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Brief Communication

The Heterogeneity of Ovomucoid-Specific IgE Idiotype Is Associated With Egg Allergy Symptom Severity

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ABSTRACT

Immunoglobulin E (IgE)-mediated egg allergy presents as one of the most common food allergies. The level of specific IgE (sIgE) antibody is widely used as an important *in vitro* diagnostic indicator. However, sIgE antibody levels are often inconsistent with the clinical manifestations of patients. The heterogeneity of egg-specific IgE idiotypes (sIgE-IDs) may help reflect clinical egg allergy severity. Eight peptides were synthesized, corresponding to the linear epitopes of ovomucoid (OVM). The sIgE-IDs of egg-allergic patients were detected by enzyme-linked immunosorbent assay. Fresh peripheral blood was collected from patients with different heterogeneity strength of sIgE-ID, and egg extract was used as a stimulus to the basophil activation test (BAT). RBL-2H3 cells were sensitized with serum with different strength of sIgE-ID heterogeneity and the release rate of β-hexosaminidase was calculated. Among 75 patients with egg allergy, 24% had sIgE for all epitopes and 85% had sIgE for at least one epitope. Analysis of individual patients revealed differences in epitope recognition patterns among the patients, that is, heterogeneity in sIgE-ID. More importantly, the number of IgE-positive peptides had a strong correlation with allergic symptoms in egg-allergic patients ($r = 0.706$). BAT and RBL-2H3 cell degranulation confirmed that higher sIgE-ID heterogeneity strength was more effective in inducing effector cell responses. Our results suggest that the greater the heterogeneity strength of OVM-sIgE-ID, the more severe the allergic symptoms.

Keywords: Egg allergy; ovomucoid; epitopes; immunoglobulin E; food allergy; immunosorbent assay; basophil activation test

INTRODUCTION

Allergen-specific immunoglobulin E (IgE) is an important indicator for the *in vitro* diagnosis of allergic diseases.[1-4] However, specific IgE (sIgE) antibody levels are often inconsistent with the clinical manifestations of patients. In recent years, researchers have turned their attention to the study of epitope-sIgE. They found a broader diversity of IgE binding in patients with

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Disclosure

There are no financial or other issues that might lead to conflict of interest.

persistent food allergy than in the tolerant group.[5,6] A microarray immunoassay of the main peanut allergens Ara h 1, Ara h 2, and Ara h 3 indicated that the diversity of IgE antibodies was related to the severity of allergic reactions.[7,8]

According to previous studies, there are individual differences in the number of epitopes recognized by allergen-sIgE antibodies in some food allergies. This difference is reflected in the difference in the variable region (V region) of the antibody, especially the hypervariable region (HVR), that is, "the difference in the antibody idiotype, which we define as the heterogeneity of sIgE-ID."[9] Immunoglobulin idiotype (ID) refers to the specificity exhibited by the amino acid type and the sequence of the HVR of the antibody structure, which is complementary to the spatial structure of the antigenic epitope.[10]

We hypothesized that the clinical manifestations of food allergy are related to the degree of allergen sIgE-ID heterogeneity and studied sIgE antibodies from the perspective of experimental diagnosis to explore whether allergen sIgE-ID heterogeneity could be used as an experimental indicator reflecting allergic symptoms. In this study, ovomucoid (OVM), the main allergen component of eggs, was used as the research object.[11,12] The linear epitope profile of OVM was analyzed and the association between sIgE-ID heterogeneity and clinical symptoms in egg-allergic patients was explored.

MATERIALS AND METHODS

Study population

The plasma samples of patients were obtained from the Second Hospital of Tianjin Medical University and Academy of Traditional Chinese Medicine Affiliated Hospital on the basis of detectable IgE to egg (>0.1 kUA/L measured by fluorescence enzyme immunoassay, ImmunoCAP; Phadia, Uppsala, Sweden). Enrollment required a history of either a convincing immediate allergic reaction, such as urticaria, difficulty breathing, wheezing, vomiting, or diarrhea, following the ingestion of egg, a positive skin prick test response to egg or the trigger food as documented by a patient's local physician, or both. No oral food challenges were performed considering its potential risks.[13] Excluding 32 patients with unclear history, a total of 75 patients with a confirmed history of allergy to egg were included in the study. The negative control included 15 individuals with no history of any food or environmental allergies and 15 atopic controls with food allergies other than egg. The clinical characteristics of the subjects are summarized in **Table 1** and **Supplementary Table S1**. Informed consent

