

Abnormal Histones Acetylation In Patients With Primary Sjögren's Syndrome

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Abstract

Background

pSS is an autoimmune disease characterized by an exocrine gland epithelium injury with dense lymphocytic infiltration, involvement of the peripheral nervous, pulmonary, blood, kidney, skin, and joint. Aberrant histone acetylation is increasingly thought to plays important role in the pathogenesis of AIDs. However, there is very little data on histone acetylation in pSS.

Methods

We investigated the expressions of HAT genes (*p300*, *CREEBP* and *PCAF*) by real time PCR in PBMCs from pSS patients. HAT activity and histone H3/ H4 acetylation activity measured by activity kit and histone H3/H4 acetylation verified by WB. Spearman test was utilized to analyze the association between levels of HAT activity and clinical parameters of patients with pSS.

Results

The expressions of *p300*, *CREEBP* and *PCAF* in PBMCs from pSS patients were decreased in mRNA comparison with HCs. HAT activity and histone H3/H4 acetylation were reduced in PBMCs from pSS patients. We found negatively correlations between the HAT activity and CRP and TNF- α , and positively correlations between the HAT activity and C4.

Conclusions

Histone hypoacetylation is observed in patients with pSS and involved in the disease duration of Sjogren's syndrome.

Background

Primary sjögren's syndrome (pSS) is one of complex systemic autoimmune diseases (AIDs) affecting 0.3–0.7% of the general population with a 9/1 female predisposition [1, 2]. This disease manifests dry mouth, dry eyes, and 70–80% of patients appear protean extra-glandular symptoms, such as involvement of the peripheral nervous, pulmonary, blood, kidney, skin, and joint [3, 4]. However, pathogenesis of this disease is obscure. Growing evidences suggest that epigenetic dysregulations have been linked with pSS[5]. Epigenetic modifications, including DNA methylation, histone modification and miRNA, are essential in regulating gene expression, cell development, differentiation and function[6].

Epigenetic mechanisms have a long history in the pSS, but the studies have focused on DNA methylation and miRNA. Altorok N et al. reported hypomethylated genes involved in type I interferon (IFN) pathway in

naive CD4 + CD45RA + cells in pSS patients[7]. Another study further identified prominent hypomethylation of IFN-regulated genes in tissues of pSS including whole blood, salivary gland and CD19 + B cell[8]. The global DNA hypomethylation in blood and Epithelial cells from salivary gland (SGEC) was associated with autoreactivity, lymphocyte infiltration and SSB expression [9, 10]. In recent reports, there were a lot of differences in miRNA expressions between T cells and B cells in pSS and HCs, such as *miR-155*, *miR-146a*, *miR-378a* and *miR-30b-5p* [11–15]. The expression of *hsa-miR-30b-5p* is correlated with the expression of B-cell activating factor (BAFF) in B cells of pSS[16]. DNA hypomethylation and miRNA may contribute to pSS pathophysiology.

Histone acetylation, the same as DNA methylation and miRNA, is also one of important pattern of epigenetic modifications to regulate gene transcription. However, there is very little data on histone acetylation in pSS. A study found that TNF- α restrains aquaporin 5 (AQP5) expression in human salivary gland acinar cells through repression of histone H4 acetylation [17]. The results of this study indicate that histone acetylation may involve in SS salivary gland dysfunction.

The histones acetylation is mainly catalyzed by two opposite groups of enzymes: histone acetyltransferase (HAT) and histone deacetylase (HDAC)[18]. HAT involves the transfer of an acetyl group to the ϵ -amino group of lysine residues in histone and non-histones[19]. A variety of HAT proteins are clear, such as P300 and P300/CBP associated factor (PCAF), p300 / CREB binding protein (CREBBP), and so on[20]. The main role of HDAC is regulating transcription by the removal of acetyl groups from lysine residues of histone tails. It is important to maintain a balance between HAT and HDAC.

Histones include H2A, H2B, H3 and H4, which are part of the nucleosome. Histones undergo post-translational modifications that alter their interaction with DNA and histones. The H3 and H4 histones have long tails protruding from nucleosome, which can be more easily covalently modified by acetylation, methylation and phosphorylation than H2A and H2B[21]. We investigated the alterations in global histone H3/H4 acetylation status and the expression of HAT (P300, CREBBP and PCAF) in pSS. We assessed the aberrant H3/ H4 acetylation in PBMCs of pSS patients.

