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Establishment and validation of a drug-target microarray for SARS-CoV-2

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ABSTRACT

COVID-19 has become one of the worst epidemic in the world, currently already more than four million people have been infected, which probably co-exist with human beings, and has a significant impact on the global economy and political order. In the process of fighting against the epidemic in China, the clinical value of a variety of herbal medicines has been recognized and written into the clinical application guide. However, their effective molecular mechanism and potential targets are still not clear. Pathology and pharmacology research will gradually attract attention in the post-epidemic outbreak term. Here, we constructed a COVID-19 protein microarray of potential therapy targets, which contains the main drug targets to the SARS-CoV-2 virus and the anti-virus, anti-inflammatory cellular targets of the host. Series of quality controls test has been carried out, which showed that it could be applied for drug target screening of bio-active natural products. The establishment of this microarray will provide a useful tool for the study of the molecular pharmacology of natural products.

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1. Introduction

Data from Johns Hopkins University updated that Coronavirus Disease-2019 (COVID-19) has infected more than fourteen million people, which has become a rare pandemic in human history (<https://www.jhu.edu/>). In the early stage of the outbreak, diagnostic methods and virus traceability are the research focus. With the development of the disease, COVID-19 is likely to become a kind of infectious disease coexisting with human beings. Vaccine development, drug screening and related pharmacology will gradually become the research hot areas.

In China, through thousands of years clinical application, herbal medicine has accumulated valuable experiences in the use of fighting against plague [1]. This includes artemisinin extracted from *Artemisia* for malaria treatment [2]. In the early outbreak stage, the modern drug research and development system has also shown its disadvantages: it is impossible to develop new drugs and complete registration of clinical trials in a short period for a novel virus [3]. Herbal medicine (experienced clinical safety evaluation) has the

characteristics of compound multi-targets, non-specific adjustment of the human immune system and antiviral effect, which makes it possible for its rapid application [4]. In the practice of anti-COVID-19, three Chinese patent drugs were written into the guideline for the treatment of COVID-19 in China. Lianhuaqingwen capsule has been shown to significantly inhibit severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) replication [5–8]. The pharmacological studies of herbal drugs and active natural products often lack compare to modern drugs [9]. The target and mechanism of clinically effective natural products are still largely unknown [10].

For protein target research of traditional Chinese medicine, it is usually carried out through network pharmacology and bioinformatics, for example, by obtaining the main active ingredients of Lianhuaqingwen capsule, molecular docking method was used to predict the possible targets [11,12]. However, the researches that were not based on the real world are still not enough to reveal their mechanism of action by molecular pharmacology.

Protein microarray is a high-throughput drug screening platform, which can be used to screen protein targets of active natural products [13]. Generally, the proteomic proteins come from one species, human proteomics, or virus. Host-based whole proteomic microarrays are often high cost, and composed of many redundant proteins, which are not suitable for efficient drug screening of anti-virus targets. In this

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Abbreviations

COVID-19 Coronavirus Disease-2019
 CTSB Cathepsin B
 RBD Receptor Binding distict
 NSP Non-structural protein
 SARS Severe acute respiratory syndrome

study, we designed a new protein microarray for COVID-19 which contains both potential virus targets and host targets. This protein microarray may provide a new way to solve the problem of target selection of traditional Chinese medicine compounds.

