunohistochemical staining for Cytc. The fixed biopsy specimen slides were fixed in Carnoy's fixative and embedded in paraffin wax. Paraffinized tissues were sectioned to 4 μm thickness, deparaffinized in xylene and rehydrated through a series of concentrations of ethanol. Antigen retrieval was carried out using an electric pressure cooker (110˚C, 20 min) in 10 mM citrate buffer (pH 6.0). The sections were immersed in blocking solution (Dako Corp., Carpinteria, CA, USA) for 10 min at room temperature followed by washing with phosphate buffered saline (pH 7.4) plus 0.1% Tween-20 for blocking the endogenous peroxidase activity. The sections were incubated with rabbit anti-human Cytc (BD Pharmingen, Santiago, CA, USA) overnight at 4˚C which were diluted 1:50. The slides were washed for 5 min, sections were then incubated with peroxidase-conjugated goat anti-rabbit secondary antibody for 1 h at room temperature; both of which were diluted 1:200. The reactions were developed using a DAB substrate kit, with hematoxylin as counterstain. Each slide was evaluated by one of the authors under a microscope (Nikon, Tokyo, Japan).

At higher magnification (x400), five visual field were selected randomly, the expression positive signal was analyzed by Image-proplus software. Compared the level of Cytc protein in pSS group and healthy control group of labial salivary gland according to the integral optical density (IOD) as a parameter for semi-quantitative detection.

Determination of the content of Cytc. The tissue homogenate was preparation and the mitochondria was extracted: Reference Zydowo *et al* methods (13). Labial gland tissue were weight after rinsed with cold saline, 10% tissue homogenate were made with precooling medium homogenate (0.1 mol/l Tris-HCl, 1 mol/l KCl, 0.25 mol/l sucrose, pH 7.4). At low temperature with 15 min, centrifugation of 600 g was conducted. Take the supernatant, centrifugation of 1,800 x g was conducted again. The cold slurry medium was added with

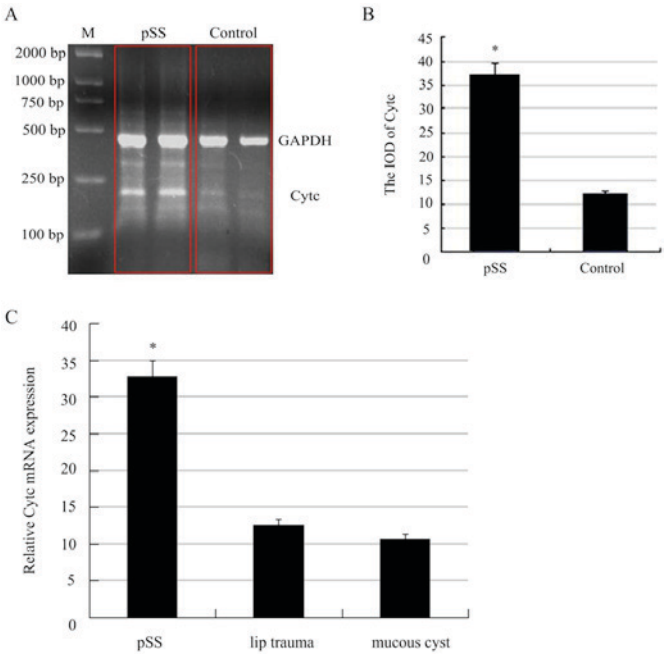

Figure 1. Result of RT-PCR and qRT-PCR. (A) The levels of Cytc mRNA and GAPDH was expressed by electrophoresis bands. (B) Expression levels of Cytc mRNA were examined by RT-PCR and normalized against the expression of the GAPDH gene. (C) Expression levels of Cytc mRNA were examined by qRT-PCR and normalized against the expression of the GAPDH gene. *P<0.05 vs. healthy controls. Cytc, cytochrome *c*.