Table 1. Characteristics of the participants according to egg-allergic and non-allergic individuals

Characteristic	Egg-allergic individuals (n = 75)	Atopic individuals (n = 15)	Healthy individuals (n = 15)
Age (y)	7 (0.5–49)	4 (1–15)	3 (0.9–10)
Sex, male	45 (60.0)	9 (60.0)	7 (46.7)
Total IgE (kUA/L)	285.39 (64.42–794.66)	319.23 (93.86–584.55)	< 0.1
sIgE to egg (kU_A/L)	2.00 (0.36–43.00)	< 0.1	< 0.1
Ovomucoid-sIgE (OD 450)	0.225 (0.134–1.228)	0.160 (0.121–0.201)	0.173 (0.150–0.199)
Allergic symptoms			
Cutaneous	69 (92.0)	13 (86.7)	0
Digestive	26 (34.7)	8 (53.3)	0
Respiratory	33 (44.0)	5 (33.3)	0

Values are expressed as number (%) or medians (range).
IgE, immunoglobulin E; sIgE, specific immunoglobulin E; OD, optical density.

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Table 2. Location of the IgE-binding epitopes in ovomucoid

Number	Position	Amino sequence	Length of sequence	Purity/%
Peptide 1	1–10	AEVDCSRFPN	10	90
Peptide 2	9–20	PNATDKEGKDVL	12	90
Peptide 3	31–44	GTDGVTYTNDCLLC	14	90
Peptide 4	46–59	YSIEFGTNISKEHD	14	90
Peptide 5	91–104	FNPVCGTDGVTYDN	14	90
Peptide 6	101–114	TYDNECLLCAHKVE	14	90
Peptide 7	113–124	VEQGASVDKRHD	12	90
Peptide 8	175–186	NGTLTLSHFGKC	12	90

was obtained from all subjects, and this study was approved by the Ethics Committees of Tianjin Medical University (TMUHMEC2017008).

Peptide synthesis

According to previous research,[5,14-17] 8 identified OVM-related IgE sequential epitopes were selected for biotinylation in N-terminal and commercially synthesis (Sangon Biotech, Shanghai, China) (**Table 2**). Peptides were resuspended in phosphate-buffered saline (PBS) to a final concentration of 1 mg/mL and titrated to optimal concentrations before a formal experiment.

Detection of sIgE-ID heterogeneity

For the measurement of sIgE-ID heterogeneity of OVM, a novel method of indirect enzyme-linked immunosorbent assay (Indirect-ELISA) was used. First, 96-well plates were coated overnight with 2.5 μg/well biotinylated bovine serum albumin in 0.05 M carbonate buffer (pH 9.6) at 4°C. Then, microplates were washed 3 times with PBS containing 0.05% (v/v) Tween-20. Streptavidin with 0.1 μg/well (100 μL in PBS) was added and incubated for 1 hour at 37°C. After blocking, the samples were detected by the classic Indirect-ELISA method. Optical densities (ODs) were measured at a wavelength of 450 nm with an automated ELISA reader. The mean OD value (A_{450}) plus 2 standard deviations of the negative control sera for the respective peptide was chosen as the cutoff point.

Basophil activation test (BAT)

Patients with different numbers of positive epitopes recognized were randomly selected to evaluate the activity of IgE antibodies. Heparinized whole blood from 6 egg-allergic patients and a non-allergic subject were collected by venipuncture. Whole blood aliquots (500 μL) were incubated with equal volumes of stimulation buffer (RPMI + IL-3 at 2 ng/mL) alone (negative control) or with the addition of OVM in stimulation buffer at serial 10-fold dilutions (from 1×10^2 to 1×10^{-2} ng/mL total protein), or anti-IgE antibody (1 μg/mL; positive control) at 37°C for 30 minutes. Degranulation was stopped by transferring tubes on ice, and cells were harvested and washed twice with RPMI. Cells were then resuspended in 100 μL of RPMI and stained with previously titrated amounts of anti-CD123-PE, anti-HLA-DR-PerCP and anti-CD63-FITC (BD Biosciences, San Jose, CA, USA) for 20 minutes at 4°C. Unstained and compensation controls were also prepared from the same donor cells. Finally, whole blood was lysed, washed, resuspended and analyzed within 2 hours using a FACSCanto II flow cytometer (BD Biosciences). Unactivated and activated basophils were identified as $CD123^+HLA\text{-}DR^-CD63^-$ and $CD123^+HLA\text{-}DR^-CD63^+$ cells, respectively. Data were analyzed by using FlowJo (BD Biosciences).

