Identification of Der f 23 as a new major allergen of *Dermatophagoides farinae*

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Abstract. House dust mites (HDM) are common allergen sources worldwide. At present, 32 of the 37 internationally recognized HDM allergen groups have been identified in *Dermatophagoides farinae*. The present study study describes the identification of the first known *D. farinae* Group 23 allergen (Der f 23). Recombinant Der f 23 protein (rDer f 23) was cloned, expressed and purified. The open reading frame of rDer f 23 was 525 base pairs and encoded a 174-amino acid protein (GenBank accession no., KU166910.1). ELISAs indicated that 72/129 HDM allergic serum samples (55.8%) had specific immunoglobulin E (sIgE) binding activity to rDer f 23. Additionally, 3/10 patients with HDM allergies (30%) exhibited positive skin prick test reactions to rDer f 23. IgE western blot analysis data suggested that only 4/11 HDM allergic sera had a positive sIgE binding result. Sequence homology analysis revealed an extra P2 region (Ser56-Thr117) in Der f 23 that was not present in the *D. pteronyssinus* homolog, which may affect sIgE binding. Der f 23ΔP2 demonstrated binding with HDM allergic sera, whereas the P2 peptide alone did not. The sIgE binding ability of Der f 23 ΔP2 (Der f 23 with a truncated P2 region) was more marked compared with that of Der f 23 in an IgE ELISA. These data indicate that P2 region in Der f 23 attenuates IgE binding ability. In conclusion, the results of the present study indicate that Der f 23 is a major HDM allergen with predominantly conformational sIgE binding epitopes. The allergenic identification of Der f 23 and its inclusion in World Health Organization/International Union of Immunological Societies database contributes to the theoretical basis underlying the diagnosis and treatment of HDM allergic diseases.

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Abbreviations: HDM, house dust mite; Der f, *Dermatophagoides farinae*; Der p, *Dermatophagoides pteronyssinus*; Der f 23, Group 23 allergen of *Dermatophagoides farinae*; Der p 23, Group 23 allergen of Dermatophagoides pteronyssinus; IPTG, isopropyl-β-D-thiogalactopyranoside; HRP, horseradish peroxidase

Key words: *Dermatophagoides farinae*, Der f 23, house dust mite, novel allergen

Introduction

House dust mites (HDMs) are common indoor sources of allergens (1,2). The primary species of HDM are *Dermatophagoides pteronyssinus*, *D. farinae*, *Euroglyphus maynei* and *Blomia tropicalis* (3,4), with the former two being ubiquitous in home dust samples in temperate and tropical regions (5,6). HDM allergens constitute a major cause of allergic diseases (7,8), with one-half of allergy sufferers exhibiting an allergic reaction to HDM allergens (9,10).

Clinicians use HDM allergen proteins to diagnose and treat HDM allergies (11,12). The HDM antigens used clinically are obtained from a crude HDM extract (13,14). As these crude extracts are a mixture of a small portion of allergens and a number of unrelated impurities, their effects are highly varied, including adverse side effects in certain patients (15). Of the 39 HDM allergen groups recognized in the World Health Organization and International Union of Immunological Societies (WHO/IUIS) allergen database, 33 include identified *D. farinae* allergens and only 23 include *D. pteronyssinus* allergens (16). The identification of HDM allergens, particularly the detection and naming of novel HDM allergens, has direct significance for the diagnosis and treatment of HDM-induced allergic diseases.

Previous studies have suggested that the major HDM allergens belong to Group 1 (17,18), Group 2 (19,20), Group 23 (21,22) and Group 24 (23). A Group 23 allergen from *D. pteronyssinus* (Der p 23) was identified to be a major allergen present in HDM feces in dust (24,25). Der p 23 has been demonstrated to react with specific immunoglobulin Es (sIgEs) from 74% of patients with *D. pteronyssinus* allergies, which is smaller proportion compared with the positive reaction rates of the two previously recognized major HDM allergens, namely Der p 1 and Der p 2 (21). Der p 23 is a

small, globular protein stabilized by 2 disulfide bonds that is structurally similar to other allergens, including Blot 12, in that it contains carbohydrate-binding domains that bind chitin (26).

To the best of our knowledge, the Group 23 allergen in *D. farinae* (Der f 23) had not been identified and characterized prior to the present study. The aims of this study were firstly to confirm the existence of Der f 23, and secondly to characterize the sIgE binding activity of Der f 23, if such an allergen was able to be isolated. *In vitro* IgE binding was determined by IgE western blot analysis, dot blot assays and ELISAs; *in vivo* reactivity was assayed with a skin prick test (SPT). The identification of a novel Der f 23 allergen would be clinically useful for the diagnosis and treatment of HDM-induced allergic diseases.