2. Methods and materials

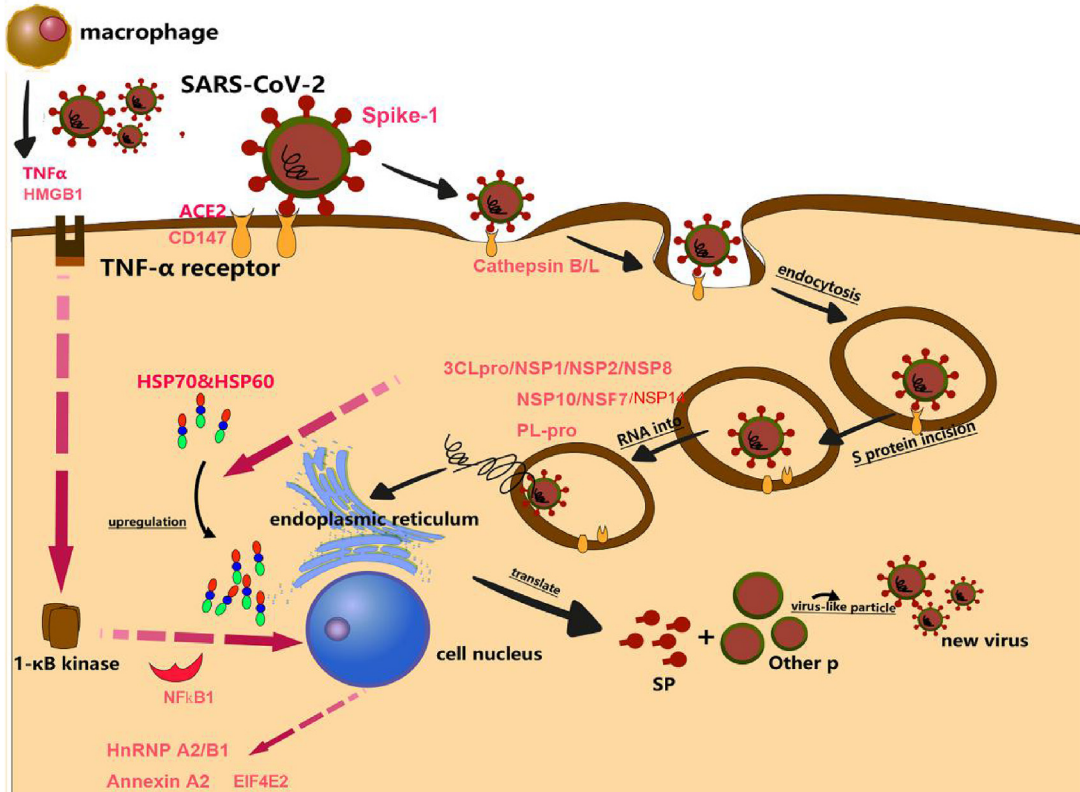
2.1. Target proteins

There are two sources of proteins used in the study. One source

A

BSA-Cy3	BSA-Cy3	BSA-Cy3	PBS	PBS	PBS	CD147	CD147	CD147
TNF-α	TNF-α	TNF-α	HSP70	HSP70	HSP70	HSP60	HSP60	HSP60
NFκB1	NFκB1	NFκB1	EIF4E2	EIF4E2	EIF4E2	HMGB1	HMGB1	HMGB1
BSA-cy3	BSA-cy3	BSA-cy3	HnRNPA 2/B1	HnRNPA 2/B1	HnRNPA 2/B1	Anexin A2	Anexin A2	Anexin A2
ACE2	ACE2	ACE2	CTSB	CTSB	CTSB	CTSL	CTSL	CTSL
Spike-1	Spike-1	Spike-1	Spike-1-2	Spike-1-2	Spike-1-2	sRBD	sRBD	sRBD
BSA-Cy3	BSA-Cy3	BSA-Cy3	3CL Proteinase	3CL Proteinase	3CL Proteinase	NSP8	NSP8	NSP8
NSP2	NSP2	NSP2	NSP7	NSP7	NSP7	NSP10	NSP10	NSP10
PL-pro	PL-pro	PL-pro	NSP1	NSP1	NSP1	NSP14	NSP14	NSP14
BSA-Cy3	BSA-Cy3	BSA-Cy3	Human-IgG	Human-IgG	Human-IgG	BSA-Cy3	BSA-Cy3	BSA-Cy3

