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Occupational Allergy to Peach *(Prunus persica)* Tree Pollen and Potential Cross-Reactivity between *Rosaceae* Family Pollens

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ABSTRACT

Orchard workers in north China are highly exposed to orchard pollens, especially peach and other *Rosaceae* family pollens during pollination season. The aim of this study was to investigate whether occupational allergy to peach tree pollen as a member of *Rosaceae* family is IgE-mediated and to evaluate the cross-reactivity among *Rosaceae* family pollens.

Allergen skin test and conjunctival challenge test were performed; enzyme linked immune-sorbent assay(ELISA), inhibiting ELISA, western immunoblotting and inhibiting western immunoblotting were done with *Rosaceae* family orchard pollens, including peach, apricot, cherry, apple and pear tree pollens. Mass spectrometry was also performed to probe the main allergen component and cross-reactive protein.

Sensitizations to peach pollen were found in both skin test and conjunctival challenge in the patients. Serum specific IgE to three pollens (peach, apricot and cherry) were detected through ELISA. When peach pollen used as solid phase, ELISA inhibition revealed other four kinds of pollens capable of inducing partial to strong inhibitions (45% to 87%), with the strongest inhibition belonging to apricot pollen (87%). Western blotting showed predominant IgE binding to a 20 KD protein among these pollens, which appeared to be a cross-reactive allergen component through western blotting inhibition. It was recognized as a protein homologous to glutathione s-transferase 16 from *Arabidopsis thaliana.*

Peach and other *Rosaceae* family tree pollen may serve as a potential cause of IgE mediated occupational respiratory disease in orchard workers in north China.

Keywords: Allergy; Glutathione S-transferase; Occupational asthma; Peach tree; Pollen allergies

INTRODUCTION

Allergic sensitization to workplace antigens can result in occupational asthma, rhinitis and dermatitis.[1] Agriculture workers are exposed to a variety of allergens, such as pollens, molds and mites.[2] Occupational asthma to *Rosaceae* family pollens (rose pollen,[3] strawberry pollen[4]) among agricultural workers

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has been recorded. Workers are repeatedly exposed to peach tree pollen during artificial pollination and are likely to inhale a substantial amount of pollen, which may induce clinical manifestations of allergy, and in the same period, subjects may be exposed to other *Rosaceae* family pollens in the same Orchard i.e. apple (*Malus spectabilis*), apricot (*Armeniaca vulgaris*), pear (*Pyrussorotina*), cherry (*Prunus pseudocerasus*). Allergens and cross-reactive allergens in peach and other *Rosaceae* family fruits have been documented.[5] Cross-reactivity between peaches (Pru p3), apricots (Pru ar3), plums (Pru d3), and apples (Mal d3) has been demonstrated in inhibition experiments using serum pools from patients with fruit allergy.[5, 6]

As far as we know, there are no publications on the involvement of peach tree pollen as an aeroallergen in causing hypersensitivity reactions in the work environment. Only one case of peach leave induced asthma and rhinitis has been reported.[7] In the present study, we address the question of whether occupational allergy to peach tree pollen is IgE-mediated and investigate the presence of cross-reactivity among *Rosaceae* family pollens and other potential cross-reactive allergens.

MATERIALS AND METHODS

Patient

A 39-year-old female was referred to the allergy clinic of Peking Union Medical College Hospital to treat allergy-induced respiratory symptoms. For 13 years, she had worked in Beijing Academy of Agriculture and Forestry Science as a peach breeder. Her main job was to facilitate artificial pollination. Since April 2003, the patient experienced sneezing and rhinorrhea. Her most severe symptoms coincided with the flowering phase of peach blossoms (from mid April to the end of April). The patient controlled her allergic symptoms with antihistamines. During her visit to our clinic in 2012, she displayed upper airway symptoms and severe coughing. Further investigation showed that she was also exposed to four other *Rosaceae* family pollens (apple (*Malus spectabilis*) pollen, apricot (*Armeniaca vulgaris*) pollen, pear (*Pyrus sorotina*) pollen and cherry (*Prunus pseudocerasus*) pollen) that pollinate during the same period as peach pollen. The patient showed that she tolerated peach and other *Rosaceae* fruits fairy well.

