

Monoclonal antibody against H1N1 influenza virus hemagglutinin cross reacts with hnRNPA1 and hnRNPA2/B1

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Abstract. Following influenza A vaccination, certain individuals exhibit adverse reactions in the nervous system, which causes a problem with the safety of the influenza A vaccine. However, to the best of our knowledge, the underlying mechanism of this is unknown. The present study revealed that a monoclonal antibody (H1-84mAb) against the H1N1 influenza virus hemagglutinin (HA) protein cross-reacted with an antigen from brain tissue. Total brain tissue protein was immunoprecipitated with this cross-reactive antibody, and mass spectrometry revealed that the bound antigens were heterogeneous nuclear ribonucleoprotein (hnRNP) A1 and hnRNPA2/B1. Subsequently, the two proteins were expressed in bacteria and it was demonstrated that H1-84mAb bound to hnRNPA1 and hnRNPA2/B1. These two proteins were expressed in three segments and the cross-reactivity of H1-84mAb with the glycine (Gly)-rich domains of hnRNPA1 (195aa-320aa) and hnRNPA2/B1 (202aa-349aa) was determined using ELISA blocking experiments. It was concluded

that the Gly-rich domains of these two proteins are heterophilic antigens that cross-react with influenza virus HA. The association between the heterophilic antigen Gly-rich domains and the safety of influenza A vaccines remains to be investigated.

Introduction

Influenza A infection may cause central nervous system complications, including multiple sclerosis, febrile seizures, encephalopathy and Reye's syndrome, as well as other neurological abnormalities, high mortality, poor prognosis and sequelae in the majority of survivors (1-5). Vaccination is the most effective method of preventing and controlling influenza. However, due to certain factors, including the immunological characteristics of the influenza vaccine itself, a small number of influenza vaccination subjects may develop diseases, including Guillain-Barré syndrome and narcolepsy, while obtaining immunoprotection. At present, to the best of our knowledge, the causes of these serious adverse reactions remain unclear (6,7).

In our previous study, 84 monoclonal antibodies (mAbs) against hemagglutinin (HA) were prepared. When identifying their characteristics, it was revealed that the H1-84mAb not only binds to the HA antigen, but also cross-reacts with human brain tissue, suggesting that H1N1 influenza virus HA and human brain tissue have a heterophilic antigen (8). Heterophilic antigens are a class of common antigens that are unrelated between species, and exist in humans, animals and microorganisms (9). When studying microbial infection immunity, it has been revealed that *E. coli* O14 lipopolysaccharide and human colon mucosa possess heterophilic antigens, leading to the occurrence of ulcerative colitis (10). Antibodies against the enterovirus Coxsackie VP1 protein may cross-react with mitochondrial proteins of β -islet cells, and this may be associated with infection-induced diabetes (11).

The presence of heterophilic antigens between influenza HA and human brain tissue may be an important factor affecting the safety of the influenza A vaccine. Therefore,

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it is important to find and identify heterophilic antigens recognised by H1-84mAb. It has been previously identified that H1-84mAb recognises a nine-peptide linear epitope of influenza HA (12). The present study used H1-84mAb as a research tool to confirm its cross-reactivity with heterophilic antigens from brain tissue and to provide experimental data for subsequent studies investigating the pathogenic mechanism involving these antigens.

