

Original Article

Characterization and analysis of a cDNA coding for the group 29b (Der f 29b) allergen of *Dermatophagoides farinae*

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Received August 17, 2015; Accepted December 22, 2015; Epub February 15, 2016; Published February 29, 2016

Abstract: This study aims to acquire a recombinant allergen of Der f 29b by cloning and expression, and to identify its immunogenicity. In this study, the total RNA of *D. farinae* was extracted, cloned and expressed based on the Der f 29b gene. The molecular characteristics of Der f 29b was analyzed by the procedures of Bioinformatics. The allergenicity of recombinant Der f 29b protein was examined by western-blotting, ELISA, Immune inhibitory assays and skin prick test. The gene of Der f 29b consisted of 495 bases, derived from its nucleic acid sequence and encoded 164 amino acids. Positive responses to r-Der f 29b were shown in 24.3% by means of skin prick testing with 37 DM-allergic patients. The immunoblotting assays demonstrated that serum IgE from allergic patients reacted to r-Der f 29b protein. The IgE reactivity of r-Der f 29b in the serum from r-Der f 29b allergic patients was increased by more than 2 folds compared with healthy subjects. Immune inhibition assays showed that the IgE cross-reactivity was between r-Der f 29b and DME. Bioinformatics analysis predicted four peptides (13-17, 67-71, 104-109 and 147-155) as the B cell epitopes and five peptides (5-14, 16-31, 35-43, 52-63 and 87-97) as the T cell epitopes. Secondary structure prediction of Der f 29b with software PSIPRED identified two α -helices and seven β -sheets in Der f 29b. In conclusion, Derf 29b protein was identified as a novel subtype of dust mite allergen.

Keyword: *Dermatophagoides farinae*, Der f 29b, western-blotting, ELISA, skin prick test

Introduction

Allergic diseases affect 30% to 40% of the population, the prevalence of allergic diseases is increasing rapidly in the recent decades, and have become a global health problem [1, 2]. Allergens from the house dust mites are one of the major environmental factors inducing allergic diseases, such as asthma, rhinitis, and atopic dermatitis [3, 4]. It is estimated that 20% of the population in the world and more than half patients with allergic diseases are allergic to dust mites [5, 6]. Skin prick test has demonstrated that there are 59.0% and 57.6% allergic patients sensitive to *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* respectively [7].

Currently, desensitization treatment with the crude allergen extracts is one of the major remedies used for the dust mite allergic disease.

However crude extracts of house dust mites not only contain allergens, but also contain inflammatory molecules, such as ceramides, kallikreins and the ever popular endotoxin [8]. Therefore, the recombinant allergens are regarded as a substitute for crude mite extracts used in the clinical immunotherapy, which may improve the efficacy and safety of the house dust-mite-specific immunotherapy.

Two groups of mite allergens (group 1 and 2) have been proven as the major allergens in *Dermatophagoides farinae*, including a cysteine protease and a epididymal protein, respectively. More than 80% of humans with house dust mite allergy have high serum levels of IgE antibody to the group 1 and more than 90% to the group 2 [9-12]. It is estimated that there are at least 30 different allergens in the extracts of *D. farinae*, which have high IgE binding activity, but these allergens usually present at low and vari-

Der f 29b analysis

able concentrations in mite extracts, less than 1% of the group 1 and 2 allergens [7, 13].

In this study, we cloned, expressed and purified the Peptidyl-prolyl cis-trans isomerase (r-Der f 29b) from HDM, and evaluated the allergenicity of r-Der f 29b.

Materials and methods

Material

Main reagents Total RNA extraction kit and reverse transcription kit were purchased from QIAGEN. Prokaryotic expression vector pET-28a(+) were obtained from Novagen. pMD-19 T vector, Taq DNA polymerase, restriction endonuclease EcoRI and XhoI. Plasmid DNA extraction kit and Agarose gel DNA recovery kit were purchased from Omega. Ni Sepharose and pillar were purchased from GE. The sera of patients with allergic disorders were obtained from the First Affiliated Hospital of Guangzhou Medical University, Escherichia coli Top10 and Escherichia coli BL21 were recovered from the deposits of our laboratory.

Der f 29b gene synthesis and plasmid construction

Der f 29b gene sequences were obtained through analyzing the full genome sequence of dust mite. To generate a His-tagged Der f 29b expression plasmid, the PCR products of Der f 29b cDNA was obtained from pMD19-T -Der f 29b cloned by Shenzhen Huada Gene Research Institute, and cloned into the EcoRI and XhoI sites of pET-28a(+), and the recombinant plasmid was verified by DNA sequencing. The cloned amino acid sequence of Der f 29b was comparable with the relevant homologous sequence from Genbank.

