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The Potent Chinese Herbs for Inhibiting Cytotoxicity of Eosinophil Cationic Protein

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Abstract The eosinophil cationic protein (ECP) is cytotoxic to bacteria, viruses, parasites and mammalian cells. The cells were damaged *via* the processes of pore-forming, permeability change and membrane leaking. Some clinical studies reported that ECP gathered in the bronchial tract of asthma patients, the bronchial and the airway epithelial cells were damaged and thus resulted in breathing tract inflammation. Therefore, inhibition of the cytotoxicity of ECP may serve as an approach for treatment of airway inflammation. To reach the purpose, reduction of the ECP/cell interaction is rational. In Chinese populations, herbs and prescriptions have been used to mitigate the airway inflammation with the chronic or acute symptoms. In this work, the relative binding ability between ECP and Beas-2B bronchial epithelial cells was measured by cell-based ELISA with or without adding Chinese herbs. Eighty three Chinese herbs or prescriptions were tested and 5 and 6 effective herb and prescription candidates were selected, respectively. According to the combinatory network generated from single-herbal drugs and prescriptions, a combinative network was established, and we found that a single herb, Gan-cao (甘草), served as a node connecting 5 prescriptions. In addition, Sheng-di-huang (生地黃), Dang-guei (當歸) and Mu-tong (木通) appeared in 5, 4 and 3 kinds of prescriptions, respectively. After further characterization, the extracts of these 3 herbs indeed effectively inhibit the interaction between ECP and Beas-2B cells. Since these Chinese herbs can reduce the binding affinity between ECP and cells, they are potential for

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mitigating the airway inflammation symptoms.

Keywords Chinese herb, prescription, eosinophil cationic protein (ECP), drug screen

1 Introduction

Eosinophil cationic protein (ECP) which belongs to RNase superfamily [1] is a protein secreted into biological fluid under the inflammatory condition. In the clinical diagnosis, it has been used as a biomarker for determination of the serverity of inflammatory disease, such as asthma [2]. In airway inflammation, ECP is a common feature to indicate epithelial cell damage [3].

Structurally, ECP folds topology containing 3 α-helices and 5 β-strands [4]. It is a highly positively-charged protein attributed to its high arginine content (pI=10.8). The high pI promotes the interaction between ECP and the molecules with negative charges on the cell surface [5, 6]. Dependent on the interaction with organism surface, ECP translocates into cells and causes the cell damage [7]. The mechanisms of ECP-triggered cell damage are hypothesized that ECP destabilizes the cell membrane *via* the processes of pore-forming, permeability change and membrane leaking [6]. ECP attributes to the tissues damage as it is released by activated eosinophils, for example, with regards to bronchial asthma or the intestinal mucosa in Crohn's disease [8]. ECP also possesses cytotoxicity against bacteria, single strand RNA viruses, helminth parasites and mammalian cells [9-11].

For a long time**,** Chinese people use many herbs and prescriptions to treat some diseases including asthma and airway inflammation. In Chinese medical science, several prescriptions have been used for supportive care to asthma, availing against the diseases by the patients' own immune system. The asthma patients could be treated by supporting approaches to regulate the whole body immunity, but these strategies are too slow to respond the emergent events. In Chinese prescription, the herbs are separated into 4 parts, Jun, Chen, Zuo and Shi; Jun is the major drugs to target the disease, Chen is supportive to Jun or reduces the side effect of Jun, Zuo makes Jun and Chen stronger, reduces the adverse effects or eliminates the toxicity of Jun and Chen; Shi harmonizes the total herbs or guides the herbs to the target effectively [12].

In this paper, 83 kinds of Chinese prescriptions and herbs recommended by the Chinese medical doctors were screened using the ELISA method. The decrease in binding affinity between ECP and bronchial epithelial cells could be determined. Some prescriptions and herbs were verified for preventing the interaction of ECP and epithelial cells. After further investigation, some of them have the potency for anti-asthma or anti-inflammation treatments.