Materials and methods

Experimental materials. A total of 5 Male Sprague Dawley (SD) rats (weight, 250–300 g) were purchased from the Experimental Animal Centre of the Fourth Military Medical University (Xi'an, China) in order to prepare paraffin sections and total protein extracts of rat brain tissues. The 6–8-week SD rats received humane care and were raised in the same clean environment, with ambient temperature at 26°C, humidity of 50±5%, and a 12-h light/dark cycle. In addition, the standard food and water available *ad libitum* to the animals was sterilized. Following the experiments, the animals were anesthetized with ether, and clinical manifestations included loss of consciousness, loss of systemic pain, inhibition of reflexes, and skeletal muscle relaxation. The animals were euthanized by cervical dislocation. Cell culture supernatant of the H1-84mAb against influenza virus hemagglutinin was maintained in our laboratory (titre, 1:1,000; <https://doi.org/10.1007/s12250-019-00100-9>). A horseradish peroxidase-labelled goat anti-mouse secondary antibody (cat. no. B141027) and a tissue immunohistochemical staining kit (cat. no. QN2755) were purchased from OriGene Technologies, Inc. Bovine serum (cat. no. 16000-044) for cell cultures was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. RIPA lysis buffer (cat. no. P0013C) and BeyoECL Plus (cat. no. P0018S) were purchased from Beyotime Institute of Biotechnology. The SP2/0 hybridoma cells were purchased from Hangzhou Lianke Meixun Biomedical Technology Co., Ltd. (cat. no. YB-ATCC-2224). BL21(DE3)pLysS competent cells, >10⁶ cfu/μg, were purchased from Promega Corporation (cat. no. L1191). Protein A/G PLUS agarose (cat. no. GS4780) was purchased from Santa Cruz Biotechnology, Inc. The total RNA extraction kit (cat. no. DP433), cDNA first-strand synthesis kit (cat. no. KR104) and bicinchoninic acid (BCA) protein assay kit (cat. no. P0012S) were purchased from Tiangen Biotech Co., Ltd. PCR polymerase (cat. no. C10966-018), the pMD19-T vector (cat. no. 6013) and DM5000 DNA Marker (cat. no. 116899) were purchased from Takara Biotechnology Co., Ltd., and a prokaryotic expression vector was kept at our laboratory. Primer synthesis and sequencing were performed by Beijing Liuhe Huada Gene Technology Co., Ltd.

Preparation and identification of mAbs. mAbs against the H1N1 influenza virus HA protein, including H1-84mAb, were prepared in our laboratory. The titre of the antibody was determined using the indirect ELISA method, and the reactivity of the antibody with the HA antigen was determined by western blotting (8). It has been previously determined that H1-84mAb binds to a nine-peptide linear epitope (191-LVLWGIIHP-199) on HA (12).

Immunohistochemistry. In brief, SD rat brain tissues were obtained to generate paraffin sections. Immunohistochemical staining was performed according to the kit instructions. Paraffin sections were dewaxed in xylene, rehydrated with alcohol at gradient concentration, and finally soaked in distilled water. Citrate buffer (pH 6.0) was used for antigen retrieval at 60°C microwave. Subsequently, 3% hydrogen peroxide was used to block endogenous peroxidase activity at room temperature for 20 min, followed by blocking in 3% sheep serum at 37°C for 30 min. The sections were incubated with H1-84mAb (dilution, 1:50) at 4°C overnight. Subsequently, the sections were rewarmed to room temperature for 60 min, followed by three washes with phosphate-buffered saline (PBS). The horseradish peroxidase-labelled goat anti-mouse secondary antibody (dilution, 1:500) was added and incubated at 37°C for 40 min, followed by three washes with PBS. Colour development was performed with diaminobenzidine and haematoxylin counterstaining at room temperature for 10 min. A conventional dehydrated transparent neutral gum mounting medium was used. Negative control was established.

Immunoprecipitation and mass spectrometry. As previously described (13), total protein was extracted from rat brain tissues using RIPA lysis buffer, and lysed on ice or at 4°C for 30 min. The supernatant was collected following centrifugation at 12,000 × g for 30 min at 4°C. The protein content was determined using a BCA protein assay kit. A total of 500 μg of the collected protein was transferred to a 1.5-ml microcentrifuge tube, and 2 μg H1-84mAb was added and incubated at 4°C for 1 h. Next, 20 μl of a resuspended volume of protein A/G PLUS agarose was added. This was incubated overnight at 4°C with gentle agitation. Following immunoprecipitation, the samples were centrifuged at 1,500 × g for 5 min at 4°C. The protein A/G beads were centrifuged to the bottom of the tube, the supernatant was carefully aspirated, the protein A/G beads were washed 3–4 times with 1 ml lysis buffer, and finally 15 μl 2X SDS loading buffer was added and the samples were boiled for 10 min. Samples were then analysed by SDS-PAGE, western blotting and mass spectrometry.

Western blotting. The sample was mixed with SDS electrophoresis sample buffer and the protein was transferred onto a nitrocellulose membrane following SDS-PAGE on a 12% separation gel. Then the protein gel was transferred to the NC film and blocked overnight with 5% skimmed milk at 4°C. H1-84mAb was used as the primary antibody (dilution, 1:100). A goat anti-mouse enzyme-labelled secondary antibody, as mentioned above, (dilution, 1:2,500) was used. Enhanced chemiluminescence (ECL) was used to develop the colour.