Materials and methods

Materials. A *D. farinae* cDNA library preserved by the School of Medicine, Shenzhen University (Shenzhen, China) was employed. *E. coli* BL21 (DE3) plysS cells were purchased from Merck KGaA, and the pMD 19-T vector was purchased from Takara Biotechnology Co., Ltd. The pET-His and pET-His-DsbA vectors were purchased from Wuhan Miaoling Bioscience & Technology Co., Ltd. The DNA sequence encoding the Der f 23Δ P2 protein (P2) was synthesized by Nanjing GenScript Biotech Corp. The Primer STAR HS DNA polymerase was purchased from Takara Biotechnology Co., Ltd. The lysozyme was purchased from Sangon Biotech Co., Ltd. The nitrocellulose and polyvinylidene fluoride (PVDF) membranes were purchased from Merck KGaA.

Serum samples from HDM-sensitive individuals, referred to as HDM allergic sera, and non-allergic individuals were provided by the First Affiliated Hospital of Guangzhou Medical College. Sera from non-allergic individuals were used for control group. A cohort of 129 subjects (65 males and 64 females; age range, 18-55 years) were enrolled. Samples from 31 non-allergic individuals were used as negative controls. The HDM-specific IgEs within the sera samples were assayed using an ImmunoCAP system (Thermo Fisher Scientific, Inc.). Ethical approval was obtained from the First Affiliated Hospital of Guangzhou Medical College (approval no. 2012-51). All procedures involving human participants were in accordance with the ethical standards of the committee of the First Affiliated Hospital of Guangzhou Medical College. All participants voluntarily agreed to participate, and all provided written informed consent.

Methods

cDNA cloning, protein expression and purification. Der f 23 open reading frame (ORF) cDNA was amplified by polymerase chain reaction (PCR) using the Primer STAR HS DNA polymerase with the *D. farinae* cDNA library. The primers used were: Forward, 5'-ATGAAATTCAACATAACTAT CGC-3'; and reverse, 5'-TTATGTACATGTTAATTCTTTT TCA-3'. The PCR thermocycling conditions were 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 50 sec, for 30 cycles. The PCR product, confirmed by DNA sequencing (GenScript Biotech Corporation), was subcloned into a pET-His vector, and then transformed into *Escherichia coli* (*E. coli*) BL21 (DE3) plysS. *E. coli* were grown overnight in Luria-Bertani medium (Thermo Fisher Scientific, Inc.) containing 100 mg/l ampicillin at 37˚C, and were induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Following cultivation for additional 3 h at 37˚C, *E. coli* cells were harvested by centrifugation at 9,600 x g for 5 min at 4˚C. Following mixing with the protein extraction buffer (20 mM PB, 150 mM NaCl and 1 mg/ml lysozyme), the harvested cells were sonicated for 3 sec with 5-sec intervals in an ice bath for a total of 20 min, and then centrifuged again at 9,600 g for 20 min at 4˚C. The recombinant protein in the soluble portion was purified with a Ni-NTA column (cat. no. 17040303; GE Healthcare) and gel filtration (HiLoad Superdex 16/600; cat. no. 28-9893-33; GE Healthcare).

IgE western blot analysis and IgE dot blot assays. The protein concentration was determined by Bradford assay (Sangon Biotech Co., Ltd). The protein samples were diluted to 1 mg/ml and subjected to electro-transfer onto the PVDF membrane through 12% SDS-PAGE (20 μg/lane) for the IgE western blot analysis. Subsequently, the membranes were blocked overnight at 4˚C with 5% skim milk in TBS + 0.05% Tween-20 (TBST). Then, the membranes were incubated with allergic sera (diluted in 1:10 vol/vol in PBS buffer) for 2 h at 37˚C. Following washing, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated anti-human IgE antibody for 2 h at 37˚C (1:2,000; cat. no. 9160-05; SouthernBiotech). The proteins were visualized for 10 min using 3,3'-diaminobenzidine (1:10; cat. no. 34002; Thermo Fisher Scientific, Inc.) at room temperature.

The IgE dot blot assay was performed as previously described (23). The protein samples were diluted to 1 mg/ml, and 2 μl samples were added to the nitrocellulose membrane for the IgE dot blot assay. Subsequently, the membranes were dried and blocked overnight at 4˚C with 5% skim milk in TBST. Then, the membranes were incubated with allergic sera (diluted in 1:10 vol/vol in PBS buffer) for 2 h at 37˚C. Following washing, the membranes were incubated with the HRP-conjugated anti-human IgE antibody for 2 h at 37˚C (1:2,000; cat. no. 9160-05; SouthernBiotech). The proteins were visualized for 10 min using 3,3'-diaminobenzidine (1:10; cat. no. 34002; Thermo Fisher Scientific, Inc.).

