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Research Paper

HnRNP A1 is Involved in Deep Vein Thrombosis Patients with Behçet's Disease

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ABSTRACT

Objective: The aim of this study was to verify the hypothesis originated from bioinformatics and literature reviews that hnNRP A1 may be a new immune target of Behçet's disease (BD).
Methods: First, bioinformatics was used to show the correlation between hnRNP A1 and A2/B1 in amino acid sequences and three dimensional structures. Second, hnRNP A1 was expressed, purified, and immunologically confirmed by systematic immunology methods including: Western blotting, immunoprecipitation and Dot-ELISA. Then, ELISA was used to screen the anti-hnRNP A1 autoantibodies in newly confirmed clinical samples and the clinical significance was compared between anti-hnRNP A1 antibody positive and negative groups. Finally, the endothelial cells antigen profile of one anti-hnRNP A1 antibody positive BD patient was detected using immunoprecipitation with liquid chromatography tandem mass spectrometry (LC–TMS).
Results: In total 720 subjects enrolled and tested in this study. Our results demonstrated hnRNP A1 as a new immune target of BD. The reactivity of BD serum IgG antibodies against hnRNP A1 was significantly higher than healthy controls ($P < 0.0001$), and deep vein thrombosis (DVT) showed a significant higher in the anti-hnRNP A1 antibodies positive group ($P < 0.05$).
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1. Introduction

Behçet's disease (BD) is a chronic systemic autoimmune disease mainly characterized by recurrent oral ulceration, genital ulceration and uveitis (Pineton de Chambrun et al., 2012; Mendes et al., 2009), which can also affect all kinds of blood vessels, joints, nervous and gastrointestinal systems (Mendes et al., 2009). Vasculitisis considered playing a key role in BD pathogenesis, resulting from most of the symptoms observed during the process of this disease. However, the pathogenesis of BD is not clear, and the diagnosis is based on a typical clinical syndrome, no serum test specific for BD is yet available (Mor et al., 2002).

Like other classical autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), BD also exhibits a great diversity of clinical manifestations, indicating the co-existence of a large number of autoantigens (Hsieh and Yu, 2013; Sherer et al., 2004; Nell et al., 2005). Endothelial function has a close relationship with BD pathology (Balta et al., 2014a, 2014b, 2014c). Anti-endothelial cell antibodies (AECA), which are related to autoimmune vascular injury, have been found involved in many rheumatism diseases (Yoshio, 1993; Yoshio et al., 1994). They have also been detected in BD patients and proven to be associated with vasculitis symptoms (Dinc et al., 2003; Aydıntug et al., 1993). Researchers have emphasized the key role of AECA in BD in the past decades, for example, hnRNP A2/B1 was successively identified in human dermal microvascular endothelial cells as an AECA autoantigen of BD (Cho et al., 2012; Liang et al., 2015). And subsequent experiments proved that this valuable antigen play an important role in this disease's pathological process (Cho et al., 2013). Recently, at least four new targets of AECA in BD, like prohibitin, HSP27, ETFB and annexin A2 have been successfully identified by our group (Xun et al., 2014; Chen et al., 2015a, 2015b, 2015c). These findings, not only added new information to explain the AECA related pathology, but also further confirmed the key role of AECA in the process of BD. In patients with BD, vascular involvement

Abbreviations: BD, Behçet's disease; DVT, deep vein thrombosis; SLE, systemic lupus erythematosus; AECA, anti-endothelial cell antibodies; LC–TMS, liquid chromatography tandem mass spectrometry; PVDF, polyvinylidene fluoride membranes; PBS, Phosphate buffered saline.

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Table 1
The characteristics of samples used in this study.

Group (number)	Average age (Range)	Gender (Female)
Behçet's disease (186)	37(14–80)	78
Systemic lupus erythematosus (184)	37(15–75)	152
Rheumatoid arthritis (176)	34(14–68)	135
Healthy controls(174)	28(25–35)	114

and thrombotic tendency is a potentially life risk condition (Balta et al., 2014a, 2014b, 2014c). Deep vein thrombosis (DVT) is the most common manifestation but other organs may also be involved (Balta et al., 2015a, 2015b). Thrombotic events may be associated so many conditions and its treatment may cause serious complications (Balta et al., 2015a, 2015b). More useful details should be explored to differential diagnosis BD patients with DVT and guide its treatment.

Bioinformatics as a useful protein analysis tool has been widely used in the research of autoimmune diseases. The possible role of HSPs proteins were analyzed by bioinformatics approach, suggesting that microbial HSPs, which cross-react with host related proteins and trigger immune responses are possible pathogenic factors involved in the development of BD (Ghasemi et al., 2012). HLA B*5101, which is generally accepted as a genetic marker of BD, can be highly recognized by several short sequences through computerized programs (Baharav and Weinberger, 2012).

