# Identification of hnRNP C1/C2 as an Autoantigen in Patients with Behcet's Disease

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## ABSTRACT

**Background:** Ribonucleoproteins particles that form the spliceosomes are among the most frequently targeted molecules of the autoimmune response. In the last few years, autoantibodies against all A/B hnRNP proteins have been found in the sera of patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), and serve as diagnostic markers for several rheumatic diseases. However, the functional role of hnRNP C1/C2 in autoimmune diseases is still not clearly understood. **Objective:** To identify hnRNP C1/C2 as an autoantigen in patients with Behcet's Disease (BD). **Methods:** First, HaCaT and EA.hy926 cells were cultured and RNA was extracted. Second, amplification of the corresponding gene by RT-PCR, cloning, and purification techniques was applied to acquire the recombinant protein hnRNP C1/C2. Third, the target protein band was excised from gel electrophoresis, digested with trypsin, and analyzed by (MALDI-TOF/). Finally, Western blotting and ELISA were performed to verify the immunoreactivity of BD serum with recombinant hnRNPC1/C2. **Results:** Results demonstrated that the reactivity of BD serum against recombinant hnRNP C1/C2 protein was significantly higher as compared to healthy control ($P<0.001$). **Conclusion:** hnRNP C1/C2 can be considered as a self antigen which might be involved in BD pathology in Hans Chinese population.

*Hussain M, et al. Iran J Immunol. 2018; 15(2):133-141.*

**Keywords: Autoimmunity, hnRNP-C, Immunogenicity, Nuclear RNPs**

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## INTRODUCTION

Behcet's disease (BD) is a rare chronic autoinflammatory condition of unknown origin, which is caused by vasculitis that can damage blood arteries and veins throughout the body. It is a multi-systemic disease with triplet symptoms of oral, genital ulcer, and ocular inflammation (1). The disease was named in 1937, after its first identifier HulusiBehcet, a Turk dermatologist. Geographically BD is distributed all over the world but more prevalent along the old Silk Road, extending from the Middle East to China (2,3). BD has a variety of clinical expressions, indicating the co-existence in their autoantigens(4,5). Similarly, BD has shown an association with various autoimmune and inflammatory disorders such as polymyositis, Sjögren syndrome (SS), Crohn's disease (CD), Systemic lupus erythematosus (SLE), and Rheumatoid arthritis (RA) (6,7). Endothelial cells dysfunction (8,9) and vasculitis (10,11) are major pathological findings in BD, although the etiopathogenesis of the disease is still obscure. Anti-endothelial cell antibodies (AECA) play a part in the pathology of vascular injury (i.e., vasculitis), which is the major cause of autoimmune diseases (12,13). Heterogeneous nuclear RNPs are a group of about 30 proteins known as hnRNPs (A through U). These proteins are present in the nucleoplasm and some of them have cytoplasmic expressions such as A1, D, F/H, and K (14). Nucleoplasmic hnRNPs are associated with RNA polymerase II with newly transcribed pre-mRNA and form a large complex of RNA binding proteins that play a vital role in the biogenesis of mRNA (15). Moreover, hnRNPs involve in other different cellular functions such as chromatin remodeling, translocation, telomere elongation, DNA repair, pre-mRNA 3-end processing, mRNA stability, translation, regulation of proteins implicated in mediating cellular growth, apoptosis, splicing, and nuclear-cytoplasmic shuttling (16,17). Heterogeneous nuclear RNP proteins are important targets of autoimmune responses such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and mixed connective tissue disease (MCTD) (18).hnRNPs also are reported as autoantigens (19). Among the core hnRNPs, C1 and C2 have a similar structure with except that 13 amino acids in addition near the middle of the C2 protein (20). Both C1 and C2 are nuclear proteins that are restricted to the nucleus. They probably retain the incompletely processed pre-messenger RNA (mRNA) involved in cleavage at the 5′ splice site and formation of the spliceosome, which is the targeted molecule of autoimmune responses (15). Antibodies to hnRNP C1/C2 and R have been detected in sporadic cases (21,22). Our group has already described hnRNP A1 and A2/B1 biomarkers in patients with BD (23,24).