IgE ELISA. The IgE ELISA assay developed in the present study was performed as previously described (23). The isolated protein was added to the ELISA plate at 100 ng/well and coated overnight at 4˚C. The coated plate was blocked with 5% skim milk in PBS + 0.1% Tween-20 (PBST) for 2 h at 37˚C. Then, the plate was incubated with allergic sera (diluted 1:10 vol/vol in 1% skim milk-PBST) for 2 h at 37˚C. IgEs were detected with the HRP-conjugated mouse anti-human IgE antibody (1:2,000 in 1% skim milk-PBST; cat. no. 9160-05; SouthernBiotech). Binding reactions were visualized by adding tetramethyl benzidine substrate, and absorbance at 450 nm was measured by a microplate reader (Bio-Rad Laboratories, Inc.).

SPTs. SPTs were performed with rDer f 23 protein (10 mg/ml) and standardized *D. farinae* extract (ALK Abelló A/S) in 10 patients with allergic rhinitis and/or asthma and 10 healthy controls (Table I) (23). The response was observed 15 min after

Table I. Skin reactivity to *Der f* extracts and rDer f 23 protein (10 μg/ml).

		Skin prick tests[a]		
Patient no.	Sex/age	Clinical history	*Der f* extracts	rDer f 23
1	Female/39	AR	3+	2+
2	Female/38	AR+BA	3+	-
3	Male/25	AR	3+	-
4	Female/40	AR	3+	-
5	Male/52	AR	4+	2+
6	Female/28	BA	3+	-
7	Male/24	AR	4+	2+
8	Female/23	AR	3+	-
9	Male/38	BA	3+	-
10	Male/44	AR+BA	3+	-

[a]Skin reaction scoring method: ≥2+, positive; -, negative. AR, allergic rhinitis; BA, bronchial asthma; *Der f*, *Dermatophagoides farinae*; rDer f 23, recombinant *Dermatophagoides farinae* 23 protein.

pricking. The result was considered positive when the prick spot developed a wheal with a surrounding fleck; no visible response was considered a negative result.

Sequence homology. The nucleotide sequences of Der f 23 (GenBank: KU166910.1) and Der p 23 (GenBank: KP895831) were imported into DNAMAN 8 software (version 8.0; Lynnon Biosoft) for alignment. The sequences were saved in FASTA format for subsequent analysis.

Expression and purification of Der f 23ΔP2 and P2 proteins. Der f 23ΔP2 and P2 region gene sequences were synthesized by Nanjing GenScript Biotech Corp., and each was subcloned into the pET-His-DsbA vector. The recombinant proteins were expressed and purified as aforementioned. Specifically, the Der f 23ΔP2 protein was expressed in inclusion bodies and was dissolved in 6 M urea with 25 mM β-mercaptoethanol. For the renaturation process, the Der f 23ΔP2 protein was dialyzed with PBS at 4°C for 24 h using a dialysis membrane (cat. no. F132550; Sangon Biotech Co., Ltd.). Additionally, soluble recombinant DsbA-P2 protein was obtained. The purified protein Der f 23 ΔP2 and DsbA-P2 were subjected to *in vitro* IgE binding assays.

Statistical analysis. Quantitative data are presented as means ± standard error of the mean. Analyses were performed using GraphPad Prism 7 software (GraphPad Software, Inc.). Differences between the allergic and control groups were determined by one-way ANOVA followed by Dunnett's post-hoc test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Amino acid sequence homology between Der f 23 and Der p 23. The Der f23 ORF sequence was cloned using the *D. farinae* cDNA library as a template, and specific Der f 23 sequence primers were designed based on the *D. farinae* genome in the National Center for Biotechnology Information (NCBI) database (Genome ID: 9138). DNA sequencing confirmed the sequence of the ORF cDNA of Der f 23 to be 525 base pairs, and the sequence was registered in the NCBI library (GenBank: KU166910.1). A homology comparison conducted in DNAMAN 8 software indicated that Der f 23 has 173 amino acids, whereas Der p 23 has 97 amino acids, with the additional amino acids constituting 2 extra regions, namely P1 (Phe26-Pro38) and P2 (Ser56-Thr117), as demonstrated in Fig. 1.