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Measurement of RBL-2H3 cell degranulation

The human FcεRIα-transfected rat basophilic leukemia cell line RBL-2H3 was a gift from the same laboratory. After one passage, adjust the cell concentration to 5×10^4 /mL and 5,000 event/well (100 μL in minimum essential medium [MEM]) were added to 96-well, flat-bottom, tissue-culture plates and incubated overnight at 37°C in a 5% CO_2 atmosphere to allow cells to adhere. Then, cells were sensitized passively by overnight incubation with participants' sera (diluted 1:10). Here, we randomly selected 10 patients with high or low heterogeneity strength to make serum pools as well as serum from a healthy individual as a negative control. Subsequently, cells were washed and treated with equal volumes of MEM medium alone (blank control), or with the addition of OVM in MEM medium at serial 10-fold dilutions (from 1×10^2 to 1×10^{-2} ng/mL total protein), or anti-IgE antibody (1 μg/mL; positive control) for 1 hour. Three replicates were operated. After incubation with allergen, supernatant and cell pellet were separated. Cell pellets were lysed in 100 μL of media culture containing 0.5% Triton X-100 for 30 minutes. Then, β-Hexosaminidase levels were measured in cell pellets as well as in supernatants by adding 100 μL of β-hexosaminidase substrate (4-Nitrobenzene-N-acetyl-beta-D-glucosamine) in 0.1 mol/L citrate buffer (pH 4.5) for 1 hour. The reaction was stopped by addition of 100 μL of 0.1 mol/L sodium carbonate buffer (pH 11.0), and ODs were measured at 405 nm with an automated ELISA reader.

Statistical analysis

The Mann-Whitney *U* test was used to compare results between egg-allergic patients and control, and reported *P* values are 2-tailed, with *P* values < 0.05 considered statistically significant. Analysis was performed with GraphPad Prism (GraphPad, San Diego, CA, USA) and SPSS (IBM, New York, NY, USA).

RESULTS

Serum OVM-sIgE antibodies in the egg-allergic and non-egg-allergic groups

The egg-allergic group and negative control showed significant differences in OVM-sIgE levels (**Fig. 1**). Compared to the non-egg-allergic group (n = 30, blue violin), the egg-allergic group (n = 75, red violin) had significantly higher mean levels of sIgE antibodies to OVM.

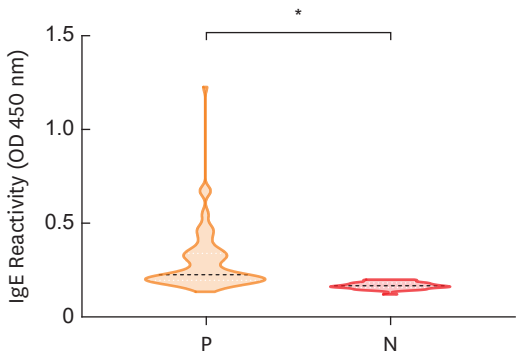

Fig. 1. Comparison of serum specific IgE against ovomucoid in different groups.
IgE, immunoglobulin E; OD, optical density.
*$P < 0.001$.

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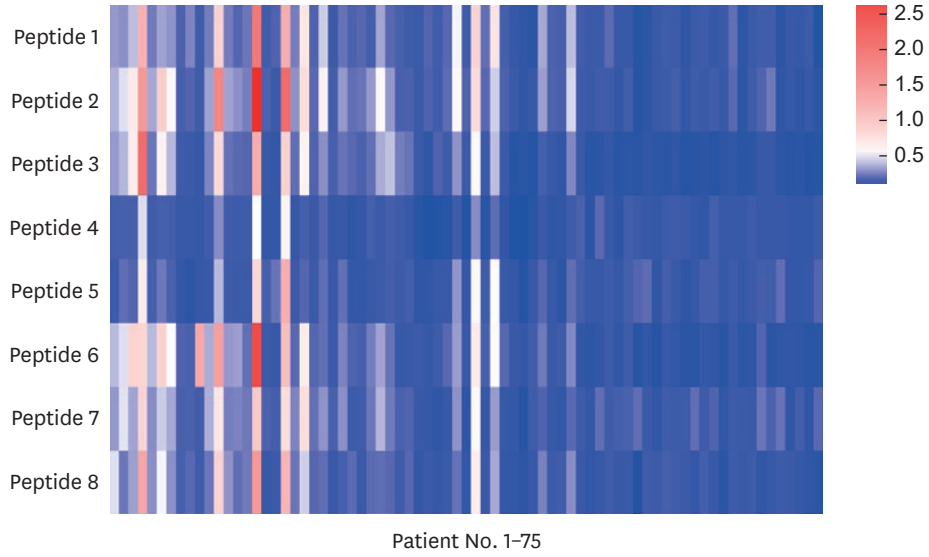

