

# IgE and IgG<sub>4</sub> responses to shrimp allergen tropomyosin and its epitopes in patients from coastal areas of northern China

JUNPU LI<sup>1,2\*</sup>, ZHIWEI LI<sup>2\*</sup>, DEYU KONG<sup>3</sup>, SHAOSHEN LI<sup>4</sup>, YANG YU<sup>2</sup> and HUIQIANG LI<sup>2</sup>

<sup>1</sup>The Clinical Laboratory of Tianjin Chest Hospital, Tianjin 300222; <sup>2</sup>Department of Clinical Laboratory, School of Medical Laboratory, Tianjin Medical University, Tianjin 300203; <sup>3</sup>Department of Laboratory Medicine, Tianjin Port Hospital, Tianjin 300456; <sup>4</sup>Department of Laboratory Medicine, Academy of Traditional Chinese Medicine Affiliated Hospital, Tianjin 300120, P.R. China

Received July 13, 2019; Accepted March 17, 2020

DOI: 10.3892/mmr.2020.11084

**Abstract.** Sensitization to allergens and their peptides varies among patients due to geographical or ethnic differences. The present study aimed to investigate immunoglobulin (Ig)E and IgG<sub>4</sub> responses to tropomyosin and its peptides in shrimp allergic patients from northern China. A total of 92 subjects were studied, including 35 shrimp allergic patients, 29 patients with house dust mite (HDM) and/or cockroach allergic patients and 28 healthy volunteers. Serum IgE and IgG<sub>4</sub> antibodies to recombinant shrimp tropomyosin (rPen a 1) and its peptides were measured by means of a light-initiated chemiluminescent assay. A total of 9 major sequential epitopes of Pen a 1 reported in the literature were synthesized. Of 35 shrimp allergic patients, 25 (71.4%) had positive Pen 1-specific IgE (sIgE) antibodies and 22 (62.9%) contained measurable rPen a 1-specific IgG<sub>4</sub> (sIgG<sub>4</sub>) antibodies. A strong IgG<sub>4</sub> response accompanied the presence of IgE to Pen a 1. None of the patients with HDM and/or cockroach allergy demonstrated IgE reactivity to rPen a 1. The reaction frequency of IgE binding epitope was 20–48%, while that of IgG<sub>4</sub> binding epitope was 63.6–90.9%. The IgE and IgG<sub>4</sub> recognition patterns of the tropomyosin peptides demonstrated high interpatient heterogeneity. Diversity of IgE binding epitopes was positively correlated with Pen a 1 sIgE levels. In the study population, tropomyosin was a major allergen recognized by the majority of shrimp allergic patients, which is consistent with previous reports. However, none of the 9 epitopes are major (reaction

frequency >50%) IgE-binding regions, indicating the epitopes profile may be different in other regions.

## Introduction

Shellfish, a major sensitizing component of seafood, is widely consumed worldwide due to its nutritional value. Therefore, the increase in immunoglobulin (Ig)E-mediated seafood allergy reports is particularly related to shellfish (1). The prevalence rates of shellfish allergy range from 0.5 to 2.5% depending on geographic locations, dietary habits and age. Unlike most other food allergies, a shellfish allergy persists for life in up to 90% of patients and is often associated with severe systemic anaphylactic reactions. Seafood-associated shellfish includes crustaceans and mollusks. The majority of species that cause allergic reactions are the crustaceans, with shrimp being by far the most frequently involved (1–3).

Tropomyosin, a protein from muscle, was the first major allergen identified in shrimp (4,5). Sensitization to the major allergen tropomyosin has been found in 80% of shrimp-allergic patients (6,7). Tropomyosin is also reported as being a panallergen of numerous other species such as lobster, crab and mollusks (8). Due to evolutionary lineages, there is a 78–98% amino acid homology of tropomyosin between crustaceans and mites, and 80–97% between crustaceans and cockroaches (3). Consequently, the highly conserved amino acid sequence of tropomyosin is responsible for clinical cross-reactivity among shellfish species (9). Tropomyosin is also considered to be responsible for cross-reactivity between food and aeroallergens of animal origin such as dust mites or cockroaches (10,11).

Tropomyosin is important for the diagnosis of seafood allergy. A recombinant tropomyosin from *Penaeus aztecus*, Pen a 1, has been commercially available for the component-resolved diagnostics (CRD) of shrimp allergy and could provide a more accurate diagnosis (12,13). Clearly, research on food allergens can be useful for the treatment of food allergy (14,15). Mapping the IgE and IgG<sub>4</sub> epitopes of tropomyosin may reveal relevant information about antigen structure, on the basis of which a safe hypoallergenic agent can be designed to treat shrimp allergy (16).

