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A study of cross-reactivity between citrus fruit and pollen allergens in oral allergy syndrome and food-dependent exercise-induced anaphylaxis in Japan

Nobue Takamatsu[1], Yasuto Kondo, MD, PhD[2], Ikuya Tsuge, MD, PhD[1], Yoichi Nakajima, MD, PhD[1], Norihiko Naruse, MD[2], Kenichi Tanaka, MD, PhD[2], Chisato Inuo, MD[1], Takeshi Hayashi, PhD[3], Tsukasa Matsuda, PhD[4], Tetsushi Yoshikawa, MD, PhD[1], Atsuo Urisu, MD, PhD[2]

[1]Department of Pediatrics, Fujita Health University School of Medicine, Toyoake, Aichi, Japan, [2]Department of Pediatrics, Fujita Health University, The Second Teaching Hospital, Nagoya, Aichi, Japan, [3]Department of Nutrition and Food Science, Beppu University, Beppu, Oita, Japan, [4]Graduate School of Bioagricultural Sciences and School of Agricultural Sciences, Nagoya University, Nagoya, Aichi, Japan

Abstract

Objectives: Oral allergy syndrome (OAS) associated with citrus fruits has been previously reported. However, in Japan, although rare, citrus allergy is also associated with food-dependent exercise-induced anaphylaxis (FDEIA). This study was done to determine whether different allergens may be involved in these two responses, and to examine cross-reactivity between popular citrus fruits and local pollen allergens.

Methods: Twelve patients were studied who had a history of OAS (8 cases) or FDEIA (4 cases), and had positive IgE antibody titers or tested positive with a skin prick test to citrus antigens. Serum immunodetection assays and immunoblot inhibition assays were performed with extracted Valencia orange (rCit s 2), selected antigens in white birch pollen (WBP) and orchard grass pollen (OGP).

Results: Immunodetection assays demonstrated an allergen associated with OAS as the 14-kDa protein, Cit s 2. An as yet unidentified 54-kDa protein was demonstrated in FDEIA patients. Cross-reactivity between orange and pollen antigens was confirmed by the following results: 1) Significant correlations between serum specific IgE levels for orange and OGP, 2) Immunoblot inhibition with WBP and OGP pollen antigens against rCit s 2. In Immunoblot inhibition, WBP and OGP both pollen antigens inhibited against rCit s 2.

Conclusion: The main allergen of OAS induced by orange was Cit s 2, which has cross-reactivity with WBP and/or OGP in Japanese patients with orange allergy. IgE-binding protein pattern in patients with FDEIA induced by orange were different from that in OAS. A 54-kDa protein was assumed to be a candidate for clinically relevant allergens in the development of the FDEIA.

Keywords: orange allergy, oral allergy syndrome, food-dependent exercise-induced anaphylaxis, Cit s 2, cross-reactivity

Introduction

Citrus fruits are widely produced worldwide. In Japan, they are the second most commonly consumed type of fruit, following bananas, and are included in the items recommended to be labeled as processed food containing allergens by the Japanese Food Sanitation Act. In the botanical classification, citrus fruit belongs to *Citrus* of Rutaceae in Sapindales. The main *Citrus* species include the orange group (*Citrus sinensis*), such as Valencia and navel, the mikan group, such as mikantangerine (*Citrus unshiu*) and mandarin (*Citrus reticulata*), the flavorful acid citrus fruit group, such as grapefruit (*Citrus x paradisi*), lemon (*Citrus lemon*), and yuzu (*Citrus junos*), and the other citrus group, such as Hassaku orange (*Citrus hassaku*). The most popular citrus species consumed in Japan is the tangerine.