In the present study, we identified hnRNP C1/C2 autoantibodies in Behcet's disease patients by matrix-assisted laser desorption-ionization (MALDI-TOF) tandem mass spectrometry (MS), after in-gel digestion with trypsin. Results were further confirmed by Western blotting and homemade ELISA, which showed the involvement of hnRNP C1/C2 in BD pathology.

## MATERIALS AND METHODS

**Collection of Serum Samples.**176 subjects were enrolled in this study. Samples were categorized into four groups (1) BD: Experimental group (n=44) with age range of 16-62 years, (2) SLE: Diseased control group (n=44) with age range of 18-43 years, (3) RA: Diseased control group (n=44) with age range of 34-83 years, and (4) HC: Healthy

control group) (n=44) with age range of 25-38 years. BD diagnosis was carried out in accordance with the international criteria (International Study Group for Behçet's Disease, 1990). Peking Union Medical College Hospital ethical committee approved the sample collection and usage of human specimens from consenting patients. Serum samples were aliquoted and kept at-80$^{O}$C until used.

**Cell Lines.** Human umbilical vein endothelial cells (HUVEC) and human immortalized non-tumorogenic keratinocyte cells (HaCaT) are established cell lines, which were purchased from American Type Culture Collection (Manassas, VA) and supplied by Eppelheim, Germany. EA.hy926 and HaCaT cells were cultured in DMEM (HyClone, UT) containing 10% fetal bovine serum (HyClone, UT) as described in our previous studies (25). The integrity of cells was tested carefully before the experiment to ensure its purity. Culturing conditions of these cell lines were set as per our previous studies.

**Cloning, Expression, and Purification of hnRNP C1/C2.**This method was performed as per our standard lab protocol (26). In this procedure, mRNA was extracted with TRIzol reagents (Invitrogen, CA) from EA.hy926 and HaCaT cells. Forward and reverse primers were designed as mentioned below and were amplified by RT-PCR as per kit's instruction (Fermentas, MD).

Forward (EcoR I) 5-CCGGAATTCCGGAACTCCCGTGTATTCATTG-3, Upstream
Reverse(Xho I) 5-CCGCTCGAGCGG TCTCCTTCCTCAGCCTCT-3, Downstream

After enzyme digestion, PCR product of hnRNP C1/C2 was ligated with vector pET-28($a^{+}$), transformed into *E.coli* DH5α, and the transformed *E.coli* DH5α strain was recovered in 400 μL Super Optimal Broth (SOC) medium. After 1.5 h, the same was transferred to LB medium containing antibiotic kanamycin (50μL/mL). Extracted recombinant plasmid from *E.coli* DH5α was transformed into the high expression engineering strain *E.coli* BL21. Under optimal conditions (a temperature and shaking speed of 37$^{O}$C and 200 rmp, respectively), the cloned gene was achieved by adding IPTG (1 mM) when the level of optical density (OD) reaches 0.4-0.6. Finally, the recombinant hnRNP C1/C2 with N-terminal hexahistidine-tag was purified by Ni-NTA resin (Qiagen, Hilden, Germany). Finally, the concentration of recombinant hnRNP C1/C2 protein was determined by BCA assay (Biosynthesis Biotechnology, Beijing, China), and stored at -80$^{O}$C for further testing.

**Identification of hnRNP C1/C2 by Mass Spectrometry.** This method we applied and described in detail in our previous studies (23). In brief, the band of purified recombinant protein (hnRNP C1/C2) was excised from the gel, destained with a mixture of 25 mM $NH_4HCO_3$ and 50% acetonitrile. Gel pieces were dehydrated in vacuum centrifugation and soaked in 10 mMdithiothreitol for almost 2 h. Then, an equal amount of 25 mM $NH_4HCO_3$ and 55 mMiodoacetamide was added in dithiothreitol solution and incubated at room temperature for 45 min in dark. The digested and dried gel pieces were mixed with 20 μL (0.05 mM $NH_4HCO_3$) buffer containing trypsin (Sigma, MO) for overnight at 37$^{O}$C. Finally, the target protein was identified by mass spectrometer MALDI-TOF (Applied Biosystems, model 47000, Foster City, CA) and the data were searched with Mascot database software (Matrix Sciences, London, UK).