Expression and purification of recombinant protein Der f 29b

pET-28a (+)-Der f 29b were transformed into E. coli BL21 which by the calcium chloride characteristic. The transformed bacteria were grown on LB plates containing kanamycin (100 µg/ml) at 37°C overnight. The single colony was inoculated into 5 mL LB containing kanamycin and cultured at 37°C overnight. Then 1 mL bacteria was taken into fresh LB medium and grown to an OD600 of 0.6 to 0.8. The expression of the

recombinant protein was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 4 h incubation, E. coli cells were harvested by centrifugation at 10,000 rpm for 15 min, and resuspended in 50 mM Tris-HCl, 100 mM NaCl, pH 7.5. The cells were lysed by sonication at an amplitude of 38% for 5 min (1 s pulse on and 1 s spulse off) followed by centrifugation at 10,000 rev/min at 4°C for 20 min. Finally, the recombinant protein was purified by affinity chromatography using the Ni⁺ as solid phase. After vigorous washing with washing buffer (50 mM Tris, 40 mM imidazole and 0.5 M NaCl, pH 8), the protein was eluted slowly using elution buffer (50 mM Tris, 0.3 M imidazole and 0.2 M NaCl, pH 8).

Skin prick test of restructured dust mites Der f 29b

SPTs with purified and endotoxin removed r-Der f 29b was dissolved in PH 7.4 phosphate buffer. Glycerin was added to 50% final concentration. The Sample concentration was 0.01 mg/ml controlled with both histamine phosphate (0.1%; positive control) and saline (negative control). The SPT results were checked 20 minutes after. The judgement of the result: if the prick spot became a wheal and fleck surrounding the wheal, it was positive (+). 4+: the response was stronger than histamine control; 3+: the response was almost the same as histamine control; 2+: the response was weaker than histamine, but stronger than negative control; 1+: the response was significantly weaker than histamine, but slightly stronger than the negative control; negative: No response.

Approval to conduct these studies was obtained from the ethics committee at Guangzhou Medical University. A written and informed consent was obtained from each human subject.

Immunoblotting analysis with sera from allergic patients to dust mites

The r-Der f 29b protein were prepared with PBS. The proteins were fractioned by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and electro-transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) for immunoblotting analysis. The membrane was blocked for 2 hours with 3% BSA (Bovine Serum Albumin) at room

Der f 29b analysis

temperature, incubated with the sera from patients sensitized to dust mite (diluted in 5% BSA-PBST; PBST: PBS containing 0.05% Tween 20) for 1 h under constant agitation on a rotary shaker at room temperature. After washing three times with TBS-T (Tris-buffered saline containing triton X-100), the membrane was incubated with the secondary antibodies (mouse anti human IgE) for 60 minutes. In the end, after washing with TBS-T for three times, the membrane was developed with DAB kit (Invitrogen, USA). Stop developing reaction with ddH₂O.

Enzyme-linked immunosorbent assay (ELISA)

Sera IgE antibodies specific for purified allergens were measured by indirect ELISA. Briefly, the ELISA microtiter plates were coated overnight at 4°C with 100 µl of r-Der f 29b at a concentration of 1 µg/mL in Carbonate buffered solution (CBS pH9.5). Then blocked at 37°C with 200 µl 3% bovine serum albumin (BSA) in PBS for 120 min. The serum (diluted for 5 times, used as primary antibodies) or BSA (using as a negative control) was added to each well (100 µL/well) and incubated for 60 min at room temperature. After IgE binding, the plates were incubated with peroxidase-labeled goat anti-human IgE (1:2000) for 60 min at 37°C. Each incubating step was followed by 3 washes with PBST. The color was developed by adding tetramethylbenzidine (TMB; 100 µL/well) and stopped by the addition of 2M H₂SO₄ (50 µL/well). The absorbances at 450 nm were then measured on a microplate reader, and the data were analyzed.

Der f 29b and crude extract inhibition assays

For the inhibition assay, serum allergic to r-Der f 29b were used for inhibition experiment to detect the cross reaction among r-Der f 29b and dust mites crude extract. Patients sera (diluted 1:50 in 2% BSA, 0.05% Tween 20 in PBS) were pre-incubated with purified r-Der f 29b or DME (final concentration: 0.0001, 0.001, 0.01, 0.1, 1 g/ml) at 4°C overnight; and then were added to the microtiter plates pre-coated with r-Der f 29b or DME (0.1 µg/well). Data from 3 measurements were accumulated. The inhibition rates were calculated according to the following formula: inhibition (%)=(OD₀-OD_{inhibitor})/(OD₀-OD_{BSA}), where OD₀ is the optical density of antigen without any inhibitor, OD_{inhibitor} is the optical density after adding

an inhibitor and OD_{BSA} is the optical density with only BSA in the plate.