Fig. 2. Heat map of immunoglobulin E binding to ovomucoid peptides in egg-allergic patients.

Individual patients have heterogeneous patterns of IgE binding

As presented in **Fig. 2**, there were heterogeneity of sIgE-IDs, as manifested as both the number of epitopes recognized, and the pattern of recognition varied considerably among 75 egg-allergic individuals. The level of sIgE for each epitope in the same patient was different, and the level of sIgE for the same epitope was also different among individual patients. Eleven (15%) sera showed no epitope reactivity, while 18 (24%) sera had the positive IgE binding to all epitopes. Sixty-four (85%) sera had the positive IgE binding to at least one epitope. The mean number of IgE-binding epitopes recognized per individual was 4 (range, 0–8). In addition, the study found a moderate correlation between the number of IgE-positive peptides and serum egg-sIgE antibody levels (r = 0.414/OVM-sIgE antibody levels (r = 0.581).

The response frequency of each peptide in egg-allergic patients is illustrated in **Fig. 3**. The response frequencies of 4 peptides (#1, #2, #6, and #7) were higher than 50%. AA1–10 and AA9–20 had the highest response frequencies at 60% and 59%, respectively. AA31–44 and AA175–186 had the lowest response frequency at 44%.

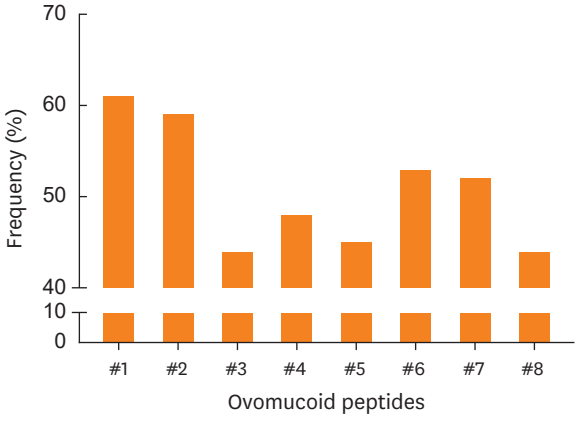

Fig. 3. The recognition frequency of the 8 peptides of ovomucoid in the serum of egg-allergic patients.

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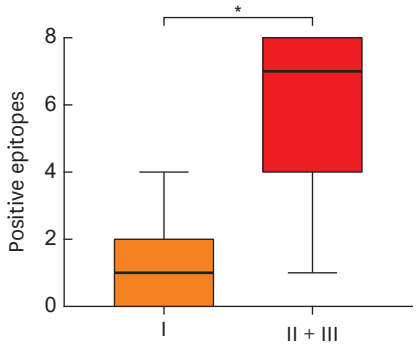

Fig. 4. The recognition number of the 8 peptides of ovomucoid in different groups. Grade II + III (multiple organ system reactions) recognized more egg allergen epitopes than grade I (cutaneous symptoms alone).
*P < 0.001.

Serum sIgE-ID heterogeneity corresponds to symptom severity

According to the World Health Organization's classification of anaphylaxis,[18] we divided egg-allergic patients into 2 groups: one with cutaneous symptoms alone (grade I, n = 28) and the other with multiple organ system involvement (grade II + III, n = 47). The patients with a history of cutaneous symptoms alone recognized a significantly fewer number of epitopes than those with a history of more severe multiple organ system reactions, which had greater ovomuciod sIgE-ID heterogeneity strength (median, 1 vs. 7, respectively; P < 0.001; **Fig. 4**).

In addition, we found that there was a moderate correlation between the number of IgE-positive peptides and OVM-sIgE levels in egg-allergic patients (r = 0.581, P < 0.01). Egg-sIgE antibody levels and OVM-sIgE antibody levels had no correlation with allergic symptoms in the same population (r = 0.066 and 0.152, respectively), while the number of IgE-positive peptides had a strong correlation with allergic symptoms (r = 0.706, P < 0.01). Therefore, we hypothesized that greater sIgE-ID heterogeneity strength might be a marker for more severe reactions.