HnRNP A1 and A2/B1 were also coexisted in many rheumatic diseases as hnRNP A/B complex antigens. For examples, autoantibodies for A and B proteins of the hnRNP complex have been detected in RA in late 1980s (Caporali et al., 2005). Among 10 hnRNPs, the highest identity was observed between hnRNP A1 and A2/B1 in many systemic rheumatic diseases (Op De Beéck et al., 2012). Using bioinformatics tools, we found that hnRNP A1 and A2/B1 have high protein sequence and structure homology, and many antigen epitopes were shared between them. Theoretical and experimental phenomenon enlightens us that hnRNP A1 may be an autoantigen of BD, too. Above all, traditional immunology Western blotting was used to address this hypothesis, the immunoprecipitation and Dot-ELISA further confirmed the preliminary result.

2. Subjects and Methods

2.1. Subjects

In this study, serological criteria were evaluated through the assessment of 720 subjects in total, the data about epidemiology were shown in Table 1. Including 1) BD patients: 186 BD patients were used for doing large set test. 2) Disease control: 184 SLE patients; 176 RA patients. 3) Healthy control: Blood samples of 174 healthy people were provided by our clinical part. The diagnosis for BD is fulfilled by the criteria proposed by the International Study Group (International Study Group for Behçet's Disease, 1990). The BD patients were collected from the Chinese PLA General Hospital and Shanxi DaYi Hospital, and the disease and healthy controls were collected from the Chinese PLA General Hospital. This study was approved by the ethical committee of the Chinese PLA General Hospital and Shanxi DaYi Hospital, and each patient involved gave informed consent. Samples were collected, dispensed and stored at −80 °C before further tests.

2.2. Antigenic Determinant Prediction

As aforementioned, since hnRNP A2/B1 was a probable antigen of BD, hnRNP A1 and A2/B1 were selected to perform sequence alignment. Sequence alignment was performed with default parameters using Gonnet 250 protein weight matrix by Clustalx software (EMBL, Heidelberg, Germany) (Altschul et al., 1990). Then Bepipred Linear Epitope Prediction (Larsen et al., 2006) was used to predict epitopes of two proteins. Potential common epitopes of two proteins were selected with standards that amino acid lengths were no less than 8 and co-occurred in both hnRNP A1 and A2/B1. The protein structure was obtained from published literatures. Similarity analysis of protein structures was used flexible structure alignment by chaining aligned fragment pairs allowing twists (FACAT) method (Ye and Godzik, 2003 ,2004), which is a method for flexible protein structure comparison. It simultaneously addresses the two major goals of flexible structure alignment, optimizing the alignment and minimizing the number of rigid-body movements in the reference structure.

2.3. Protein Cloning, Expression and Purification

Total RNA was isolated from EA.hy926 cell using TRIzol reagent (Invitrogen, CA). RT-PCR was carried out according to the manufacturer's instruction (Fermentas, MD). Human hnRNP A1 protein was overexpressed in *Escherichia coli* BL21, followed by the purification of recombinant proteins using Ni-NTA resin (CWBIO, Beijing, China). The concentration of protein was determined by BCA assay kit (Biosynthesis Biotechnology, Beijing, China). Purified recombinant protein was confirmed by mass spectrometry (Applied Biosystems, Foster City, CA).

2.4. Western Blotting

Human umbilical vein cell line (EA.hy926) was used in this study. The EA.hy926 was cultured in DMEM (HyClone, UT) containing 10% fetal bovine serum (HyClone, UT). Cell lysates were loaded into the wells of a 12%