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*Correspondence to:* Professor Huiqiang Li, Department of Clinical Laboratory, School of Medical Laboratory, Tianjin Medical University, 1 Guangdong Road, Hexi, Tianjin 300203, P.R. China  
E-mail: lhq@tmu.edu.cn

\*Contributed equally

**Key words:** shrimp allergy, recombinant tropomyosin, immunoglobulin E, immunoglobulin G<sub>4</sub>, peptides, light-initiated chemiluminescent assay

However, there are still some problems with the clinical use of tropomyosin, which requires further study. One problem is that previous studies demonstrated that sensitization to allergens and their peptides varies among patients due to geographical or ethnic differences. First, the tropomyosin sIgE frequencies vary. Asero *et al* (17) found that less than half (41.6%) of the Italian adult patients with shrimp allergy reacted to tropomyosin (Pen a 1). The variability depends on the route and dose exposure to allergens and individuals of different ages from different ethnic backgrounds (18,19). CRD has revealed that these sensitization profiles might show geographical differences with clearly distinct clinical outcomes (20). Second, the major sequential IgE binding epitopes of tropomyosin (Pen a 1) have been identified using overlapping peptide mapping by SPOTs membrane-based immunoassays to elucidate sensitization profiles (21,22), but previously published results have demonstrated great heterogeneity in the number of epitopes and their locations for the same allergens (23,24). These differences are probably related to the technology used, the overlapping peptide length and the populations selected (25-27). Third, the role of IgG<sub>4</sub> may be different in different species and the role of IgG<sub>4</sub> in shrimp allergy is not fully understood. IgG<sub>4</sub> epitopes have been reported to be associated with immunologic tolerance to milk and peanuts (28). On the other hand, IgG<sub>4</sub> has also been considered to be associated with atopy and allergic sensitization (29).

Limited work has been conducted regarding the potential allergenicity and antigenicity of tropomyosin and its peptides in patients from coastal areas of northern China (30). The object of the present study was to determine the frequency of IgE and IgG<sub>4</sub> antibodies reactivity to shrimp tropomyosin (Pen a 1) in the northern Chinese population. The present study also investigated the IgE and IgG<sub>4</sub> specificity and diversity to sequential epitopes of Pen a 1 in Pen a 1-positive patients.

## Materials and methods

**Patients.** A total of 92 subjects were consecutively recruited from Tianjin Port Hospital and Academy of Traditional Chinese Medicine Affiliated Hospital between January 2018 and November 2018. Patient characteristics are shown in Table I. Upon study entry, all participants underwent a detailed medical examination and clinical history review. Clinical allergy was diagnosed by an experienced allergologist using the following criteria: i) A convincing history of acute allergic reactions after contact (including urticaria, abdominal pain and wheezing) and ii) increased sIgE levels [cutoff: >0.35 kU/L, measured by fluorescence enzyme immunoassay (ImmunoCAP, Phadia AB)] as defined by the European Academy of Allergy and Clinical Immunology guidelines (31). The study protocol was approved by the Ethics Committees of Tianjin Medical University (grant no. TMUHMEC2017008) and written informed consent was obtained from the patients and volunteers prior to study entry.

**Preparation of recombinant tropomyosin.** The gene coding Pen a 1 protein sequences (GeneBank NO. DQ151457) was synthesized and cloned into the *Bam*H I and *Hind*III sites of PET28a (+) expression vector using the DNA Ligation kit (cat.

no. D6020A; Takara Bio, Inc.), according to the manufacturer's protocol. The resulting plasmid containing the Pen a 1 coding regions and a poly-histidine affinity purification tag (6XHis) at the N-terminus was subsequently transformed into *E. coli* BL 21 (DE3) competent cells (Tiangen Biotech Co., Ltd.) using the heat shock transformation method. Briefly, 5 µl pET28a-Pen a 1 plasmid was transformed into 100 µl *E. coli* BL21 cells (DE3) and incubated on ice for 30 min, prior to being heated in a water bath at 40°C for 60 sec, followed by an ice bath for 2 min. The transformants were streaked on LB agar plate supplemented with kanamycin (50 mg/ml). When cultures reached an optical density at 600 nm level of 0.4-0.6, the expression of Pen a 1 was induced by adding 1 mM isopropyl-B-D-thiogalactoside (Invitrogen; Thermo Fisher Scientific, Inc.) and then incubated for 3 h at 37°C and 220 rpm on an orbital shaker. *E. coli* cells were harvested by centrifugation at 4,000 x g for 5 min at 4°C, resuspended in phosphate-buffered saline (PBS, 0.01 M, pH 7.4), and sonicated at 60 kHz, with 10 sec pulse-on and 10 sec pulse-off for five cycles on ice. The recombinant protein was purified from the culture supernatant using Ni<sup>+</sup>-NTA affinity column chromatography (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, prior to the purification, 6 ml binding buffer (50 mM PBS, 0.3 M NaCl) was used to prepare the 10 ml purification column. Then, 5 ml lysate was added into the column and incubated for 30-60 min at 4°C using gentle agitation to keep the resin suspended in the lysate solution. The column was washed three times with washing buffer (20 mM imidazole, 50 mM PBS, 0.3 M NaCl) to remove the proteins that were not bound to the resin. Finally, the target protein was eluted with elution buffer (250 mM imidazole, 50 mM PBS, 0.3 M NaCl); the column flow rate was ~1.0 ml/min and all of the obtained eluted fractions were collected for further analysis. Then the concentration of purified recombinant tropomyosin was determined using a bicinchoninic acid assay and further characterized by SDS-PAGE gel electrophoresis and mass spectrometry. The purity of the recombinant allergen was determined by ImageJ software version 1.8.0 (National Institutes of Health). SDS-PAGE was performed using the Bio-Rad Mini Protean II cell (Bio-Rad Laboratories, Inc.). 25 µg recombinant protein was boiled for 5 min in loading buffer before being separated using 12% polyacrylamide gels. Following the protein separation, the protein was visualized using Coomassie Brilliant Blue R-250 staining (Sigma-Aldrich; Merck KGaA).

