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Identification of heat shock protein 27 as a novel autoantigen of Behçet's disease

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ABSTRACT

Objective: The aim of this study was to identify candidate pathogenic autoantigens of Behçet's disease (BD) in pathogen-stimulated target cells.
Methods: First, three cell lines were used as target cells to screen autoantibody. Second, selected target cells were simulated with pathogens. Third, western blotting was used for detecting the auto-antigens in cell extracts. Next, immunoprecipitation was performed and the amino-acid sequences of target antigens were analyzed by LC-MALDI-TOF/TOF. Then, the potential target antigen was expressed, purified, and immunologically confirmed. And finally, an ELISA kit was developed and clinically validated through the assessments of 456 clinical samples with BD.
Results: One antigen with a molecular weight of approximately 27-kDa was identified as heat shock protein 27 (HSP27). The reactivity of serum IgG against recombinant human HSP27 was detected in 52 of 91 BD patients (57%), 66 of 92 rheumatoid arthritis (RA) patients (72%), 32 of 90 Sjogren syndrome (SS) patients (36%), 22 of 92 systemic lupus erythematosus (SLE) patients (24%) and 0 of 91 healthy controls (HC). The reactivity of BD serum IgG antibodies against HSP27 was significantly higher than SLE ($P < 0.0001$) SS ($P < 0.0001$) and HC ($P < 0.0001$).
Conclusions: This study identified HSP27 as a candidate endothelial cell autoantigen of BD, which is interesting and probably worth further exploration.

1. Introduction

Behçet's disease is a multisystem chronic systemic vasculitis, which was characterized by recurrent oral, genital ulcerations and iritis [1,2]. Like other classical autoimmune diseases, such as SLE and RA [3,4], many organs including skin, gastrointestinal and nervous system are usually involved in this disease [5], which determine the various signs and symptoms of BD, indicating a large number of autoantigens may be co-existed in this disease. So far, many autoantigens including retinal S-antigen, IRBP, HSP70, a-tropomyosin, kinectin, annexin V, Mtch1 [6–11] have been successfully examined. On the other hand, anti-endothelial cell antibodies (AECA) are closely related to autoimmune vascular injury and have been found in many autoimmune diseases [12,13]. AECA have also been detected in BD patients and have been proven to be associated with vasculitis symptoms [14,15]. Thus, the importance of AECA in the pathogenesis of BD has been emphasized in the past decade, and α-enolase, hnRNP-A2/B1, Sip-1, RLIP-76, prohibitin have been identified as AECA autoantigens of BD [16–20].

These findings greatly expanded current knowledge of BD, however, there are still many questions remain: the etiology of BD is still unclear and so far no commercial test kit for BD is available. Specific autoantigens that could be up-expressed have been found when target cells suffered external stimulation. HnRNP A2/B1 was over expressed in the membrane of HDMEC cells after stimulated with *streptococcus sanguis* [21]. HSP70 was also observed up-expressed in apoptosis HUVEC cells [22]. This phenomenon indicates that pathogenic autoantigens might be up-expressed when target cells pathogens infection or in stress conditions. It reminds us new pathogenic autoantigens of BD might be more likely identified in target cells after pathogen stimulation. Based on our previous research strategies [20,23], this study was

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designed to identify new candidate autoantigens in stimulated target cells by *Escherichia coli* (one of potential pathogens of BD [24]), which might be more likely involved in the early pathological process of BD.

2. Materials and methods

2.1. Subjects

In this study, serological criteria were evaluated through 456 sample assessments, including (1) Experiment group: 91 BD patients with an average age of 38 years (range 14–66, 38 female and 53 male) who all fulfilled the criteria proposed by the International Study Group for BD [25]. (2) Disease control: 92 SLE patients with an average age of 35 years (range 16–59; 83 female and 9 male patients); 92 RA patients with an average age of 37 years (range 15–70; 50 female and 42 male patients); 90 sjogren syndrome (SS) patients with an average age of 50 years (range 19–70; 87 female and 3 male patients). (3) Healthy control: 91 healthy donors (25 years old: range 21–33; 69 female and 22 male). Initially, the first batch of 5 BD patients was collected in July 2013 for western blotting detection. The other samples were collected from Sep. 2013 to Jun. 2014 for a large-scale test using the ELISA method. All of the patients involved in the study were treated at the Chinese PLA General Hospital. This study was approved by the ethical committee of Chinese PLA General Hospital, and each patient involved gave informed consent. Samples were collected, dispensed and stored at −80 °C for further testing.