Cloning, expression and purification of Der f 23. To obtain the Der f 23 protein, a prokaryotic pET-His vector was constructed and transformed into *E. coli* for expression and purification. The Der f 23 ORF DNA sequence obtained by PCR amplification with specific primers, as demonstrated in Fig. 2A, was cloned into a pET-His vector. The pET-His-Der f 23 vector was identified by double digestion with *Bam*H I and *Hin*dIII (Fig. 2B). Subsequently, the pET-His-Der f 23 vector was transformed into *E. coli* BL21 (DE3) plysS cells for expression and purification. SDS-PAGE analysis demonstrated that Der f 23 (~30 kDa) was successfully expressed in the supernatant following IPTG induction and purification with Ni-NTA-resin (Fig. 2C and D).

IgE binding of Der f 23. The IgE ELISA indicated that HDM allergic sera from 72/129 patients with HDM allergies (55.8%) exhibited sIgE binding activity with Der f 23 (Fig. 3A). None of the serum samples from 31 non-allergic individuals were reactive (Fig. 3A). Data from the IgE dot blot assays suggested that Der f 23 protein exhibited a good sIgE binding capacity for HDM allergic sera, but not for sera from non-allergic individuals. Der f 1/2 fusion protein was used as positive control (Fig. 3B). In the IgE western blot analysis, only 4/11 selected IgE ELISA reactive sera exhibited sIgE reactivity to rDer f 23 (Fig. 3D), suggesting that sIgE binding epitopes in Der f 23 may be conformation-dependent. As demonstrated in Table I, 3/10 (30%) of patients with HDM allergies exhibited a positive *in vivo* reaction to Der f 23 in the SPTs. Based on these

Figure 1. Amino acid sequence homology between Der f 23 and Der p 23. The common amino acids are shaded in blue. The different regions, P1 (Phe26-Pro38) and P2 (Ser56-Thr117), are highlighted in blue and red boxes, respectively. Der f, *Dermatophagoides farinae;* Der p, *Dermatophagoides pteronyssinus*.

results, the WHO/IUIS Allergen Nomenclature subcommittee published this allergenic HDM protein as Der f 23 (16).

Involvement of P2 region (Ser56-Thr117) in sIgE binding. The P2 region peptide and Der f 23 ΔP2 protein were expressed in *E. coli*. DsbA-Der f 23ΔP2 was observed in the form of inclusion bodies with a molecular weight of ~38 kDa and DsbA-P2 was soluble with a molecular weight of ~35 kDa (Fig. 4A). The IgE ELISA demonstrated that rDer f 23 and DsbA-Der f 23ΔP2 exhibited significant binding activity, with all 8 HDM allergic sera tested, whereas P2 exhibited no IgE binding reactivity with any of the sera (Fig. 4B). The DsbA protein served as a negative control for sIgE binding activity (data not shown). Notably, within the same positive sera, the optical density value of DsbA-Der f 23ΔP2 was increased compared with that of rDer f 23 ($P<0.05$). Similarly, the dot blot assay results confirmed that DsbA-Der f 23 ΔP2 exhibited significant sIgE binding reactivity, while the P2 peptide did not. The color reaction was more marked for DsbA-Der f 23 ΔP2 compared with the Der f 23. These data indicated that the P2 region of Der f 23 may affect the Der f 23 protein structure and thereby attenuate the IgE binding ability of Der f 23 in sera.

Discussion

The present study cloned, expressed and purified rDer f 23 protein using an *E. coli* expression system. The resultant recombinant protein exhibited sIgE binding activity *in vitro* and *in vivo*, and was therefore included as Der f 23 in the WHO/IUIS allergen database (16). The present study identified that, in comparison with Der p 23, Der f 23 contained an extra P2 region that affected its IgE binding ability. In the IgE ELISAs, 72/129 positive sera (55.81%) from patients with HDM allergies exhibited marked sIgE binding activity to rDer f 23 protein. These data confirmed that Der f 23 is a major allergen, consistent with previous results for Der p 23 (21).

The allergen components of the 2 HDM species *D. farinae* and *D. pteronyssinus* are highly homologous, encompassing 39 HDM allergen groups (16). Indeed, the Der p 23 allergen identified in the present study had 71% amino acid sequence homology with Der f 23. Additionally, it was identified that Der p 23 had 2 extra regions not present in Der f 23, namely a P1 region (Phe26-Pro38) and a P2 region (Ser56-Thr117). These sequence differences may be the underlying cause of a differential allergenic effect between Der f 23 and Der p 23 proteins, and therefore an allergic distinction between the 2 HDM species of *D. farinae* and *D. pteronyssinus*. In light of recent evidence demonstrating the existence of multiple Der f 23 isoforms (27), we hypothesized that the sequence difference between Der f 23 and Der p 23 may be due to the insertion of an intron in the gene that encodes Der f 23.