Physical examination of the patient was normal without rhonchus or moist rale. Spirometric parameters were performed, with a force vital capacity (FVC) of 3.82L, a forced expiratory volume in the first second (FEV1) of 2.19 L, and an FEV1/FVC ratio of 79%. FEV1 was elevated for more than 200 ml with an improvement rate greater than 12% on two sprays of albuteral.

A healthy individual with no history of allergy and with negative responses to pollens and food allergenic extracts was included as nonatopic control. The study was approved by the ethical review board and participating subjects gave their written consent.

Allergen Extracts

Five *Rosaceae* family pollens *(Prunus persica, Malus spectabilis, Armeniaca vulgaris, pyrus sorotina, Prunus pseudocerasus*) were provided by the patient herself. 10mg pollens were extracted with 100ml phosphate-buffered saline (PBS, pH=7.0) for 2h at 4°C .The extracts were centrifuged for 20min at 10000g. After centrifugation, the supernatants were freeze dried overnight and stored at -80°C until further use. To prepare the SPT extracts, freeze-dried material was dissolved in the saline and then centrifuged for 20min at 10000g. Protein concentrations were determined using Bradford's method [8]. The concentration of pollens' extracts was 1.5mg/ml (peach pollen), 1.5mg/ml (apricot pollen), 0.5mg/ml (cherry pollen), 0.73mg/ml (apple pollen), and 1.11mg/ml (pear pollen), respectively.

Skin Test

Skin tests were carried out according to the instruction explained by Osterballe et al,[9] and performed by allergy nurses. Reactions were expressed in a mean wheal diameter (adding the longest diameter to the orthogonal diameter and dividing it by 2). A mean wheal diameter of 3 mm or more than the negative control was considered positive. Intracutaneous tests were done with a battery of common aeroallergens *(house dust, summer-autumn pollens I, polyvalent molds I, Artemisia sieversiana, spring pollen II, Dermatophagoides farina, polyvalent molds II, polyvalent molds III, Sabina chinensis, Cockroach, Cladosporium cladosporioides, cat hair, Fraxinns Americana, Kochia* scoparia, *Ailanthus alteissima, Platanus acerifolia, Humulus scandens, Alternatia alternate*). (Xinhualian®, Beijing) Skin tests

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were performed with the prick-to-prick method for *Rosaceae* fruits (peach, cherry, apple, and pear) and for the five *Rosaceae* pollens (peach pollen, apricot pollen, cherry pollen, apple pollen, pear pollen). For positive and negative controls, we used histamine chlorhydrate at 10mg/ml and 0.9% saline solution. Skin reactions were interpreted at 15 minutes after skin testing and found to be positive when the diameter of the wheal was >5 mm with local erythema. The following grading system was used according to Ye ST[10]**:** (1) wheal<5 mm and no or small erythema = negative; (2) wheal 5 ~ 10 mm and small erythema=1+; (3) wheal 10 ~ 15 mm and erythema>10 mm=2+ (4) wheal>15mm and erythema >10 mm or with pseudopod formation=3+; (5) local response as grade 3+, accompanying with systemic allergic reaction=4+.

CAP-RAST

Total IgE and specific IgE to common inhalant allergen were determined by the ImmuoCAP system (Phadia250 Detection System, ImmunoCAP, Phadia AB, Sweden).