Cloning of heterogeneous nuclear ribonucleoprotein (hnRNP) A1 and hnRNPA2/B1. As previously described (14), total RNA was extracted from brain tissue total protein and then reverse transcribed into cDNA. Subsequently, PCR was used to amplify hnRNPA1 and hnRNPA2/μl. The 6 μl purified PCR product was subcloned into the 2 μl pGEM®-T vector (concentration ratio 3:1). The correctly sequenced plasmid was converted into the pET-28a-SUMO vector (SUMO tag size, 15 kDa) and then was transformed into BL21(DE3)pLysS competent cells by thermal shock for 90 sec at 42°C.

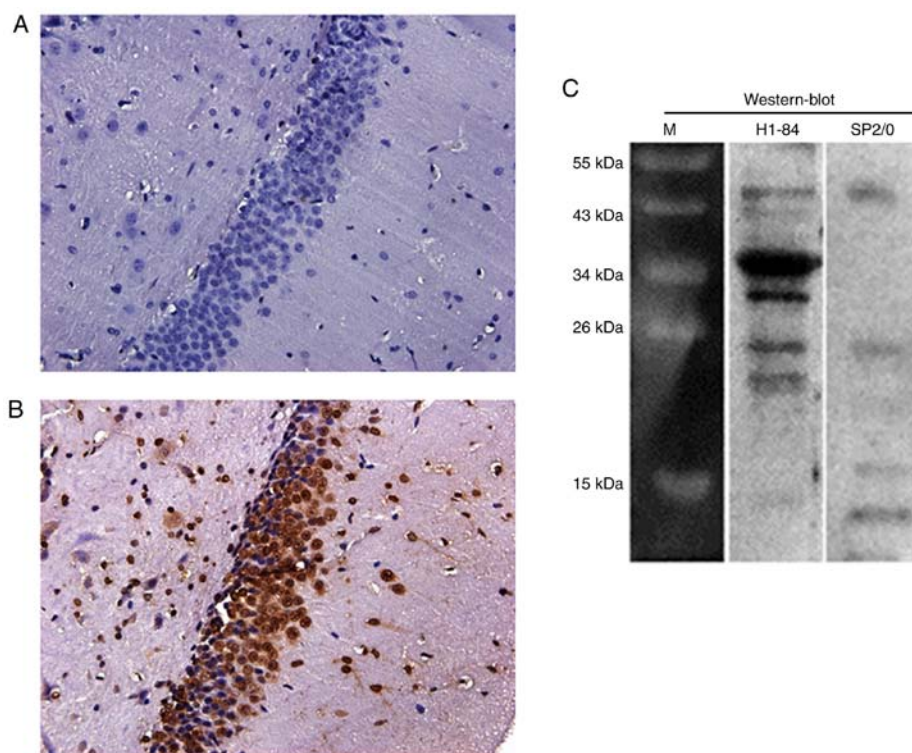


Figure 1. Identification of H1-84mAb combined with rat brain tissue. (A) SP2/0 as a negative control did not respond to brain tissue cells. Magnification, x400. (B) H1-84mAb was bound to rat brain tissue and parts of the cerebral cell nucleus were stained brownish yellow. Magnification, x400. (C) H1-84mAb reacted with the protein components of brain tissue, and the molecular weight was ~35 kDa (lane 2); SP2/0 was used as negative control (lane 3).

Recombinant hnRNPA1 and hnRNPA2/B1 were expressed by adding isopropyl β -D-thiogalactopyranoside (IPTG; 0.5 mM) to an *E. coli* strain, purified using Ni-NTA and verified on Coomassie-stained gels.

Segmental expression and identification of hnRNPA1 and A2/B1. The antigenicity of hnRNPA1 and hnRNPA2/B1 were analyzed theoretically. Using hnRNPA1 and hnRNPA2/B1 as templates, PCR was used to amplify truncated hnRNPA1 and hnRNPA2/B1, using the aforementioned steps. Truncated hnRNPA1 and hnRNPA2/B1 were expressed by adding IPTG (0.5 mM) to an *E. coli* strain, purified using Ni-NTA and verified on Coomassie-stained gels. The expression product was identified by SDS-PAGE and western blotting.