Based on the results of the SDS-PAGE, the spot identified in the Coomassie G250-stained gel that matched the position of the theoretical molecular weight was excised and submitted for analysis by MS/MS at the proteomic service provided by Sangon Biotech Co., Ltd. Briefly, prior to MS/MS analysis, the purified recombinant fusion protein was digested in-gel into smaller peptides using trypsin (Beijing Solarbio Science & Technology Co., Ltd). A tandem matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF/TOF) mass spectrometer (Model 4800; Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for peptide sequencing and the acquired data was further analyzed using ABI GPS Explorer and MASCOT software (<http://www.matrixscience.com>). Databases, including the NCBI database (<http://www.ncbi.nlm.nih.gov>), were searched to characterize the protein.

Table I. Demographic and clinical characterization of subjects.

Patient characteristics	Shrimp allergic, n=35	HDM/cockroach allergic, n=29	Control, n=28
Sex, no. (%)			
Male	119 (54.3%)	12 (41.4%)	16 (57.1%)
Female	116 (45.7%)	17 (58.6%)	12 (42.9%)
Age, median with range (years)	26 (2-67)	17 (5-45)	32 (10-61)
sIgE, median with range (kU <sub>A</sub> /l)			
Shrimp	6.80 (0.36->100)	<0.35	<0.35
Dust mite	4.3 (<0.35->100)	10.21 (0.91->100)	<0.35
Cockroach	5.7 (<0.35->100)	2.64 (0.36-20.74)	<0.35
Allergic symptoms (most frequent only)			
Cutaneous	23 (65.7%)	2 (6.9%)	0
Digestive	16 (45.7%)	1 (3.4%)	0
Respiratory	7 (20.0%)	27 (93.1%)	0
Anaphylaxis	3 (8.6%)	0 (0%)	0
Other reported allergies	19 (54.3%)	12 (41.4%)	0

HDM, house dust mite; s, Pen 1-specific; Ig, immunoglobulin.

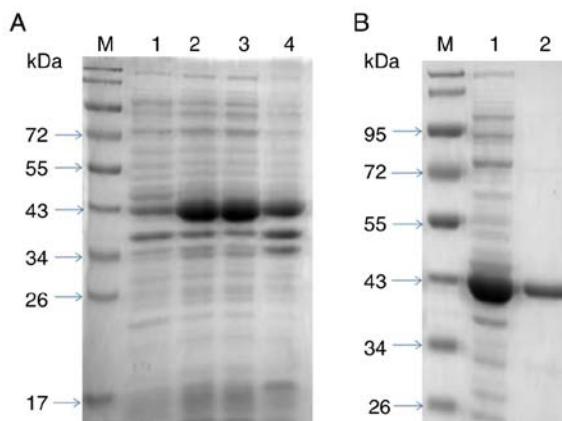


Figure 1. SDS-PAGE analysis of recombinantly expressed and purified tropomyosin rPen a 1. (A) M: Molecular weight marker; Lane 1: Total cell lysate from un-induced bacteria; Lane 2: Total cell lysate from induced bacteria; Lane 3: Lysate supernatant; Lane 4: Lysate pellet. (B) M: Molecular weight marker; Lane 1: Total cell lysate from induced bacteria; Lane 2: Purified recombinant tropomyosin rPen a 1.

**Preparation of biotinylated recombinant Pen a 1.** EZ-Link Sulfo-NHS-LC-LC-Biotin was purchased from Thermo Fisher Scientific, Inc. Recombinant Pen a 1 was biotinylated according to manufacturer's protocol. Briefly, the protein was dissolved in PBS. The appropriate volume of biotin reagent solution was added to the protein solution at a molar ratio of 20:1 and incubated on ice for 2 h. Calculations were performed based on the product instructions. To remove unreacted biotin, the solution was desalting or dialyzed against PBS buffer at 4°C. Finally, the conjugate was stored at 4°C until further use.

**Preparation of Pen a 1 peptides.** A total of 65 linear epitopes of *Penaeus aztecus* tropomyosin (Pen a 1) were found in the Immune Epitope Database (<http://www.iedb.org>). All 9 major epitopes of Pen a 1 as reported in the literature (21,22)

were chosen for the present study. A 'major epitope' of an allergen is defined as an IgE-Binding region recognized by >50% of allergic patients (21,32) whereas a 'minor epitope' was recognized by <50% of allergic patients.

The 9 peptides and their corresponding biotinylated peptides with a purity of >95% by high performance liquid chromatography, consisting of 15-27 amino acids in length and corresponding to the primary sequences of tropomyosin, were commercially synthesized (Sangon Biotech Co., Ltd.). Peptides were suspended in PBS at 1 mg/ml and stored at -70°C until use. The sequences of synthetic peptides are shown in Table SI.

**A light-initiated chemiluminescent assay.** The allergen specific IgE and IgG4 antibody levels are usually low in serum and not easy to detect by routine methods such as ELISA. Sandwich fluoro-immunochemical, lumino-immunochemical and radioimmunologic are common methods to increase the detection sensitivity (33).