Various citrus fruit allergens have been reported. Specifically, Cit s 1 of orange (23–24-kDa germin-like proteins: GLP),[1,2] Cit s 2 (13–14-kDa actin-binding proteins: profilin),[1,3] Cit s 3 (9-kDa lipid transfer proteins: LTP),[4] Cit l 3 of lemon,[4] and Cit r 3 of mandarin orange[5] have been identified as allergens. Minor allergens include isoflavone-reductase, which shows cross-reactivity with Bet v 6, a birch allergen.[6,7]

GLP belongs to the cupin super family, and germin is a heat-resistant glycoprotein produced in the early phase of wheat germination. Germin-analogous proteins are widely present in plants[8] and have been reported as allergens inducing celery-birch-mugwort-spice syndrome, in addition to an orange allergen[9]. Profilin is a protein present in the eukaryotic cytoskeleton, which causes cross-reactivity between plants, such as pollen and fruit. Clinical relevance has been suggested for the association between pollinosis and food allergy.[10,11] LTP belongs to the PR-14 family of pathogenesis-related PR proteins, and has been widely reported as a fruit, vegetable, and pollen allergen. It is stable against heat and digestive enzymes, and induces systemic symptoms in many cases.[12]

In Europe, orange allergy has been reported, and it has been demonstrated that almost all of the patients were sensitized to Cit s 1 or Cit s 2.[1-3] However, there were no reports about these sensitization rates in Japan. Most of these patients had pollinosis, for which the clinical relevance of grass or olive[3,7] or birch pollen (Bet v 2)[13] has been suggested. According to these reports, few patients presented with anaphylaxis and food-dependent exercise-induced anaphylaxis (FDEIA).[14,15] Recently,

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Corresponding author : Nobue Takamatsu
Department of Pediatrics, Fujita Health University School of Medicine, 1-98 Dengakugakubo Kutsukake-cho, Toyoake, Aichi 470-1192, Japan
E-mail: mxe03340@fujita-hu.ac.jp

oral allergy syndrome (OAS) caused by orange intake has been occasionally noted in Japan. In addition, FDEIA cases have been infrequently reported in Japanese journals.[16]

We examined Japanese orange allergy patients with two different phenotypes: OAS and FDEIA. The sensitization patterns of orange-specific allergens were compared, and the correlation between the serum levels of specific IgE to orange and pollen, as well as their cross-reactivity, was investigated.

Methods

Patients

The subjects were outpatients who had been treated from 2010 to 2012 at Fujita Health University Hospital, Fujita Health University Second Teaching Hospital, National Hospital Organization Fukuoka Hospital, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Ehime Seikyo Hospital, and Aichi Children's Health and Medical Center. Patients with a history of allergic symptoms due to citrus ingestion and specific class 2 or above IgE antibody titers of orange measurable with ImmunoCAP® (Thermo Scientific, Uppsala, Sweden) were included. The patients who gave their consent underwent sublingual challenge and exercise load tests depending on their symptoms to examine the reproducibility of their symptoms. The study protocol was reviewed by the Epidemiological and Clinical Study Ethics Committee of Fujita Health University (10-216), and written informed consent was obtained from all patients.

Oral food challenge (OFC), sublingual challenge, and exercise load tests

The subjects were diagnosed with OAS and FDEIA based on their histories. Of these patients, consenting OAS patients (Cases 4, 6, 7, and 8) underwent a sublingual challenge test, and FDEIA patients (Cases 9, 11, and 12) underwent an exercise load test to examine the reproducibility of symptoms. The sublingual challenge test was conducted using an open challenge method according to the Japanese Guidelines for Food Allergy 2014.[17] Specifically, in the sublingual challenge test, 5 g of citrus pulp was placed under the tongue for 15 min, and was directly swallowed if symptoms were mild. Subsequently, 70 g (equivalent to one fruit) was ingested if possible.

The exercise load test was conducted according to the Japanese Pediatric Guidelines for Food Allergy 2012.[18] Specifically, Case 9 ingested 70 g of Valencia orange and underwent treadmill exercise for 20 min and, on a different day, ingested 70 g of Valencia orange after aspirin preadministration. Subsequently, Case 10 ingested 200 ml of mikan juice and underwent exercise for 60 min. Case 12 ingested 70 g of mikan and underwent free running until the heart rate reached 160 bpm. However, Case 11 did not undergo an exercise load test because consent could not be obtained.