Statistical analysis. The results were analyzed using SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA). Data are expressed as the mean \pm SEM. One-way analysis of variance, followed by Tukey's *post hoc* test, was used to determine the significance of differences in multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cross-reactivity of influenza virus HA mAb with rat brain tissue. In our previous study, 84 mAbs against influenza virus HA were obtained and screened using a human tissue microarray. H1-84mAb cross-reacted with human brain tissue, and western blotting revealed that H1-84mAb bound to the HA antigen (8).

Due to the limitations of medical ethics, the present study used rat brain tissues instead of human brain tissues for subsequent

experiments. To further confirm the cross-reactivity of H1-84mAb, immunohistochemical staining of paraffin sections of rat brain tissues was performed. The results revealed that the control antibody (cell culture supernatant of the SP2/0 hybridoma) was negative (Fig. 1A). Additionally, H1-84mAb reacted with the rat brain (Fig. 1B). Furthermore, western blotting demonstrated that H1-84mAb reacted with the protein components of brain tissue. The molecular weight of the reactive protein was ~35 kDa (Fig. 1C).

Immunoprecipitation and mass spectrometry. H1-84mAb bound to the protein in rat brain tissue in immunoprecipitation experiments. The cell culture supernatant of the SP2/0 hybridoma served as a negative control. Western blotting revealed that the molecular weight of the target antigen reacting with H1-84mAb was ~35 kDa (Fig. 2A). Specific reaction bands were excised from the SDS-PAGE gel and analysed by mass spectrometry.

Verification of cross reactivity. Immunoprecipitation combined with mass spectrometry indicated that H1-84mAb bound to hnRNPA1 and hnRNPA2/B1 from brain tissues. The two proteins were expressed separately using an *E. coli* expression system (Fig. 2B). Subsequently, the cross-reactivity of the expressed proteins with H1-84mAb was demonstrated by western blotting. The results revealed that H1-84mAb cross-reacted with the two purified recombinant proteins (Fig. 2C).

Fine localisation of H1-84mAb binding to brain tissue proteins. The functional regions of the hnRNPA1 and hnRNPA2/B1 proteins were analysed (Figs. 3A and 4A); they were divided into three segments and prokaryotic expression