Conjunctival Provocation Test

The concentrated extract for the conjunctival provocation test was prepared at 1mg/ml and serial dilutions were prepared at 0.0001, 0.001 and 0.01mg/ml. The test started by administering one drop of dilution into the right conjunctival sac and one drop of 0.9% saline solution to the left eye as the negative control. Ocular symptoms were monitored within 20 minutes. If no reaction was observed within 20 minutes, then the next concentration was repeated up to 0.01 mg/ml, unless a positive response was detected. The conjunctival provocation test was considered positive under one of the following conditions : 1) chemosis; 2) erythema>50% of the bulbar conjunctival area; 3) erythema <50% of the bulbar conjunctival area plus itching or epiphora.[11]

SDS-PAGE

All these extracts were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli.[12] The extract was separated in a discontinuous buffer system in an SDS-PAGE gel (5% stacking gel and 15% resolving gel). The extracts were diluted 1:2 in sample buffer (Tris 10% SDS, DTT, 50% glycerol,1% Bromphenol blue) at 100°C for 5 min, with 20µg of protein applied per lane. After separation, parts of the gel containing molecular weight markers and extract were stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA, USA) for 90 min. Some parts were electrophoretically transferred onto a Polyvinylidenefluoride(PVDF) membrane (0.45µm) (Immobilon-P, Millipore ,MA, USA) using a trans-blot cell form at 1.5mA/cm^2 for 1h at room temperature.

Immunoblotting and Inhibition Tests

Five types of Rosaceae pollens' extracts were analyzed by SDS-PAGE (15% homogeneous gel 100X70X1.5mm, 5% stacking gel) under reducing conditions. The proteins in the PAGE were transferred to PVDF membrane. The PVDF membrane was then cut into strips and saturated with a 5% bovine serum albumin (BSA) in Phosphate Buffered Saline with Tween-20 (PBST) for 1 hr at room temperature and incubated for 18 hrs at a patient's serum dilution of 1:5. To inhibit immunodetection, the serum was pre-incubated for 1h with 10 ug of peach pollen, apricot pollen, cherry pollen, apple pollen, and pear pollen. As a negative control, a PVDF strip with peach pollen proteins was incubated overnight with a health serum dilution of 1:5. For a blank control, a PVDF strip with each of the peach pollen proteins was incubated with diluents (5% milk and 0.05% Tween-20 in PBS). The strips were then washed with 0.05% Tween in PBS and incubated at room temperature for 2 hrs at a 1:1000 dilution of horseradish peroxidase (HRP) conjugate anti-human IgE (Abcam, Cambridge, UK). The strips were then incubated in streptavindin-peroxidase (BM Chemiluminescence Blotting Substrate, Roche Molecular Biochemicals, USA). As a final step, the strips were washed and the IgE-binding proteins were detected using a chemoluminescence method provided by the manufacturer's instructions (Sage Creation, Beijing, China).

ELISA and ELISA Inhibition

Allergen-specific IgE to peach pollen was determined by ELISA. Ten microgram of the extract in coating buffer (50mM carbonate buffer, pH=9.5, 0.1% NaN3) was incubated in wells of a microtiter plate for 24hrs at 4°C. After 3 washes with PBST, 0.2ml PBST 1% bovine serum albumin was added to each well.

After a 2hr-incubation time at 37°C, subsequent washes with PBST were done, and 0.1ml of the serum

Table 1. Results of skin tests and serum specific IgE

Skin tests	Patient	Control
Intracutaneous tests(common aeroallergens)		
Artemisia vulgaris	++++	ND
Fraxinns Americana	++	ND
Kochia scoparia	++	ND
Skin prick tests(*Rosaceae* pollens extracts)		
Peach pollen	++++	Neg
Apricot pollen	+++	Neg
Cherry pollen	++++	Neg
Apple pollen	+++	Neg
Pear pollen	++	Neg
Prick to prick (*Rosaceae* fruits)		
Peach	Neg	Neg
Cherry	Neg	Neg
Apple	Neg	Neg
Pear	Neg	Neg
CAP-RAST, IgE (kUA/L)		
Total-IgE	87.2	ND
Ragweed	1.99	ND
Mugwort	13.0	ND
Peach	<0.35	ND
Mugwort	13.0	ND
Ragweed	1.99	ND
rPru p1	<0.35	ND
rPru p3	<0.35	ND