2.2. Cell lines and strain

Human Umbilical Vein Endothelial Cells (HUVEC), primary human oral epithelial cells (CPkq), human immortalized non-tumorogenic keratinocyte cell line (HaCaT) were selected as representatives of typical organ involvement of BD. The HUVEC cells were purchased from American Type Culture Collection (Manassas, VA). The HaCaT was supplied by Cell Lines Service (Eppelheim, Germany). The primary oral epithelial cells were isolated from a healthy donor and labeled as CPkq according to his name. The HaCaTs were cultured in DMEM (HyClone, MA) containing 10% fetal bovine serum (HyClone, MA). HUVECs were cultured as described previously [20]. The *E. coli* strain (ATCC 25922) used in this study was stored in our lab.

2.3. Indirect immunofluorescence assays

Cells were placed on cover slips, fixed with 4% paraformaldehyde. Indirect immunofluorescence assays were then carried out as follows. The BD and HC sera were incubated with the slides for 1 h at 37 °C. After washing 3 times, the slides were incubated with FITC-conjugated goat anti-human IgG secondary antibody (ImmunoHunt, Beijing, China) for 1 h at 37 °C. The slides were then examined under fluorescence microscopy (AMG, WA). Total cell fluorescence was obtained by Image J software (NIH, MD), and compared between 3 patients in each group.

2.4. E. coli stimulation

The *E. coli* cells were prepared in F-12K medium (HyClone, MA) including 10% fetal bovine serum (HyClone, MA), 0.1 mg/mL heparin (Sigma, MO), 0.03–0.05 mg/mL endothelial cell growth supplement (Sigma, MO) to obtain bacterial suspensions containing about 1×10^5 colony-forming units (CFU)/mL. Next, HUVECs were co-cultured with 5 μL of bacterial suspension for 12 h (the time was set according to literature [21] also as our recent study [23]). HUVECs were cultured without bacterial suspensions as controls.

2.5. Western blotting

The western blotting was performed as described elsewhere [26] with slight modifications. The cell membrane proteins were extracted by RIPA buffer (Beyotime, Jiangsu, China) with 1% complete protease inhibitor cocktail (Sigma, MO). Cell lysates were loaded into the wells of a 15% polyacrylamide gel and separated. The gel was then transferred onto polyvinylidene fluoride membranes (Merck Millipore, MA) which were then blocked with 5% nonfat milk in PBS at 4 °C overnight and incubated with BD sera (1:500 dilution) or sera from healthy controls at 4 °C for 12 h. Next the membranes were extensively washed 4 times with 5% PBST buffer to remove unbound antibodies. Last, the membranes were incubated with horseradish-peroxidase-conjugated goat anti-human IgG (ImmunoHunt, Beijing, China) for 1 h at 37 °C, and ECL detection was carried out in accordance with the product instructions (Applygen, Beijing, China). HSP27 expression in *E. coli* stimulated HUVECs was also examined by western blotting using rabbit anti-human HSP27 antibody (Proteintech, Chicago, IL).