2 Materials and Methods

2.1 Recombinant Protein Purification

The pMAL-c2X-ECP was transformed into *E. coli* BL 21(DE3) pLysS (Novagen, USA) for protein expression. Twenty milliliters of the culture grown overnight were

inoculated into 500 ml of LB media containing 100 μg/ml carbenicillin (Sigma, USA) and 50 μg/ml chlororamphenicol (Sigma, USA), and incubated at 37 °C for 6 h till the OD_{600} reached to 0.4-0.8. Subsequently, IPTG (Ameresco, German) was added to a final concentration of 1 mM, and the bacteria were harvested after 4 hr induction at 30 °C. Recombinant MBP-ECP was fractioned in the soluble portion of bacterial lysate. MBP-ECP was purified using amylose affinity chromatography (NEB, UK), and dissolved in PBS buffer. To remove the impurity, heparin affinity chromatography was employed. Then, MBP-ECP was eluted using 100 mM phosphate buffer (pH 7.4) containing 1 M NaCl. The purified MBP-ECP was concentrated and the buffer was changed to PBS by Amicon Ultra-15. The protein concentration of MBP-ECP was determined by BCA assay kit (Pierce, USA) and the MBP was also purified and served as the negative control for cell-based ELISA.

2.2 Preparation of Chinese herbs

The extracts of Chinese herbs were viscous, so that they were diluted into distilled water to a final concentration of 10% (w/v). Afterwards, the mixture was centrifuged at 12,000 *g* for 20 min at 4 °C, and the supernatant was transferred to a new tube and stored at -20 °C.

2.3 Cells and Cell Culture

Beas-2B, a human bronchial epithelial cell line (ATCC CRL-9609), was cultured in RPMI-1640 medium (Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, USA) at 37 °C in an incubator under 5% CO_2 and 95% air.

2.4 Cell-based ELISA

The binding affinity between MBP-ECP and Beas-2B cells in the presence of various Chinese herbs was measured using cell-based ELISA. One microliter of the processed herbs was diluted into 49 μl serum-free RPMI-1640 medium containing 200 nM MBP-ECP. The Beas-2B cells with 70% confluent monolayer in each well of a 96-well plate (20,000 cells/well) were washed with PBS and blocked with 2% BSA/PBS at 4 °C for 1 h. Next, the cells were incubated in 50 μl serum-free media and mixed with 50 μl herb/MBP-ECP mixture at 4 °C for 1 h. After washed with PBS, fixed with 2% PFA/PBS, quenched with 50 mM NH_4Cl and reblocked with 2% BSA/PBS, the MBP-ECP was probed by mouse anti-MBP mAb (Santa Cruz, USA) at 25 °C for 1 h and anti-mouse HRP-conjugated secondary antibody (Jackson, USA) was used to probe the anti-MBP mAb. One hundred microliters of TMB (KPL, USA) were added into each well prior to incubation for 10 min in dark. After terminated the reaction by adding 100 μl 2N HCl, the absorbance for 450 nm was measured using ELSA reader (Biotek, Australia). The A_{450} value indicating interaction of MBP-ECP and cells was set as 100% and the relative fold of ECP/cell binding reduction was calculated by Microsoft Office Excel 2003.

2.5 Generation of combinative network

The combinative network was generated according to the ELISA screening results. Each single herb of the prescriptions was listed and boxed. The network was

drawn using CellDesigner 4.0.1 [13].

3 Results and Discussions

3.1 The pre-screening of Chinese herbs

In the first step, thousands of Chinese herbs and prescriptions were prescreened by Chinese medical doctors. After prescreening, two herbal sets containing 90 and 83 kinds of herbs or prescriptions were selected and purchased from Sun Ten Pharmaceutical Co. Ltd. and Chuang Song-Zong Pharmaceutical Co. Ltd., separately. Because of the manufacture process, the single-herbal drugs and prescriptions from Chuang Song-Zong Company were boiled, cooled and concentrated to viscous formulation; however, the drugs from Sun Ten Company appeared clear. After ELISA analysis, the drugs from Sun Ten Company showed less effect on inhibiting the ECP-cell interaction, possibly due to the difference between the two manufacture processes. So that, the herbs produced by Chuang Song-Zong Company were screened for determination of the effective candidates which reduced the binding affinity of ECP and cells.