The levels of sIgE and sIgG<sub>4</sub> to rPen a 1 as well as its peptides were measured using a light-initiated chemiluminescent assay (LICA) carried out as previously described (34,35). LICA is based on the formation of nanoparticle pairs and luminescence oxygen channeling immunoassay technology (36,37). Studies have shown that this new method possesses excellent performance characteristics with high sensitivity, broad analytical range and rapid turnaround cycles and is suitable for immunodetection of allergen sIgE and sIgG<sub>4</sub> (34,35). Two-step assay procedures were performed. Briefly, test serum samples (25 µl) diluted 1:20 in PBS containing 1% human serum albumin (Beijing Solarbio Science & Technology Co., Ltd.) were added to the 96-well plates. A 50 µl mix of anti-human IgE (cat. no. ab7382; Abcam) and/or IgG<sub>4</sub> antibody (cat. no. ab238320; Abcam)-chemibeads (Beyond Biotech, Co., Ltd.) (33,34), diluted 1:1,000, and biotinylated rPen a 1 and/or peptides, diluted 1:200 in PBS/HSA, was then added to each

Table II. Recombinant tropomyosin from *Penaeus aztecus*, rPen a 1 identified by Tandem mass spectrometry and Mascot database searches.

GenBank protein	Protein description	Theoretical molecular weight/D	Score	Matching peptide number
AAZ76743	<i>Penaeus monodon</i> , tropomyosin	32830	292	4
CDW59661	<i>Trichuris trichiura</i> , elongation factor tu	24465	171	2
KZC10872	<i>Dufourea novaeangliae</i> tropomyosin-1	35055	108	2

well and incubated at 37°C for 30 min with mild agitation. Subsequently, 150 µl streptavidin-coated sensibeads (Beyond Biotech, Co., Ltd.) were added. The reaction mixture was incubated at 37°C for 10 min in the dark. Finally, the chemiluminescence (CL) signal was measured on a LICA instrument (LICA series 500; Beijing Chemclin Biotech Co., Ltd.) and expressed as Relative Light Units. This immunoassay was run with samples in duplicate.

The healthy nonatopic sera were processed in parallel as negative controls. If the CL value of the tested sera was higher than the cut-off values, determined by adding two standard deviations to the mean CL value of the negative control, the reactivity was considered positive.

**Peptide inhibition experiment.** A peptide inhibition assay for IgE binding was performed as described above except that the serum pool from 3 patients was preincubated with several peptides unconjugated with biotin (the first, the third, the fifth, the seventh and the ninth peptide) at a concentration of 5 µg/ml at 37°C for 30 min with mild agitation. The same serum pool without peptides addition was analyzed in parallel and incubated under the same conditions as a control.

**Statistical analysis.** Mean values for each serologic parameter and log-transformed antibody levels (Igs binding to rPen a 1 and its peptides) were compared among patients and control subjects. Between the two groups, differences were compared with two-tailed Student's t-test for parametric data and/or with the Mann-Whitney U test for non-parametric data. The frequency of Igs responses to Pen a 1 and its peptides between patients and control subjects were compared using a  $\chi^2$  test. Spearman's correlation was used to evaluate the correlation. P<0.05 was considered to indicate a statistically significant difference. All statistical computations were performed using GraphPad Prism Version 7.0 (GraphPad Software, Inc.) and R Version 3.6.1 (R core Team; <https://cran.r-project.org>) software.

## Results

**Patient characteristics.** The demographic and clinical characteristics of the subjects are presented in Table I. According to their clinical reactivity, 35 individuals were diagnosed with a shrimp allergy based on a clear-cut clinical history of allergic reactions immediately following shrimp ingestion and positive shrimp-sIgE of >0.35 kU<sub>A</sub>/l. Furthermore, 29 subjects were allergic to HDM and/or cockroaches with a convincing history of a recent hypersensitivity reaction, including allergic rhinitis and/or asthma, to HDM and/or cockroaches and increased

serum sIgE antibodies. As control group, 28 nonatopic healthy subjects (no convincing history of clinical reaction and negative serum sIgE level) were also included for providing negative serum. None of the patients received immunotherapy. Glucocorticoid or antihistamine drug such as loratadine or were used to alleviate symptoms.

**Expression and purification of rPen a 1.** The recombinant shrimp tropomyosin, rPen a 1, was produced in *Escherichia coli* and purified as described above (Fig. 1, Tables II and SII). With respect to purified rPen a 1, a unique band with the expected MW (~37 kDa) was observed in SDS-PAGE analysis. The 95-99% purity was obtained by further analyzing SDS-PAGE results using ImageJ software.

**rPen a 1-sIgE and sIgG<sub>4</sub> responses.** The IgE and IgG<sub>4</sub> binding ability of rPen a 1 was evaluated by LICA. The comparison of IgE and IgG<sub>4</sub> binding between patients and control subjects is shown in Fig. 2A and B. The shrimp allergic and nonatopic groups demonstrated significant differences regarding rPen a 1-sIgE and sIgG<sub>4</sub> levels. rPen a 1-sIgE and sIgG<sub>4</sub> levels were significantly increased in shrimp allergic patients than in controls (P<0.0001 for each). As shown in Fig. 2C, 25 (71.4%) shrimp allergic patients were sensitized to rPen a 1 and 22 (62.9%) contained sIgG<sub>4</sub> to rPen a 1. In addition, sIgE to rPen a 1 was significantly correlated with sIgE to shrimp extract ( $r=0.668$ , P<0.0001) and sIgG<sub>4</sub> to rPen a 1 ( $r=0.484$ , P=0.003, Fig. 2D).