**Western Blotting.**In this step, 12% polyacrylamide gel was prepared and samples were loaded into the wells to separate and identify the recombinant human hnRNP C1/C2 protein. Human IgG protein was also loaded as a positive control to relocate the gel on polyvinylidene fluoride membranes (Merck Millipore, MA). Skim milk (5%) was used to block the PVDF membranes. Next, 5 BD, 5 RA, 5 SLE, and 5 healthy control sera

(1:500 dilution) were added and incubated at 4℃ for 12 h. The unbound antibodies were removed after washing the membranes 5 times with PBST 1%. The secondary antibody, i.e., HRP/IgG goat anti-human (ImmunoHunt, Beijing, China), was added and incubated for 60 min at 37℃. Finally, ECL mix was prepared (Solutions A and B) and the membranes were incubated according to the kit instruction (Applygen, Beijing, China).

**In-house Development of ELISA.** Microtiter plates (Corning, NY) were coated with purified recombinant hnRNP C1/C2 and incubated for a whole night at 4ºC. Next day, the solution was washed three times with PBST and then the wells were blocked with 200 µL goat serum (5%) and incubated for 1 h at 37ºC. Subsequently, the serum samples were added to dilution buffer with the ratio 1:100, followed by incubation at 37ºC for 2 h. The plates were washed with PBST three times and then 100 µL anti-human conjugated HRP/IgG (ImmunoHunt, Beijing, China) was added to each well to incubate it again for an additional 1 hr at temperature 37ºC. The liquid was dispensed and 100 µL TMB substrate was added to the solution and incubated at room temperature for 10 min in dark place. Finally, 50 µL of 2M $H_2SO_4$ (stop solution) was added and the absorbance was measured at 450/620 nm with a plate reader (Tecan, Hombrechtikon, Switzerland).

**Statistical Analysis.** To prove the validity of our results, the data were analyzed by Fisher and Wilcox's test using the SPSS software (Version 21 Chicago, IL). $P<0.05$ was taken as the significance level. The statistical software MedCalc (Version 9.2) was used to diagnose healthy/diseased groups and plot the graphs.

## RESULTS

**Cloning, Expression, and Purification of hnRNP C1/C2.**

Human hnRNP C1/C2 gene and expression vector was amplified by Polymerase Chain Reaction (PCR) technique and were by double restriction enzymes by Gel Electrophoresis (1% agarose) as mentioned in (Figure. 1A). A target band was obtained at 800 bp and no other specific amplification appeared (Figure. 1B). The chromatogram of pET-28 a $^{(+)}$ -hnRNPC1/C2 is shown in (Figure. 1C).Human hnRNPC1/C2 was over-expressed in E.coli BL21 as a fusion protein with His-Tag expression level and was assessed by adding IPTG (1mM) (Figure. 2A). Then, recombinant hnRNP C1/C2 was overexpressed as a fusion protein with N-terminal hexahistidine-tag and purified with Ni-NTA resin kit. The presence of eluted target protein was confirmed by SDS-PAGE, as shown in Figure. 2B.

**Mass Spectrometry Analysis.**

Purified recombinant human hnRNP C1/C2 was reassured by mass spectrometry. Mass spectrum identification showed individual ions score is greater than ≥28 (Figure. 2C), suggesting that the putative protein has a homologous identity or extensive homology and significant p-value ($P<0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits, as shown in PMF analysis (Figure. 2D).

**Western Blotting of hnRNP C1/C2 with patients' sera.**

Recombinant hnRNP C1/C2 associated sera of 5 BD, 5 RA, 5 SLE, and 5 HC were randomly selected from the repository of sera bank. In this study, 1 BD sera out of 5 BD patients demonstrated antigen-antibody positive reaction and all others including 5 healthy people sera showed no reaction, indicating that hnRNP C1/C2 is a probable

autoantigen of BD patients (Figure. 3A).