Bioinformatics analyses of the cloned Der f 29b

Three immunoinformatics tools including bioinformatics predicted antigenic peptides (BPAP) system, DNASTar protean system and Bepi-Pred 1.0 server were used to predicate the B cell epitopes of Der f 29b. The ultimate consensus epitope results were obtained by combining the results of the three tools together with the method published earlier [14]. The Bepi-Pred 1.0 server and BPAP system and just need the amino acid sequence and provide straightforward results which are combined with physicochemical properties of amino acids such as hydrophilicity, accessibility, flexibility, exposed surface and turns [15]. In the DNASTar protean system, four properties (antigenicity, hydrophilicity, accessibility and flexibility) of the amino acid sequence were chosen as parameters for epitopes prediction.

T cell epitopes are principally predicted indirectly by identifying the binding of peptide fragments to the MHC molecules. In this study, human asthma related allelic genes HLA-DRB1*0301 and HLA-DRB1*0401 were used to predict HLA-DR-based T cell epitope prediction. Five immunoinformatics tools including Immune Epitope Database (IEDB), ProPre, SYFPEITHI, Net-MHCII 2.2 Server and NetMHCII pan 3.0 Server were used to predicate the T cell epitopes of Der f 29b.

Der f 29b secondary structural elements were predicted by PSIPRED, and identified with the result obtained with DNASTar and NetSurfP ver 1.1. Phosphorylation site analysis by NetPhos 2.0.

Statistics

All data were expressed as mean ± standard deviation and processed with SPSS statistical software. T-test was used for the mean differences between two groups. *p* value < 0.05 was considered as significant.

Results

Cloning and sequence alignment of the dust mite Der f 29b gene

The amplification products showed a bright band at around 500 bp by agarose gel electro-

Der f 29b analysis

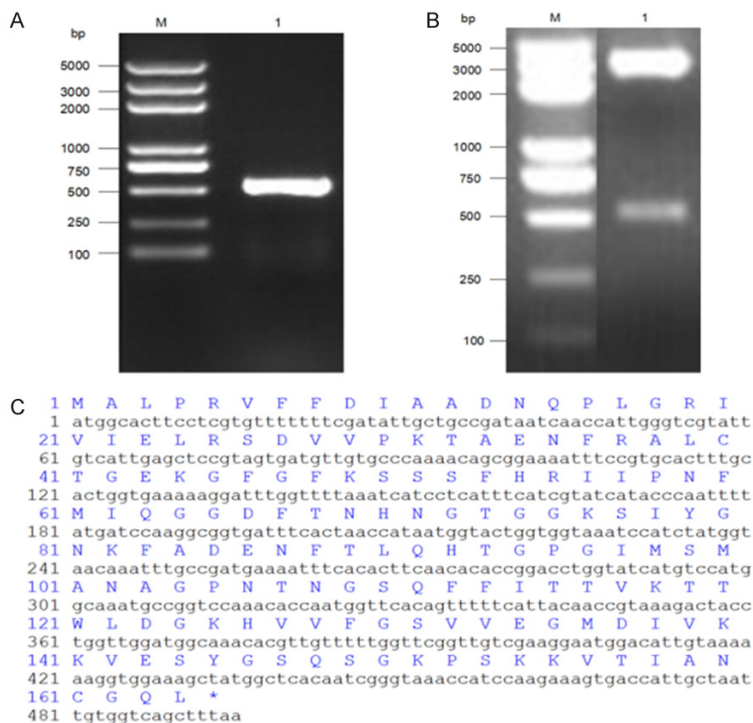


Figure 1. Cloning and sequence alignment of the Der f 29b gene. A. Cloning and PCR of Der f 29b cDNA (1: DNA Marker; 2: Der f 29b gene); B. Restriction enzymatic digestion results of recombinant Hyastatin-pET-28a(+) plasmid (1: DNA Marker; 2: Der f 29b DNA); C. The amino acid sequences of Der f 29b.

phoresis (**Figure 1A**) and double enzymatic digestion (**Figure 1B**). The cloned dust mite Der f 29b genes consist of 495 bases (including termination codon), derived from its nucleic acid sequence that encoded 164 amino acids (**Figure 1C**).