**Cross-reactivity study.** Cross-reactive responses to tropomyosin between crustaceans and HDM and/or cockroaches were frequently observed. However, in the present clinical and serological study, the HDM and/or cockroach allergic patients and controls did not show significant differences in rPen a 1 sIgE levels (P=0.196), which indicated that no IgE to rPen a 1 was detected among patients with HDM and/or cockroach allergy (Fig. 3).

**IgE and IgG<sub>4</sub> binding regions of rPen a 1.** Individual sera from 25 patients with positive rPen a 1 sIgE levels and 22 patients with positive sIgG<sub>4</sub> levels together with 4 healthy volunteers were used to identify IgE and IgG<sub>4</sub> binding areas on Pen a 1. The frequency of positive IgE and IgG<sub>4</sub> binding to each peptide of Pen a 1 is shown in Fig. 4. All synthetic peptides were recognized by between 28-48% of the sera tested for IgE binding. None of the peptides were major epitopes in this population and 9 peptides were identified as IgG<sub>4</sub> epitopes because the majority of patients (>50%) had positive IgG<sub>4</sub> antibodies to all synthetic peptides. The most frequent IgG<sub>4</sub>

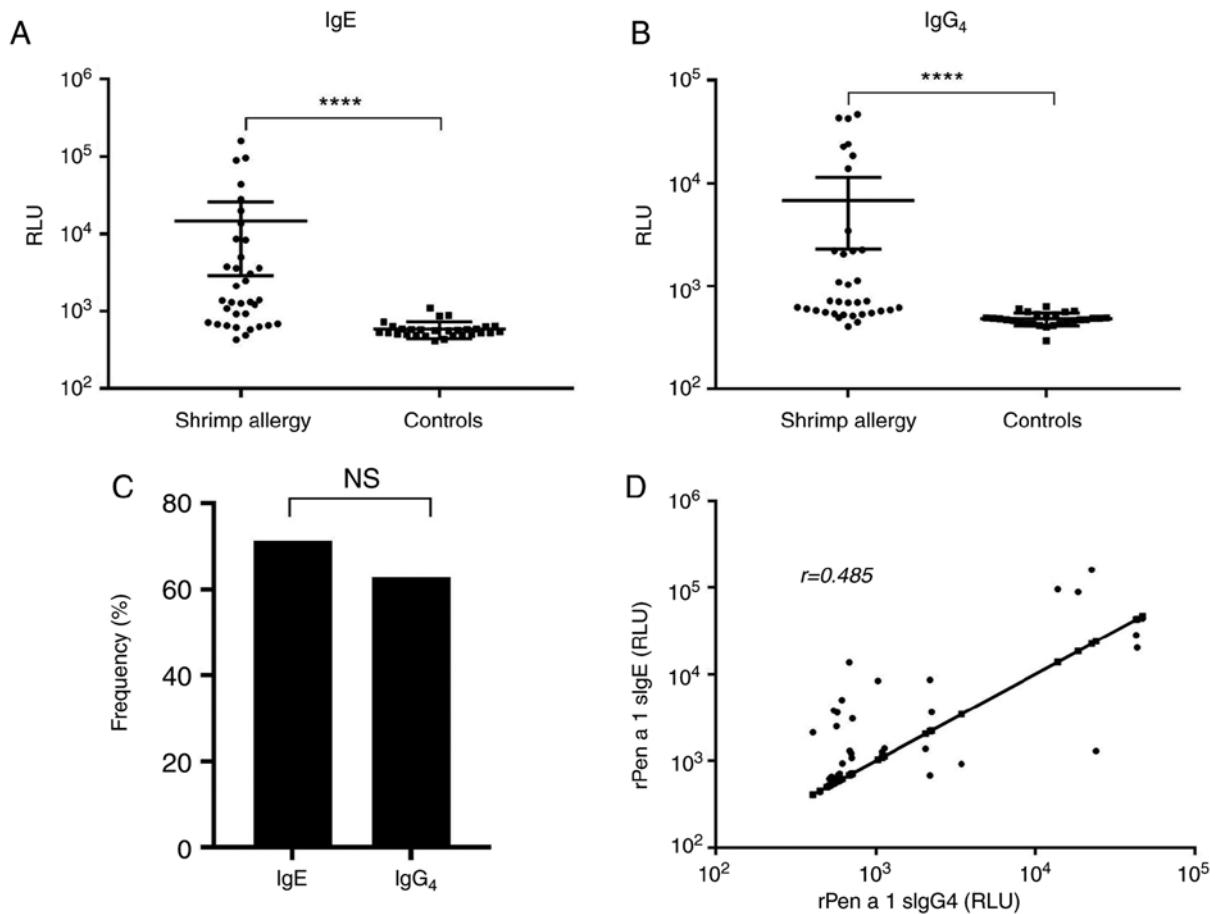


Figure 2. Serum sIgE and sIgG4 responses to rPen a 1. rPen a 1 (A) sIgE and (B) IgG<sub>4</sub> levels in shrimp allergic (n=35) and control (n=28) groups. (C) Proportions of shrimp allergic patients (n=35) with reactive sIgE and IgG<sub>4</sub> antibodies against rPen a 1. (D) Correlation between serum levels of rPen a 1 sIgE and rPen a 1 slgG4. \*\*\*P<0.0001. Measurements are shown on a log<sub>10</sub> scale. s, Pen 1-specific; Ig, immunoglobulin; NS, not significant; RLU, relative light units.