Methods

Patients and Healthy Donors

A total of 46 patients who were fulfilled the 2016 ACR/EULAR classification criteria for pSS and 46 ages and sex matched HCs were recruited from the outpatient clinic of the department of Rheumatology[22]. All patients were primarily diagnosed as pSS without any treatment such as corticosteroids and disease modifying anti-rheumatic drugs (DMARDs) at the disease onset. The study was approved by the Ethics Committee of the First Affiliated Hospital of Xiamen University and performed by the Key Laboratory of Rheumatology and Immunology in Xiamen University. All patients signed the informed consent from. Table 1 summarizes the demographic and clinical characteristics of the patients with pSS and HCs.

Table 1
Demographic and clinical characteristics of the patients with pSS and HCs.

	pSS patients(n = 48)	HCs (n = 48)
Age, mean (range) years	53 (20–71)	51.9 (26–67)
No. of women/no. of men	41/5	39/7
Anti-SSA (anti-Ro)-positive%	91.3%	
Anti-SSB (anti-La)-positive%	41.3%	
ANA- positive %	78.3%	
FS(foci/4mm ²)%	60.9%	
C3 (g/L)	1.025(0.592–1.54)	
C4 (g/L)	0.194(0.072–0.464)	
IgG (g/L)	15.4(8.09–33.9)	
IgA (g/L)	2.72(0.905–6.97)	
IgM (g/L)	1.15(0.281–2.87)	
ESR (mm/h)	23.5(2–91)	
CRP (mg/L)	1.77 (0.1–63)	0.8 (0.2–1.9)
Disease duration, mean (range) months	10.5 (1.3–17.8)	
ANA = Antinuclear antibody, LFS = Focal sialadenitis, C3/4 = Complement3/4, Ig = Immunoglobulin, ESR = Erythrocyte sedimentation, CRP = C-reactive protein		

Cell And Serum Preparation

PBMCs were isolated by standard density gradient centrifugation from sodium heparin vacutainer blood samples over Ficoll-Paque Plus (Axis-Shield PoC AS, Oslo, Norway). PBMCs were washed and resuspended in phosphate buffered saline (PBS) and removed red blood cells by treating with red blood cell lysis buffer. PBMCs in fetal bovine serum (Thermo Fisher) with 10% dimethyl sulfoxide (DMSO, Solarbio) were frozen and stored at – 80 °C. Separated serum from the blood by centrifugation at 300 relative centrifugal force (RCF) for 10 minutes and stored at -80°C.

Reverse Transcription-polymerase Chain Reaction (RT-PCR) And Quantitative (qRT-PCR) Analysis

Total RNA was extracted by TRIzol Reagent (Ambion by Life Technologies) from PBMCs and reverse transcribed to cDNA according to the manufacturer's instructions with reverse transcription reagent kits (Bio-Rad, Hercules, CA, USA). The expression of *P300*, *PCAF*, *CREBBP*, β -*actin* and *GAPDH* were determined by qRT-PCR. The specific primer sequences were listed in Table 2. A 25 μ l SYBR Green II PCR reaction mixture was used containing 12.5 μ l of SYBR master mix (TaKaRa Shuzo), 1 μ l of sense primer, 1 μ l of antisense primer and 2 μ l of cDNA. QRT-PCR was performed using MyiQ™ Real-Time PCR Detection Systems (Bio-Rad, Hercules, CA, USA), and relative gene expression was normalized to internal control as *GAPDH*. The method of calculated was with the $2^{-\Delta\Delta Ct}$.