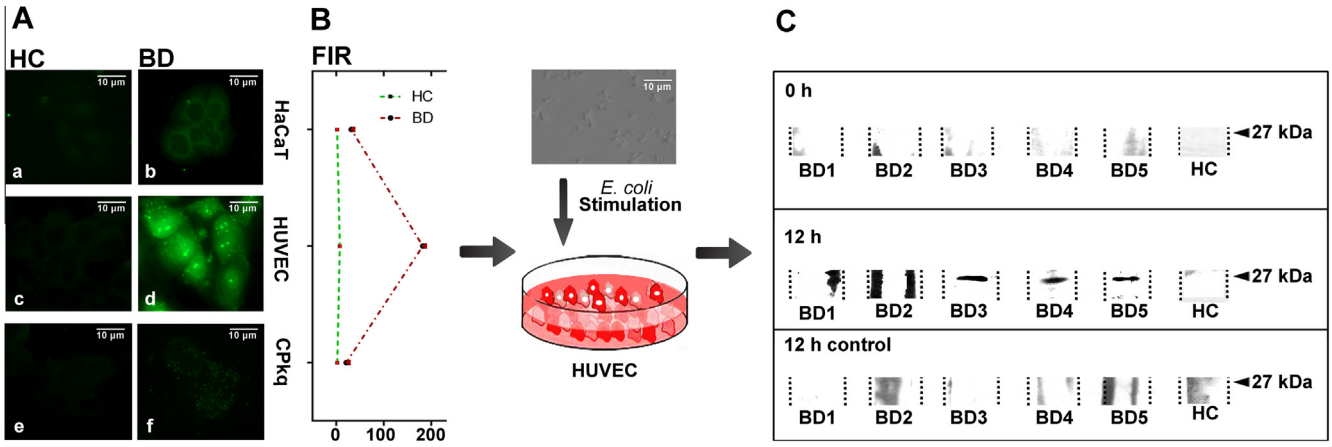
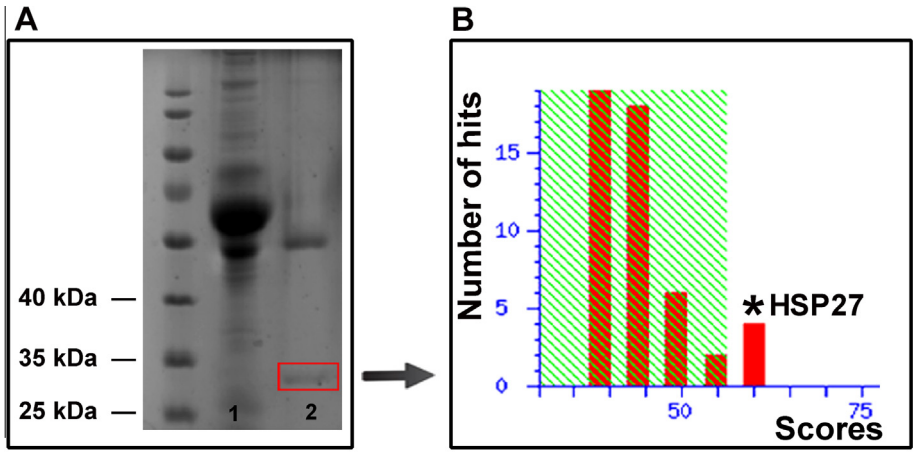


Fig. 1. Detection of autoantigens. (A) Three cell lines were used to carry out an indirect immunofluorescence assays. (a and b) HaCaT cells, (c and d) HUVEC cells, (e and f) CPkq cells. (B) The cell fluorescence intensity ratio was obtained using Image J software (NIH, MD). Error bars were shown. (C) Western blotting for HUVEC extracts (after stimulated with *E. coli* for 12 h) with the sera from 5 BD patients (the first batch of samples) showed a positive band (≈27-kDa) in 3 patients, but not in 0 h (primary HUVECs), 12 h control (HUVECs cultured for 12 h without stimulation), or healthy controls. BD: Behçet's disease, HC: healthy controls, FIR: fluorescence intensity ratio.



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Peptide Origin	Peptide Sequence
Peptide 05-12	R.RVPFSLLR.G
Peptide 06- 12	R.VPFSLLR.G
Peptide 13 - 20	R.GPSWDPFR.D
Peptide 21- 27	R.DWYPHSR.L
Peptide 28 - 37	R.LFDQAFGLPR.L
Peptide 80 - 89	R.QLSSGVSEIR.H
Peptide 97 - 112	R.VSLDVNHFAPDELTVK.T
Peptide 128 - 136	R.QDEHGYISR.C
Peptide 172 - 188	K.LATQSNEITIPVTFESR.A

Fig. 2. Identification of specific autoantigens. (A) Immunoprecipitation revealed a protein band with about 27-kDa in HUVECs (after stimulated with *E. coli* for 12 h) reacting with sera from BD patients (band 2), but not in supernatant (band 1). Positions of protein molecular weight standards are shown on the left. (B) The band was identified by LC-MALDI-TOF–TOF mass spectrometry (NCBI number, NP_001531.1; MASCOT score, 60). (C) Nine unique peptides matched HSP27 were identified by mass spectrometry.