B



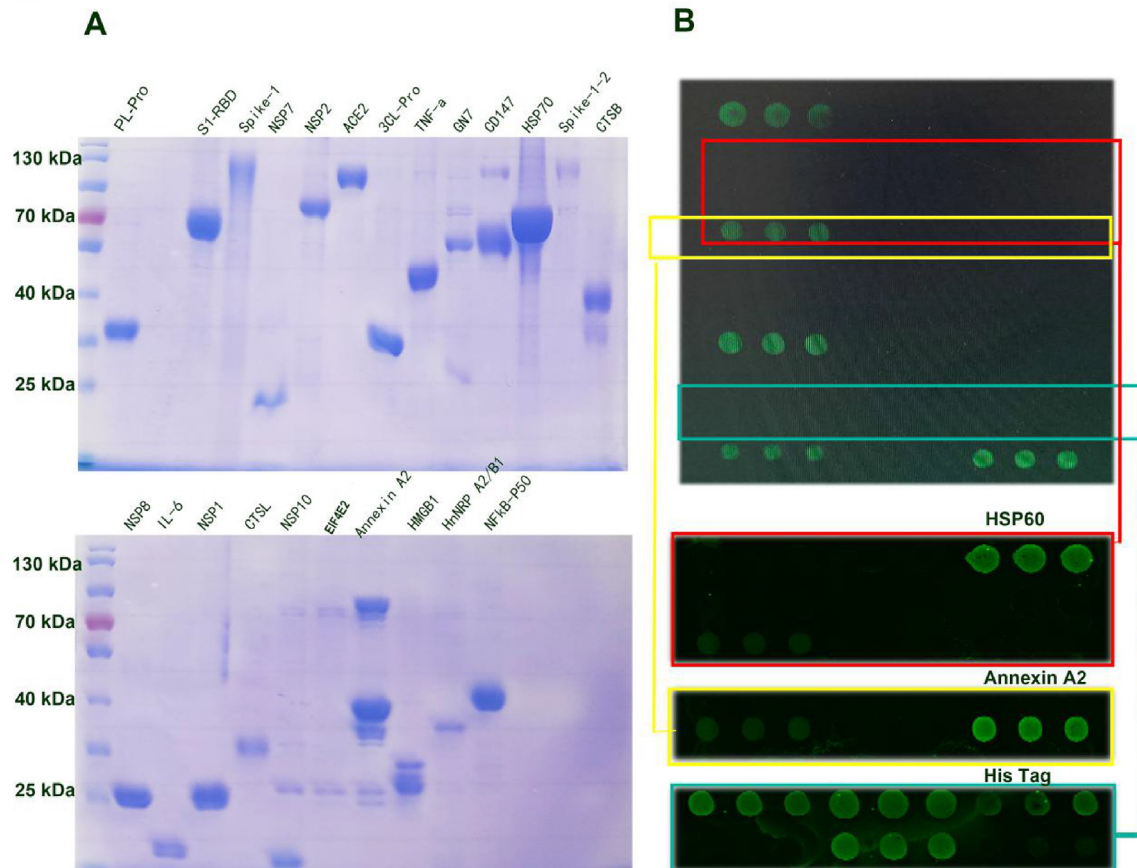


Fig. 2. Protein and microarray quality control.

A: The molecular weight and purity of all chip proteins were determined by SDS-PAGE.

B: Detection of protein by specific antibody in microarray. HSP60, annexin a2 and his tag antibodies were shown in the frame. GN7: Guanine-N7-methyltransferase.

was purchased from the company, including human ACE-2 (from HEK293), human Cathepsin L (CTSL) (from HEK293), human Cathepsin B (CTSB) (from HEK293), SARS-CoV-2 Guanine-N7-methyltransferase, Non-structural protein (NSP) 14 (from *E.coli*), SARS-CoV-2 NSP1 (from *E.coli*), SARS-CoV-2 NSP8 (from *E.coli*), SARS-CoV-2 Spike 1 protein (from HEK293), SARS-CoV-2 Spike 1-Receptor Binding distict (RBD) (from HEK293), SARS-CoV-2 3C-like Proteinase (from *E.coli*), SARS-CoV-2 Papain-Like Protease (PLpro) (from *E.coli*), SARS-CoV-2 NSP10 (from *E.coli*), SARS-CoV-2 NSP2 (from *E.coli*), SARS-CoV-2 NSP7 (from *E.coli*) purchased from novoprotein (Shanghai, China); Human CD147 (from HEK293), HSP70 (from HEK293), HSP60 (from HEK293), TNF- α (from HEK293) from sinobiological (Beijing, China). SARS-CoV-2 Spike 1–2 protein (from HEK293) purchased from Jinmotang biotechnology (Qingdao, China). Another protein source were over-expressed in *E.coli* from the laboratory, the human Anneixn A2, HMGB1, HnRNP A2/B1, EIF4E2, NF κ B1-P50 genes were inserted into the Pet-28a vector with His-tag. Bacteria were lysed by ultrasound and proteins purified by nickel column. All proteins were under unified quality control, which was carried out by gel electrophoresis.

Fig. 1. Protein sample distribution in protein microarray.