Specific IgE antibody titer, skin prick test

Specific IgE antibody titers against Japanese cedar pollen (*Cryptomeria japonica*; JCP), orchard grass pollen (*Dactylis glomerata*; OGP), white birch pollen (*Betula pendula*; WBP), and grapefruit, along with orange, were determined with ImmunoCAP® (Thermo Scientific, Uppsala, Sweden).

The skin prick test was performed using raw citrus fruits. Saline was used as a negative control, and 10 mg/mL histamine phosphate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as a positive control. The skin on the flexion side of the forearm was pricked once with the sample using PRICK-LANCETTER (Ewo Care AB, Gislaved, Sweden), and the reaction was judged after 15 min. In this judgment, the major axis of the wheal and the diameter vertical to it were measured, and the mean was compared with those of wheals induced by the controls. The reaction was graded as 3+ or 4+ when the wheal size was equivalent to or exceeded that of the positive control-induced wheal, respectively, 2+ when the size was 1/2 of that of the positive control-induced wheal, and 1+ when the size was smaller than 2+, but greater than that of the negative control-induced wheal; a grade of 2+ or higher was regarded as positive. As a patient control, two patients without subjective symptoms induced by the ingestion of citrus fruit and their processed food underwent the same test, and they were confirmed to be negative for a reaction.

Valencia orange and pollen extraction

Valencia orange was purchased at a supermarket. Antigens were extracted from the raw pulp of Valencia orange, excluding flavedo (peels). After grinding the flesh, the sample was mixed 1:5 with sucrose buffer [1 M sucrose, 2% (wt/vol) polyvinylpolypyrrolidone (Sigma–Aldrich Co., St. Louis, MO, USA), 2 mM Ethylenediamine-N,N,N′,N′-tetraacetic acid disodium salt dehydrate, 10 mM sodium N,N-diethyl dithiocarbamate trihydrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan)], and the pH was adjusted to 8.0 with NaOH. After gently stirring at 4°C overnight, the mixture was centrifuged (4°C, 20,000 × g10,000 rpm, 20 min) and filtered through MF-Millipore 0.45 μm (Merck Millipore, Billerica, MA, USA), and the supernatant was used as a sample.[19] Protein in the sample was measured using bovine serum albumin as a standard, by the Bradford method.[20]

Regarding pollen extract, WBP was purchased from Allergon AB (Ängelholm, Sweden), and OGP was purchased from TORII Pharmaceutical Co., Ltd. (Tokyo, Japan). Pollen was dissolved with 0.125 M ammonium bicarbonate (Wako Pure Chemical Industries, Ltd., Osaka, Japan), stirred overnight, centrifuged, and the supernatant was collected as a sample, similarly to the fruit extract preparation.

Preparation of recombinant Cit s 2 (rCit s 2)

rCit s 2 was prepared using the pET system (Novagen, Darmstadt, Germany). Cit s 2 (gene accession number: AJ865015) was synthesized by Eurofins Genomics (Ebersberg, Germany). Cit s 2 was inserted into the *Nde* I/*Bam*H I restriction enzyme sites of the pET–15b expression vector. A His-tag was attached to the C-terminus of the target gene. rCit s 2 was expressed in *Escherichia coli* BL21(DE3) pLysS. The cells were harvested by centrifugation at 4℃ and 4500 × g for 10 min, washed with sonication buffer (50 mM Tris–HCl, pH 8.0, 300 mM NaCl), and then resuspended in the same buffer. The cells were disrupted by sonication, followed by centrifugation at 20,000 × g for 30 min at 4℃. The supernatant of the cell extract was loaded onto a Ni-NTA His-Bind Resin (Novagen, Darmstadt, Germany) and rCit s 2 was eluted by stepwise increments in the imidazole concentration in the sonication buffer. The fractions containing r Cit s 2 were dialyzed (SnakeSkin Pleated Dialysis Tubing 3500 MWCO; Thermo Fisher Scientific, Rockford, IL, USA) against sonication buffer.