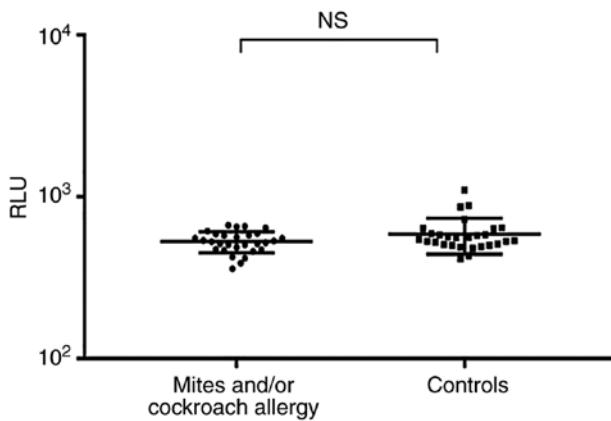


Figure 3. Comparison of serum sIgE against rPen a 1 between house dust mite and/or cockroach allergic (n=29) and nonatopic (n=28) groups. Levels of IgE binding to Pen a 1 were normalized by <sup>10</sup>log-transformation. s, Pen 1-specific; Ig, immunoglobulin; NS, not significant.

recognized antigenic areas were the second and the eighth peptides (AA 85-105 and AA 259-273), both of which were recognized by 90.9% of the patients. In addition, IgE and IgG<sub>4</sub> binding regions of Pen a 1 largely overlapped.

**Epitope profiles of individual patients.** As demonstrated in Fig. 5, the number of IgE and IgG<sub>4</sub> positive peptides per

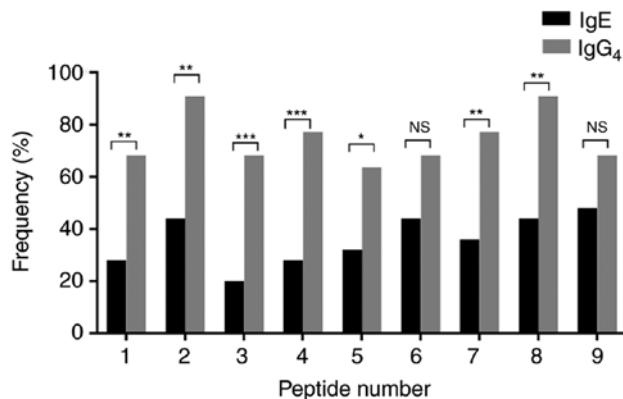


Figure 4. Frequency of IgE and IgG<sub>4</sub> recognition against each peptide. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001. s, Pen 1-specific; Ig, immunoglobulin; NS, not significant.

subject and recognition patterns varied considerably among 25 shrimp-allergic, rPen a 1-reactive individuals. A total of 28% (7/25) of the sera demonstrated no peptide sIgE binding reactivity, while 16% (4/25) sera exhibited positive IgE binding to all peptides. The mean number of IgE binding peptides recognized per individual was 3.2 (range, 0-9). Serum sIgE levels to rPen a 1 was positively correlated with the number of IgE positive peptides ( $r=0.680$ ,  $P<0.001$ ). The number of sera which recognized IgG<sub>4</sub>