The molecular evolutionary tree was constructed by software MEGA5. 1. The results showed that *D. farina* Peptidyl-prolyl cis-trans isomerase has a close relationship with *Maconeilicoccus hirsutus*, *Locusta migratoria* and *Tribolium castaneum* Peptidyl-prolyl cis-trans isomerase (**Figure 2A**). Compared the cloned Der f 29b amino acid sequence with other species, we found its homology with *Maconeilicoccus hirsutus* peptidyl-prolyl cis-trans isomerase (NCBI accession number ABM55516.1) was 85%, homology with *Locusta migratoria* peptidyl-prolyl isomerase-1 (NCBI accession number AGC84404.1) was 84%. Homology with *Tribolium castaneum* hypothetical protein TcasGA2_TC004820 (NCBI accession number EEZ98965.1) was 84% (**Figure 2B**).

The expression and purification of recombinant Der f 29b gene

To obtain r-Der f 29b protein for the subsequent experiments, we cloned Der f 29b cDNA into pET-28a(+), expressed and purified r-Der f 29b. After the His-tag Der f 29b were successfully expressed by induction with IPTG, the high purity of r-Der f 29b was obtained after purification by Ni²⁺ affinity chromatography and molecular sieve chromatography (**Figure 3A**).

Immunoreactivity to IgE

To determine the allergenicity of Der f 29b protein, immunoblotting was performed using serum from five DME-allergic patients. The results showed that serum IgE from allergic patients reacted to r-Der f 29b protein. IgE-binding bands with molecular weight around 18 kDa (r-Der f

29b) were positive against the serum from allergic patients, but completely negative in healthy volunteers (**Figure 3B**). The specific immunoreactivity of IgE against purified r-Der f 29b was further confirmed using ELISA. Compared with the serum from healthy subjects, the IgE reactivity of r-Der f 29b from allergic patients was increased by more than 2 folds (**Figure 4A**). For ELISA inhibition assay, Serum allergic to r-Der f 29b was chosen. Different concentrations of r-Der f 29b or DME were incubated with the serum. They inhibited the patients' serum IgE binding to the coated DME or r-Der f 29b in a dose-dependent manner. At the concentration of r-Der f 29b was 0.1 mg/ml, the inhibition rate of r-Der f 29b against DME was about 65% (**Figure 4B**).

These results suggested that Der f 29b may be allergens from dust mite allergen family that leading to type I hypersensitivity.

Skin prick testing

The allergic activity of Der f 29b was evaluated by SPT. The results (**Table 1**) showed that 9