Immunodetection assays

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS-PAGE) was performed using the NuPAGE SDS-PAGE Gel System (Life Technologies Co., Carlsbad, CA, USA), comprised of NuPAGE Precast Gel (4-12% Bis-Tris; Life Technologies Co., Carlsbad, CA, USA), MOPS Buffer (NuPAGE MOPS SDS Running Buffer; Life Technologies Co., Carlsbad, CA, USA), and reducing agent (NuPAGE Antioxidant; Life Technologies Co., Carlsbad, CA, USA).

The extracted Valencia orange (12 μg of protein/2D well) was separated on SDS-PAGE (200 V, 50 min), and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon, 0.45 μm; Merck Millipore, Billerica, MA, USA) employing Western blotting (250 mA, 60 min). The PVDF membrane was incubated with 20 μL of serum diluted 1:30 with B114 buffer (PBS containing 0.1% BRIJ; Sigma–Aldrich). After the reaction, goat anti-human IgE antibody (1:2,000 dilution; KPL, Gaithersburg, MD, USA) was added to the membrane and left to stand for 3 h at room temperature. The color was developed by reaction with 5-bromo-4-chloro-3-indolyl-phosphate/ nitro blue tetrazolium (BCIP/NBT) substrate (KPL, Gaithersburg, MD, USA). Cord serum was used as a negative control.

Immunoblot inhibition

For immunoblot inhibition, 50 μl each of antigen solutions of extracted Valencia orange (15μg of protein), rCit s 2 (1.8 μg of protein), and OGP and WBP pollen (10 μg of protein) were mixed with 20 μl of the patient's serum prior to incubating on a PVDF membrane with Valencia orange antigens transferred as described above, for the detection of IgE-binding protein in the same manner as in immunoblotting.

Statistics

Statistical analysis was performed using SSRI STAT2010 (Social Survey Research Information Co., Ltd., Tokyo, Japan). Antibody titers of >100 U_A/ml and <0.35 U_A/ml were determined to be 100 U_A/ml and 0.35 U_A/ml, respectively.

Results

Twelve patients (10 females and 2 males) aged 6–22 years (median 11 years) were included (Table 1). Allergic reactions were determined by interview Cases 1–8 were only OAS. Cases 9–12 showed no acute symptoms after ingestion, and were diagnosed with FDEIA, with systemic urticaria, hypotension, and dyspnea induced by exercise after ingestion. Accordingly, we divided these cases into OAS and FDEIA groups. The FDEIA group showed symptoms due to exercise after ingestion. In addition, these patients were also allergic to other types of fruit beside citrus (e.g., peach, apple, and kiwifruit)

Challenge test

Among the consenting cases of 3, 4, 6, 7, and 8, cases 3, 4, and 7 showed OAS in the sublingual challenge test with 5 g of Valencia orange. The test was discontinued because they refused further ingestion. Cases 6 and 8 showed OAS after ingestion of an additional 70 g.

Valencia orange IgE-binding proteins in each case

Valencia orange IgE-binding proteins were detected at 14, 18, 24, 38, 52, and 54 kDa (Figure 1 and Table 2). These IgE-binding proteins were not stained with negative control serum on immunoblot, and they disappeared upon immunoblot inhibition by serum reacted with Valencia orange extract beforehand, confirming that these were Valencia orange-specific bands.

Cases 1–7 responded to the 14-kDa protein. Cases 6, 9, and 10 responded to the 18-kDa protein. Cases 1, 4, 5, 8, and 10 responded to the 24-kDa protein. Cases 6, 9, 10, and 12 responded to the 38-kDa protein. Cases 6, 9, and 10 responded to the 52-kDa protein. Cases 6-12 responded to the 54-kDa protein.