ND: not done; Neg: negative;

was added, diluting to a ratio of 1:7 in PBST solution. After the 2hr incubation at 37°C and the 3 subsequent washes with PBST, 0.1ml anti-IgE (Abcam, Cambridge, UK) diluted 1/1000 in PBST, 1% bovine serum albumin was added to each well. After an additional hour of incubating at 37°C and an additional 3 washes with PBST, the assay was developed with substrate solution (Sigma, USA) in the dark. The enzymatic reaction stopped after 30 minutes of substrate incubation by adding 0.5M sulfuric acid. Absorbance was measured at 405 nm using a Spectrophotometer (Spectra MAX 250 Molecular Device Sunnyvale, USA). The positive/negative (P/N) value of each sample was computed, and samples with a P/N value ≥2 were considered positive. For inhibition of the peach pollen ELISA, 4 other *Rosaceae* pollens extracts were diluted in PBST to a final concentration of 0.1mg/ml. One hundred microlitre of these extracts were incubated with 2ml serum at 37°C. Then, 0.1ml of this mixture was tested in the peach pollen ELISA as described above.

Identification of Protein Bands as Allergens by Mass Spectrometry

The protein in gel-digestion and mass spectrometry experiments were performed by Protein Research Center, Beijing, China. Briefly, the protein of interest was identified by means of nanobore liquid chromatograph-mass spectrometer/mass spectrometer (nanoLC-MS/MS.) The band selected was cut out from the gel with a sterile scalpel and subjected to in-gel trypsin digestion. The in-gel digests were purified. Peptides were analyzed by using a micrOTOF-Q II (Bruker, Germany). Protein identification was performed by searching a nonredundant protein sequence database (National Center for Biotechnology Information) with the Mascot program (http://www.matrixscience.com).

RESULTS

Skin tests and CAP-RAST

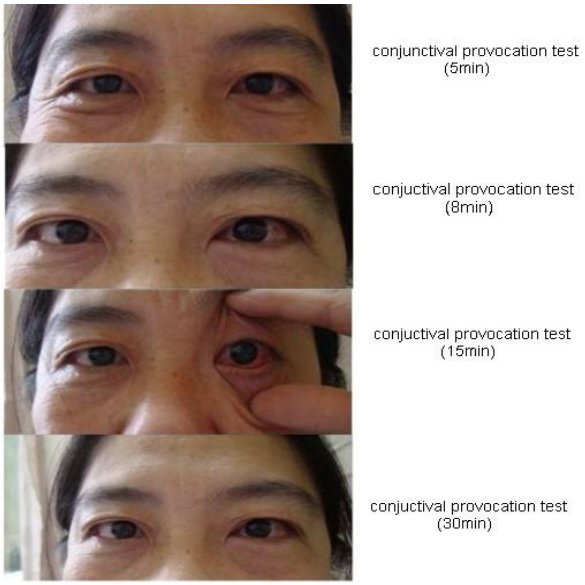

Figure 1. Conjunctival provocation test with pollen peach extracts (0.01mg/ml). Right eye: peach pollen extract, 0.05ml; Left eye: 0.9% saline solution, 0.05ml

Table 1 shows the results of skin testing and serum specific IgE. Intracutaneous tests with common aeroallergens were positive to mugwort (*Artemisia vulgaris).* Skin prick tests with the 5 *Rosaceae* pollens extracts were positive in the patient. Prick-to-prick testing with the 4 *Rosaceae* fruits showed negative results. Skin tests with *Rosaceae* pollens and fruits were negative in the control subject.

Conjunctival Provocation Testing

Conjunctival provocation testing in this patient was positive at a peach pollen extract concentration of 0.01mg/ml. (Figure 1)

SDS-PAGE Immunoblotting and Immunoblotting Inhibition

SDS-PAGE of these five pollens showed multiple protein bands with an apparent molecular mass ranging from 10 to 110kD (Figure 2a). As shown in Figure 2b, sera from the patient showed clear IgE binding to 20kD, 60kD, and 80kD proteins in peach pollen extract. Similarly the patient's sera recognized apricot pollen proteins of 20kD, 55kD, and 80kD. The sera showed weak IgE binding to 20kD and 80kD components of cherry pollen, and the IgE reactivity protein in apple pollen appeared to be at 80kD and 110 kD. Compared with the control sera, the faint IgE binding bands (80kD) in pear pollen extracts with the patient's sera was non-specific.