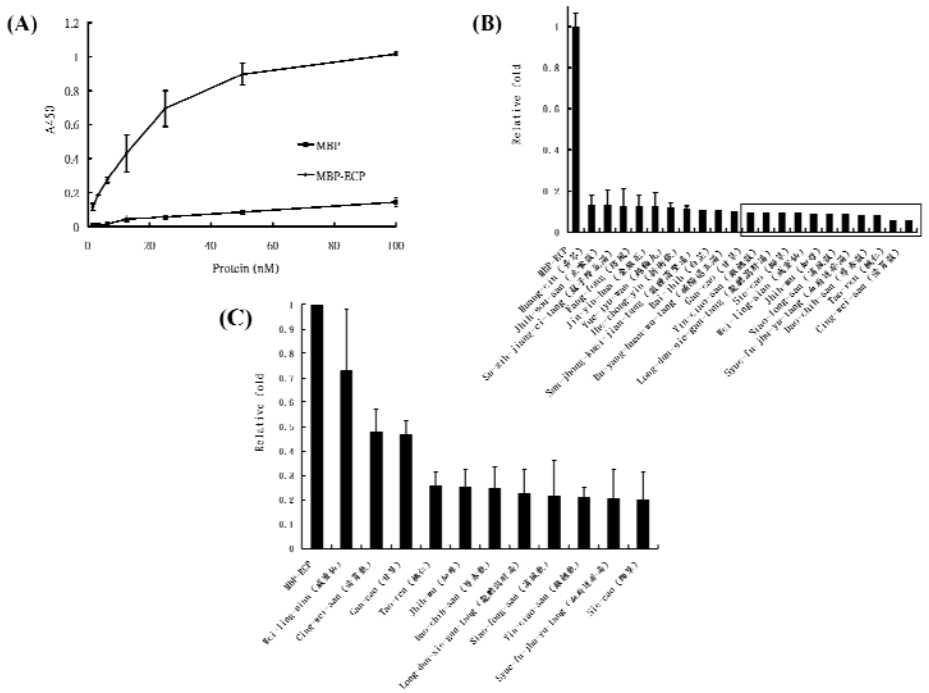

Figure 1: Herbal extract effects on ECP-cell binding affinity. The comparison of MBP and MBP-ECP interaction with Beas-2B cells (A). Partial results of MBP-ECP-cell interaction in the presence of 0.1% (B) and 0.01% herbs (C). The box shown in (B) means the relative fold less than 10%.

3.2 Eleven herbs and prescriptions reduced 90% of the ECP-cell affinity

The cell-based ELISA platform is employed to screening for the drugs which can reduce or inhibit the interaction between ECP and Beas-2B cells. It is expected that the cytotoxicity of ECP can be inhibited *via* blockage of the ECP-cell interaction. ECP was tagged with MBP for antibody probing in the ELISA process. As shown in Fig. 1A, MBP-ECP, as compared with MBP, significantly bound to cells with dependence on the MBP-ECP concentration. Before ELISA, 83 herbs or prescriptions were individually mixed with 100 nM MBP-ECP recombinant proteins to a final concentration of 0.1% (w/v) in serum-free media. The relative binding affinity normalized by MBP-ECP-cell binding strength was measured and calculated (Fig. 1B). As shown in Fig. 2, the herbs and prescriptions were classified into 5 groups according to the relative reductive folds of ECP/cell binding affinity. Five single herbs and 6 prescriptions reduced 90% affinity of the ECP/cell interaction. However, Fig. 1B indicated that although 11 herbs inhibited ECP-cell interaction most effectively, some more herbs also reduced the interaction significantly. In order to verify the most effective drugs, the central 11 herbs or prescriptions listed in Fig. 2 were analyzed afterwards. The 11 drugs were diluted to a final concentration of 0.01%. Most of the drugs excluding Wei-ling-sian (威靈仙) still reduced ECP-cell interaction significantly (Fig. 1C).