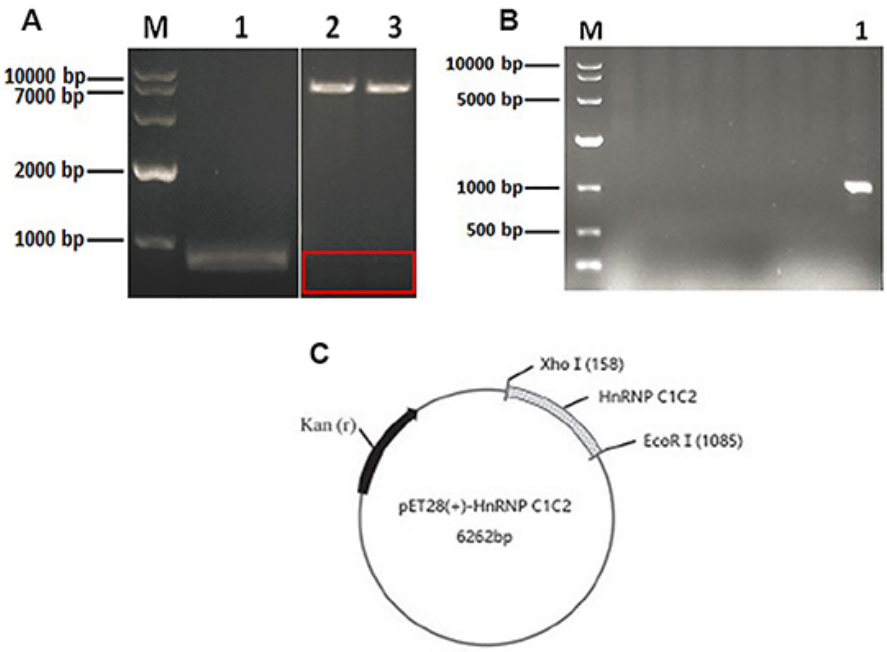


**Figure 1. Cloning, Expression and Purification of hnRNP C1/ C2):(A)** Lane 1: hnRNP C1/C2 gene band having 800bp molecular weight, Lane 2&3 having band 7000 bp molecular weight of plasmid (pET-28(a+), M**:** DNA marker, **(B)** PCR band position M: DNA marker band, Lane 1: hnRNP C1/C2 band **(C)** Confirmed structure of recombinant plasmid pET-28(a+) –hnRNP C1/C2 whose total sequence is 6262 bp.

**ELISA based measurement of anti-hnRNP C1/C2 autoantibodies.**

To determine the titer of anti-hnRNP C1/C2 autoantibodies in BD patients, the reactivity of BD serum with recombinant protein was performed by homemade ELISA. For this purpose, 76 samples having 44 for each category (BD, SLE, RA, and Healthy control) were analyzed with the same operating procedure. Initially, these samples with anti-IgG, IgA, and IgM conjugated with HRP antibodies were tested.

Only IgG secondary antibody showed an elevated reaction with BD serum samples. In this test, recombinant hnRNP C1/C2 autoantibodies were detected in 17 of 44 BD (38%), 3 of 44 RA patients (6%), and 4 of 44 SLE (9%) while 2 of 44 (4%) serum reaction in healthy control (HC) were measured. Statistically, BD patients showed statistically significant p-values ($p<0.001$) compared with healthy control. Anti-hnRNP C1/C2 antibodies in all groups are described in a scatter plot, which shows a clear difference between BD, SLE, RA, and healthy control (Figure. 3B).

**Statistical Analysis.**

MedCalc software was used to calculate the area under the curve (AUC), which discriminate BD and healthy groups. Scatter plot showed statistically significant results for BD compared with HC ($P<0.001$). The cut-off value was 0.37, which is significant at 100-Specificity. The accuracy of the test was determined by the area under ROC curve (AUC=0.84) with CI=95%. By this mean, we clearly classified the two groups (BD and HC), as depicted in (Figure. 3C).