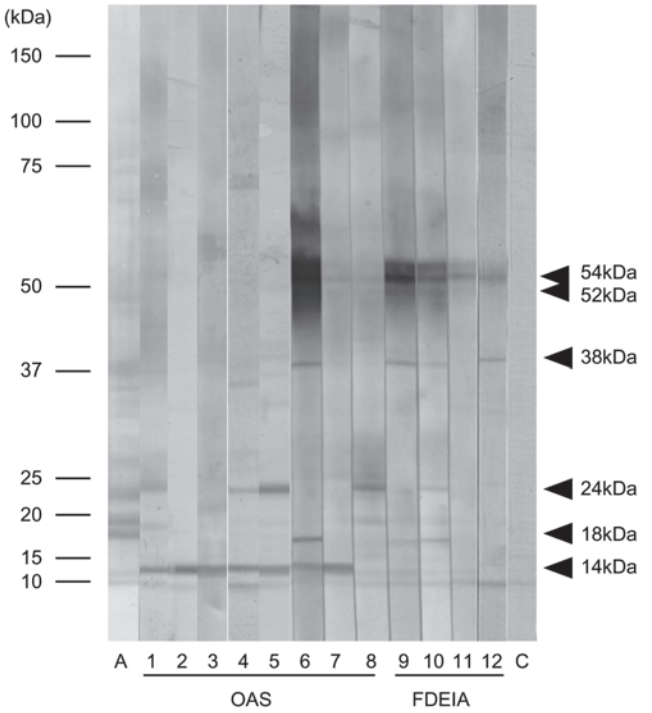
The 24-kDa protein is assumed to be Cit s 1 based on its molecular weight. The 14-kDa protein was considered to be Cit s 2 because its band disappeared in immunoblot inhibition using patient's serum pre-incubated with rCit s 2. Positive rates were 50% and 88% for Cit s 1 and 2, 38% for the 54-kDa protein in the OAS group, 25% and 0% for Cit s 1 and 2, and 100% for the 54-kDa protein in the FDEIA group, respectively.

Table 1. Patient background and specific IgE antibody titer(ImmunoCAP®) and skin prick test to citrus group

Group	No.	Age	Sex	Allergy to orange and/or mikan		Allergen		Pollinosis	ImmunoCAP®(UA/ml)		SPT		
				Symptoms by orange	Challenge test	Citrus	Other fruit and vegitables		Orange	Grapefruit	Orange (*Citrus sinensis*)	Mikan (*Citrus unshiu*)	Grapefruit (*Citrus ×paradisi*)
OAS	**1**	6	M	OAS	—	Orange	Tomato, Cucumber, Lettuce	+	3.22	4.65	2+	1+	2+
	2	11	F	OAS	—	Mikan	Tomato, Persimmon	—	3.74	10.6	2+	ND	ND
	3	12	F	OAS	○	Orange, Mikan	Kiwifruit, Melon, Peach	+	7.3	24.5	2+	3+	2+
	4	14	F	OAS	○	Orange, Mikan	Kiwifruit, Melon, Peach	+	1.21	8.86	2+	2+	—
	5	16	F	OAS	—	Orange, Mikan	Kiwifruit, Melon, Peach	+	0.83	1.86	—	—	—
	6	9	F	OAS	○	Orange, Mikan	Melon, Banana, Peach	+	0.7	1.53	2+	2+	2+
	7	11	F	OAS	○	Orange, Mikan	Kiwifruit, Melon, Peach	+	2.59	9.72	2+	2+	2+
	8	11	F	OAS	○	Orange, Mikan	Melon, Grape, Pineapple	+	2.79	3.59	2+	2+	2+
FDEIA	**9**	10	F	FDEIA:(swelling of eyelids, dyspnea, generalized erythema)	○	Orange, Mikan	Apple	+	0.85	<0.35	2+	3+	3+
	10	11	M	FDEIA:(generalized urticaria, dyspnea, hypotension)	○	Mikan	Peach, Apple	+	1.07	0.92	ND	ND	ND
	11	22	F	FDEIA:(generalized urticaria, hypotension)	—	Orange, Mikan	—	+	3.25	1.11	ND	ND	ND
	12	15	F	FDEIA:(wheezing, facial swelling, generalized urticaria)	○	Orange, Mikan	Peach, Apple	+	2.51	2.09	2+	2+	2+