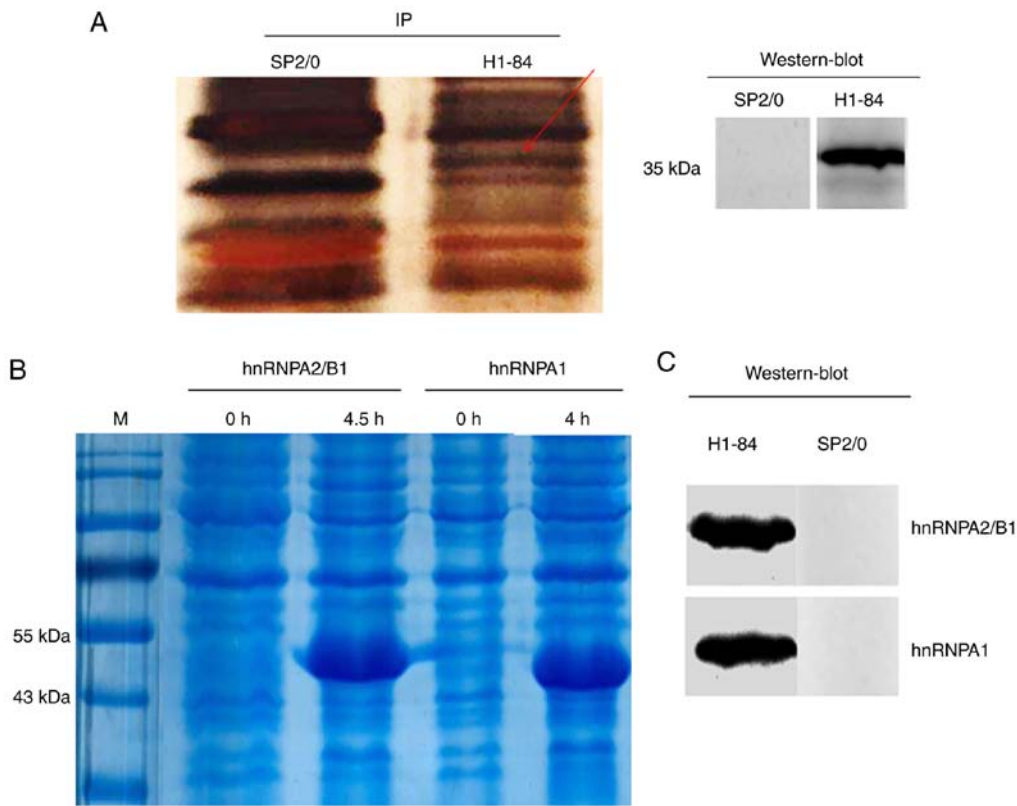


Figure 2. Immunoprecipitation and identification of hnRNP2/B1 and hnRNP1 protein expressed in bacteria. (A) Western blot analysis of brain tissue protein following immunoprecipitation. SP2/0 (lane 1) and H1-84 (lane 2) were used as the primary antibodies. The red arrow indicated specific banding, which was performed for mass spectrometry. Lane 3 and lane 4 show that the target antigen, which specifically reacted with H1-84mAb, was ~35 kDa. (B) Coomassie-stained gels revealed that hnRNP2/B1 and hnRNP1 expressed in bacteria were ~50 kDa (SUMO tag size was 15 kDa). (C) Western blot analysis demonstrated that H1-84mAb bound to the recombinant protein hnRNP2/B1 and hnRNP1.

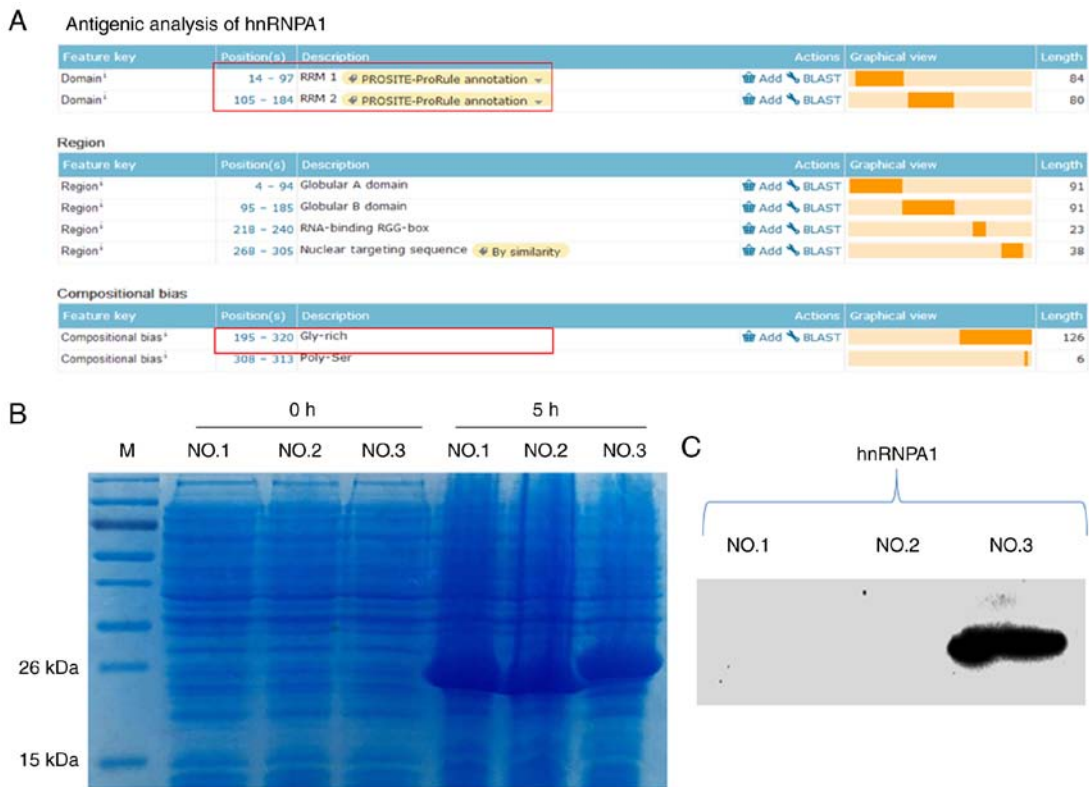


Figure 3. Fine localization of H1-84mAb bound to the hnRNP1. (A) Antigenic analysis of hnRNP1. (B) hnRNP1 was expressed in stages, which were NO.1 (14-97aa), NO.2 (105-184aa), and NO.3 (195-320aa). All three proteins were correctly expressed. (C) Western blot analysis revealed that H1-84mAb bound to the NO.3 of the hnRNP1. NO.1, stands for RRM1 domain; NO.2, stands for RRM2 domain; NO.3, stands for Gly-rich domain.