The IgE binding ability of HDM allergens is based primarily on B-cell epitopes of allergens, which may occur in conformational or linear form. Conformational epitopes are more favorable for IgE binding compared with linear epitopes (28). Conformational changes in IgEs contribute to a decrease in their dissociation rates from high-affinity IgE receptors (29). B-cell epitopes are primarily conformational, including some that are discontinuous, in which polypeptide chain folding draws distant amino acid segments in the primary structure into close proximity on the surface of the molecule (30,31). These regions are able to form complementarity-determining regions recognized by

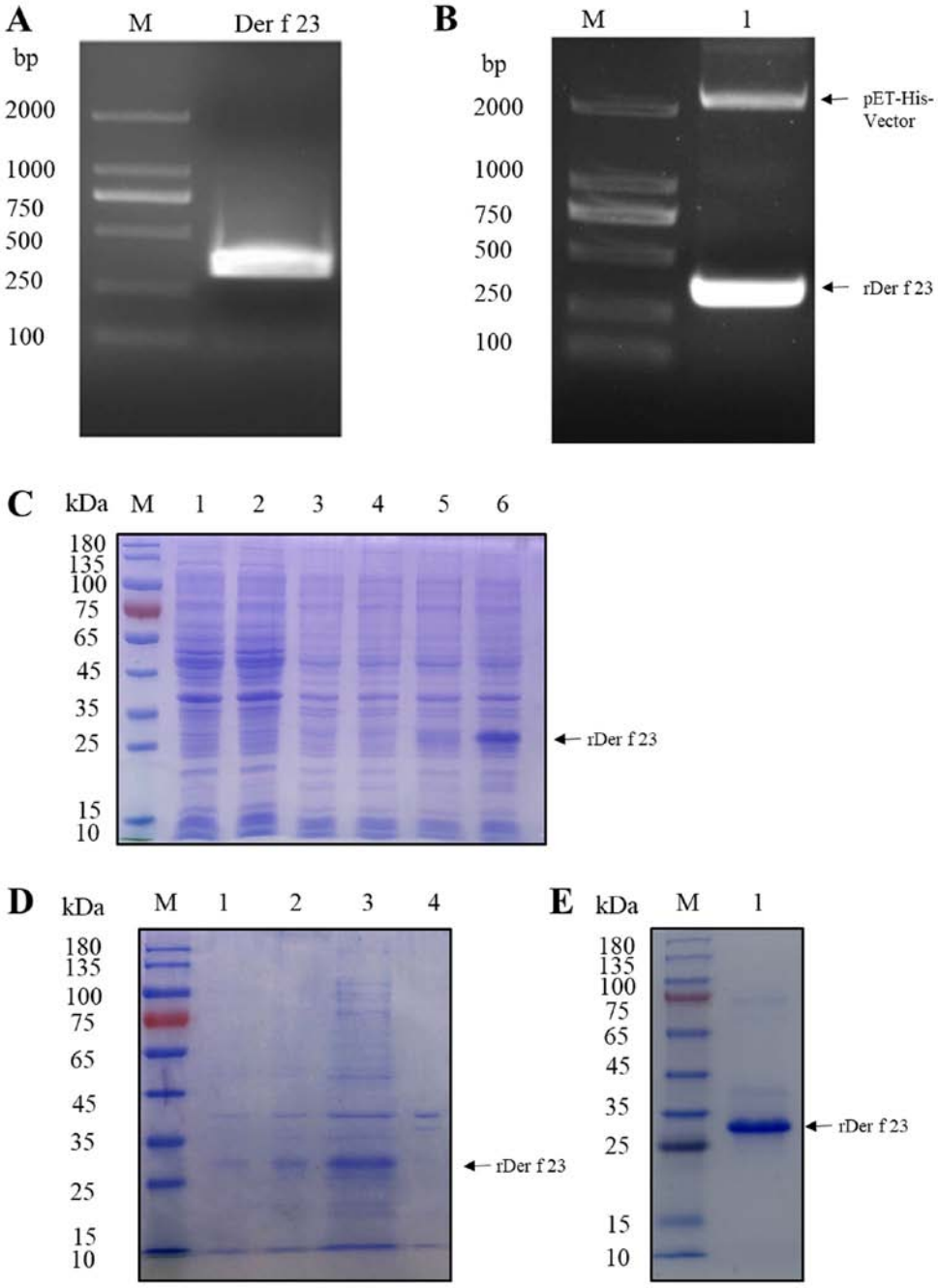

Figure 2. Cloning, expression and purification of rDer f 23. (A) Cloning of Der f 23 cDNA by polymerase chain reaction. Lane M, DL2000 marker; lane 1, Der f 23 cDNA PCR product. (B) Analysis of expression vector pET-His-Der f 23 by double enzyme digestion. Lane M, DL2000 Marker; lane 1, pET-His-Der f 23 vector digested by *Bam*H I/*Hin*dIII. (C) Analysis of purification of pET-His-Der f 23 by SDS-PAGE. Lane M, protein marker; lane 1, cell lysate of *E. coli* without induction; lane 2, cell lysate of *E. coli* following IPTG induction; lane 3, *E. coli* transformed with pET-His-vector without induction; lane 4m, cell lysate of pET-His-vector following IPTG induction; lane 5, *E. coli* transformed with pET-Der f 23 prior to IPTG induction; lane 6, *E. coli* transformed with pET-Der f 23 following IPTG