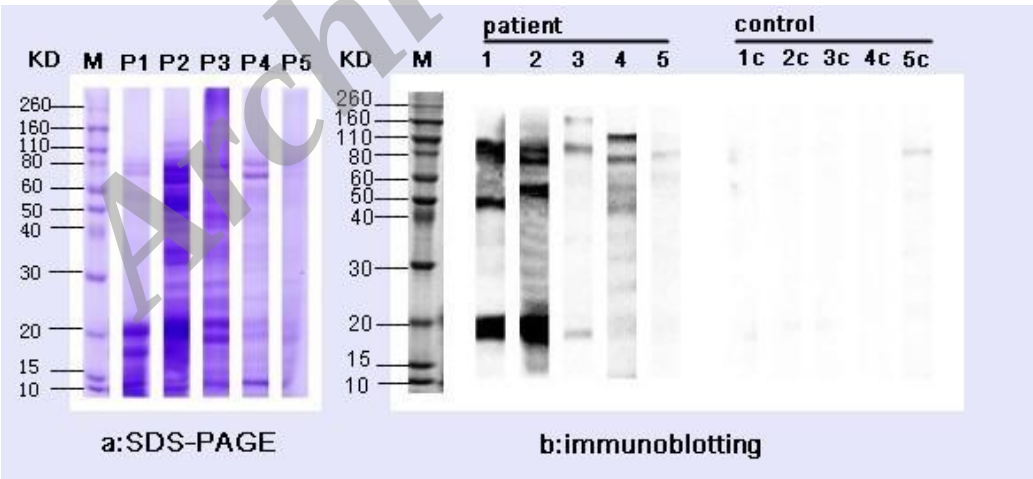

Figure 2. SDS-PAGE analysis and immunoblotting. (a): SDS-PAGE analysis. P1: peach pollen extract; P2: apricot pollen extract; P3: cherry pollen extract; P4: apple pollen; P5: pear pollen extract; (b): immunoblotting with five pollens. Molecular weight bands are shown to the left. (kD); lane1-5: serum from patient with immunoblotting with 5 pollens. lane1: peach pollen extract; lane2: apricot pollen extract; lane3: cherry pollen extract; lane4: apple pollen;lane5: pear pollen extracts. Lane 1c-5c: serum from control with immunoblotting with 5 pollens. lane1c: peach pollen extract; lane 2c: apricot pollen extract; lane3c: cherry pollen extract; lane4c: apple pollen extract; lane5c: pear pollen extract