Table 2
Related Primer Sequences

Gene	Primer	Sequences 5'→3'
P300	forward	CATCTACCAGACTTGGCACC
P300	reverse	CACTGTCCACAAACCTTGCT
PCAF	forward	ATGAATATGCAATTGGATAC
PCAF	reverse	CTCCTTCATAATCCTTGATA
CREBBP	forward	CTGCACACGACATGA CT
CREBBP	reverse	GAAGTGGCATTCTGTTG
GAPDH	forward	GATTCCACCCATGGCAAATT
GAPDH	reverse	TCTCGCTCCTGGAAGATGGT

Cell Lysate Extraction

Isolation of nuclear and cytoplasmic extracts was performed using the nuclear/cytosol extraction kit according to the manufacturer's directions (Thermo Scientific, Rockford, IL, USA). Cells were washed with wash buffer and then vortexed the tube vigorously on the highest setting for 15 seconds to fully suspend the cell pellet. Incubate the tube on ice with cytoplasmic isolation buffer for 10 minutes. Centrifuged the tube for 5 minutes at maximum speed in a microcentrifuge ($\sim 16,000 \times g$) and collected cytoplasmic extracts. Washed the nuclear pellets twice in wash buffer, spun and incubated for 40 min on ice with nuclear isolation buffer. Centrifuged the tube for 10 minutes at maximum speed in a microcentrifuge ($\sim 16,000 \times g$) and collected nuclear extract. Protein concentration in nuclear was determined by the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). The cytoplasmic extracts and nuclear protein were stored at -80°C .

HAT Activity Assay

The HAT activity assay kit (Enzo Life Sciences, Koropi, Greece) was used to measure HAT activity in the nuclear extract according to the manufacturer's instructions. The nuclear extract used for HAT activity assay was 50 ug, respectively. HAT fluorescence signal was detected with 440 nm using a fluorescence microplate reader (BD, USA).

Western Blot Analysis

PBMCs were washed with PBS and resuspended in RIPA buffer (Solarbio) including protease inhibitors (Roche). Cell lysates were centrifuged (12000 g revolutions per minute at 4 °C). Protein concentration was determined as described. 20ug of protein in each sample was subjected to a 15% SDS-PAGE gel and transferred into immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking with Tris-buffered saline contains 0.1% Tween 20 and 5% non-fat dry milk, the membranes were incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used: Anti-acetyl-Histone H3 (rabbit polyclonal, 1:2000 dilution) purchased from Merck Millipore; Anti-Histone H4 antibody (rabbit monoclonal, 1:10000 dilution) purchased from Abcam; goat anti-rabbit secondary IgG antibodies purchased from Cell Signaling Technology. Protein detection was performed using the chemiluminescence reagent (Millipore, Billerica, MA, USA). Quantification of target proteins was normalized to β -actin. Proteins were quantified using Image Lab.

Global Histone H3 And H4 Acetylation Assay

Acetylated histone H3 and H4 proteins were extracted according to the manufacturer's protocol (Epigentek Group Inc). Histone H3 and H4 concentration was determined as described. We used the Global Histone H3 Acetylation Assay Kit and Global Histone H4 Acetylation Assay Kit (Abnova) to measure histone acetylation in histone extraction from PBMCs. Adjust the concentration of histones to be 200 ng/ul-400 ng/ul, add 5 ul (1–2 ug of histone) per well. Histone H4/H3 acetylation were detected with 450 nm using a microplate reader (BD, USA).

Enzyme-linked Immunosorbent Assay (ELISA)

The concentration of TNF- α were measured using ELISA kit (Quantikine assay, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The concentration of TNF- α was detected with 450 nm and 620 nm using an ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

The statistical significance of the data was analyzed with Prism 6 software (GraphPad Software, San Diego, CA). The HDACs, HAT mRNA expression, HDAC activity, HAT activity, total histone H3 and H4 acetylation levels between pSS and HCs were compared by the Mann Whitney test. Spearman test was

utilized to analyze the association between levels of HAT activity and clinical parameters of patients with pSS. P values < 0.05 were considered significant.

Results

Decreased expression of HAT (P300, PCAF, and CREEBP) in PBMCs from patients with SS. To assess whether histone acetylation played a role in human pSS, we first detected the mRNA expression of HAT (*P300*, *PCAF* and *CREEBP*) in PBMCs from patients with pSS and HCs by qRT-PCR. As shown in Fig. 1A, the average relative expression level of *P300* was 3.0-fold higher for HC than pSS patients ($p < 0.0001$). *PCAF* and *CREEBP* expressions were 2.0-fold higher for HC than pSS patients (Fig. 1B and 1C, $p < 0.0001$).