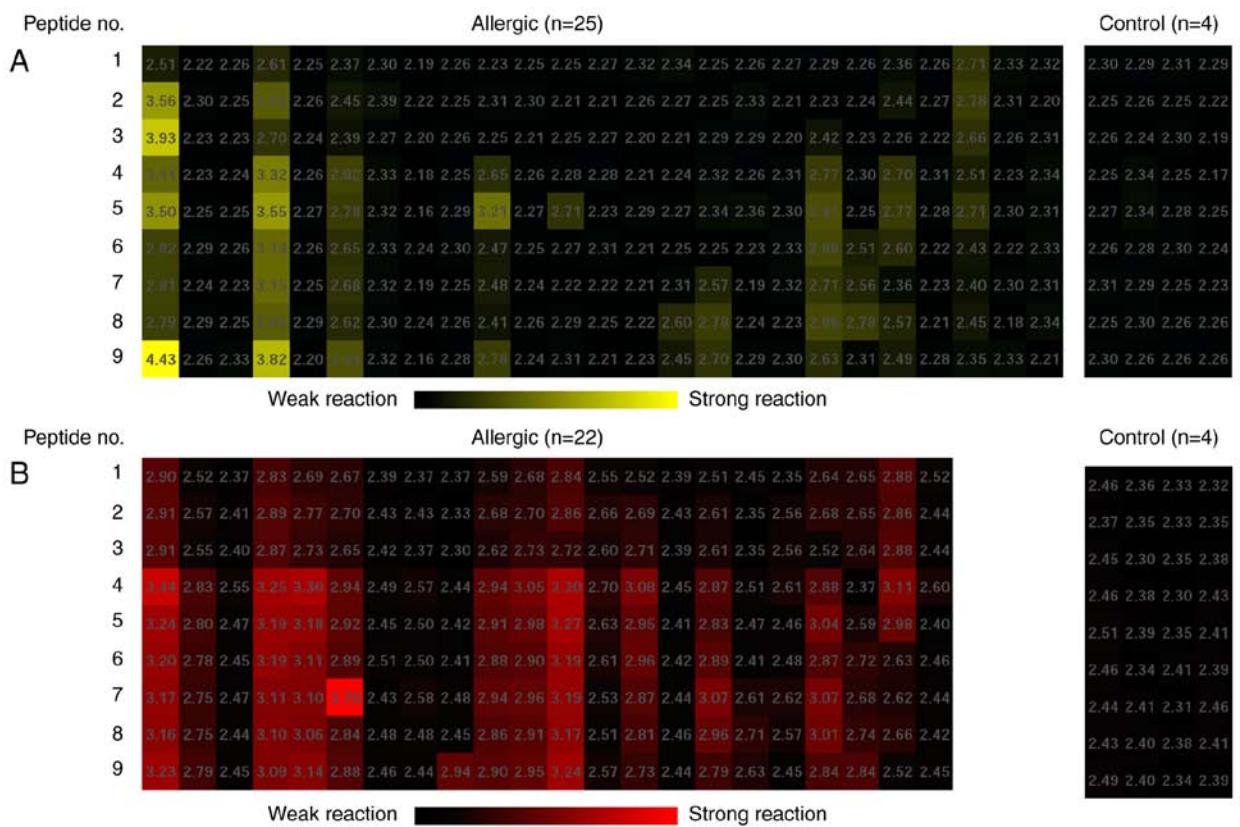


Figure 5. IgE and IgG<sub>4</sub> binding to peptides corresponding to a linear sequence of Pen a 1. (A) IgE binding to peptides of Pen a 1 and (B) IgG<sub>4</sub> binding to peptides of Pen a 1. Peptides are in rows and individual sera of shrimp allergic patients and control subjects are in columns. Data are present as logarithmic values by using R software with pheatmap package. s, Pen 1-specific; Ig, immunoglobulin.

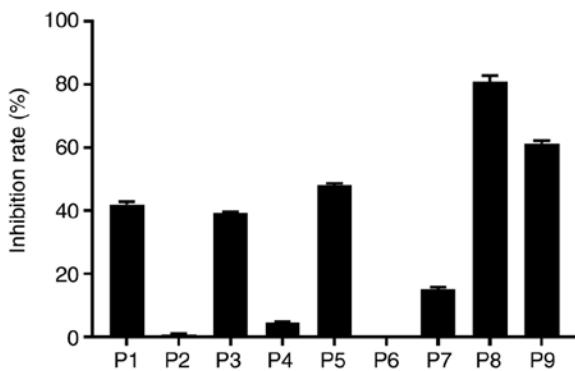


Figure 6. Validation of allergenic epitope peptides by inhibition assay. P1-P8: peptide number. All data were presented as the mean  $\pm$  standard deviation (n=2).

binding peptides varied from 0 (0%) for 2 peptides to 8 (67%) for 9 peptides.

The specificity of IgE binding was assessed by a peptide inhibition assay. As shown in Fig. 6, peptides preincubated with the serum pool partially inhibited the IgE binding to the same peptide or neighboring peptide, indicating that the detected binding was due to epitope-sIgE antibodies.

## Discussion

The present study analyzed profiles of IgE and IgG<sub>4</sub> against shrimp tropomyosin, Pen a 1 and its epitopes among patients

from coastal areas of northern China using LICA. It was found that Pen a 1 is a major allergen recognized by the majority of shrimp allergic patients. The data from the present study demonstrated that 71.4% of shrimp-allergic patients in this population exhibited IgE reactivity to shrimp tropomyosin. The current study demonstrated that Pen a 1 was the major shrimp allergen, which was consistent with previous studies (6,7) and confirmed its clinical diagnostic value in patients from coastal areas of northern China. However, none of the 9 epitopes are major (reaction frequency >50%) IgE-binding regions, indicating that the epitope profile may be different for other regions.

In particular, 62.9% of allergic sera had increased IgG<sub>4</sub> antibodies to Pen a 1 compared with the non-atopic healthy group. A strong IgG<sub>4</sub> response accompanied the presence of IgE to Pen a 1. This was consistent with previous studies which revealed that levels of sIgG and IgG subclasses were significantly higher in individuals with allergic sensitization and could develop in parallel with or invariably preceding IgE responses (38,39). However, the role played by allergen sIgG and sIgG<sub>4</sub> antibodies in allergic reactions, unlike that of IgE, remains controversial. On one hand, allergen-related IgG<sub>4</sub> antibodies have been proposed to be associated with the development of clinical tolerance due to their induction by allergen-specific immunotherapy (40,41). On the other hand, a previous study reported peanut-specific IgG<sub>4</sub> is significantly higher in the avoidance group compared with the peanut-eating group in peanut-sensitized children and supposes that IgG<sub>4</sub> may not indicate tolerance from sensitization (38). There is also a suggestion that allergen-related IgG antibodies are an epiphenomenon with no functional relevance (42). From

the point of view of the present study, it is the difference between natural- and therapy-induced tolerance that causes these contradictions. In therapy-induced tolerance, allergen-specific IgG<sub>4</sub> should be considered as a protective factor rather than the cause of sensitization (28). In natural tolerance, there may be two mechanisms: One is IgG<sub>4</sub>-associated tolerance (43) and the other is IgG<sub>4</sub>-independent tolerance (38).