A: The target proteins contained in protein microarray. Green background color protein represents the human origin, blue background part from the virus. Protein with yellow font contains His-Tag. B: Functional map of protein target selected in this study. In red are related proteins on microarray chips, and in black are related cellular components and some viral components. The black arrows represent functions directly involved by the virus, and the red arrows represent functions indirectly affected by the virus. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The reasons of target proteins selection are as follows: ACE2 and CD147 are the main receptors of the virus [14]; Host cell protease Cathepsin L/B is the key element of the lysosomal pathway [15], and almost all of them are in lysosomes. 3C-like Proteinase is main proteases of novel coronavirus [16,17]. PLpro is necessary for the virus to process polyproteins as mature subunits. Guanine-N7-methyltransferase, NSP1/2/7/8/10 were involved in RNA transcription and replication [18]. NF κ B1 and HMGB1 are important lung inflammatory regulators [19,20], anneixn a2, HnRNP A2/B1 were proved as transcription regulators associated with pneumonia [21–23]. The latest research showed EIF4E2 as an important host interaction protein with virus, potential to be a drug target for COVID-19 [24].

2.2. Microarray printing

The protein was mixed with sample buffer (Capital-bio, Beijing, China), then put into 384 well plates (Axygen, CA), and made with microarray printing device (Capital-bio, Beijing, China). Polymer 3D substrate (Capital-bio, Beijing, China) was selected, for each protein by three times of repetition. The proteins in the microarray after

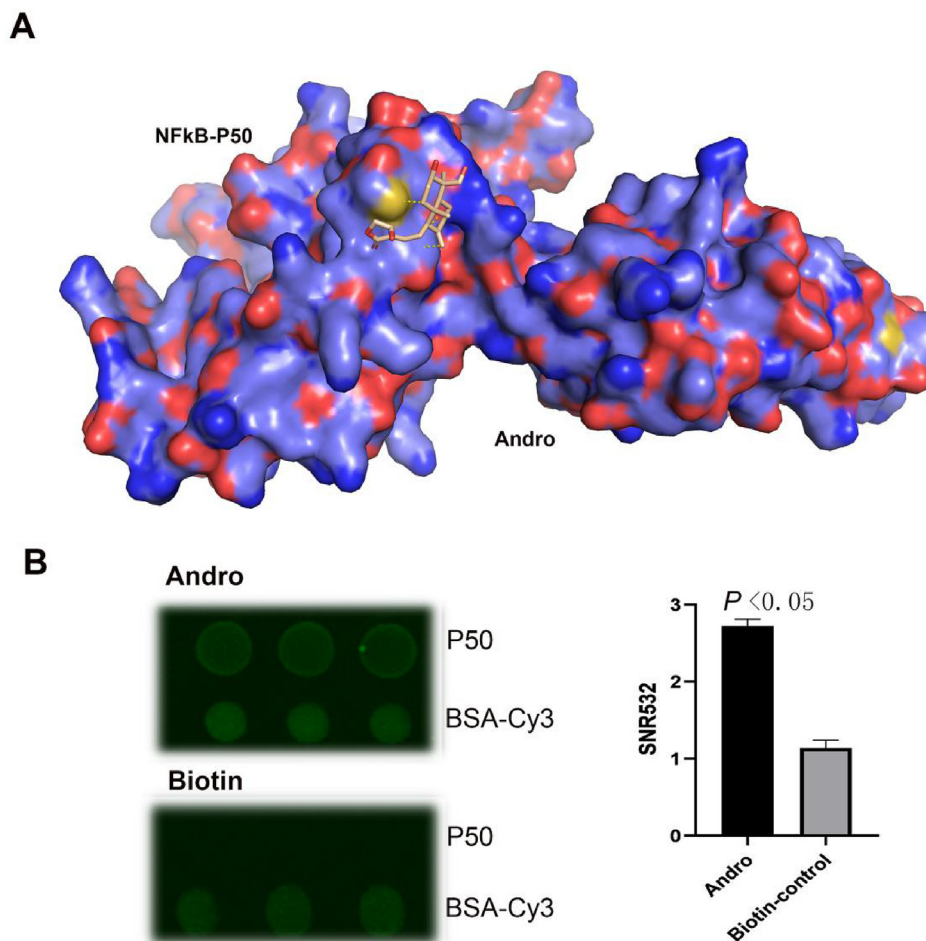


Fig. 3. Application confirmation of the microarray.

A: The binding sites of andrographolide and NFκB1-P50 protein were displayed
 B: Using the biotin labeled andrographolide to test the reactivity with the P50 protein, the protein on the chip clearly showed the reaction signal. After the gray value of the signal extracted, statistical analysis has significant differences. Andro:andrographolide.

printed were quality checked by antibodies.