Decreased nuclear HAT activity in patients with pSS. Because the mRNA expressions of *P300*, *PCAF* and *CREEBP* in PBMCs from patients with pSS were decreased, we speculated that HAT activity is abnormal in pSS. So, we have further studied HAT activity in human pSS and HCs. As we expected, HAT activity was decreased in pSS patients ($n = 11$) compared to HCs ($n = 10$, $P = 0.0037$) (Fig. 2). Therefore, the decreased HAT activity may promote histone hypoacetylation.

Abnormal histone acetylation in pSS. Whether the reduction in HAT activity causes histones hypoacetylated, we further verified by acetylation histone H3 and H4 WB and histone H3 and H4 acetylation activity assay. Acetylation histone H3 and H4 protein were measured in pSS patients and HCs by WB. As shown in Fig. 3A, global histone H3 and H4 acetylation were reduced in pSS compared with HCs. Quantification of histone H3 and H4 were normalized to β -actin, the average relative expressions of acetylation histone H3 and H4 were 3.0-fold higher for HC than pSS patients ($p = 0.0068$ for H3, $p = 0.0089$ for H4; Fig. 3B). Histone acetylation activities were measured in 15 pSS patients and 15 healthy controls. Global histone H3 and H4 acetylation activities were reduced in PBMCs of pSS patients compared with HCs ($p = 0.0005$ for H3, $p = 0.0033$ for H4; Fig. 3C). These data showed decreased histone H3 and H4 acetylation in the PBMCs of patients with pSS.

The correlation about HAT activity and pSS disease characters. Our data suggested that histone hypoacetylation might involve in the pathogenesis and progression of pSS. To further demonstrate the association between histone hypoacetylation and pSS, we analysed the relationship between HAT activity and pSS disease characters. We also detected the expression of TNF- α in pSS serum by ELISA. We found negatively correlations between the HAT activity and CRP (Fig. 4A, $p = 0.040$) and TNF- α (Fig. 4B, $p = 0.012$), and positively correlations between the HAT activity and C4 (Fig. 4D, $p = 0.041$). No correlation between the HAT activity and C3 (Fig. 4C, $p = 0.118$) may be the reason for the small amount of data.

Discussion

Histones and their accompanying post-translational modifications are receiving increasing attention because they can affect chromatin structure, regulate gene expression, and participate in other nuclear

modification processes[23]. It has been found that there are imbalances in HAT and HDACs expression and abnormal histone acetylation in various AIDs such as rheumatoid arthritis (RA) [24, 25], systemic lupus erythematosus (SLE) [26] and multiple sclerosis[27]. And there are many studies on the mechanism of histone acetylation in these diseases and related potential therapeutic effects, but there is little study on the histone acetylation in of patients with pSS. This study demonstrates the histone acetylation is aberrant in human pSS.

In an earlier study, the researcher used stable isotope labeling combined with mass spectrometry to demonstrate histone H3 and H4 hypoacetylation in MRL-lpr/lpr mice compared to control MRL/MPJ mice. Therefore, this study establishes a link between abnormal histone codes and the pathogenesis of SLE, demonstrating that HDAC inhibition(HDACi)trichostatin A (TSA) can reset the aberrant post-translational histone modifications in vivo[28]. Nan Hu et.al reported that global histone H3 and H4 were less acetylated in active lupus CD4 + T cells than in the controls, while expression of *CREBBP* and *P300* were decreased in patients with active lupus. They also found that the degree of histone H3 acetylation was negatively correlated with disease activity SLEDAI in lupus patients [26]. Given that pSS and SLE are similar AIDs, we hypothesize that histone acetylation imbalance is involved in the pathogenesis of pSS.

In this study, at first, we detected the mRNA expression of HAT (*P300*, *PCAF* and *CREBBP*) by qPCR. The results showed that the mRNA expressions of HAT (*P300*, *PCAF* and *CREBBP*) were reduced in pSS patient compared to HCs. In order to further clarify the histone acetylation, we next detected the HAT activity. The data showed that the activity of HAT in patients with pSS were lower than this in HCs. At the same time, Western blot and activity assay were used to detect the acetylation of histone H3/H4 in PBMCs. We certificated global histone H3/H4 hypoacetylation in pSS compared with HCs, which were consistent with the HAT activity. We aslo investigated the correlation between HAT activity and disease characters (CRP, C3/C4, TNF- α). In addition, we observed a negative correlation between the HAT activity and TNF- α and CRP, a positive correlation between the HAT activity and C3/4. Taken together, these suggest that the aberrant histone acetylation might play an important role in the pathogenesis of pSS.