In the present study, the IgE reactivity to rPen a 1 was not observed in HDM and/or cockroach allergic patients with no sensitization to shrimp. This meant tropomyosin did not appear to be the markers of cross-reactivity with other arthropods in this population, which appears to conflict with previous studies (8,10,11,44). One possible explanation could be the difference between the recombinant allergens and their native counterparts due to lack of posttranscriptional modifications (27). Conformational epitopes play an important role in allergenicity, particularly for globular inhaled allergens (45,46). However, this was unconvincing as Reese *et al* (6) found the recombinant tropomyosin (Pen a 1) behaved similarly to its native counterpart. Another possibility could be that tropomyosin was a minor allergen in HDM and/or cockroaches since Hu *et al* (47) reported the frequency of Der p 10 was only 10% in mite allergic patients in China, so tropomyosin may not be the major cause of cross-reactivity between shrimp and other arthropods. Similarly, Pascal *et al* (48) recently described that individuals allergic to HDM and/or cockroaches, with no sensitization to shrimp, recognized arginine kinase and hemocyanin. Immunoblotting using HDM extract should be included in future studies to confirm a presence of sIgE against HDM tropomyosin in HDM and/or cockroach allergic patients. Despite the above, the detection of specific IgE to allergenic components could provide higher specificity for allergy diagnosis (48); accurate allergy diagnosis without oral food challenge test remains a challenge (31,49) because it is heavily influenced by cross-reaction. When considering cross-reactions, it is important to distinguish whether a specific allergen is the cause of allergic symptoms (50). For instance, tropomyosin has been proved to be non-allergenic in the majority of vertebrate foods although ~1/2 of the amino acid sequences between invertebrate and vertebrate tropomyosin are similar (3). That is, the biologic activity of cross-reactive allergens is often relatively low and analysis of recognition profiles of specific epitopes on allergen could better diagnose allergies (32).

The peptides of Pen a 1 were further evaluated by LICA to identify IgE and IgG<sub>4</sub> binding regions. It was worth noting the following points: First, the IgE and IgG<sub>4</sub> epitope mapping of Pen a 1 are fundamental for designing safe hypoallergenic agents for the treatment of shrimp allergy. Effective immunotherapy for food allergy with IgE binding epitope modified hypoallergens has been reported (16,51): 28% (7/25) of patients were sensitized to tropomyosin but did not recognize any major epitope of Pen a 1. In addition, none of the 9 peptides were the major epitopes recognized by the north Chinese population. This indicated that there may be geographical or ethnic differences with regard to the epitopes profiles of tropomyosin. The major epitope of a certain allergen in one population may be a minor epitope in another population. This means an immunotherapy product designed for one region may have no effect in another. Individual allergen specific immunotherapy should be

recommended as a plan. Secondly, it was found that the number of peptides recognized by IgE was positively correlated to level of allergen sIgE ( $r=0.680$ ,  $P<0.001$ ), which was similar to the result of a previous study (25). This indicated that the diversity of IgE binding epitopes is positively correlated with rPen a 1 IgE levels. However, according to the present study, there was no association between the number of IgE epitopes of Pen a 1 and shrimp-allergic symptoms. Other studies have demonstrated that identification of allergen epitopes is of great value in the diagnosis of food allergies (52-55). IgE-recognizing certain sequential immunodominant regions as well as broad IgE epitope diversity correlate with the severity of an allergic reaction (48,56). One possible explanation as to why the present study arrived at a different conclusion could be that the number of tropomyosin peptides synthesized was insufficient and the epitopes of other major allergens in shrimp were not tested. Another explanation could be that IgG<sub>4</sub>-related tolerance may alleviate the symptoms. On the last point, the epitope recognition regions of IgE and IgG<sub>4</sub> largely overlapped, which was in agreement with previous studies (48,57). Coincident shared IgE and IgG<sub>4</sub> binding epitopes could be critical for the development of tolerance by blocking of the sIgE antibodies to the same allergen by sIgG<sub>4</sub> antibodies (58).

There were some limitations to the present study. For financial reasons, not all the 65 epitopes of Pen a 1 were analyzed. Although double blind, placebo-controlled food challenge is considered the gold standard for diagnosis of food allergy, the present study did not conduct provocative studies because it is an *in vivo* test involving a relatively complex procedure (59).

In conclusion, the present study used, for the first time to the best of our knowledge, LICA technology to determine the IgE and IgG<sub>4</sub> responses to Pen a 1 and its peptides among shrimp allergic patients from coastal areas of northern China. It was identified that 71.4% of patients with shrimp allergy were sensitized to Pen a 1 and 62.9% had increased IgG<sub>4</sub> antibodies to Pen a 1. Pen a 1 might not be responsible for cross-reactivity between shrimp and other arthropods. The results of the present study indicated that a strong IgG<sub>4</sub> response accompanied the presence of IgE to Pen a 1. It was also found that there may be geographical or ethnic differences with respect to the IgE and IgG<sub>4</sub> recognition patterns of the Pen a 1 peptides. The present study suggested that understanding the molecular basis of clinical reactivity will be useful for the accurate diagnose of shrimp allergy and to further implement better immunotherapy.

## Acknowledgements

Not applicable.

## Funding

The present study was funded by the National Natural Science Foundation of China (grant no. 81772259).

## Availability of data and materials

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

## Authors' contributions

JL participated in all stages of the study, including preparing and performing the experiments, creating the figures and analyzing the data. ZL performed the recombinant protein expression and LICA analysis. YY performed the cross-reactive experiments, participated in the drafting of the manuscript and made critical revisions. DK and SL collected serum and patient information and performed the peptide inhibition assay. HL conceived and designed the study. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The study protocol was approved by the Ethics Committees of Tianjin Medical University (approval no. TMUHMEC2017008) and written informed consent was obtained from the patients and volunteers prior to study entry.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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