3.3 Gan-cao, Sheng-si-huang, Dan-guei and Mu-tong were potential for inhibiting the ECP-cell interaction

The 83 drugs were divided into two groups, single herb and prescription. Cross comparison provided useful information to verify the effective single herb for further investigation. According to the ELISA results, Gan-cao and Sheng-di-huang appeared in 5, Dan-guei in 4 and Mu-tong in 3 of the 6 prescriptions (**Fig. 3**). Among which, Gan-cao was the most potential. Gan-cao was one of the components of Dao-chih-san (導赤散), Long-dan-sie-gan-tang (龍膽瀉肝湯), Siao-fong-san (消風散), Sie-fu-jhu-yu-tang (血府逐瘀湯) and Yin-ciao-san (銀翹散), and it could reduce 90% of ECP-cell affinity individually. Although in the Chinese medicine concept, Gan-cao is “Shi” which plays a role in assisting the sound effects of “Zuo” herbs, it may play a novel role in mitigating the symptom of inflammation. Besides Gan-cao, Sheng-di-huang also appeared in 5 prescriptions. Sheng-di-huang also reduced 80% of the ECP-cell binding affinity. Hence it may serve as a new candidate for inflammation treatment. Dan-guei and Mu-tong were two nodes in the herbal network. However, they were not selected by Chinese medical doctors in the prescreening processes. After the cell-based ELISA was performed, Dan-guei and Mu-tong exactly decreased the affinity between ECP and Beas-2B cells to 24.4% and 16.8%, respectively (**Fig. 4**).

4 Conclusion

We identified the extract of Gan-cao inhibited the interaction of ECP and Beas-2B

cells. It may serve as a potential drug to rescue the airway epithelial cells damaged by ECP. However, it is necessary to identify the effective HPLC fractions or compounds using metabolomics approaches.

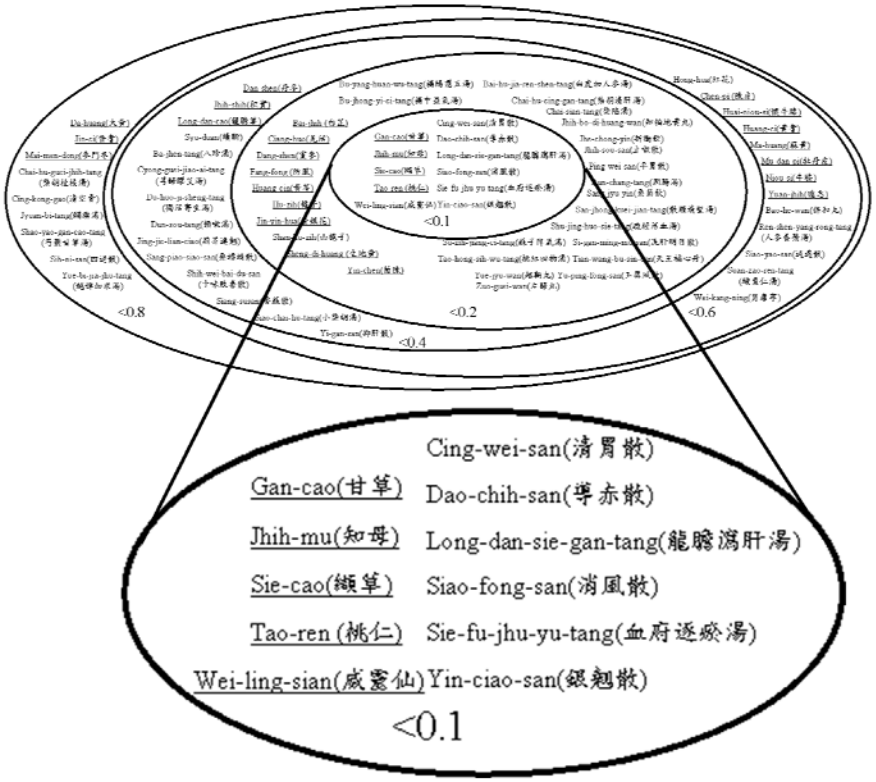

Figure 2: The classification of the Chinese herbs for inhibiting the interaction between ECP and Beas-2B cells. The inhibitory effect was classified into 5 groups from less than 10% to 80% as indicated. The most effective drugs containing 5 single herbs (underlined) and 6 prescriptions were zoomed in illustrated,.