Der f 29b analysis

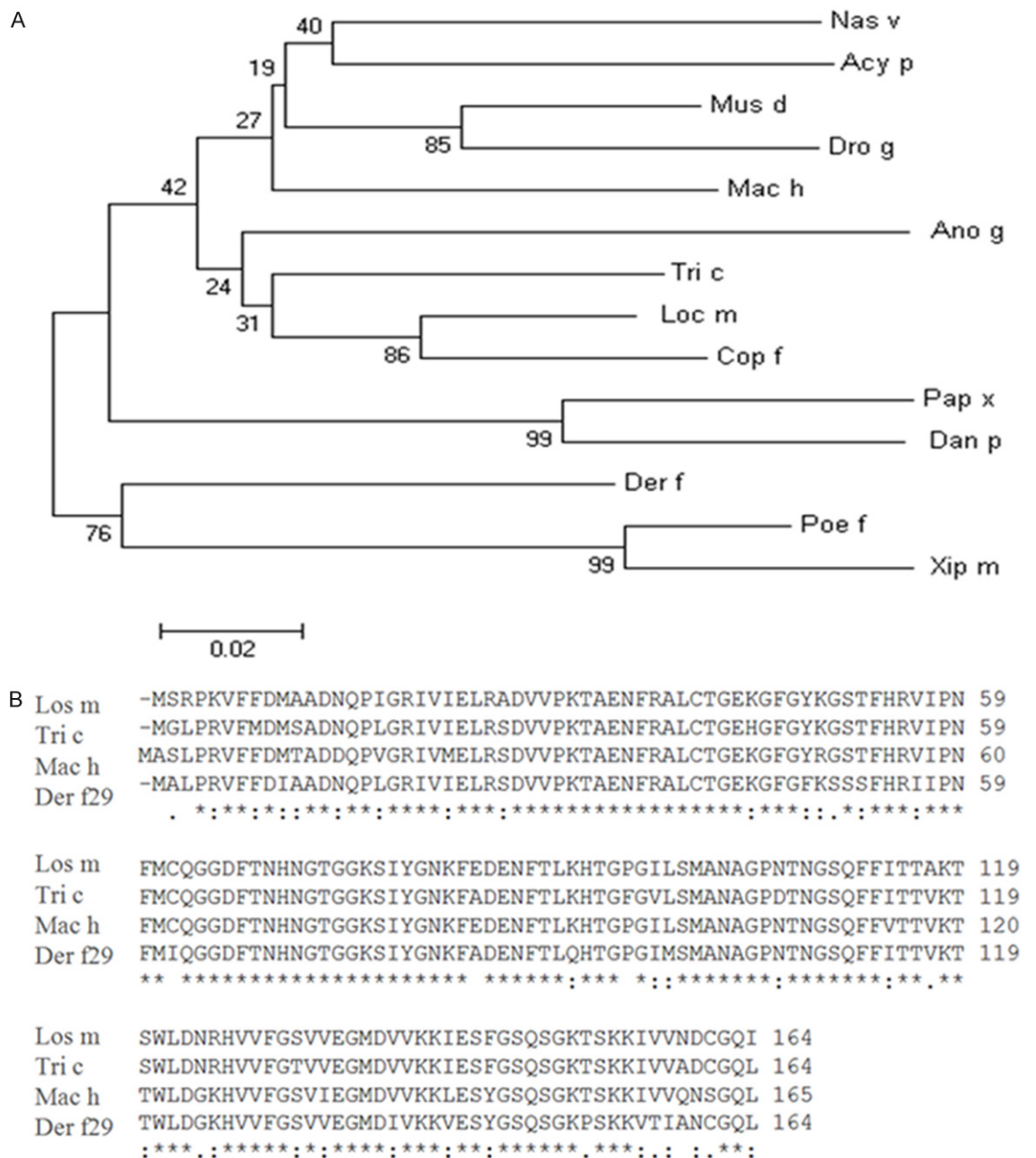


Figure 2. A. The phylogenetic tree analysis; Der f (*Dermatophagoides farinae*); Mac h (*Maconellicoccus hirsutus*); Loc m (*Locusta migratoria*); Tri c (*Tribolium castaneum*); Nas v (*Nasonia vitripennis*); Mus d (*Musca domestica*); Pap x (*Papilio xuthus*); Poe f (*Poecilia formosa*); Acy p (*Acyrtosiphon pisum*); Dro g (*Drosophila grimshawi*); Xip m (*Xiphophorus maculatus*); Ano g (*Anopheles gambiae* str. PEST); Cop f (*Coptotermes formosanus*); Dan p (*Danaus plexippus*). B. Alignment of Der f 29b from different sources.

(24.3%) out of 37 allergic patients showed positive reaction to r-Der f 29b (Table 1).

Structural and functional prediction

Based on these sequence properties, the final predicting functional regions of DNASTar were

13-17, 50-53, 67-71, 78-84, 104-109, 141-143, 147-155. Also, the predicted results of the BPAP system were 16-30, 48-56, 110-116, 123-135, 138-144, 152-159 and Bepi-Pred 1.0 server were 12-17, 28-35, 45-48, 66-79, 83-85, 89-97, 102-109, 143-156. Furthermore, the final potential B cell epitopes

Der f 29b analysis

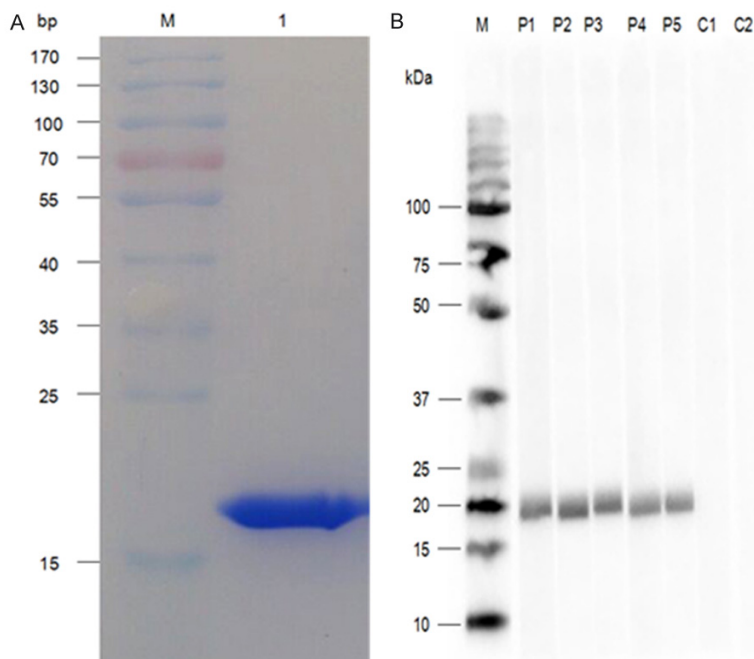


Figure 3. SDS-PAGE and Western blot analysis of recombinant protein Der f 29b. A. The protein band indicates the purified Dermatophagoides farinae Der f 29b, M: protein ruler (kDa); 1: The recombinant der f 29b purified by affinity chromatography. B. The Western blots indicate that the r-Der f 29b binds to IgE in the sera from patients allergic to Dermatophagoides farinae, Lanes P1-P3: serum from allergic subjects. Lane C1 and C2: serum from healthy subjects as negative control.