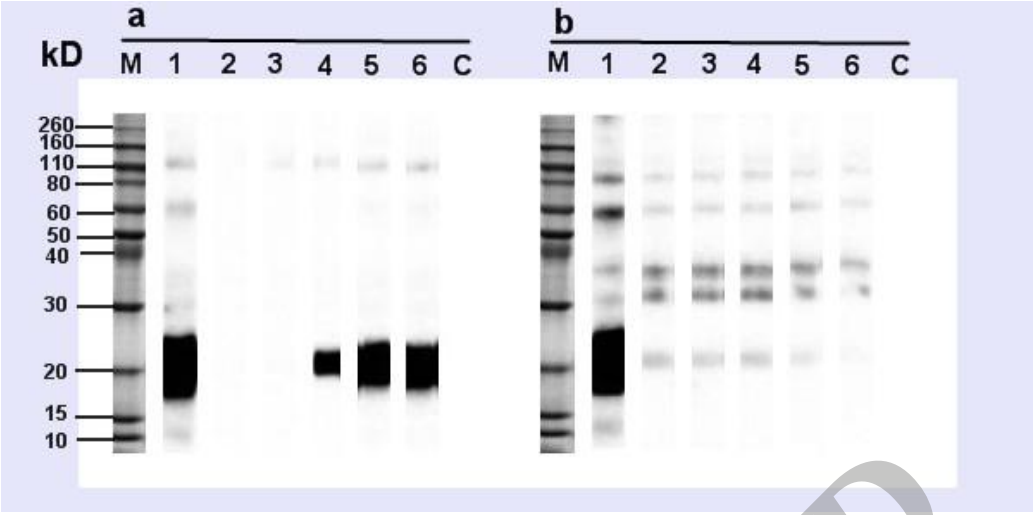

Figure 3 SDS-PAGE immunoblotting inhibition assays. (a) lane M:molecular weight. lane 1 patient's serum preincubated with bovine serum albumin(negative control of inhibition) ; lane 2: patient's serum preincubated with 10μg peach pollen extract(homologous inhibition; positive control of inhibition);lane3-6: serum form patient with immunoblotting with peach pollen extract after preincubation with 10μg apricot pollen extract(3),10μg cherry pollen extract(4), 10μg apple pollen extract(5),10μg pear pollen extract(6), respectively. Lane C is control serum. (b): lane M: molecular weight. lane 1 patient's serum preincubated with bovine serum albumin(negative control of inhibition) ; lane 2-6: serum form patient with immunoblotting with peach pollen extract after preincubation with 0.625μg apricot pollen extract(2), 1.25μg apricot pollen extract(3),2.5μg apricot pollen extract (4),5μg apricot pollen extract (5),10μg apricot pollen extract(6), respectively. Lane C is control serum

SDS-PAGE immunoblotting inhibition showed nearly complete inhibition when the patient's sera was pre-incubated with apricot pollen extract (10μg) and partial inhibitions when the sera was pre-incubated with cherry pollen (10μg), apple pollen (10μg) and pear pollen(10μg) (Figure 3a). Figure 3b showed the inhibition with different concentrations of apricot pollen extract.

ELISA and ELISA Inhibition

Specific IgE antibody against three pollens (peach, apricot and cherry) could be demonstrated in the patient (Figure 4). Using peach pollen as a solid phase, cross-reactivity studies by ELISA inhibition performed with the patient's sera showed that the other four pollens induced partial inhibitions ranging from 45% to 87%, with apricot pollen causing the strongest inhibition. When apricot pollen was used as a solid phase, 31%~86% inhibition was observed after pre-incubation of the serum with the four kinds of pollen, with peach pollen as the strongest inhibitor. When cherry pollen was used as solid phase, it showed that the other four kinds of pollens produced partial inhibitions ranging from 33% to 78%, with peach pollen and apricot pollen showing a strong and nearly equivalent inhibition rate of 78% (Figure 5).