OAS=Oral allergy syndrome NT=not tested FDEIA=food-dependent exercise-induced anaphylaxis

Figure 1



Immunoblot with patient's sera incubated with Valencia orange (Citrus sinensis) pulp extract; A (Amide black), C (non-allergic control)

Table 2. Immunoblot with IgE from patient's serum to the extracts from Citrus sinensis pulp

		Valencia orange(*Citrus sinensis*)					
		Immunoblot(kDa)					
Group	No.	14	18	24	38	52	54
OAS	**1**	+		+			
	2	+					
	3	+					
	4	+		+			
	5	+		+			
	6	+	+		+	+	+
	7	+					+
	8			+			+
FDEIA	**9**		+		+	+	+
	10		+	+	+	+	+
	11						+
	12				+		+

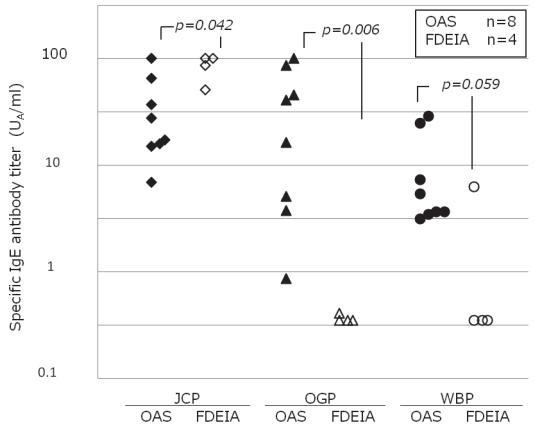
Relationship between orange and pollen

Antibody titers against each type of pollen are shown in (Table 3). These antibody titers were compared between the OAS and FDEIA groups (Figure 2). The specific IgE antibody titers against OGP were higher in the OAS group than in the FDEIA group (p=0.006, Mann–Whitney U test).

Table 3. Specific IgE antibody titer(ImmunoCAP®) of Japanese cedar (*Cryptomeria japonica* :JCP), orchard grass(*Dactylis glomerata* :OGP), and white birch(*Betula pendula* :WBP)

		ImmunoCAP®(UA/ml)		
Group	No.	JCP	OGP	WBP
OAS	**1**	36.8	40.9	5.35
	2	17.3	86	24.7
	3	27.6	>100	28.7
	4	65.2	16.3	3.67
	5	15.9	0.86	3.13
	6	6.91	5.12	3.67
	7	>100	45.2	7.3
	8	14.9	3.74	3.47
FDEIA	**9**	>100	<0.35	<0.35
	10	50.4	0.41	<0.35
	11	85.4	<0.35	<0.35
	12	>100	<0.35	6.28

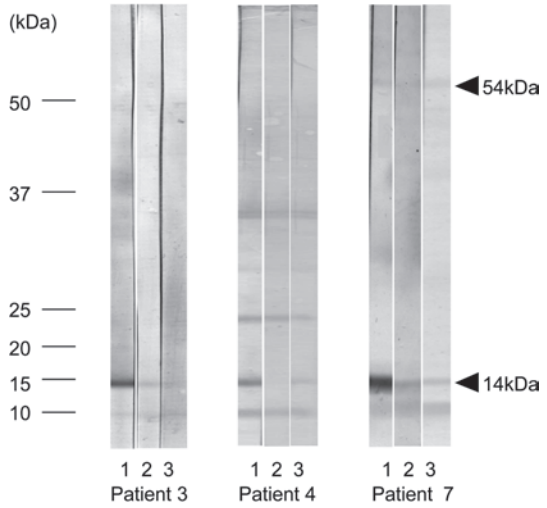
Figure 2



Comparison of serum-specific IgE levels between OAS and FDEIA groups

In addition, the relationship between orange and pollen IgE antibody titers was examined in the OAS group, demonstrating a significant correlation between OGP or WBP, and orange IgE (OGP: r=0.762, p=0.044, WBP: r=0.744, p=0.049, Spearman's rank correlation coefficient). However, unlike OGP and WBP, JCP did not correlate with orange (JCP: r=0.286, p=0.450). To examine the antigenicity common in OGP or WBP that correlated with orange (Cases 3, 4, 6, and 7), immunoblot inhibition was conducted, demonstrating the suppression of IgE binding to the 14-kDa protein (Figure 3).