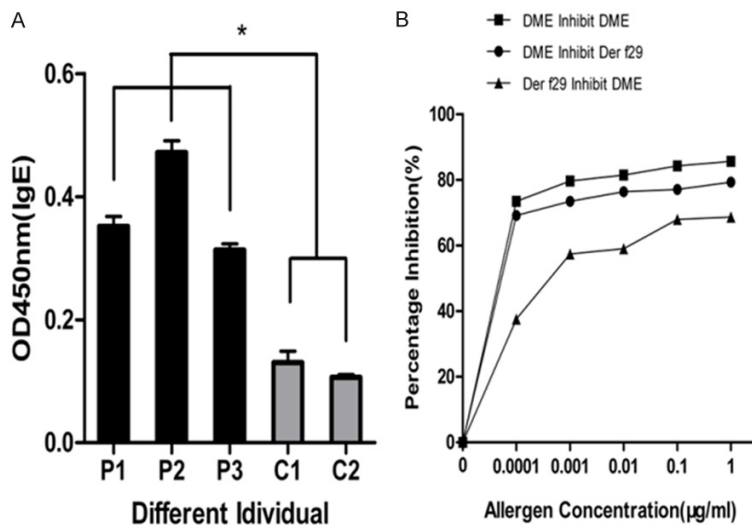


Figure 4. Enzyme-linked Immunosorbent Assay (ELISA) and ELISA Inhibition. A. The specific IgE reactivity to allergen r-Der f 29b by ELISA. P1-P3, the serum from r-Der f 29b positive patients; C1 and C2, the serum from healthy subjects. B. IgE binding inhibition to extracts or r-Der f 29b. Serum allergic to r-Der f 29b were previously incubated with inhibitors of different concentrations and then reacted with coated allergen. Inhibitors were used at the concentrations of 0.01, 0.1, 1, 10, 100 and 1000 µg/ml.

of Der f 29b were selected based on the results of these three tools. The ultimate results of the three immunoinformatics tools finally predicted four peptides (13-17, 67-71, 104-109, 147-155) and these peptides were shown in **Figure 5**.

Immune Epitope Database (IEDB), Propred, SYFPEITHI, NetMHCII 2.2 Server and NetMHCII pan 3.0 Server were used to predicate the T cell epitopes of Der f 29b. The final potential T cell epitopes of Der f 29b were selected based on the results of these five tools. The ultimate results of the five immunoinformatics tools finally predicted five peptides (5-14, 16-31, 35-43, 52-63 and 87-97) (**Table 2** and **Figure 5**).

Secondary structure prediction of Der f 29b with PSIPRED identified two α -helices and seven β -sheets (**Figure 5**) in Der f 29b. Alternatively, Net-SurfP v1.1 predicted two α -helices and eight β -sheets. DNASTAR predicted one α -helices and five β -sheets. These results were predicted by different servers and have subtle distinction. Phosphorylation sites including two Ser (147 and 153) residues were predicted and showed in **Figure 5**.

Discussion

House dust mites are important indoor allergens, eliciting allergic diseases such as asthma, rhinitis, and atopic dermatitis and other allergic diseases [16]. In China, house dust mites were the most prevalent allergens in patients with asthma and/or rhinitis [17]. Application of molecular