Previous experiment demonstrated aberrant global hypoacetylation in the histone H3/ H4 with SLE. *P300*, *PCAF* and *CREBBP*, capable of acetylating histone in vitro and possibly in vivo, were expression decreased[29]. Our results also show that *P300*, *PCAF* and *CREBBP* mRNA is significantly decreased and hypoacetylation in histone H3/H4 in pSS compared with HCs. The reduction expression of *P300*, *PCAF* and *CREBBP* may explain the significant reduction in total histone H3 and H4 acetylation observed in PBMCs in pSS. *CREBBP* and *P300* are key regulators of RNA polymerase II-mediated transcription and also are used to disrupt activator and repressor complexes, when they expression alterations will be linked to human diseases [30–32] .

Yamamura Y's research indicated that TNF- α destroys the acinar structure in pSS, and that TNF- α inhibits the expression of water channel aquaporin-5 (AQP5) in human salivary gland acinar membrane. The gene promoter was associated with the epigenetic mechanism of histone H4 acetylation, and the acetylation can change with the inflammatory stimuli (such as IL-1 β and TNF) [17]. This study found that

HAT activity is correlated with CRP, C4 and TNF- α , indicating that HAT activity is associated with disease activity in patients with pSS and may be involved in the inflammatory cytokine expression. We considered that HAT activity decreased in pSS as a factor contributes to the pathogenesis of pSS. HAT activity can be used as an inflammatory factor to monitor the pSS.

Based on our results, we believe that the histone hypoacetylation is involved in the pathogenesis of pSS. Current studies have tested drugs that modulate epigenetic responses in a variety of rheumatic diseases in vitro and in animal models[33]. HDACi may be a good method to treatment pSS. HDACi has become a potential anti-inflammatory agent that regulates the function of immune cells. The application of HDACi leads to a wide ranged acetylation of histones and thus reduces the expression level of those inflammatory cytokines, such as IL-2, interferon (IFN)- γ , and IL-6[33, 34]. PLGA-based SAHA (suberoylanilide hydroxamic acid) micropheres, a specific HDACi released in the lacrimal gland, can reduce the expression of proinflammatory cytokines and increase the expression of FoxP3 in the lacrimal glands in DED mice [35]. It indicates that HDACi might be a potential target in the pSS therapy.

Conclusions

In our study, the global histone H3 and H4 hypoacetylation is the result of the decreased HAT activity and HAT (*CREBBP*, *P300* and *PACF*) expression. These findings will assist us to elucidate the epigenetic pathogenesis of pSS and the potential reversibility of these epigenetic abnormalities. HAT activity can be used as an inflammatory factor to monitor the pSS. We need to ascertain what triggers the abnormal histone codes in patients with pSS, as these may represent a novel target for therapeutic intervention in pSS.

Abbreviations

pSS: primary sjögren's syndrome; AID: autoimmune diseases; HAT: histone acetyltransferase; HDAC: histone deacetylase; PCAF: P300/CBP associated factor; CREBBP: p300/CREB binding protein; ANA: Antinuclear antibody; FS: Focal sialadenitis; C3/4: Complement3/4; CRP:C-reactive protein; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus.

Declarations

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Data Availability

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Author contributions

Yan Li and Xiuying Lv designed, performed, and analyzed the experiments and data. Mi Zhou, Ying Wang and Yan He performed the laboratory work. Yan Li, Jingxiu Xuan contributed to paper writing. Guixiu Shi planned experiments and wrote the paper.