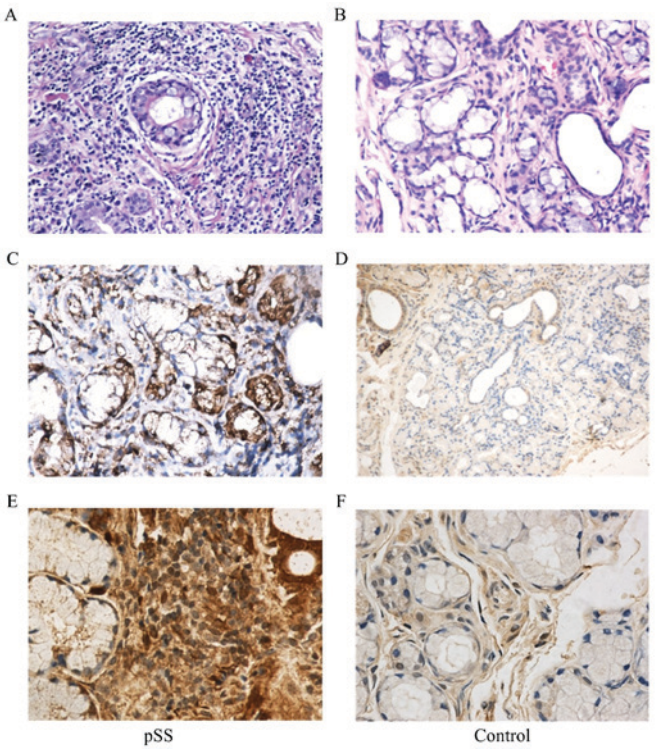

Figure 2. (A) Photomicrograph showing the features of histopathological in pSS (magnification, x200). (B) Photomicrograph showing the features of histopathological in healthy controls (magnification, x200). The labial salivary glands of pSS patients exhibit increased Cytc expression. (C and E) Shown is the expression of the Cytc in the labial salivary glands of pSS (magnification, x200; magnification, x400) and (D and F) healthy controls (magnification, x200; magnification, x400), as determined by immunostaining using specific antibodies. *P<0.05 vs. healthy controls. Cytc, cytochrome *c*.

the precipitation, the mitochondria suspension was made and saved -20˚C.

Determination of Cytc: 1 ml Cytc standard (80 mg/ml) dilution was taken, add sodium sulfate when vibration, the density of sodium sulfatewas measured at 520 nm. The standard curve was draw with the concentration of the standard for horizontal ordinate, optical density for longitudinal coordinate. The concentration of sample were calculated by the standard curve.

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software. The data are presented as the median ± interquartile. The Mann-Whitney rank-sum test were used to compare Cytc mRNA and Cytc protein between the pSS and the healthy controls. In addition, correlations between the levels of IOD of Cytc protein with clinical and laboratory variables were assessed using Spearman's rank correlation. P<0.05 was considered to indicate a statistically significant difference.

Results

RT-PCR and qRT-PCR. To determine the mRNA expression level of Cytc, the specimen from the pSS patients and the healthy controls were separated. The total mRNA was isolated and the mRNA expression level of Cytc was investigated with RT-PCR. Using SPSS 17.0 software, the data are presented as the fold-change in the gene expression normalized against GAPDH. As shown in Fig. 1A and B, there was a 3.45-fold increase in the relative mRNA expression of Cytc in the pSS patients prior to the control.

We also used qRT-PCR to explore the mRNA expression level of Cytc. In this experiment, we divided the control group into two groups according to the source of the sample. As shown in Fig. 1C, there was a 2.66-fold increase in the relative mRNA expression of Cytc in the pSS patients prior to the control from the lip trauma, there was a 2.96-fold increase in the relative mRNA expression of Cytc in the pSS patients prior to the control from the operation of mucous cyst, but there was no significant difference between the two control groups (Fig. 1C).

Immunohistochemistry staining. To evaluate the local effect of Cytc in pSS, we applied immunohistochemical staining to determine the expression of Cytc in the labial salivary glands from pSS. Freshly explanted lower lip biopsy specimens were sectioned and stained with anti-Cytc. All 35 pSS samples exhibited distinct expression of Cytc. Brown particles were mainly distributed in the cytoplasm of acinar epithelial cells, ductal epithelial cells and the infiltration of focal lymphocytic. In contrast, the labial salivary glands from the control exhibited lower expression of Cytc. It was mainly expressed in mesenchymal cells and glandular epithelial cells with a weak expression of Cytc. Overall, the expression of Cytc in pSS was stronger and more widely distributed than the control (Figs. 2 and 3A).