Der f 29b analysis

Table 1. SPT results

Subject	Gender/ Age	Diagnosis	Net wheal size (mm), Level			
			DME	Histamine	PS	r-Der f 29b
1	Male/11	BA, AR	9, +++	6	0	0, -
2	Female/50	AR	2.25, +	5	0	0, -
3	Female/25	AR	6.75, +++	3.75	0	0
4	Male/16	AR	2, +	6.5	0	2, +
5	Male/43	BA, AR	6, ++	6	0	0, -
6	Male/26	AR	5, ++	6	0	0, -
7	Female/32	AR	1.75, +	6	0	0, -
8	Male/12	AR	4.5, ++	4.75	0	0, -
9	Male/46	BA, AR	4.5, ++	8.5	0	0, -
10	Female/17	AR	4.5, +++	4.5	0	0
11	Male/45	BA	3, +	7	0	0
12	Male/19	BA	0.5, +	1	0	6.5, ++++
13	Male/38	BA, AR	6, ++	7.5	0	0
14	Male/27	BA, AR	8.5, +++	7	0	2.5, +
15	Male/15	BA	3, +	6.25	0	2.5, +
16	Female/24	BA, AR, AD, FA, DA	8.5, +++	7	0	1.5, +
17	Female/10	BA, AR	8.5, +++	5.5	0	0
18	Female/18	BA, AR, DA	10, +++	6	0	0
19	Male/54	BA, AR, AD	1.5, +	4.5	0	0
20	Female/41	BA, AR, DA	10.5, ++++	4	0	0
21	Female/13	BA, AR	7	5	0	0
22	Male/17	BA, AR, AD, FA, DA	0.25, +	1	0	0
23	Female/31	AD	9.5, ++++	2.5	0	0
24	Female/39	AR	12.5, +++	8.5	0	1.5, +
25	Male/63	AR, DA	8.5, +++	8	0	0
26	Male/15	BA, AR, FA	6.5, +++	6	0	0
27	Female/12	BA, AR	11, +++	6	0	0
28	Female/18	BA, AR	5.5, +++	4.5	0	0
29	Female/25	BA	6.5, +++	6.5	0	1.5, +
30	Female/40	BA, AR	6.5, +++	5	0	0
31	Male/75	BA, AR, FA	2.5, +	6.5	0	2, +
32	Male/11	BA, AR, FA	6.5, +++	5.5	0	0
33	Female/26	BA	1, +	5.5	0	0
34	Male/15	BA, AR, AD	7, ++	8	0	2.25, +
35	Female/14	BA, AR	6.5, +++	6.5	0	0
36	Male/8	AR, FA	1, +	4.5	0	0
37	Female/43	BA	2, +	4.5		0

The result of Skin prick tests (SPT). BA (Bronchial asthma); AR (Allergic rhinitis); DA (Drug allergy); MA (Mango allergy); FA (Food allergy); PS; Physiological saline; Positive: ≥ 1 ; Negative: 0.

biology technology to obtain recombinant allergens can provide new insight and new strategies for the development of the specific immunotherapy and the treatment of allergic diseases.

These results suggest that Der f 29b is a new type allergen of HDM.

In silico prediction has already become a useful tool for selecting epitopes from immunological

The diversity of allergens in house dust mites has not been fully identified and characterized [18]. In this study, through cloning, expression, and purification of full length recombinant Peptidyl-prolyl cis-trans isomerase (r-Derf 29b), we identified the r-Derf 29b is an allergen in *D. farinae*. r-Derf 29b is encoded by a 495 bp open reading frame, producing a predicted amino acid sequence of 164 amino acid. The recombinant protein analyzed by SDS-PAGE showed that relative MWs of r-Der f 29b protein was about 18 kDa. The recombinant products can be purified with Ni-NTA affinity chromatography since its N-terminal contained six histidine label Using SPT techniques, we proved that Der f 29b is an allergen of *D. farinae* Der f 29b reacted to sera from 24.3% of patients with *D. farinae* allergy (**Table 1**). The immunoblotting assays demonstrated that serum IgE from allergic patients reacted to r-Derf 29b protein. The IgE reactivity of r-Der f 29b in the serum from the r-Derf 29b allergic patients increased by more than 2 folds compared with healthy subjects. Immune inhibition assays showed the IgE cross-reactivity between r-Der f 29b and DME.