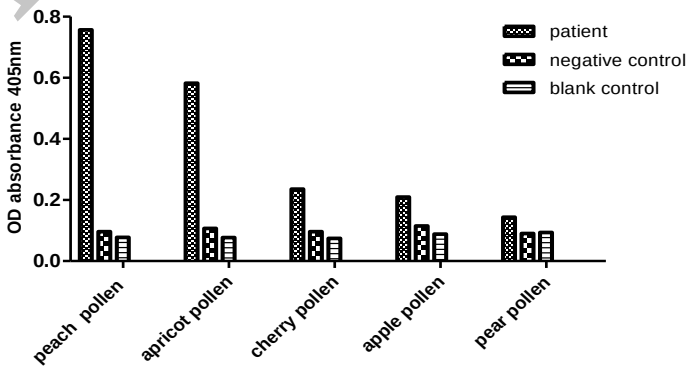

Figure 4. IgE binding to five Rosaceae pollens in ELISA

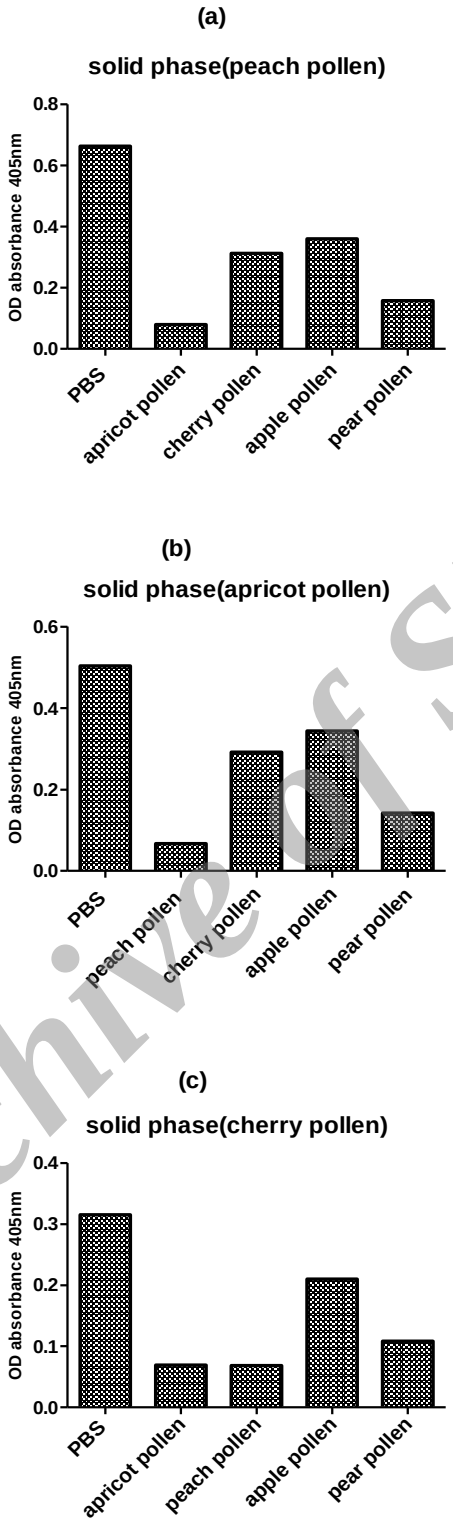

Figure 5. ELISA inhibition experiments (a) peach pollen as a solid phase, apricot pollen, cherry pollen, apple pollen, pear pollen were able to induce partical inhibitions ranging from 45%-87%, respectively; (b) apricot pollen as a solid phase, peach pollen, cherry pollen, apple pollen and pear pollen were able to inhibit ranging from 31%-86%; (c) cherry pollen as a solid phase, apricot pollen, peach pollen, apple pollen and pear pollen were able to reduce partical inhibitions ranging from 33%-78%

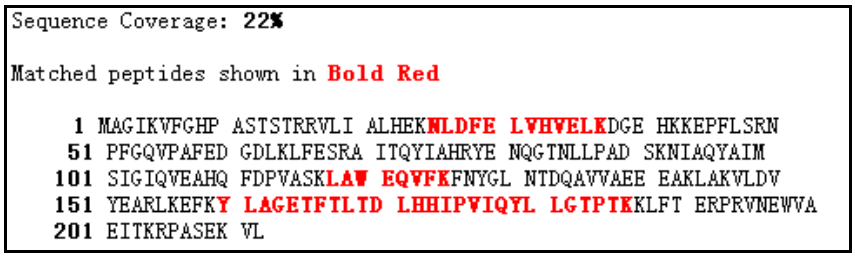

Figure 6. Sequence coverage and matched peptides to Glutathione S-transferase 16 sequence.

Mass Spectrometry

The 20 kD IgE binding band from peach pollen extract was identified by means of MS and it turned out to be homologous to Glutathione S-transferase 16. Twenty two percent coverage was obtained by means of matrix-assisted laser desorption/ionization (MALDI) analysis, with 3 peptides identified. A score of 537 was generated by using the Mascot program, which corresponds to *Arabidopsis thaliana*. The MS/MS analyses of 3 peptides NLDFELVHVELK, LAWEQVFK and YLAGETFTLTDLHHIPVIQYLLGTPTK matched with positions 26 to 37,118 to 125,159 to 186 in the Glutathione S-transferase 16 sequence (Figure 6).