Ethics approval

Patient consent was obtained. This study was approved by the Ethics Committee of the First Affiliated Hospital of Xiamen University in accordance with the World Medical Association Declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Abnormal Histones acetylation in patients with Primary Sjögren’s syndrome

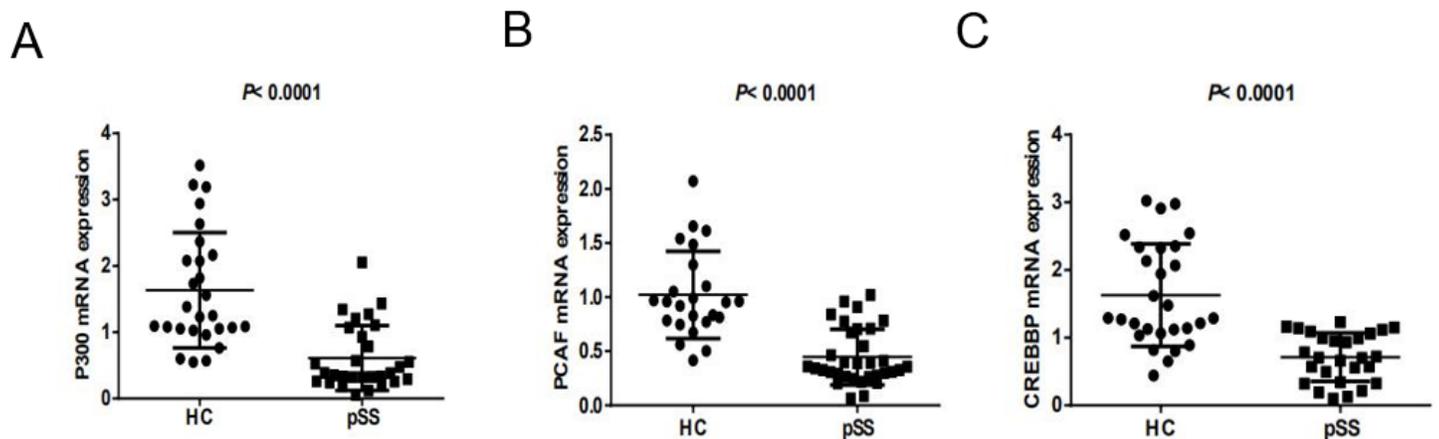


Figure 1

The mRNA expression of P300, PCAF and CREBBP were significantly decreased in pSS patients compared to those of the HCs ($p < 0.0001$). A. The mRNA expression of P300 in the human pSS ($n = 29$) and HCs ($n = 27$). B. The mRNA expression of PCAF in the human pSS ($n = 29$) and HCs ($n = 27$). C. The mRNA expression of CREBBP in the human pSS ($n = 27$) and HCs ($n = 28$).

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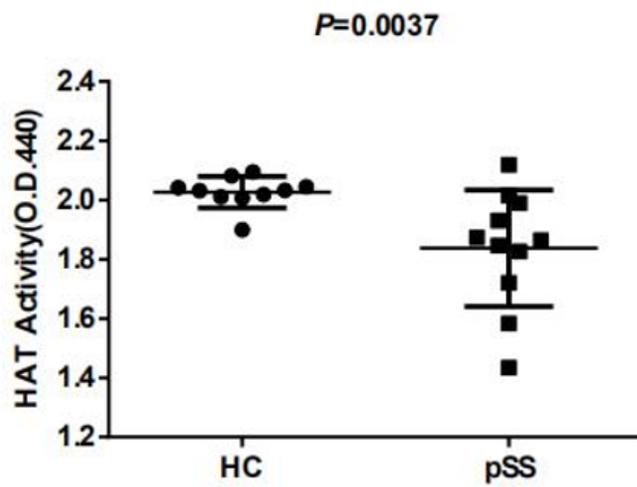


Figure 2

HAT activity in PBMCs of pSS patients and HCs. HAT activity was decreased in pSS patients (n=11) compared to HCs (n=10, $P=0.0037$).