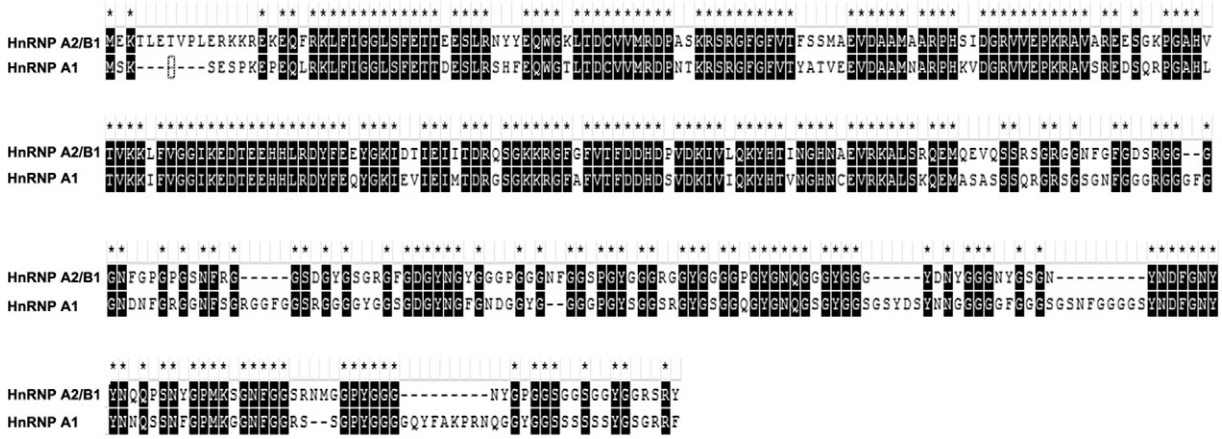


Fig. 1. Sequence alignment of hnRNP A1 and A2/B, and the similarly amino acids was traced black. High similarity between their sequences was shown.

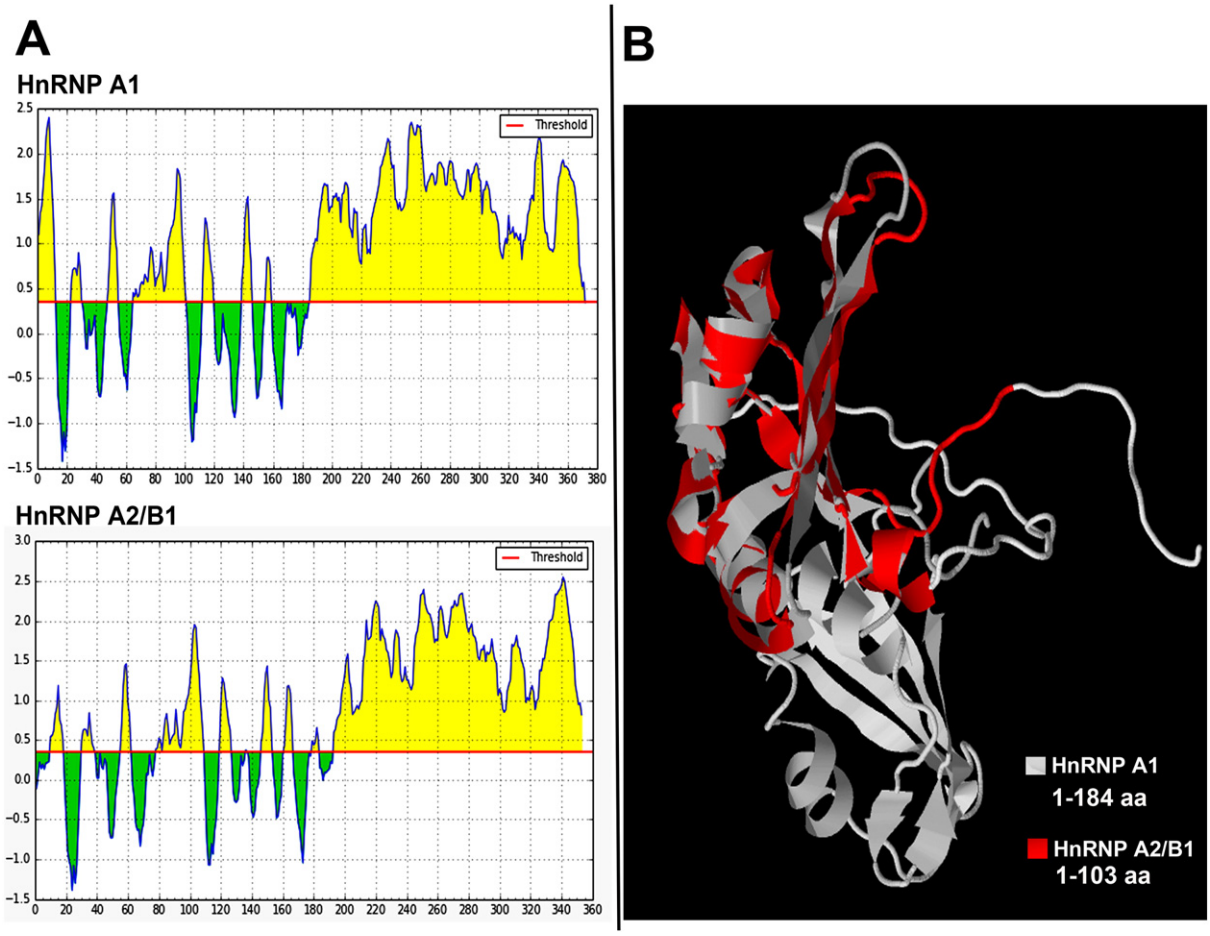


Fig. 2. Antigenic epitopes prediction and structure comparison between hnRNP A1 and A2/B1. (A) Antigenic epitopes distribution of hnRNP A1 and A2/B1. Epitope sequences were displayed beyond the underlines. Antigenic epitopes prediction result of hnRNP A1 and hnRNP A2/B1 where the thresholds of predicted residue scores are 0.898 and 0.871 respectively. The yellow indicates feasible epitopes but the green not. (B) Similar part of protein structure was shown.