was performed (Figs. 3B and 4B). Subsequently, western blot analysis was used to identify them. The results revealed that the third segments [the glycine (Gly)-rich domain] of these two proteins were the antigens that H1-84 cross-reacted with in brain tissues (Figs. 3C and 4C). Fig. 3C demonstrated that hnRNPA1-NO.3 (195aa-320aa Gly-rich domain) contained 126 amino acids. Fig. 4C demonstrated that hnRNPA2/B1-NO.3 (202aa-349aa Gly-rich domain) contained 148 amino acids. These two parts were H1-84mAb that recognized specific antigens on hnRNPA1 and hnRNPA2/B1.

Verification of H1-84mAb combined with brain tissue antigens. The hnRNPA1-NO.3 and hnRNPA2/B1-NO.3 were purified (Fig. 5A) and then these two antigens were used to block the binding of H1-84 to brain tissue proteins, respectively. The same batch of brain tissue proteins was sampled for western blot analyses, and the consistency of the sample volume was monitored with β -actin. The H1-84mAb was pre-incubated with 0, 0.2 and 0.6 μ g of the glycine (Gly)-rich domain of these two proteins, respectively, and then reacted with the brain tissue proteins on the NC membrane, * $P < 0.05$ vs. 0 μ g group. It was verified that these two partial antigens may block H1-84mAb binding to brain tissue (Fig. 5B).

Discussion

Influenza A vaccination may induce neurological adverse reactions in a small number of individuals, indicating that there is a problem with the safety of the influenza A vaccine (3,15). At present, the mechanism of this remains unclear, which limits the efficiency of clinical prevention and control, and seriously affects the treatment of patients. In 2015, Ahmed *et al* (16) reported that the nucleoproteins of influenza A vaccines stimulate the body to produce cross-reactive antibodies against hypothalamic receptor 2. This antibody blocks the hypothalamic receptors of nerve cells, leading to a sleep-wake regulation disorder, which in turn causes narcolepsy (17,18).

Our previous study demonstrated that influenza virus H1-84mAb not only bound to the HA antigen, but also reacted with human brain tissue (8). Combined with the concept of heterophilic antigens, this indicated that there was a heterotropic antigen between influenza virus HA and human brain tissue. This is an important factor affecting the safety of the vaccine. Using molecular biology and immunological methods, the present study demonstrated that the influenza virus H1-84mAb cross-reacted with heterophilic antigens in brain tissues. The heterophilic antigens were the Gly-rich domains of hnRNPA1 (195aa-320aa) and hnRNPA2/B1 (202aa-349aa) by prokaryotic expression. Although the protein expressed in the bacterial system cannot simulate the natural protein in nuclear organisms, the current research field mostly uses prokaryotic expression methods to identify heterophilic antigens. Sun *et al* (13) used a prokaryotic expression method to report that the anti-influenza virus H1-50mAb recognizes target antigen inhibin on pancreatic islet cells. Furthermore, an article published in Nature Medicine confirmed that an antibody produced by human T lymphotropic virus type 1 (HTLV-1) infection reacts with hnRNPA1 to cause myelopathy/tropical paraplegia (HAM/TSP) by prokaryotic expression (14). Therefore, the present study used the prokaryotic expression method to verify heterophilic antigens.

hnRNP is a multifunctional protein family molecule that is mainly localised in the nucleus and may be combined with newly-synthesised heterogeneous nuclear RNA. It regulates a series of important processes, including the splicing of mRNA precursors, as well as mRNA nuclear transport, translation and degradation (19). In studies investigating how viral diseases cause neurological diseases, there have been various reports on spontaneous host antibodies, and a number of studies have focused on the pathogenicity of hnRNP antibodies. Levin *et al* (20) revealed that autoantibodies to hnRNPA1 may cause neurodegenerative changes. Animal experiments have demonstrated that hnRNPA1 antibodies are associated with multiple sclerosis (21). Sueoka *et al* (22) detected antibodies in the cerebrospinal fluid of 35 patients with multiple sclerosis and reported that 32 of them had antibodies against the hnRNPA1 protein. The present experiments revealed that the H1-84mAb of influenza virus HA binds to Gly-rich domains of hnRNPA1 and hnRNPA2/B1. The proteins hnRNPA1 and hnRNPA2/B1 were screened using the IP method and were highly expressed in neurons. The Gly-rich domain of these two proteins may block the binding of H1-84mAb to brain tissue.