2.3. Microarray experiments

Human Annexin A2, HSP60, NFκB1-P50 and his-tag antibodies, goat anti-mouse IgG-Cy3, Streptavidin-Cy3 (Protech, Wuhan, China) were used to check the protein quality. After that, Andrographolide (ChemFaces, Wuhan, China) was selected as a representative of active natural products to verify the function of the microarray. Biotinylated andrographolide verified by mass spectrometry. First anti-body (1:500) incubation at 37 °C 2 h, then wash with 0.1% PBST three times, second anti-body (1:1000) or streptavidin-Cy3 incubation (1:1000) at 37 °C 0.5 h. The microarray was scanned by device (Capital-bio, Beijing, China).

2.4. Collection of small molecules and proteins for molecular docking

The protein structure files were downloaded from the PDB database, and the corresponding protein backbone was extracted, corresponding to PDBID as follows: ACE2(6ACG), Annexin A2(2hyw), Cathepsin B (3ai8), Cathepsin L (2xu1), CD147 (3qqn), ETIF4E2 (5bxy), HMGB1 (2rtu), NFκB1-P50 (1u3y), NSP3 (6w6y),

NSP12 (6m71), Spike protein (6w9c), TNF-α (3alq). Three small molecule files were downloaded from the PubChem database, corresponding to the Compound CID of polydatin (5281718), andrographolide (5318517) and chlorogenic acid (1794427). The files were downloaded in the format of SDF (3D), and then converted into PDB files by openbabel (a tools to convert sdf format to pdb format) for subsequent analysis.

2.5. Prediction of docking between small molecule and proteins

First, protein structure files (PDB files) were preprocessed with AutoDockTools (<http://autodock.scripps.edu/>), and the steps of pretreatment were “delete water”, “add hydrogen”, “compute gasteiger charges”, “assign AD4 type atoms”, and then the files were saved in PDBQT format. Then use the ADT AutoDockTools Ligand (Ver 4.2) function to preprocess ligand, steps of pretreatment which were “detect roots” and “choose torsion”, also saves the file as PDBQT format, which will handle the good file with the Auto-dock Vina in the same folder, and then write the configuration file (https://github.com/Starlitnightly/COVID-19_protein/), run Auto-dock Vina (<http://vina.scripps.edu/>) to predict docking results. Besides, it has been reported that the docking prediction accuracy of Autodock Vina is as high as 78% in the training set [25].

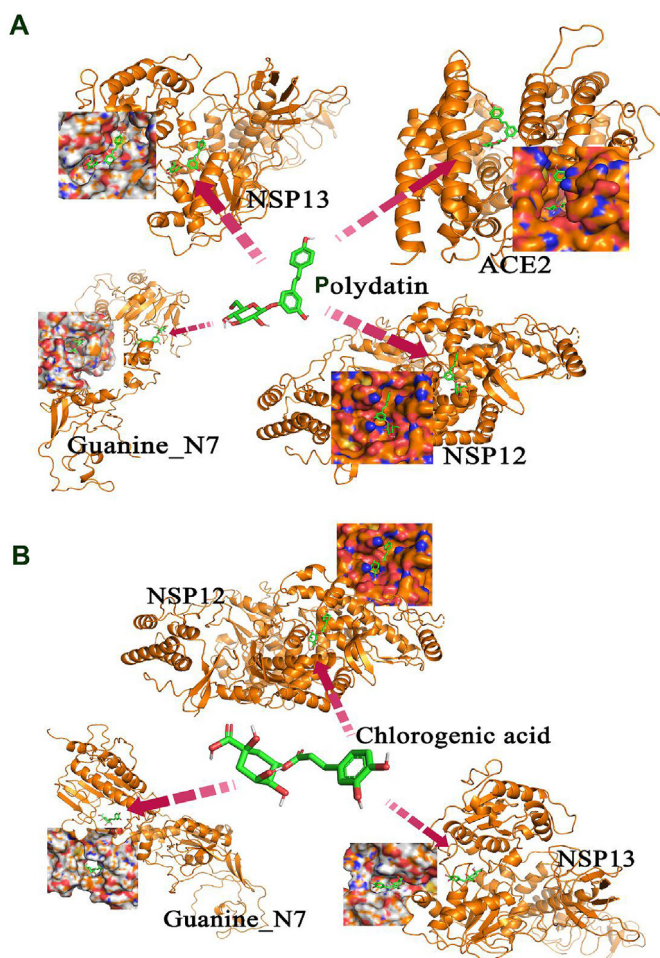


Fig. 4. The prospect of microarray application. Molecular docking results between two bioactive products polydatin and chlorogenic acid, and some target proteins. Each molecule shows classic binding results with top scores. A: polydatin; B: chlorogenic acid. (It should be noted that this molecular docking only a prediction for helping microarray experiment and does not represent the actual efficacy of these molecules.)