polyacrylamide gel and separated. The gel was then transferred onto polyvinylidene fluoride membranes (PVDF; Merck Millipore, MA) that had been washed twice with ultrapure water. The PVDF membranes were then blocked with 5% nonfat milk in PBS at 4 °C for 1 h and then incubated with 10 BD sera that were randomly selected (1:500 dilutions) or sera from random healthy controls at 4 °C for 12 h. The membranes were extensively washed 4 times with 0.5% PBST buffer to remove unbound antibodies. Last, they 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).

2.5. ELISA

The capture recombinant proteins (300 ng/mL) were used to coat the 96 well microplate (Corning, NY) overnight at 4 °C. After three washes with 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

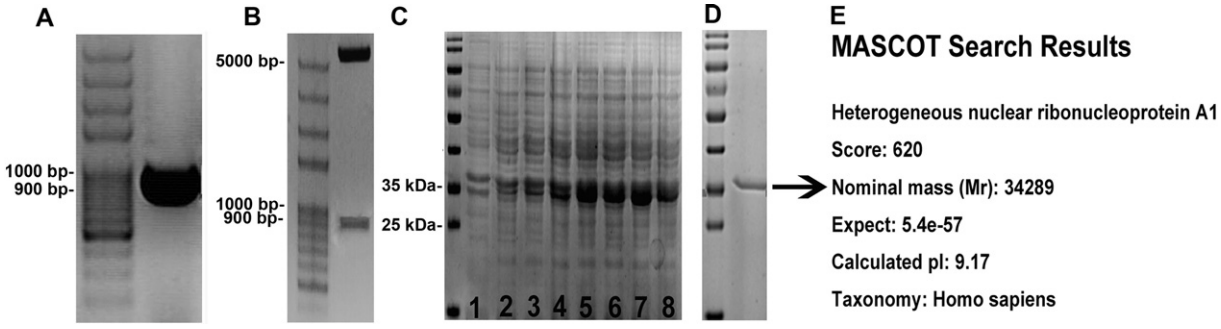


Fig. 3. Gene amplification, expression, purification of hnRNP A1. (A) The right band is the hnRNP A1 gene production after PCR amplification, left band is DNA weight marker.(B) Constructed vectors of PET-28 and hnRNP A1 were identified by double restriction enzyme digestion. 1: DNA marker; 2: The bands after double restriction enzyme digestion. (C) The IPTG inducible expression of *E. coli* BL21. Lane 1–8: the corresponding IPTG concentration is 0, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5 mM in turn. (D) Purified hnRNP A1 protein. (E) Verification of hnRNP A1 by mass spectrometry. The target protein is identified as hnRNP A1.

1:100 in PBS for 2 h at 37 °C. Three washes later, 100 μL goat anti-human IgG/HRP (ImmunoHunt, Beijing, China) was added to each well and the plate was then incubated for an additional 1 h at 37 °C. The absorbance of each well was measured with a plate reader at 450/620 nm (Tecan, Hombrechtikon, Switzerland).

2.6. Statistical Analysis

The clinical characteristics were analyzed by chi-square test and ELISA data was implemented t test with 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).

In-gel digestion and mass spectrometry analysis, dot-ELISA, immunoprecipitation, and indirect immunofluorescence assays were shown in supplementary materials.

3. Results

3.1. Bioinformatics Analysis

Sequence alignment of hnRNP A1 and A2/B was performed as described in method chapter. The homology of two proteins was 62% (Fig. 1.). The high sequence similarity indicates that they have similarity immunogenicity. In the result of Bepipred Linear Epitope Prediction, epitopes were predicted for hnRNP A1 and A2/B1 respectively (Fig. 2A.). The part three-dimensional structure proteins (hnRNP A1: 1-184aa; hnRNP A2/B1:1-103aa) were obtained from Protein Data Bank database (http://www.rcsb.org). Structure pairs with probability <0.05 are significantly similar. After alignment, the two structures are significantly similar with *P* value of 2.45e−09 (raw score is 234.45) (Fig. 2B). This indicates hnRNP A1 and A2/B1 have strong similarity in the molecular level and reveals the similarity immunity quality.

3.2. The Expression and Purification of hnRNP A1

Human hnRNP A1 gene was amplified by Polymerase Chain Reaction (PCR) technique. The production was verified by 1% Agarose Gel Electrophoresis. A target band was obtained at 900 bp and no other specific amplification appeared (Fig. 3A.). The expression vector was validated by double enzymes restriction (Fig. 3B.).