2.6. Immunoprecipitation

Total HUVEC cell (after stimulated with *E. coli* for 12 h) extracts (300 μg) were incubated with BD sera (equal volumes from 3 BD patients with positive 27-kDa band) at 4 °C overnight. Thereafter, protein A Sepharose beads (Sigma, MO) that had been washed with PBS were added and mixed at 4 °C for 2 h. To obtain the immune complexes (antigen–IgG complexes), samples were centrifuged for 5 min at 3000 rpm, the supernatant was collected. The immune complex was washed 3 times in 500 μL 0.5% PBST. Then, the immune complex and the supernatant (after the first centrifuge) were suspended in a sample loading buffer and resolved by SDS–PAGE. Immunoprecipitation was also performed with human recombinant HSP27 protein to verify whether the antigen detected was an autoantigen of BD. The gels in the two experiments were stained by coomassie blue (Sigma, MO).

2.7. In-gel digestion and mass spectrometry

In-gel digestion and mass spectrometry were performed as described in detail elsewhere [27]. Briefly, the excised gel pieces were destained with a mixture of 25 mM NH_4HCO_3 and 50% acetonitrile and dried by vacuum centrifugation. Then, the gel pieces were reduced for 2 h in 10 mM dithiothreitol. After cooling to room temperature, the dithiothreitol solution was replaced with roughly the same volume of 25 mM NH_4HCO_3 containing 55 mM iodoacetamide and incubated for 45 min at room temperature in the dark. Then, the gel pieces were covered with 20 μL of the 0.05 M NH_4HCO_3 buffer with trypsin (Sigma, MO). Digestion was performed overnight at 37 °C. Finally, the recovered target proteins were identified using LC-MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA). Mass spectrometric data were analyzed with Mascot bioinformatics database search engine (Matrix Sciences, London, UK; www.matrixscience.com).

2.8. Protein expression and purification

The procedure of protein expression and purification was performed as before [28]. In brief, total RNA was isolated from HUVEC cells using TRIzol reagent (Invitrogen, CA). RT-PCR was carried out according to the manufacturer's instructions (Fermentas, MD). Human HSP27 proteins were overexpressed in *E. coli* BL21, followed by the purification of the recombinant proteins using Ni–NTA resin (Qiagen, Hilden, Germany). The concentration of protein was determined using a BCA assay kit (Biosynthesis Biotechnology, Beijing, China). Purified recombinant protein was confirmed by SDS–PAGE (the gels was stained by coomassie blue (Sigma, MO)) and mass spectrometry.

2.9. ELISA

ELISA with human recombinant HSP27 protein was performed as described previously [26]. In brief, capture recombinant proteins (300 ng/mL) were used to coat the 96 well microplate (Corning, NY) overnight at 4 °C. After three washes with 0.5% PBST, each well was blocked in 200 μL 5% goat serum for 2 h at 37 °C. Then the plate was incubated with 100 μL sera diluted 1:100 in PBS for 2 h at 37 °C. Three washes later, 100 μL goat anti-human IgG/HRP