induction. (D) Analysis of expression of rDer f 23 in an *E. coli* system. Lane M, protein marker; lane 1, *E. coli* transformed with pET-Der f 23 prior to IPTG induction; lane 2, *E. coli* transformed with pET-Der f 23 following IPTG induction; lane 3, supernatant from *E. coli* transformed with pET-His-Der f 23 plasmid following IPTG induction, subsequent to ultrasonication; lane 4, sediment from *E. coli* samples transformed with pET-His-Der f 23 plasmid following IPTG induction, subsequent to ultrasonication. (E) SDS-PAGE of purified rDer f 23. Lane M, protein marker; lane 1, purified rDer f 23. *E. coli*, *Escherichia coli*; rDer f 23, recombinant *Dermatophagoides farinae* 23 protein; IPTG, isopropyl-β-D-thiogalactopyranoside.

antibodies (32). The results from the present study, that only 4/11 sera with a positive IgE ELISA result also exhibited a positive IgE western blot analysis result suggests that the IgE epitope of Der f 23 may be predominantly a conformational epitope, consistent with the data from Szalai *et al* (33), which suggested that the B-cell epitopes of Der p 1 and Der p 2 are conformational.

Despite their high degrees of homology, different HDM allergen components within the same group have differing IgE binding abilities determined by their amino acid sequences and protein structures. For example, although Der p 1 and Der f 1 share an extensive sequence identity, differences identified in their crystal structures may explain the differences in human IgE antibody responses to these