Protein expression and purification technology was used here according to our routine optimized method to obtain the recombinant human hnRNP A1 protein (Fig. 3C and D.). The protein obtained by Ni-NTA resin was reassured by mass spectrometry. The result shows that individual ions scores are much more than 70, indicating that the putative protein is a highly homologous identity (Fig. 3E.).

3.3. Western Blotting

To further test the ability of the sera antibodies that could bind to recombinant human hnRNP A1 protein, an SDS-PAGE analysis was performed. Recombinant human hnRNP A1 protein could successfully be recognized by three of ten BD sera by western blotting (Fig. 4A.). These results proved that hnRNP A1 was an autoantigen of BD.

3.4. Immunoprecipitation

Immunoprecipitation was performed to verify the result of the Western blotting. As shown in Fig. 4B, the band for the hnRNP A1 protein was clearly present in the immunoprecipitates, which was confirmed by mass spectrometry (Fig. 4C.), indicating that the hnRNP A1 was a real target antigen of BD. And, the dot-ELISA result of BD is consistent with the immunoprecipitation too (Fig. 4D.).

3.5. The Prevalence of the Anti-hnRNP A1 Autoantibody in BD Patients

ELISA was performed to determine the reactivity of serum IgG antibody with recombinant human hnRNP A1. The critical point for positive definition was a number with a higher value than that of the healthy controls (Mean + 3 SD). The hnRNP A1 antibody was detected in 54 of 186 BD patients (29%), 40 of 184 SLE patients (22%), 71 of 176 RA patients (40%), and 1 of 174 healthy controls (<1%). The reactivity of BD serum IgG against human recombinant hnRNP A1 was significantly higher than healthy controls ($P < 0.0001$) (Fig. 5A.). We found that the optical densities of anti-hnRNP A1 antibodies showed significant difference between DVT BD patients and normal BD patients without DVT ($P < 0.0001$) (Fig. 5B.).

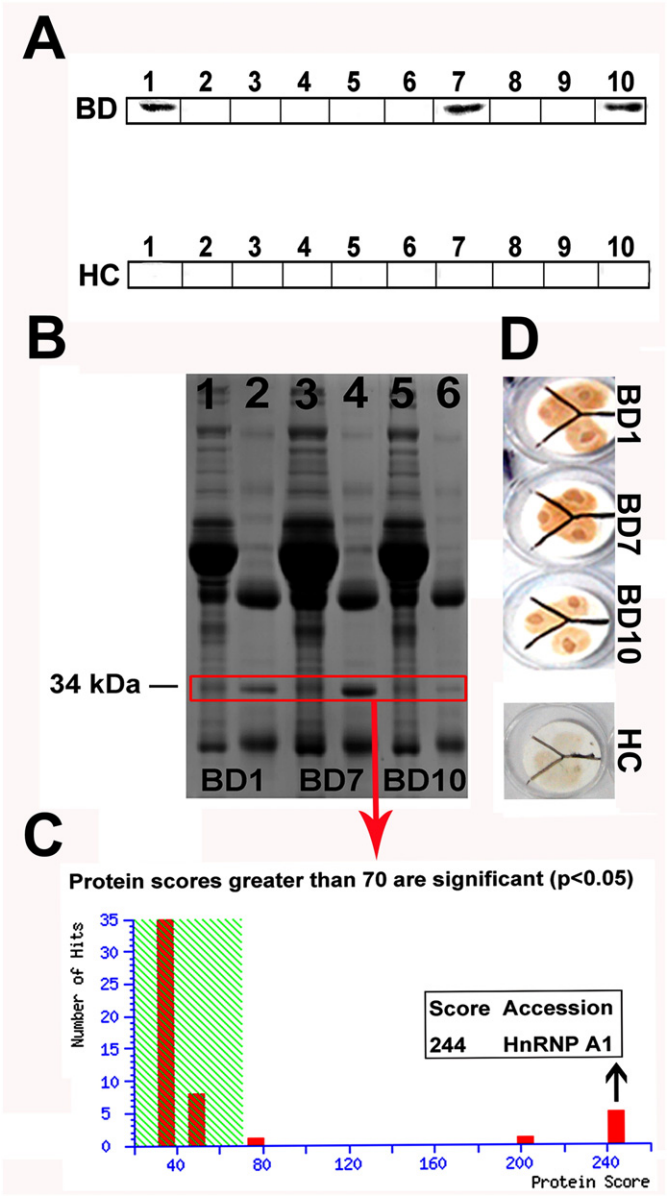
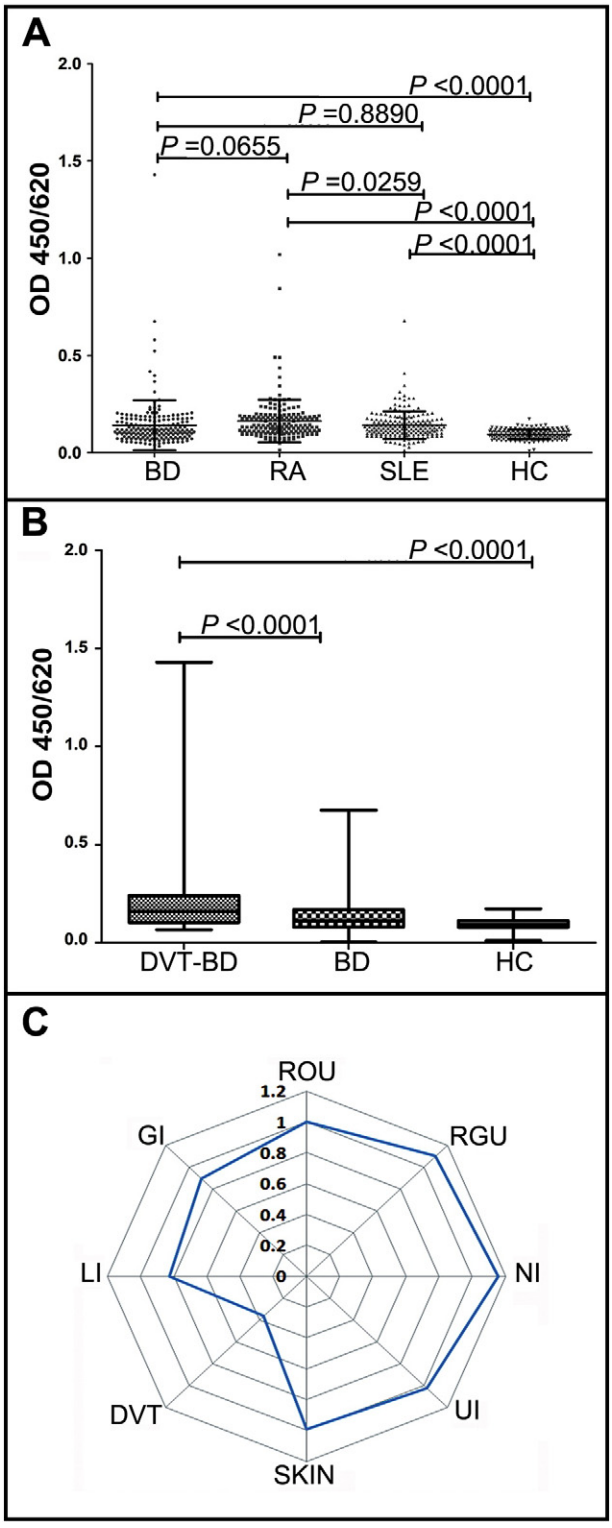


