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Cloning, expression, and immunological characterization of two novel profilins from *Artemisia annua*

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The incidence of allergic disease has increased rapidly in recent decades in both developed and developing countries.[1] In China, pollen of *Artemisia* is one of the most important outdoor allergen as reported by the first national pollen survey across all provinces.[2]*Artemisia* pollen has been found to be an important cause of seasonal asthma.[3]*Artemisia vulgaris* and *Artemisia annua* are the most important *Artemisia* species in China. *A. vulgaris* mainly grows in western regions, while *A. annua* is abundant in the densely populated northern and eastern regions.[4] Immunoglobulin E (IgE) antibodies play a key role in allergy diseases. They are synthesized and released by B cells and bind to high-affinity IgE receptors on mast cells or basophils.[5] Once the patients are exposed to corresponding allergens, the effector cells will be triggered and then release its mediator, leading to an allergic inflammation.[5] Allergen immunotherapy based on specific allergens to patients is considered a promising and effective treatment for allergic diseases.[6,7] Therefore, identification of the IgE-binding allergens will improve the diagnosis of allergy diseases and application of immunotherapy. However, previous studies have mainly investigated pollen allergens from *A. vulgaris* (Art v), such as Art v 1–6. As for *A. annua* (Art an), only two allergens have been identified as Art an 1 (defensin-like protein) and Art an 7 (putative galactose oxidase).[4] Thus, it is important to study allergens from *A. annua* pollens.

In the present study, we identified two profilins from *A. annua* by next-generation sequencing technology and the basic local alignment search tool. The complementary DNAs encoding profilin 1 and profilin 2 were amplified by polymerase chain reaction using primers designed from non-coding region sequences of two profilins obtained by transcriptome sequencing. The two profilins from *A. annua* pollen exhibited open reading frames of 399 base pair encoding 133 amino acids. The sequence data of the profilins have been deposited in Genbank under the accession numbers of MN105099 and MN105100. The amino acid sequences of profilin 1 and profilin 2 were exactly the same as those of Art v 4.0101 and Art v 4.0201 from *A. vulgaris*, respectively. Moreover, profilin 1 and profilin 2 shared approximately 65% to 90% sequence identity with other profilins from different plant pollens [Supplementary Figure 1A, http://links.lww.com/CM9/A422]. A phylogenetic tree analysis revealed that profilin 1 forms a close cluster with Amb a 8, whereas profilin 2 is close to Hel a 2 [Supplementary Figure 1B, http://links.lww.com/CM9/A422], and all these profilins were clustered into the same group.

To isolate recombinant allergens for further analysis, profilin 1 and profilin 2 genes were subcloned into pET-28a vector and expressed in *Escherichia coli* BL-21. These two profilins were expressed as the inclusion bodies, which were purified by nickel affinity chromatography under denaturing conditions. The refolded proteins showed a single band with an approximate molecular weight of 12,000 Da as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [Supplementary Figures 1C and 1D, http://links.lww.com/CM9/A422].

The IgE-binding activity of the identified allergens is essential for the initiation of allergic inflammation. Two hundred patients with allergy reactions to *Artemisia* pollen