Greater OVM-sIgE-ID heterogeneity strength increases both basophil sensitivity and maximal degranulation level

We used the BAT to assess basophil degranulation as described in the Methods section. On degranulation, CD63, a 53-kD glycoprotein, anchored in the granule membrane, fuses with and becomes exposed on the outside of the basophil plasma membrane, which perfectly correlates with histamine release.

When human basophils were sensitized with increasing relative concentrations of OVM, we found that when the sIgE-ID heterogeneity strength increased, the maximal degranulation response of sensitized basophils was increased (P < 0.001, **Fig. 5**). The RBL-2H3 cell degranulation experiment exhibited the same result and was consistent with our hypothesis (**Fig. 6**).

DISCUSSION

Numerous studies on food allergen epitopes have been reported, and the IgE-binding epitopes of various allergens have been identified, such as peanut, milk, shrimp, and soybean.[8,19-21] Studies have shown that due to differences in individual immune responses, there are individual differences in the nature and number of antigenic epitopes recognized by allergen sIgE antibodies. From the perspective of the antibody to be detected, for the same

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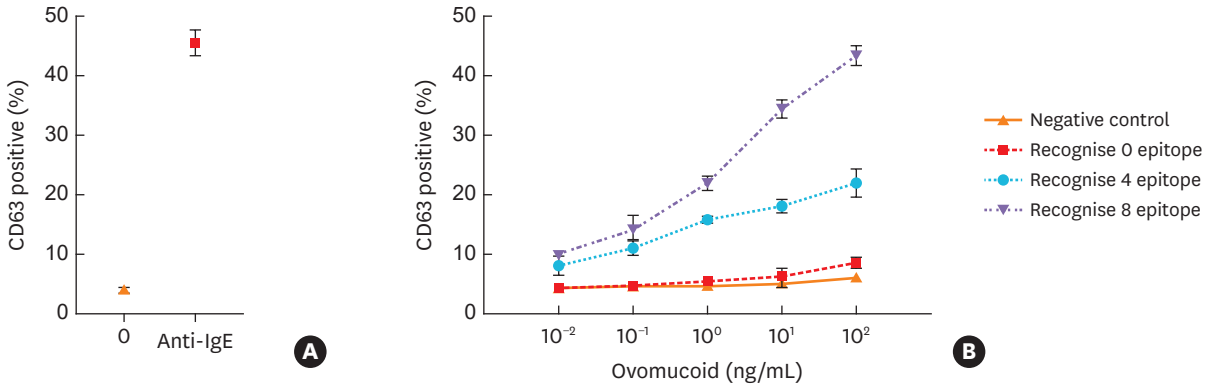

Fig. 5. Basophil degranulation from patients with egg allergy. Basophils were sensitized overnight with the sera of patients with egg allergy or control sera (volume ratio, 1:10), washed, and either stimulation buffer alone treated or exposed for 30 minutes to anti-IgE (1 μg/mL; A), or different concentrations of ovomucoid (as indicated; B). The assay was performed in triplicates; values indicate means ± 2standard deviations. Patient sera recognizing a greater number of IgE epitopes induced more degranulation of sensitized cells.
IgE, immunoglobulin E.