Fig. 4. Antigenicity of hnRNP A1 in BD patients. (A) The western blotting validation result of hnRNP A1 probed with 10 BD patient sera at dilutions of 1:500 and HC controls. (B) The hnRNP A1 protein band was clearly present in the immunoprecipitates (band 2, 4, 6), but not in supernatant (band 1, 3, 5). (C) Verification of immunoprecipitates band by mass spectrometry. The mascot score was 244 for hnRNP A1, further indicating that the hnRNP A1 protein was a potential target antigen of BD. (D) Dot-ELISA result. Three positive results were then used to perform Dot-ELISA to assure the antigenicity of hnRNP A1. Compared with healthy control (HC1), only BD sera show positive result. BD: Behçet's disease, HC: healthy controls.

3.6. Clinical Significance

The patients with detailed clinical information were analyzed. By comparing the clinical information between the anti-hnRNP A1 antibody positive and negative groups, we found 9 BD patients with DVT in 54 anti-hnRNP A1 antibody positive patients, while 8 patients with deep vein thrombosis in 131 anti-hnRNP A1 antibody negative patients. DVT BD patients showed a significant difference between anti-hnRNP A1 antibody positive group and in the negative group ($P < 0.05$). Other clinical symptoms (recurrent oral ulcers, recurrent genital ulcers, anterior uveitis, skin lesions, gastrointestinal system involvement, nervous system involvement and urinary system involvement) showed no significant differences between the anti-hnRNP A1 antibody-positive and in the negative groups (Fig. 5C.).



3.7. HnRNP A1 and hnRNP A2/B1 Were Detected Co-existing in an Anti-hnRNP A1 Antibody Positive BD Patient

Follow on ELISA test, immunoprecipitation with extracts of EA.hy 926 cells and BD sera was analyzed by liquid chromatography tandem mass spectrometry. In the antigen and antibody complex, two unique peptides of hnRNP A1 (SSGPYGGGGQYFAKPR and NQGGYGGSSSSSSYGSGR) were identified. And, hnRNP A2/B1 was also been found co-existed by hnRNP A1 (Fig. 6). This further validated our hypothesis from bioinformatics. Another important endothelial cell antigen Annexin A2 was found again, indicating that this method was reliable. In healthy control, any peptide of hnRNP A1 and A2/B1 was not found.