DISCUSSION

The present study described one patient with work-related rhinoconjunctivitis and bronchial asthma due to peach tree pollen allergy. Occupational allergy due to orchard pollen in orchard workers is rare. Teranishi et al reported occupational allergy due to artificial pollination of the Japanese pear.[13] Iraneta et al described six patients with orange tree pollen sensitization.[14]

Our study proves that IgE-mediated peach tree pollen sensitization can elicit clinical symptoms even when the patient has no clinical relevant peach allergy. Our findings were similar to the paprika or tomato pollen allergy patients [15] and the orange pollen allergy patients.[14] Van Toorenenbergen et al [15] reported 3 patients with occupational allergy to paprika or tomato pollen who had no prior allergic symptoms upon consumption of paprika or tomato fruit. Iraneta et al [14] showed that 6 patients with orange tree pollen allergy had never suffered from orange allergy before.

In our study, no evidence showed the sensitization to peach and other fruits by skin testing. In contrast, Iraneta et al [14] demonstrated the cross-reactivity between orange tree pollen and orange fruit in 6 orange pollen allergy patients, two of which have a positive skin testing to orange fruit extract. The 30 kD protein seemed to be the cross-reactivity allergen. Similarly, van Toorenenbergen et al [15] indicated the presence of IgE against tomato and paprika pollen in 3 greenhouse workers and 3 tomato and paprika allergic patients.

Our study indicated the presence of general cross reactivity among five *Rosaceae* family pollens. Cross reactivity among *Rosaceae* fruits (apple, apricot, peach, and plum) have been well documented. Non-specific lipid transfer proteins (nsLTP) was the major cross-reactivity allergen.[16] Our study suggests that the cross-reactive allergen may be different among *Rosaceae* fruits and *Rosaceae* pollens. Western blotting inhibition showed that the 20 KD IgE-binding band not only appeared to be the cross-reactivity allergen between peach pollen and other four pollens, but also turned out to be homologous to Glutathione S-transferase 16(GST16), a major allergen of *Arabidopsis thaliana* (Alt a13). GST represented a well conserved, multifunctional enzyme superfamily, present in virtually all organisms from bacteria to humans.[17] GST family allergens in mites (Der p8),[18] cockroaches (Bla g5) , [19] fungi , [20] birch pollen [21] and wheat [22] have been reported. GST as a cross reactive allergen between mites,[18] cockroaches, [23] fungi [20] and helminthes [24] has been demonstrated.

The patient had a strong skin response to mugwort pollen without clinical relativity, which suggested possible cross-reactivity between mugwort and peach pollen allergen. Studies have also showed the cross-reactivity between mugwort and peach allergens.[25] Further studies are necessary to determine the cross-reactive allergen between mugwort and peach pollen.

The strength of this study is robust. We have proved IgE mediated allergy induced by *Rosaceae* family tree pollens both in vivo and vitro testing. We also proved the undoubted allergy to *Rosaceae* tree pollens with conjunctival provocation test. In addition, we found out with western blotting method that a 20 KD protein, which appeared to be a cross-reactive allergen through

western blotting inhibition, was recognized as a protein homologous to glutathione s-transferase 16 from *Arabidopsis thaliana*. It is a new finding and has not been reported ever before.

The limitation of our study was to enroll just one patient; therefore the conclusions still need further investigation. Thus we are planning to carry out a multi-centre survey to find out more patients to confirm the findings of the current study.

In conclusion, peach and other *Rosaceae* family tree pollen may serve as a potential cause of IgE mediated occupational respiratory disease in orchard workers in north China. Further epidemiologic studies are needed to assess the importance of this aeroallergen among exposed peach orchard workers and among the general population living nearby area.

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