2.6. Statistics

SNR (signal-to-noise ratio) of three repeat proteins was calculated based on the ratio of foreground value to the background value of each protein. T test was used to compare the difference between groups and statistical significance was set at $P < 0.05$.

3. Results

3.1. The design of microarray

The proteins of the microarray come from virus and human, including the main functional targets related to the virus life cycle and host functional proteins. The microarray matrix design is shown in Fig. 1A. The functions they are responsible for are shown in Fig. 1B.

3.2. The quality control of microarray

All proteins used in microarrays were processed by gel electrophoresis and quality control. All the proteins were isolated in the gel (Fig. 2A). The results of microarray scanning showed that the proteins were well fixed, the location of positive points were

consistent with the design, and the repeat points were consistent with each other (Fig. 2B). Then specific antibodies were used to identify proteins on the microarray, as shown in Fig. 2C, the positions of annexin a2 and HSP60 are completely consistent with the matrix design. In addition, these were also confirmed by the his-tag antibodies. All these showed that the protein samples and spotter system of the microarray have high quality.

3.3. The validation of microarray application

As shown before, the microarray was used to screen protein targets of natural products with biological activity. Nfkb1-P50 protein is an important target of andrographolide, a traditional Chinese medicine product with immunoregulation function, and their binding site has been widely verified [26] (Fig. 3A). This result was repeatedly verified by the biotin-labeled andrographolide for testing this microarray. The results were shown in Fig. 3B, compared with biotin control, andrographolide and P50 protein have significant positive signals, and the signal-to-noise ratio (SNR) after quantification showed a significant statistical difference.

3.4. Prospect of microarray application using network pharmacology

Chlorogenic acid is the main antiviral and anti-inflammatory active substance in *Honeysuckle*, and it is the main herbal medicine in Lianhua Qingwen capsule, which showed the clinical therapeutic effect and the ability to inhibit virus replication in vitro [6]. Polydatin is the main active component of Chinese Traditional medicine *Polygonum cuspidatum*, which has broad-spectrum antioxidant and antiviral effects. Here, we use the method of network pharmacology molecular docking to predict the binding ability of these two natural products to the protein targets. The results showed that they have potential efficacy in the process of infection and may be used as the target for our further research. Many prediction targets of them included in our microarray with potential application value (Fig. 4). It should be noted that this molecular docking only a prediction for helping microarray experiment and does not represent the actual efficacy of these molecules.

4. Discussion

The basic principle of protein microarray is to fix proteins on solid-phase carriers such as silicon and glass substrates. These proteins will react with biomolecules that can specifically combine with them in the samples to be tested, by which we can choose those proteins critical for our research purpose through analyzing the distinct signal of proteins on the chip obtained by special equipment such as chip scanner [27]. It provides an advanced high-throughput method for modern life science, especially molecular biology research. Compared with affinity purification and separation based on mass spectrometry, protein microarray has been widely used in the field of drug target screening.

Compared with the traditional design of protein chip such as human proteome chip or virus proteome chip, this chip has its characteristic. It is not limited to the virus proteins but also combines the host targets, which expands the scope of drug screening, and many anti-inflammatory and anti-transcriptional drugs can also be applied. This idea may be more suitable to explain the pharmacological action of natural products from herb.

At present, the target selection of traditional Chinese medicine is mainly based on bioinformatics network pharmacology. The rapid screening of these drugs can provide scientific and convincing experimental pharmacological evidences for the application and promotion of these drugs. The design and manufacture of this

microarray provide a technical tool for the realization of this purpose. Because this microarray contains antigens from virus, such as S protein, also annexin a2, hnRNP A2/B1 are classical endothelial cell antigens of host [28,29]. This chip also has further value in studying the immune response after virus infection.

However, there are still some limitations on this microarray. For example, the number of host protein targets is still small, which makes some potential new drug targets maybe ignored. At present, the proteins are not all derived from eukaryotic expression, which may have the problem of protein misfolding. We will continue to improve this microarray and provide more valuable and higher quality targets for researchers. At the same time, we hope to cooperate with other laboratories with this microarray as a starting point to make contributions to the fight against the epidemic.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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