Der f 29b analysis

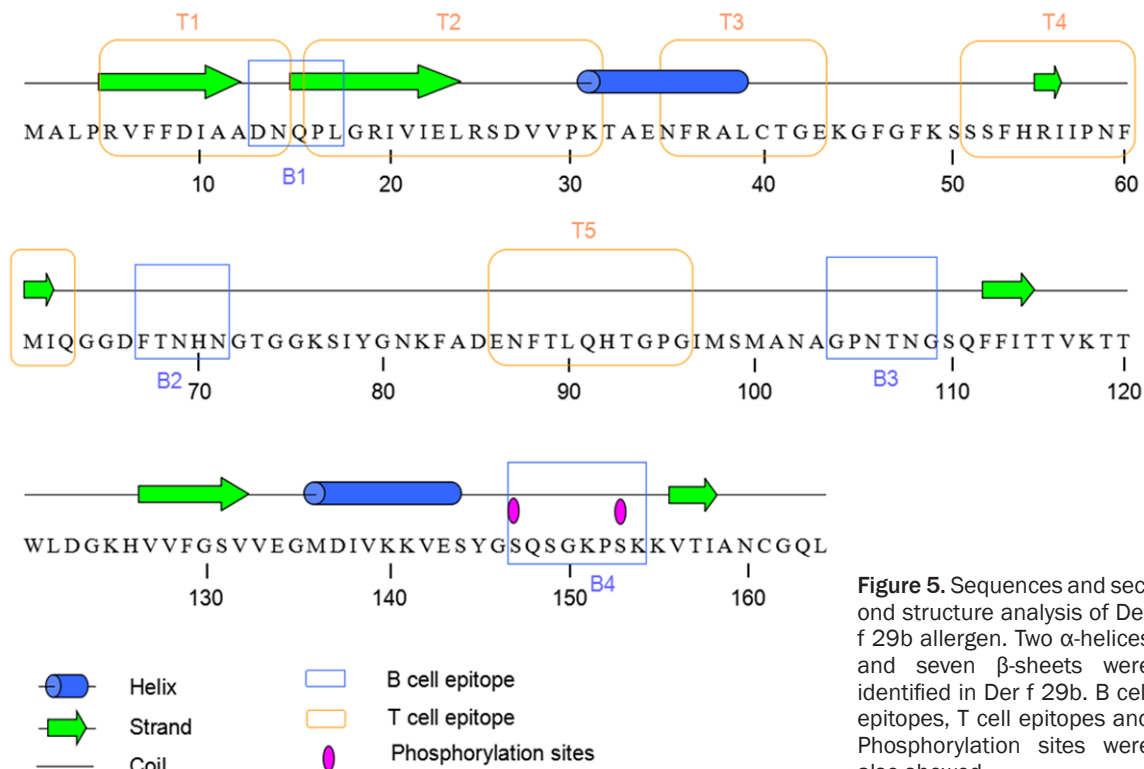


Figure 5. Sequences and second structure analysis of Der f 29b allergen. Two α -helices and seven β -sheets were identified in Der f 29b. B cell epitopes, T cell epitopes and Phosphorylation sites were also showed.

Table 2. Epitope prediction

Epitope	Sequence	SYFPEITHI	NetMHCII	NetMHCIIpan	IEDB	Preprod
(5-14) aa	RVFFDIAADN	+	+	+	+	+
(16-31) aa	PLGRIVIELRSDVVPK	+	+	+	+	+
(35-43) aa	NFRALCTGE	+	+	+	+	+
(52-63) aa	SSFHRIIPNFMIQ	+	+	+	+	+
(87-97) aa	ENFTLQHTGPG	-	+	+	+	+

Five epitope peptides in prediction of different prediction tools. "+" means the sequence was predicted as epitope in prediction tool, "-" means the sequence was not predicted as epitope in prediction tool.

relevant proteins, which can save the working time and the expense of synthetic peptides [19]. In this study, we used three algorithms (DNASstar protean system, BepiPred 1.0 server and BPAP) to predict the B cell epitopes. Previous studies showed that using bioinformatics approach to predict B cell epitopes is correlated well with the experimental approach [20]. As a result, four peptides (13-17, 67-71, 104-109 and 147-155) were predicted as the B cell epitopes. In order to predicate the HLA-DR-based T cell epitopes, five immunoinformatics tools including Immune Epitope Database (IEDB), Propred, SYFPEITHI, NetMHCII 2.2 Server and NetMHCII pan 3.0 Sever were used. The ultimate results of the five immunoinfor-

matics tools finally predicted five peptides (5-14, 16-31, 35-43, 52-63 and 87-97) as the T cell epitopes. Despite the high accuracy of these predictions, this approach has not yet been applied to peptide-based vaccine development for allergic dis-

eases and need further investigation in clinical samples.

The continuity of research of HDM allergens and the identification of allergen diversity provided information for the development of effective diagnostic and therapeutic approaches for HDM allergy [21-24]. Here we provide the evidence that Der f 29b is a new sub-group of house dust mite allergen, which may be helpful for HDM allergy specific diagnosis and therapy.

Acknowledgements

This study was supported by grants from the Natural Science Foundation of China (No.

91442118, 31328014 and 31400786), Guangdong Scientific Technology Social Development Project (No. 2013B3180002), Guangdong Foreign Scientific Technology Cooperative Project (No. 2013B051000088), Shenzhen Scientific Technology Project International Cooperative Project (GJHZ20130408174112021), Shenzhen Scientific Technology Basic Research Projects (No. JCYJ20120613100657482, JCYJ-20130329110735981, JCYJ2012061317323-3810, JCYJ20140828163633992) and Shenzhen Nanshan District Research Development Project (No. KC2014JSCX0055A).

Disclosure of conflict of interest

None.

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Der f 29b analysis

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