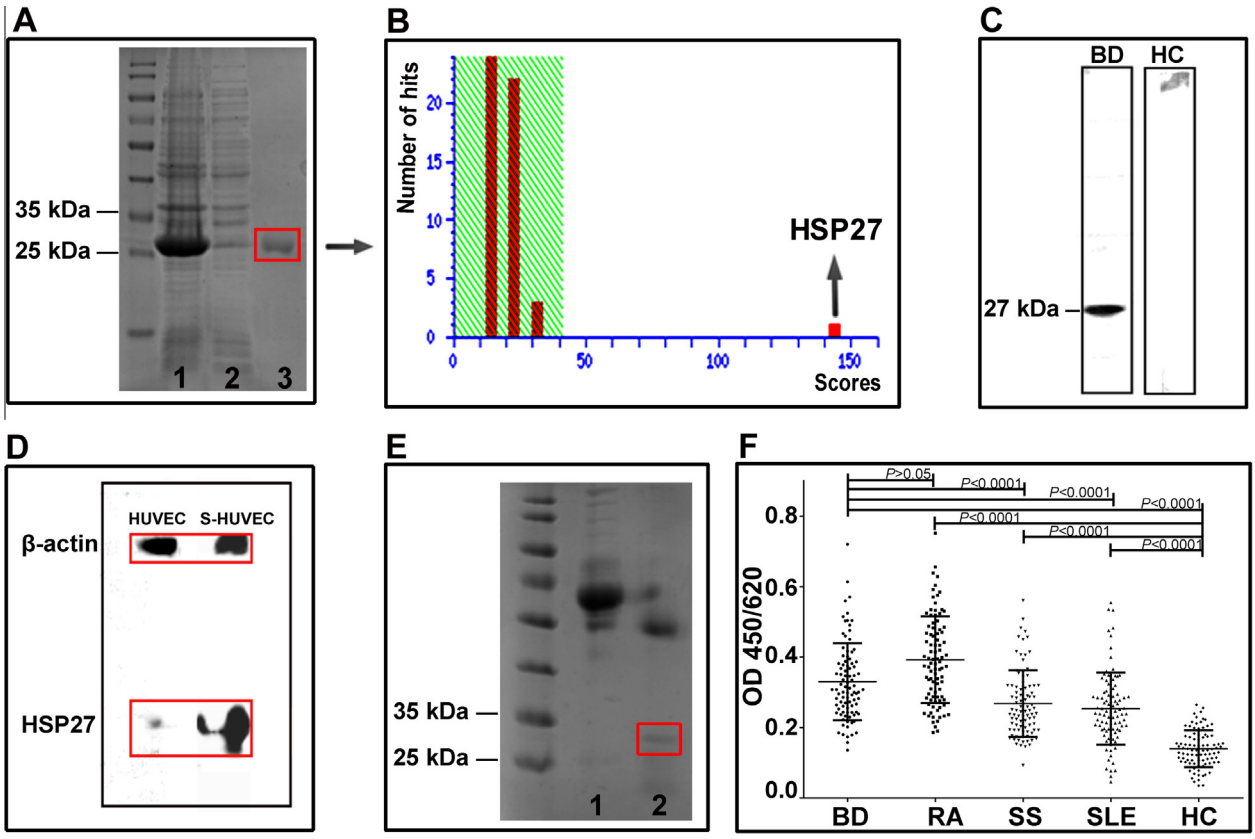

Fig. 3. Expression, purification and immunologically confirmation of HSP27 and the reactivity of sera IgG antibodies against HSP27. (A): (1) The IPTG inducible expression of *E. coli* BL21. (2) *E. coli* BL21 without plasmid transformation as blank control. (3) Purified protein. Protein marker was shown on the left. (B) Verification of HSP27 by mass spectrometry. The mascot score was 141. (C) The western blotting validation result of HSP27 probed with BD patient serum and HC controls. (D) The HSP27 expression in *E. coli*-stimulated HUVECs was examined by western blotting using anti-HSP27 antibody. The expression of HSP27 was up-regulated in HUVEC cells after *E. coli* stimulated. (E) Immunoprecipitation with human recombinant HSP27 protein with sera from BD patients was also performed to verify whether it was an autoantigen of BD. The HSP27 protein band was clearly present in the immune complexes (band 2), but not in supernatant (band 1). (F) The HSP27 antibodies were detected in 52 of 91 BD patients (57%), 66 of 92 RA patients (72%), 22 of 92 SLE patients (24%), 32 of 90 SS patients (36%), and 0 of 91 healthy controls. The reactivity of BD serum IgG antibodies against human recombinant HSP27 was significantly higher than healthy controls ($P < 0.0001$), SS ($P < 0.0001$), SLE ($P < 0.0001$). There were no significant differences between the BD patients and RA patients ($P > 0.05$). The critical point for positive definition was a number with a higher value than that of the healthy controls (Mean + 3 SD). Data are expressed as mean ± SD and *P* value analyzed by the *t* test.

(ImmunoHunt, Beijing, China) was added to each well and the plate was then incubated for additional 1 h at 37 °C. The absorbance of each well was measured with a plate reader at 450/620 nm (Tecan, Hombrechtikon, Switzerland).

2.10. Statistical analysis

The data and *t* test were analyzed by SPSS software (Version 17, Chicago, IL). *P* values less than 0.05 were considered significant. The critical point for positive definition was a number with a higher value than that of the healthy controls (Mean + 3 SD).

3. Results

3.1. HUVEC cells might be a novel target to screen autoantigens

HUVEC cells showed obvious positive binding to BD sera, which confirmed the presence of AECA in BD patients (Fig. 1 A). Fluorescent differences between three cell lines were quantified by Image J software (Fig. 1 B). Because HUVEC cells presented strongest positive fluorescence ($P < 0.0001$) in this study, they are considered promising target cells for screening autoantigens.