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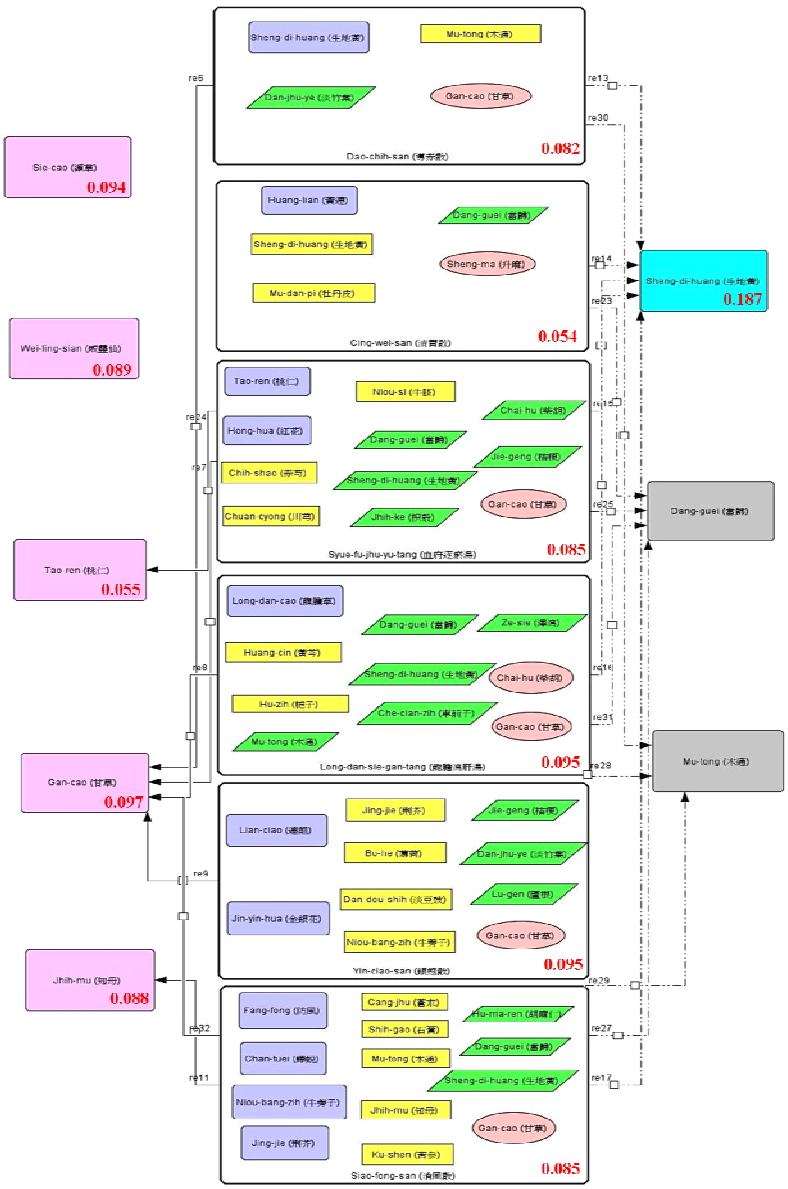

Figure 3: The network combined with herbs and prescriptions. The herbal prescriptions were gathered and boxed. Each single herb was separately shown in light purple rectangle circle, yellow rectangle rectangular, green trapezoids, and pink ellipses, which means Jun, Chen, Zuo and Shi, respectively. The consensus herbs in prescriptions were linked and connected as nodes. The relative fold of inhibition of ECP-cell interaction was shown in red numeral.

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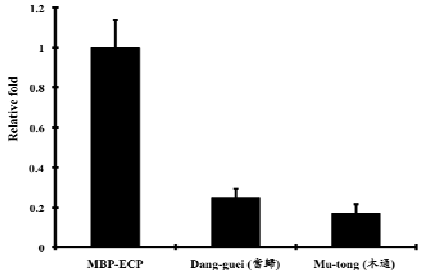

Figure 4: The binding affinity between ECP and cells are decreased by adding Dang-guei and Mu-tong.