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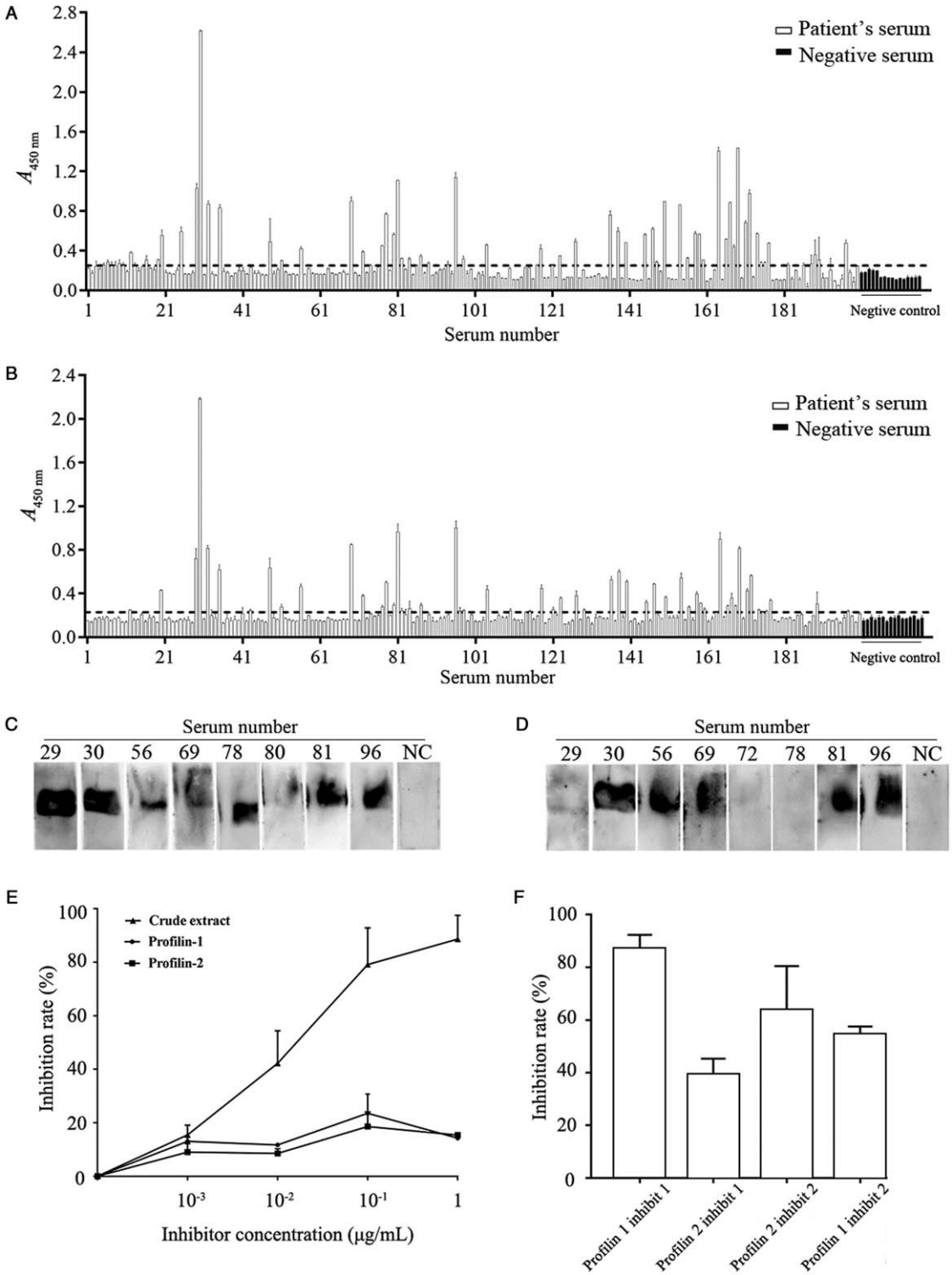

Figure 1: Immunoreactivity analysis of profilin 1 and profilin 2 by ELISA, Western blotting, and competitive inhibition experiment. ELISA analysis of profilin 1 (A) and profilin 2 (B). Two hundred *Artemisia* allergy serum samples and 16 negative control serum samples were used. The values were shown as mean ± SD. The dotted line represents the cut-off value, which was calculated as mean absorbance value of the negative control plus 3 SDs (mean + 3*SD) of the 16 negative control serum samples. Results of the Western blot analysis of profilin 1 (C) and profilin 2 (D). The serum number corresponds to ELISA serum number, which reacted to profilin 1 and/or profilin 2. NC represents negative control serum. (E) Inhibition of the IgE immunoreactivity to the *A. annua* extract by profilin 1, profilin 2, and crude extract. (F) Competitive inhibition experiment conducted to analyze the cross-reactivity between profilin 1 and profilin 2. *A. annua*: *Artemisia annua;* ELISA: Enzyme-linked immunosorbent assay; IgE: Immunoglobulin E; SD: Standard deviation.