The Gly-rich domain includes the RGG domain and the M9 shuttle domain, which are two important functional regions of hnRNP. Antibodies against these two functional domains may cause nervous system damage (23). Therefore, the specific epitope of H1-84 binding to the Gly-rich domain and the pathogenesis of neurological diseases caused by H1-84mAb binding to the Gly-rich domains of these proteins should be studied.

Although hnRNP is widely distributed in various cells and tissues of different species, H1-84mAb specifically binds to brain tissue. Furthermore, Sun *et al* (13) reported that anti-influenza virus H1-50mAb recognises a target antigen on islet cells, which has been revealed to be prohibitin. Prohibitin is an antiproliferative protein that is widely distributed in various cell types in different species, and serves an important regulatory role in cell metabolism, growth, differentiation, senescence and apoptosis (24).

The present study compared the similarity between the HA of influenza virus and the Gly-rich domain of hnRNPA1 and hnRNPA2/B1; and they were found to have no homologous sequences. Sun *et al* (13) revealed that an influenza virus mAb (H1-50) cross-reacts with islet cells, but there is no homologous sequence between HA and prohibitin. Previous studies (14,25) revealed that an antibody produced by human T lymphotropic virus type 1 infection reacts with hnRNPA1 to cause myelopathy/tropical paraplegia. Although there is no homologous sequence between the dominant epitope (KHFRETEV) and hnRNPA1, autoantibodies against hnRNPA1 may still cause neuronal damage. In a mouse model of viral-induced myocarditis, a cross-reaction was found between myocardial myosin and Cocksackie B3 virus neutralizing antibody, but no molecularly mimicked sequence was found (26). Not all cross-reactions can find homologous sequences, as they may be conformational fit.

H1-84mAb may recognize HA antigens of human 2009H1N1, seasonal H1N1, H3N2, avian influenza H5N1 and H9N2. It was reported that H1-84mAb can recognize the epitope 191-LVLWGIHHP-199 of human influenza and avian influenza HA (12). Antibodies that recognize this epitope have also been detected in patients' sera following vaccination, and our recent studies have reported that H1-84mAb may mediate nervous system damage (study under submission). Therefore,