Abnormal Histones acetylation in patients with Primary Sjögren's syndrome

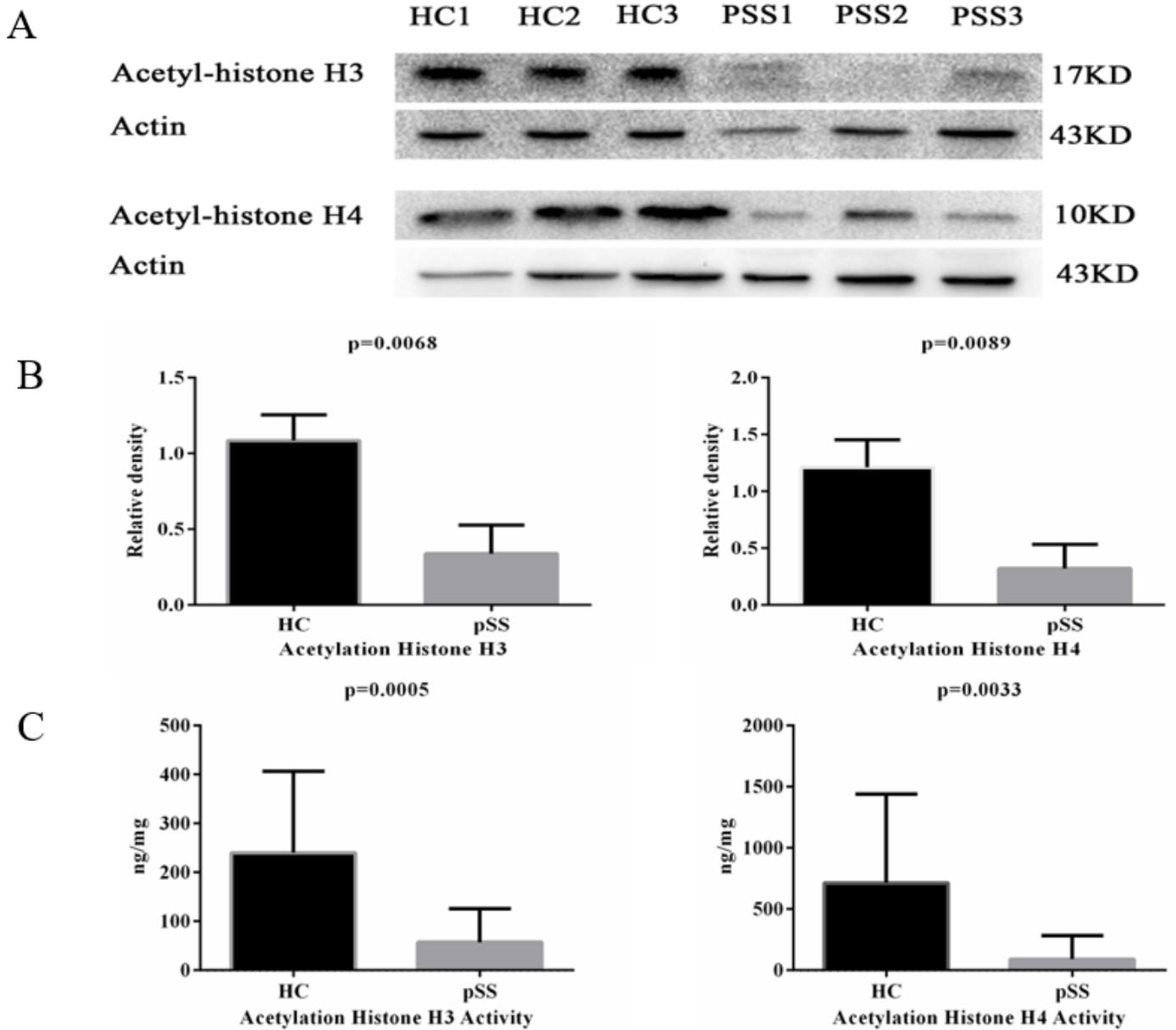


Figure 3

Total histone H3 and H4 acetylation activities were reduced in PBMC of pSS patients compared to HCs. A. Expression of acetylation histone H3 and H4 was studied by WB. B. Relative expression of acetylation histone H3 and H4 protein in PBMCs normalized to β -actin ($p = 0.0068$ for H3, $p = 0.0089$ for H4). C. Total histone H3 and H4 acetylation activities were reduced in pSS patients compared to HCs ($p = 0.0005$ for H3, $p = 0.0033$ for H4).