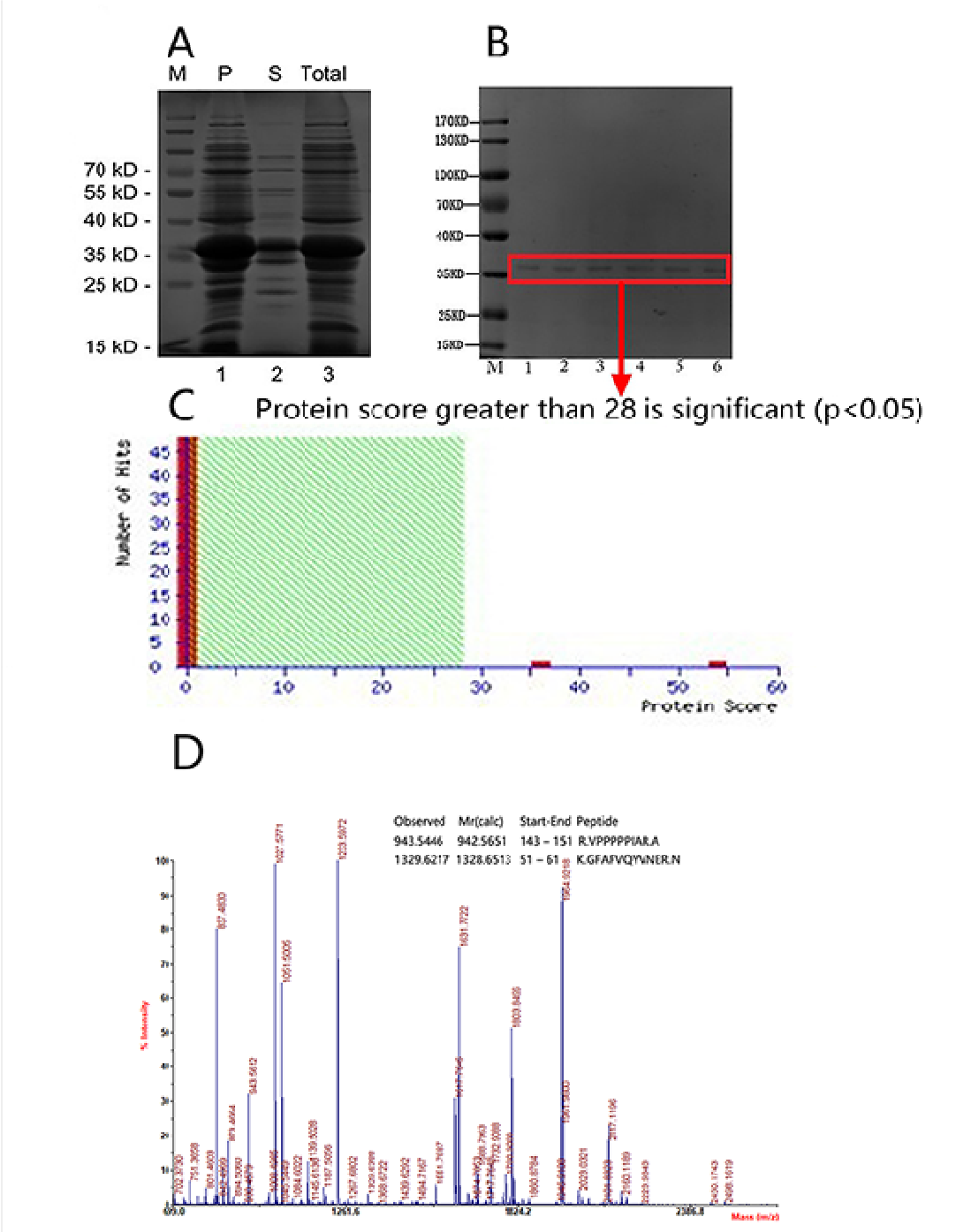


**Figure 2. Purification of Recombinant hnRNP C1/C2:** Presence of eluted protein was confirmed by SDS-PAGE **(**Histidine Tagged hnRNP C1/C2) **(A)** Lane 1(P): Precipitated Electrophoresis band, Lane 2 (S): Supernatant Electrophoresis band, Lane 3 (Total): Whole Protein hnRNPC1/C2 band, M: Protein Marker **(B)** Purified hnRNP C1/C2 was confirmed by SDS-PAGE: Lane: I-6, Purified hnRNP C1/C2 protein bands>36 kDa. M: Protein Marker **(C)** Mass spectrum analysis confirmed the score of hnRNP C1/ C2 is ≥28 indicate identity or extensive homology ($p<0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits as shown in PMF analysis**(D)**.