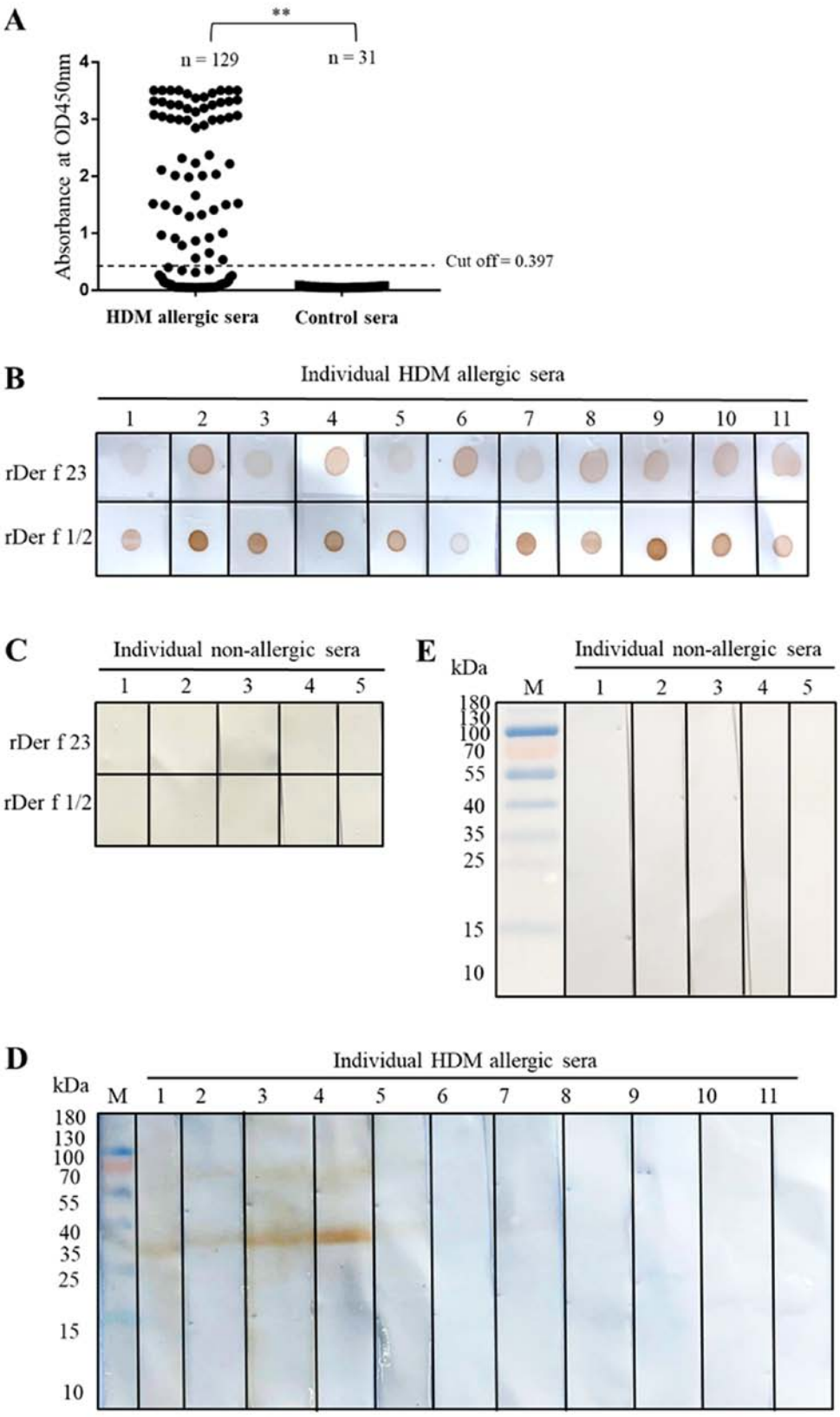

Figure 3. *In vitro* IgE binding reactivity of rDer f 23. (A) sIgE binding reactivity to rDer f 23 revealed by IgE ELISA. Dot blot analysis of rDer f 23 in (B) HDM allergic sera from individual patients and (C) sera from non-allergic individuals. Der f 1/2 was used as the positive control. IgE western blot analysis of rDer f 23 with (D) HDM allergic sera from individual patients and (E) sera from non-allergic individuals (E). $^{**}P<0.05$. rDer f 23, recombinant *Dermatophagoides farina* 23 protein; rDer f1/2, recombinant Der f 1-Der f 2 fusion protein; HDM, house dust mite; sIgE, specific immunoglobulin E; OD, optical density.

allergens (34). The IgE ELISA experiment conducted in the present study to examine the role of the P2 region of Der f 23, which is not present in Der p 23, in the allergenicity of Der f 23 indicated that the rDer f 23 and DsbA-Der f 23 ΔP2 proteins exhibited positive sIgE binding activity with HDM allergic sera, whereas DsbA-P2 did not. In addition,