and 16 healthy subjects were included in the present study. The overall clinical information of the patients is shown in Supplementary Table 1, http://links.lww.com/CM9/A423. All participants provided a written informed consent for the use of their blood samples for this study. Results of enzyme-linked immunosorbent assay (ELISA) showed that serum IgE reacted to profilin 1 in 65 of 200 (32.5%) patients [Figure 1A] and reacted to profilin 2 in 54 of 200

(27%) [Figure 1B] The sensitization rates were similar to profilins from other pollens, such as Bet v 2 (22%, 55/242)[8] and Hel a 2 (30.5%, 37/121),[9] which might be due to their structural conservation. The IgE binding activities of the two profilins were further confirmed by Western blot using serum samples from eight representative patients and one negative control. Western blot showed that of the eight positive serum samples, seven showed strong bands in profilin 1 [Figure 1C] and five showed bands in profilin 2 [Figure 1D]. However, some ELISA-positive serum samples showed no bands in Western blotting. This is because some IgE-binding epitopes of profilin 1 and profilin 2 are conformational, which are easily disrupted during the Western blot test.

A competitive inhibition experiment was performed to evaluate the immunoreactivity of profilin 1 and profilin 2, in comparison to the crude extract of *A. annua* pollen. We found that the average inhibition rate of profilin 1 and profilin 2 was 23.6% and 18.6%, respectively [Figure 1E]. A further competitive inhibition experiment was conducted to analyze the cross-reactivity between profilin 1 and profilin 2. It was showed that 54.57% of the IgE from No. 30 serum bound to profilin 2 was inhibited by profilin 1, and 39.35% of that bound to profilin 1 was inhibited by profilin 2 [Figure 1F].

Comprehensive analysis of clinical symptoms and ELISA results revealed that there was no significant difference in the immunoreactivity characteristics of profilins in patients with different allergic symptoms (asthma, rhinitis, eczema, hay fever, dermatitis, or conjunctivitis) [Supplementary Figures 1E and 1F, http://links.lww.com/CM9/A422]. Similarly, there was no significant difference in the immunoreactivity characteristics of profilins between asthma and non-asthma patient groups, and rhinitis and non-rhinitis patient groups [Supplementary Figures 1G and 1H, http://links.lww.com/CM9/A422]. These indicated that sensitization to profilins from *A. annua* was not relevant to one or more specific clinical symptoms in Chinese *Artemisia* allergic patients, which was consistent with previous studies.[10]

In summary, we identified two profilins named as profilin 1 and profilin 2 from *A. annua* pollen. These two profilins have identical amino acid sequences to that of Art v 4.0101 and Art v 4.0201 from *A. vulgaris*, respectively, but with different nucleotide sequences and non-coding regions. Moreover, alignment analysis showed that profilin 1 and profilin 2 shared high amino acid sequence identity to the previously reported profilin allergens from various species. This may contribute to cross-sensitization or polysensitization to different pollen or food allergens. The IgE-binding rates of profilin 1 and profilin 2 in *Artemisia* allergy patients were 32.5% and 27%, respectively, similar to that of pollen profilins from other species. We also found that the immunoreactivity of profilin 1 and profilin 2 in Chinese patients with *Artemisia* allergic decreased with increasing age, but this needs further confirmation in patients from different ethnicities and race. Finally, the identified novel profilins had a non-negligible influence in Chinese *Artemisia* allergic patients and would be valuable to component-resolved allergy diagnostics in the future.

Ethical statement

The study protocol was approved by the Ethical Committee of the First Affiliated Hospital of Nanjing Medical University (Ethical Approval Number: 2018-SRFA-094). Written informed consent for the use of blood samples was obtained from all participants before study.

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Conflicts of interest

None.

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