4. Discussion

HnRNP A1 has been reported as an important autoantigen of the autoimmune response in rheumatic diseases in many articles. Autoantibodies to hnRNP complex have been detected in patients with RA, SLE and mixed connective tissue disease in succession (so RA and SLE are chosen in this study as disease control) (Hassfeld et al., 1989; Isenberg et al., 1994). Recent researches indicated that hnRNP A1 can become target for autoantibodies in other autoimmune diseases, such as systemic sclerosis, Sjogren's syndrome, dermatomyositis (Generini et al., 2009; Op De Beéck et al., 2012) which supposed that hnRNP A1 may contribute in the regulation of inflammation (Noguchi et al., 2009). Compared with other classic disease specific autoantibodies (such as anti-SSA and CCP), autoantibodies to the hnRNP-A1 display a more common feature and exist in many rheumatic diseases (Steiner et al., 1996). Thus we believed that hnRNP A1 was not a candidate diagnostic marker in clinical application for all BD patients.

In this study, hnRNP A1 was firstly identified also as a target antigen of BD. About 30% BD patients have circulating antibodies to hnRNP A1 in Han Chinese (both the independent ELISA and WB assay got the similar results). By comparing the clinical information between the anti-hnRNP A1 antibody positive and negative groups, DVT showed a significant higher in the anti-hnRNP A1 antibodies positive group ($P < 0.05$). Many proteins have been proved with close relationship with BD process. Serum endocan levels as a marker of disease activity in patients with BD has been found (Balta et al., 2014a, 2014b, 2014c). However, the relationship between hnRNP A1 and DVT has not been reported.

Fig. 5. Reactivity of sera IgG antibodies against hnRNP A1. (A) ELISA was performed to detect the reactivity of serum IgG antibodies against human recombinant hnRNP A1 protein. The reactivity of BD serum IgG antibodies against human recombinant hnRNP A1 was significantly higher than healthy controls ($P < 0.0001$). (B) Anti-hnRNP A1 antibodies showed significant difference between BD patients with DVT and normal BD patients. The critical point for positive definition was a number with a higher value than that of the healthy controls (Mean + 3 SD). Data in A are expressed as mean ± SD, the plot whiskers of B mean min to max data and *P* value analyzed by the t test. (C) BD-related manifestations in IgG anti-hnRNP A1 antibody positive and negative group. Comparing the clinical information between the anti-hnRNP A1 antibody-positive and negative groups. The ratio was obtained from (Numberclinical+/Numberpositive)/(Numberclinical+/Numbernegative). The data were analyzed using SPSS software (Version 17, Chicago, IL). BD: Behçet's disease, HC: healthy controls, RA: rheumatoid arthritis, SLE: systemic lupus erythematosus. DVT: deep vein thrombosis, ROU: recurrent oral ulcers, RGU: recurrent genital ulcers, NI: nervous involvement, UI: urinary involvement, SKIN: skin involvement, DVT: deep vein thrombosis, LI: lung involvement, GI: gastrointestinal involvement.