The content of Cytc. There was great differences between the two groups (P<0.05). The cytoplasm content of Cytc in pSS was 6.07±1.84, while the control group was 1.25±1.45. The mitochondrial content of Cytc in pSS was 0.76±1.32, while the control group was 5.57±0.59 (Fig. 3B).

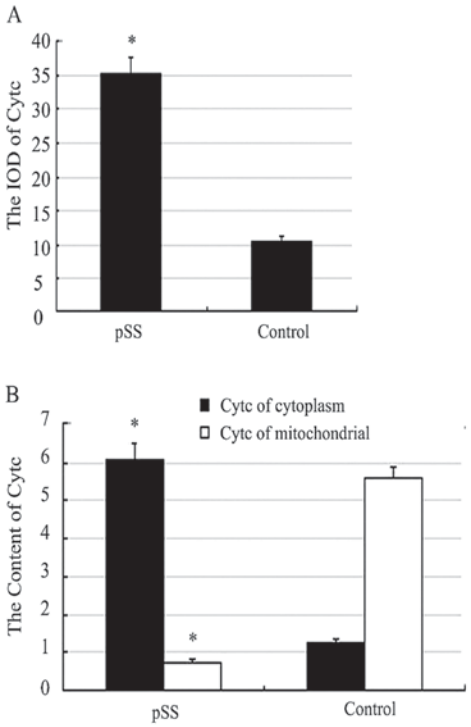

Figure 3. The result of testing the (A) IOD and (B) content of Cytc between the pSS and the control. *P<0.05 vs. healthy controls. Cytc, cytochrome *c*.

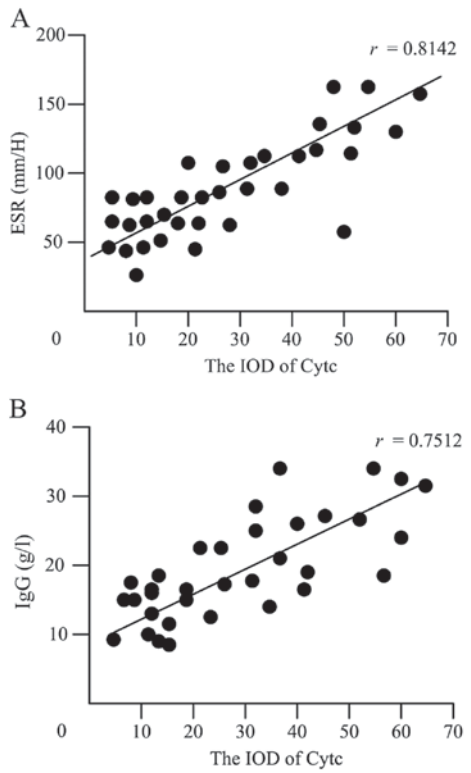

Figure 4. Correlation of the IOD of Cytc protein levels with laboratory values. Positive correlation was observed between the IOD of Cytc protein levels and ESR (A), IgG (B). ESR, erythrocyte sedimentation rate; IgG, immunoglobulin G.

The correlation between the expression of Cytc protein and clinical and laboratory variables. To further determine the relationship between the IOD of Cytc protein levels and laboratory test results including the titers of SSA, SSB, ANA, ESR, CRP, RF and IgG levels. It was found that the IOD of Cytc protein levels was positively correlated with ESR (r=0.8142, P<0.05) (Fig. 4A) and IgG (r=0.7512, P<0.05) (Fig. 4B). However, no significant correlations were found between the IOD of Cytc protein levels and the other laboratory values (data not shown). Interestingly, when patients were grouped according to test results normal or abnormal, elevated the IOD of Cytc protein levels exhibited were found in the groups with high titers of ANA (7.88±0.22 vs. 1.22±0.12, P=0.0032) or high concentration of CRP (16.11±0.12 vs. 3.22±0.45, P=0.0054), RF (7.21±2.34 vs. 1.67±0.12, P=0.0035), as shown in Table I.