Abnormal Histones acetylation in patients with Primary Sjögren's syndrome

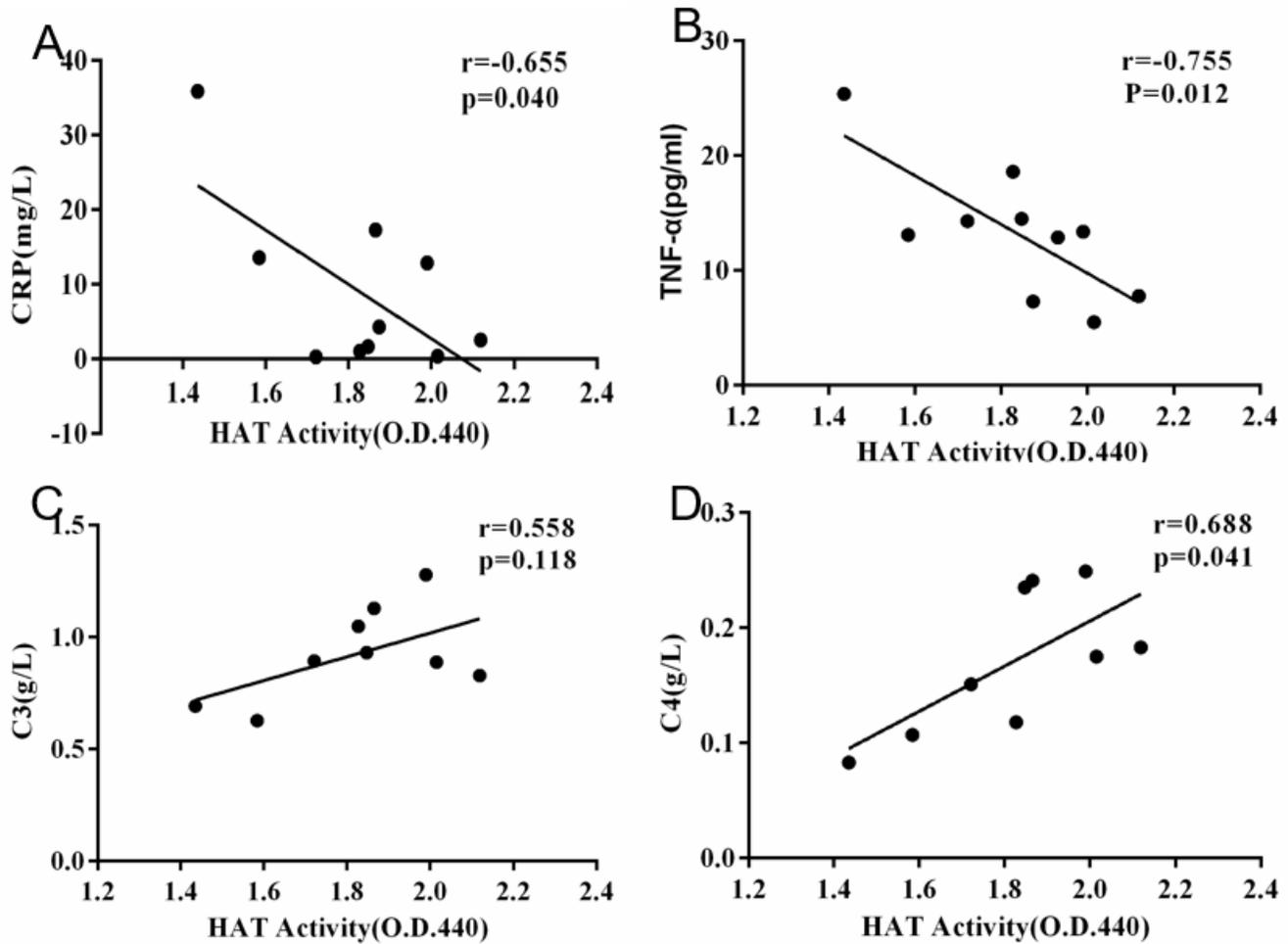


Figure 4

The correlations about HAT activity and pSS disease characters. A. HAT activity has a negative correlation with CRP (Fig.4A, $p=0.040$). B. HAT activity has a negative correlation with TNF- α (Fig.4B, $p=0.012$). C. Correlation between the HAT activity and C3 (Fig.4C, $p=0.118$). D. HAT activity has a positive correlation with C4 (Fig.4D, $p=0.041$).