3.2. Detection and identification of the autoantigens

Western blotting was performed to detect the autoantigens of BD. Detection of autoantibodies binding to HUVEC antigens was carried out and IgG autoantibodies to the 27-kDa band were detected in three out five patients with BD in HUVECs stimulated for 12 h, but no binding signal was detected for primary HUVECs, HUVECs cultured for 12 h without stimulation, and the healthy controls (Fig. 1 C). In contrast, this 27-kD band could not be detected in HaCaT cells when incubated with any BD patients' sera (data not shown).

Immunoprecipitation revealed that a protein band with about 27-kDa in HUVEC (after stimulated with *E. coli* for 12 h) could react with BD patient sera (Fig. 2 A). Then the protein band (≈27-kDa) was excised from a polyacrylamide gel and digested with trypsin. The peptide fragments were identified by LC-MALDI-TOF–TOF and then analyzed with MASCOT database, which showed that the target protein was human HSP27 (NCBI number, NP_001531.1; MASCOT score, 60; matched unique fragments, 9) (Fig. 2 B and C).

3.3. HSP27 protein is a target autoantigen of BD

Cloning, expression and purification technology was applied according to our routine optimized method to obtain the

recombinant human HSP27 protein (Fig. 3 A), and the purified HSP27 protein was confirmed by mass spectrum (Fig. 3 B).

To further test the binding ability of the serum antibodies to the recombinant human HSP27 protein, western blotting analysis was performed. 2 μg recombinant human HSP27 protein could successfully recognized by anti-27 kDa positive BD patient serum with western blotting (Fig. 3 C). These results further proved that HSP27 was an autoantigen of BD. In addition, the HSP27 expression is significantly unregulated in *E. coli* stimulated HUVECs by western blotting compared to primary HUVECs (Fig. 3 D).

Immunoprecipitation with sera from BD patients was also performed to further verify whether the detected antigen was an real autoantigen of BD. As shown in Fig. 3 E, the band for the HSP27 protein was clearly present in the immune complex, indicating that the HSP27 protein was a target autoantigen of BD.

3.4. The prevalence of the anti-HSP27 autoantibodies in BD patients

ELISA was performed to determine the reactivity of serum IgG with recombinant human HSP27. It turned out that the HSP27 antibodies were detected in 52 of 91 BD patients (57%), 66 of 92 RA patients (72%), 32 of 90 SS patients (36%), 22 of 92 SLE patients (24%), and 0 of 91 healthy controls. The reactivity of serum IgG against human recombinant HSP27 in BD patients was higher than that in SLE patients ($P < 0.0001$), SS ($P < 0.0001$) or HC ($P < 0.0001$) (Fig. 3 F). There were no significant differences between the BD patients and RA patients ($P > 0.05$).

4. Discussion

In literature, the level of HSP27 in intact vascular endothelial cells is low, and when HUVEC cells are stimulated with heavy metals, heat shock, and other stress conditions, heat shock proteins will be up-expressed [22,29]. Our result in this study also confirmed this interesting phenomenon. So HSP27 protein is hard to be identified in primary HUVEC cells with low abundance. The expression of HSP27 was up-regulated in HUVEC cells might be major important factor, which resulted a new autoantigen of BD successfully identified. The phenomenon is interesting and probably worth further exploration.

HSP27 belongs to a family of small heat shock proteins which have been proved to play an important role in the regulation of cell proliferation, differentiation and apoptosis process triggered by a variety of stimulations [30]. HSP27 overexpression has been described in many tumor tissues, including liver, stomach, and lung cancers [31]. HSPS have a closely relationship with autoimmunity regulation [32], and HSP27 was observed up-regulation in rat experimental autoimmune encephalomyelitis [33]. It has several functions involved in atherosclerosis, and anti-HSP27 antibodies have been detected in patients with coronary artery disease [34]. The presence of anti-HSP27 autoantibodies may reduce the protection function of HSP27 for vascular endothelial cells. In our study, anti-HSP27 antibody was identified as one of candidate AECA of BD, which was positively over 50% in Chinese BD patients. It may has an important role in the process of BD by inducing the dysfunction of endothelial cells, initiating inflammatory response and triggering a stronger autoimmune response.

In summary, we suggested HSP27 as a candidate vascular endothelial cell autoantigens in patients with BD. However, anti-HSP27 antibody is not restricted to BD patients, but might be also involved in multiple autoimmune and inflammatory diseases. The further investigations are necessary to elucidate the exact roles of HSP27 in BD pathogenesis, and more patient samples are needed to test and evaluate its biological role for clinical manifestations.

Conflict of interest

All co-authors have no competing interests.

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