Table I. Comparison of the IOD levels of Cytc protein between pSS patients with normal or abnormal laboratory values.

	IOD levels of Cytc protein		
Parameter	Normal mean ± SD (n)	Abnormal mean ± SD (n)	P-value
A-SSA	3.22±1.02 (5)	5.17±4.03 (30)	0.1142
A-SSB	5.22±2.21 (18)	3.53±0.12 (17)	0.6433
ANA	1.22±0.12 (5)	7.88±0.22 (30)	0.0032[b]
CRP	3.22±0.45 (25)	16.11±0.12 (10)	0.0054[b]
RF	1.67±0.12 (15)	7.21±2.34 (20)	0.0035[b]
IgM	6.23±3.20 (18)	10.08±2.14 (17)	0.0335[a]
IgA	2.16±2.11 (30)	7.24±1.24 (5)	0.0132[a]

P<0.05 means significant difference ([a]P<0.05, [b]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 limit values were defined as abnormal A-SSA, anti-Ro/SSA antibody; A-SSB, anti-La/SSB antibody; ANA, antinuclear antibody; CRP, C-reactive protein; RF, rheumatoid factor; IgM, immunoglobulin M; IgA, immunoglobulin A.

Discussion

pSS is a type of autoimmune disease concerning exocrine glands of whole body, which is characterized by the infiltration of lymphocytes and plasma cell, mainly affecting lacrimal gland as well as salivary gland. Hyposecretion of gland and some symptoms can be caused, for instance, xerostomia, xerophthalmia. On the early stage of disease, the local exocrine gland was injured, with the aggravation of local inflammation, uncontrollable immune system will induce systemic disease and develop into malignancy eventually. At present, the etiology and pathogenic mechanisms are not very clear. In recent years, from a large number of literature, we can find that pSS may related to the endogenous pathway of apoptosis (7,10). Sisto M (14) found that ectodysplasin-A2 (EDA-A2) and its receptor X-linked ectodermal dysplasia receptor (XEDAR) are overexpressed in pSS salivary gland epithelial cells in comparison with healthy individuals and that the EDA-A2/XEDAR system in these cells is involved in the induction of apoptosis via caspases

activation. Horai (15) observed that polyinosinic: Cytidylic acid induced apoptosis of primary 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*. Katsiougiannis (16) 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 relocalization of Ro/SSA and La/SSB autoantigens.

Cytc is a mitochondrial protein responsible for transferring electrons between electron transport chain complexes III and IV. Under normal circumstances, Cytc was intergrated into the inner membrane of mitochondrial. When stimulated by the apoptosis, Bax forms oligomers and intergrated into the outer membrane of mitochondrial, inducing and changing permeability of mitochondrial membrane, promoting the release of Cytc. It also contribute to the formation of apoptosome (which consists of Apaf-1, Cytc and caspase-9), make effector caspases alive to induce apoptosis (17,18). The release of Cytc from the mitochondria has been considered as a commitment step in intrinsic apoptosis (19,20).

The present study demonstrated that the expression of Cytc mRNA turns enhanced in the labial gland of pSS-patients and it has statstical signaficance when compared with the group of health patients ($P<0.05$). But it notable that the Cytc in mitochondria is lower than control-group ($P<0.05$). Cytc was released from mitochondria to cytoplasm, not only reduce the respiratory function of mitochondria but also induce the key procedure of apoptosis, activating caspases reaction and apoptosis. In addition, our investigations have revealed a close correlation of Cytc levels with the disease activity and severity in pSS patients. Our data have clearly shown that the levels of Cytc protein positively correlate with ESR and IgG. We provide new evidence indicating involvement of Cytc overactivation in the disease pathophysiology.

In conclusion, the present study demonstrated that Cytc is upregulated in pSS patients, indicating a possible role of Cytc in the pathogenesis and development of pSS.

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