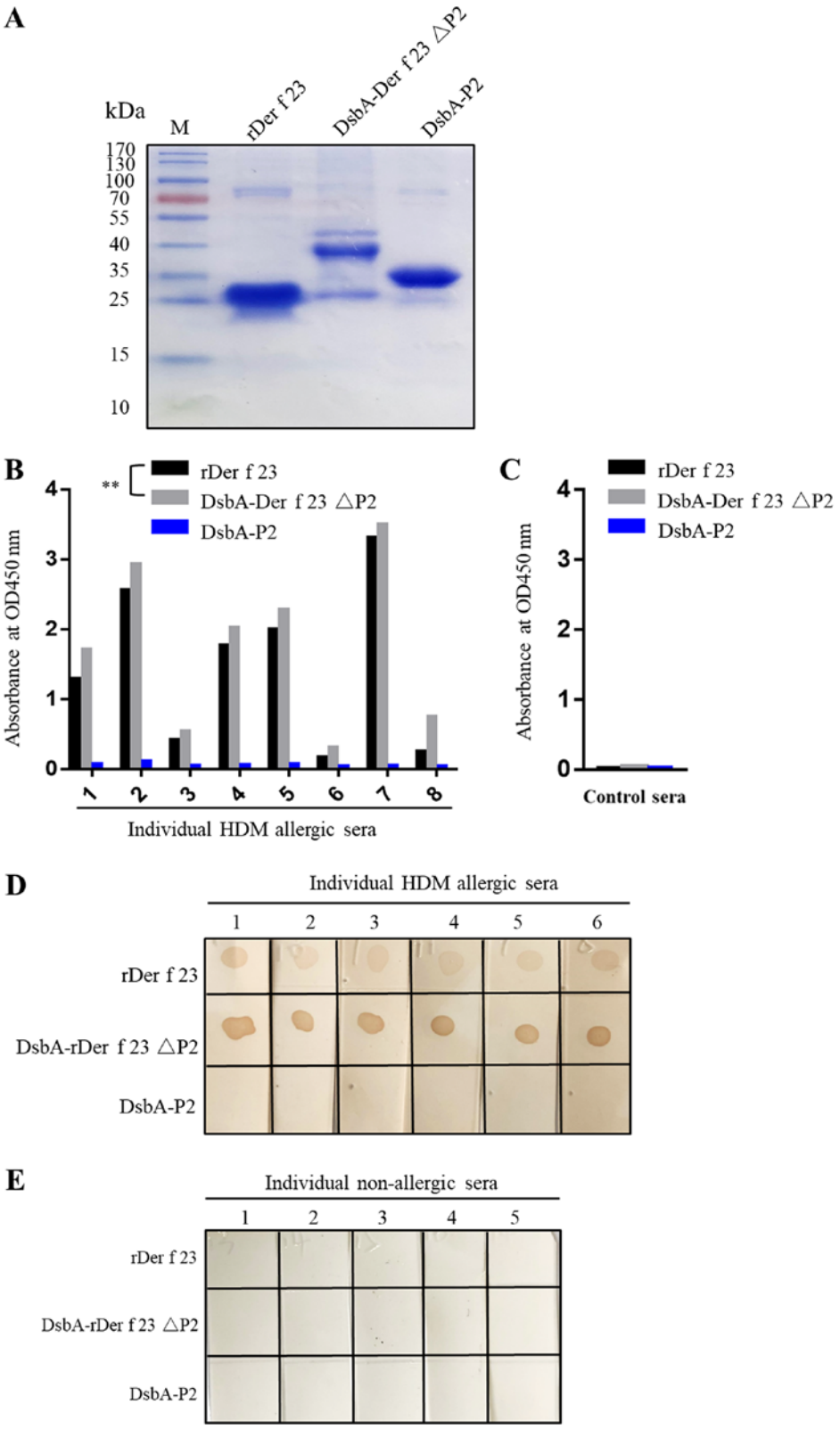

Figure 4. Effect of P2 region of Der f 23 on sIgE binding ability. The rDer f 23, DsbA-rDer f 23ΔP2, and DsbA-P2 was obtained using an *E. coli* system and subjected to IgE binding assays. (A) SDS-PAGE of purified DsbA-rDer f 23ΔP2 and DsbA-P2. IgE ELISA of rDer f 23, DsbA-rDer f 23ΔP2, and DsbA-P2 in (B) HDM allergic sera from 8 patients and (C) mixed sera from 5 non-allergic controls. sIgE binding dot blot assay of (D) HDM allergic sera from individual patients and (E) sera from non-allergic controls. $^{**}P<0.05$. P2, Der f 23 ΔP2 protein; rDer f 23, recombinant *Dermatophagoides farinae* 23 protein; sIgE, specific immunoglobulin E; HDM, house dust mite; OD, optical density.

the optical density value at 450 nm for DsbA-Der f 23 ΔP2 reacting with sIgE from HDM allergic sera was increased compared with that of rDer f 23. Furthermore, the IgE dot blot experiment revealed a more marked reaction for DsbA-Der f 23ΔP2 compared with rDer f 23 in HDM allergic sera from 6 individuals, consistent with the results of the western blot

analysis. We hypothesized that the P2 domain region affects the spatial location of the IgE binding epitope, but this requires additional studies to confirm. Taken together, these results indicate that the addition of the P2 domain has the ability to affect the allergenic capabilities of HDM allergens.

In conclusion, Der f 23 was identified and characterized using sIgE binding assays *in vitro*, and Der f 23 immunogenicity was confirmed with *in vivo* SPTs. Based on these data, Der f 23 was included in the WHO/IUIS allergen database (21). The results of the present study suggested that Der f 23 is an important allergen of *D. farinae* that interacts with sIgEs by way of conformational epitopes. An amino acid sequence difference between Der f 23 and Der p 23, namely the P2 region that is present only in the former, was demonstrated to affect the sIgE binding activity of Group 23 HDM allergens. These results contribute to the theoretical basis for the diagnosis and treatment of HDM allergies.

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Availability of data and materials

All data generated or analyzed in the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YH performed the experiments and wrote the draft manuscript. CD, YS, JialC and ZZhan participated in the experiments. JiajC and KJ designed the study and wrote the manuscript. ZZhao made revisions to the manuscript and participated in study design. All of the authors reviewed and approved the final version of the manuscript.

Ethics approval and consent to participate

Ethics approval was obtained from the First Affiliated Hospital of Guangzhou Medical College (approval no. 2012-51). All procedures involving human participants were in accordance with the ethical standards of the committee of The First Affiliated Hospital of Guangzhou Medical College. All participants voluntarily agreed to participate, and all provided written informed consent.

Patient consent for publication

All participants voluntarily agreed to participate, and all provided written informed consent.

Competing interests

The authors declare that they have no competing interests.

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