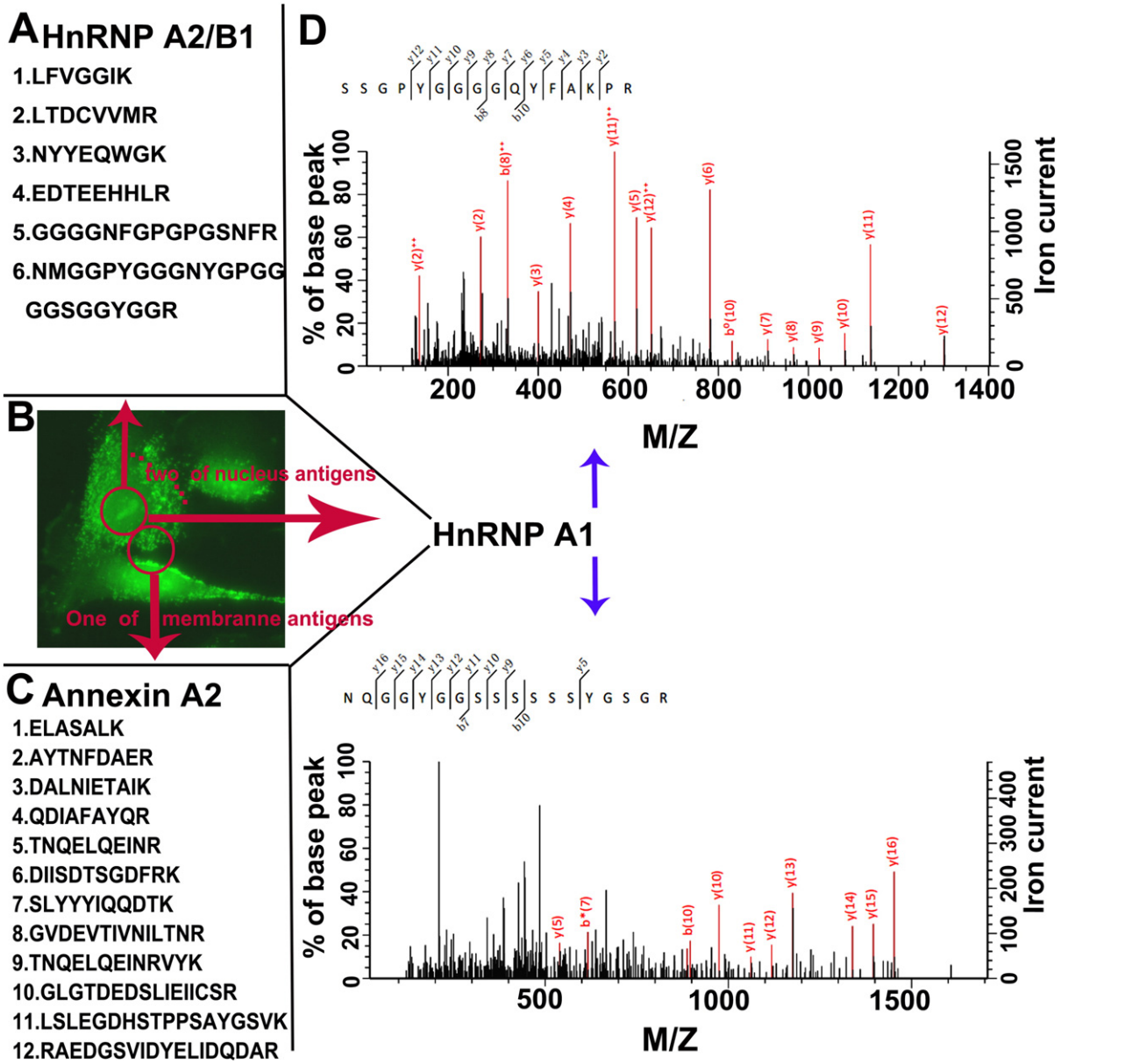


Fig. 6. HnRNP A1 and hnRNP A2/B1 antibodies were detected co-existed in one anti-hnRNP A1 antibody positive BD patients. (A) HnRNP A2/B1 was identified by mass spectrometry (SwissProt Accession number, P22626). Matched unique peptides of hnRNP A2/B1 were shown. (B) The staining pattern of EA.hy926 cells by this anti-hnRNP A1 antibody positive serum with indirect immunofluorescence method. (C)Twelve unique peptides matched one endothelial cells antigen annexin A2 (SwissProt Accession number, P07355) of BD. (D) Two matched peptides of hnRNP A1 (SwissProt Accession number, P09651) and their fragments were shown.

Intriguingly, existing literatures have proved that hnRNP A1 was related to virus infection (Lin et al., 2009; Kim et al., 2007). Many researchers believed that the onset of BD is associated with microbial infection. Sometimes anti-HSV antibody can be detected in the circulation serum of BD patients (Sohn et al., 2001), furthermore, the role of *Streptococcus sanguis* in BD immunopathogenesis has been reported in several studies before (Mizushima, 1989; Isogai et al., 1990). HnRNP A1 may be a considerable molecular in the process of BD pathology by mediating infection of pathogenic microorganisms, further inducing the endothelial cells dysfunction, initiating inflammatory response and triggering a stronger autoimmune response.

Bioinformatics was successfully used as a tool here to find new autoantigen of BD. With the application of a variety of high-throughput technologies, we received more and more data in, and one big data time about human autoimmune disease research is coming. This study is the starting point of bioinformatics applications in our team, but not a destination. More bioinformatics analysis and experiments will be performed to reveal the essential association between hnRNP A1 and A2/B1 antigens.

Future work will focus on developing high specificity *in vitro* anti-hnRNP A1 immunoassay as well as striving to extend the assay to more autoimmune disease patients from multi-medical centers. And the co-exist condition of hnRNP A1, hnRNP A2/B1 and relevant peptides will be measured.

Author's contributions

HWD and YPT designed the experiments. PC, CYZ, CL, DM performed and analyzed experiments, and wrote the manuscript. CHY checked the data of experiments and implemented statistical analyses. HWD, GYC, YPT, LYZ supervised progress and edited the manuscript.

Conflicts of interest

The authors declare no financial or commercial conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.03.009.

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