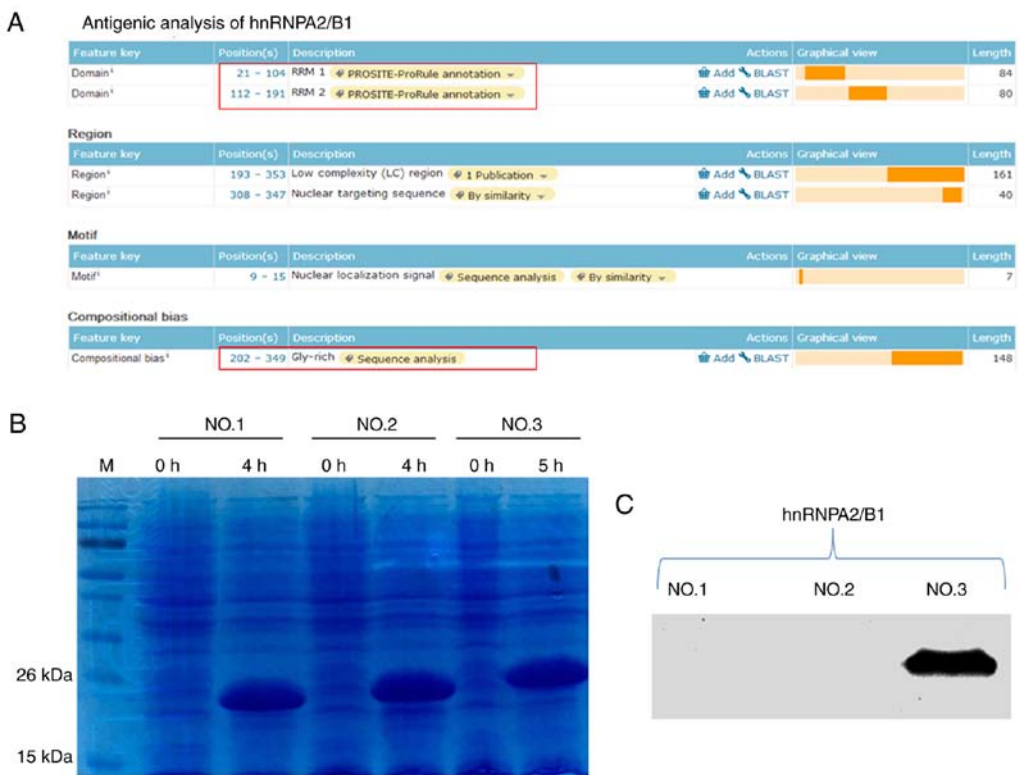


Figure 4. Fine localization of H1-84mAb bound to the hnRNPA2/B1. (A) Antigenic analysis of hnRNPA2/B1. (B) hnRNPA2/B1 was expressed in stages, which were NO.1 (21-111aa), NO.2 (112-191aa) and NO.3 (202-349aa). All three proteins were correctly expressed. (C) Western blot analysis revealed that H1-84mAb bound to the NO.3 of the hnRNPA2/B1.

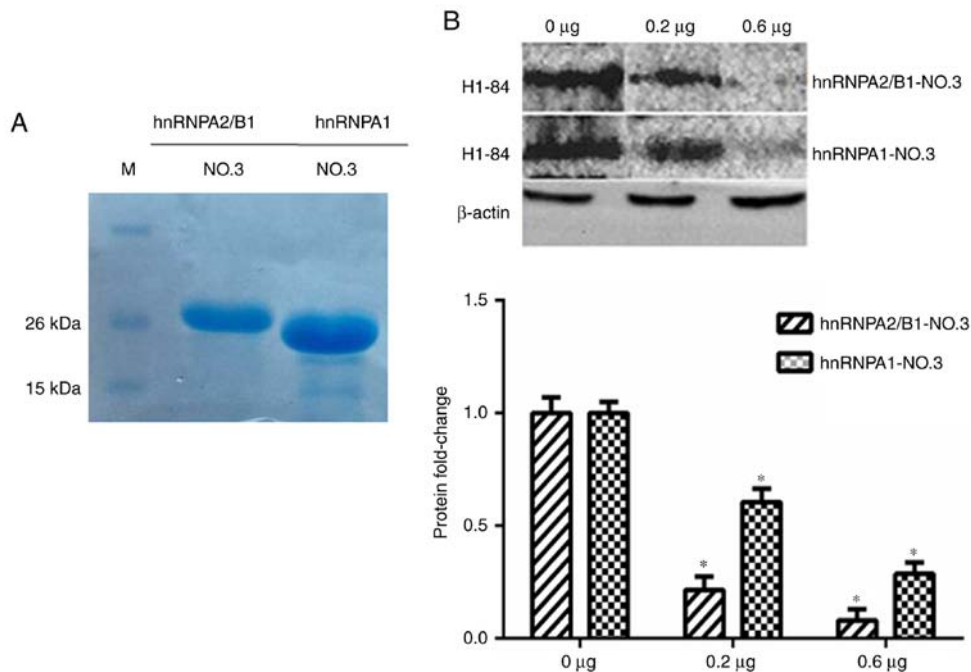


Figure 5. Verification of H1-84mAb combined with brain tissue antigen. (A) Protein purification sample. (B) Separate samples of brain tissue proteins, ensuring the same amount of sample for each well. Pre-incubation of H1-84mAb with 0, 0.2 and 0.6 μ g hnRNPA2/B1-NO.3 and hnRNPA1-NO.3, respectively. Each incubated sample was different and required separate western blot analysis with brain tissue proteins. The results showed that the NO.3 segments of these proteins were the fine localization of H1-84mAb on the brain tissue. β -actin was used as a loading control. * P <0.05 vs. 0 μ g group.

it is theoretically feasible to mutate the 191/199 epitope of HA to avoid the production of H1-84-like pathogenic antibodies, thereby improving vaccine safety.

In the future, the association between H1-84mAb, Gly-rich domains, neurological diseases and the safety of the vaccine will be investigated further. This will provide experimental

data for designing a safe influenza vaccine and will be of great significance in preventing influenza virus infection.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

CG, LS, SH and XH designed the study and performed critical revisions; CG, HH, DL, QF, YL and YF performed the laboratory measurements; CG, XX and JH performed the data collection and analysis; and CG drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments performed in the present study were approved by the Medical School of Xi'an Jiaotong University Biomedical Ethics Committee and fully meet the requirements of the animal ethics committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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