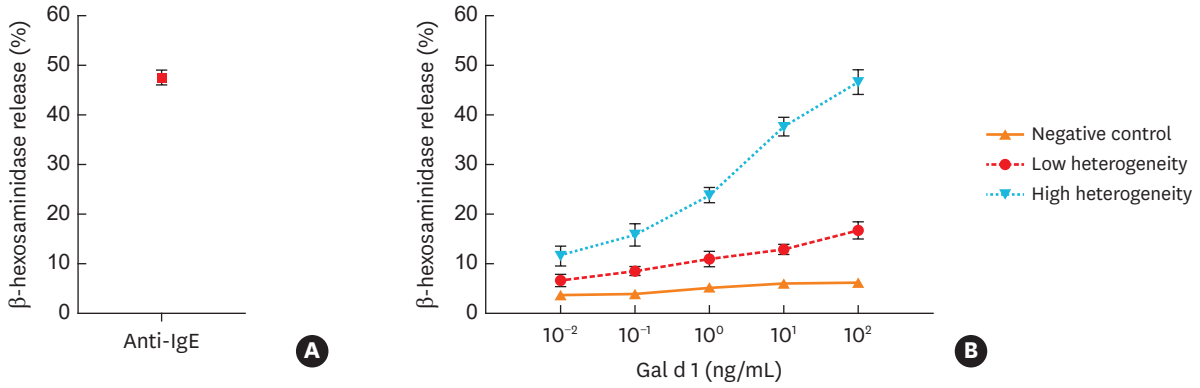

Fig. 6. The β-hexosaminidase release test from the 2 serum pools. The assay was performed in in triplicate with serum pool from 10 randomly selected egg-allergic patients or control sera (as indicated; B). The positive control was shown as (A). The patient sera of greater sIgE-ID heterogeneity induced more release of β-hexosaminidase. β-Hexosaminidase Release Rate (%) = (Supernatant OD 405 − Blank Control OD 405)/(Cell Pellet OD 405 − Blank Control OD 405) × 100%.
IgE, immunoglobulin E; OD, optical density; sIgE-ID, specific immunoglobulin E idiotype.

allergen component, the differentiated responses of different individuals ultimately manifest as differences in sIgE-ID, that is, sIgE-ID heterogeneity. It is worth noting that the pathogenic activity of sIgE antibodies *in vivo* depends on the ability of sIgE antibodies to attach to the surface of sensitized cells to bind to the allergen to form an "antigen bridge." This is closely related to the binding capacity also called "affinity" of IgE antibody V regions and antigenic epitope.[22] Studies of sIgE antibody idiotypes heterogeneity may be of great value in the diagnosis and prognosis of allergic reactions.

We analyzed the profiles of IgE sequential epitopes of OVM among patients from the coastal areas of northern China with a novel ELISA. Eight polypeptides were selected to identify the heterogeneity of ovomuciod sIgE-ID. In the egg-allergic group, the response frequency for 4 peptides (AA1–10, AA9–20, AA101–114, AA113–124) was higher than 50%, and the response frequency for AA1–10 and AA9–20 was the highest, 61% and 59%, respectively. This was consistent with previous studies,[5,14] which revealed that these epitopes may be the major IgE-binding epitopes. In addition, we found that individual patients have the heterogeneous patterns of IgE binding, that is to say, there were heterogeneous in sIgE-ID. Eighty-five percent of patients' sera recognized at least one of the peptides, and 15% of the sera showed

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no peptide-binding reactivity. One possible explanation could be that the number of ovomuciod peptides synthesized was insufficient and that these patients may recognize other linear epitopes or conformational epitopes. Plus, 24% of patients recognized all peptides synthesized, showing stronger reactivity to linear epitopes. Similar results have been found in other food allergens studied.[23]

In this study, we also found that the number of peptides recognized by IgE was moderately correlated with the level of egg-sIgE ($r = 0.414$, $P < 0.01$), which was similar to the result of the previous study.[14,24] Patients with a history of cutaneous symptoms alone recognized a significantly smaller number of epitopes than those with a history of more severe multiple organ system reactions. There was a significant correlation between the number of IgE epitopes of OVM and historical reactivity ($r = 0.706$, $P < 0.01$), epitope studies on other allergens have yielded similar results.[25,26] This indicated that the heterogeneity of OVM-sIgE-ID is associated with egg allergic symptom severity. The greater the heterogeneity of OVM-sIgE-ID, the more severe the allergic symptoms. This was confirmed in subsequent BAT and RBL-2H3 cell degranulation experiment. Patient sera with greater idiotype heterogeneity strength induced greater degranulation of sensitized cells. Double-blind placebo-controlled food challenge is the gold standard for diagnosing food allergies.[27] However, it is difficult to clinically implement due to its time-consuming, high cost and risk of allergic reactions. In this case, allergen sIgE-ID heterogeneity is expected to be used as an experimental indicator for reflecting allergic symptoms.

In conclusion, OVM-sIgE-ID heterogeneity has far-reaching implications for reflecting the severity of egg-allergic symptoms. There are also some limitations to the present study. Oral food challenge was not performed due to possible risks. The synthesis of the peptide does not cover the entire sequence of OVM, and some important epitopes may be missed. In addition, we did not perform subgroup analysis according to age, although the food allergy characteristics differ significantly according to age, which cannot be disregarded. In future research, we will focus on the age factor.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1

Characteristics of 15 patients with food allergies other than egg

Click here to view

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