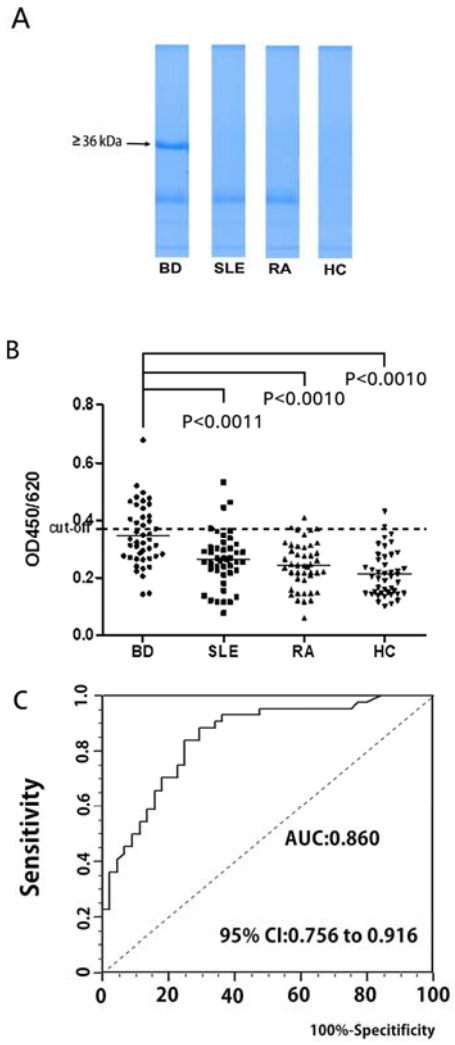


**Figure 3. Western Blotting & ELISA Results. (A)** Western blotting using 5 sera from Chinese BD, RA, SLE and healthy individuals were taken. It was detected that serum IgG can react against the recombinant hnRNPC1/C2, while such reactivity was not found in healthy controls (HC) and other diseased groups **(B)** Verification of ELISA results are demonstrated in a scatter plot which differentiate the difference of antibodies reactivity between BD, SLE, RA and HC while dotted lines show the significant cut-off value (0.3713) **(C)** ROC curve demonstrates the specificity and sensitivity levels and 95% confidence intervals (CI) between BD and healthy control.

## DISCUSSION

Heterogeneous nuclear RNPs are a family of highly conserved proteins that are involved in several human autoimmune diseases. Structurally, C1 and C2 proteins are identical with a difference of 13 amino acids insertion near the middle of the C2 protein. Similarly, groups A and B of hnRNPs constitute the core proteins of the 40S hnRNP particles (20,27). Many rheumatic diseases are associated with autoantibodies that directly react with nuclear, nucleolar, and cytoplasmic proteins and are often involved in vital cellular processes. Previously, autoantibodies against all A/B hnRNP proteins have also been found in the sera of patients with rheumatoid arthritis (RA), systemic lupus

erythematosus (SLE), and mixed connective tissue disease (MCTD) (18). The existence of anti-C1/C2 has been identified in patients with arthritis, psoriasis, myositis, and scleroderma (21). Recently, the induced autoantibodies of hnRNP A2/B1 and A1 proteins of the hnRNP family has been found by our group in patients with BD (23,24). In the present study, 17 of 44 BD patients from Han Chinese population had circulating autoantibodies to hnRNP C1/C2. In addition to BD, 4 of 44 SLE and 3 of 44 RA patients showed antibody titers against the hnRNP C1/C2 protein. Moreover, Western blotting confirmed the immune reactivity of hnRNP C1/C2 with BD patients in Han Chinese population. Although hnRNP C1/C2 might not be a great candidate as a diagnostic marker in clinical application for BD patient, this study provides a new thinking of immunological co-relationship between hnRNP C1/C2 autoantibodies belonging to the same family and their development mechanism in autoimmune diseases. However, there is still a long way to go to understand the precise functions of individual hnRNP"C" and their correlations in other autoimmune diseases. In the light of these findings, hnRNP C1/C2 in patients with BD may be an autoantigen which may be used in the early and precise diagnosis of this disease.

## ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (No. 81571592, 31371203) and China's Government Funding (No. 2018ZX10301201).

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