Figure 3



Immunoblot inhibition of Valencia orange (*Citrus sinensis*) extract by pollen extracts (10 μg of protein). Inhibitor: 1: none, 2: orchard grass (*Dactylis glomerata*), 3: white birch (*Betula pendula*). Pooled sera: patients 3, 4, 7

Antigens common among citrus fruits

The IgE antibody titers against grapefruit and skin prick test results with Valencia orange, mikan, and grapefruit are shown in Table 1. Of 10 patients, 9 were positive for Valencia orange, while 7 out of 9 patients were positive for grapefruit and mikan by skin prick test.

Discussion

Previously, Ahrazem et al. demonstrated that the sensitization to Cit s 1 was 62% and to Cit s 2 was 87%[2,3] using an *in vivo* test. By conducting skin prick tests with patients using purified Cit s 1, they demonstrated that only 10% were positive. Thus, no symptoms may develop even if specific IgE antibody to Cit s 1 is positive. The sugar chain of Cit s 1 allows non-specific IgE binding,[21] so the clinical relevance of Cit s 1 remains controversial. There were no cases sensitized to Cit s 1 alone, among our cases.

Similarly, Crespo et al.[1] demonstrated that 78% and 96% of a cohort of 23 Italian patients were sensitized to Cit s 1 and 2, respectively. In that study, it was reported that Cit s 2 was the main allergen because stimulation with orange extract caused histamine release in Cit s 2-positive patients, although some Cit s 2-positive patients present no clinical symptoms.

Our Japanese patients showed sensitization rates to Cit s 1 of 22.7% and Cit s 2 of 77.3% overall. The results demonstrated that 7 outmore than half of the 12 patients reacted to Cit s 2, yet 7 out of 8 patients in the OAS group and none in the FDEIA group reacted. Two cases (cases 2 and 3) seemed to react only to Cit s 2. Thus, this suggests that the major Valencia orange allergen responsible for OAS is Cit s 2, which had comparable sensitization rates between Japanese and European patients. However, the sensitization rate to Cit s 1 was lower in Japanese patients than that in European patients.

Furthermore, Cit s 2 has been identified as the main cause of OAS by cross-reaction with pollen,[3] for which the clinical relevance of grass and olive[3,7] and birch pollen (Bet v 2)[13] has been suggested. The correlations with IgE antibody titers and the immunoblot inhibition suggest that the source of sensitization in our cases was WBP or OGP. No reports have been published on profilin as an OGP allergen. However, we observed that in immunoblot inhibition against Valencia orange allergen, IgE binding to Cit s 2 disappeared after OGP addition, suggesting the presence of a protein comparable to profilin in OGP.

In addition, 7 out of 12 patients were positive for the 54-kDa protein (three patients in the OAS group and all patients in the FDEIA group). Thus, the 54-kDa protein may be responsible for orange FDEIA. Unfortunately, the amino acid sequence of the 54-kDa protein could not be analyzed. However, subsequent analysis with a matrix assisted laser desorption/ionization time of flight (MALDI TOF) mass spectrometer suggested that it may be an enolase-like protein (*Citrus sinensis*: gi|568856679). It is currently under investigation.

Crespo et al.[1] and Serra et al.[22] reported that the high-molecular-weight protein (around 50 kDa) observed in their orange allergy patients showed a high reaction coincidence rate with Cit s 1. However, our 54-kDa protein was not considered the same as Crespo's high-molecular-weight protein because only two out of seven patients were simultaneously sensitized to the Cit s 1 band.

Interestingly, there were reactions for the 54-kDa protein that seemed to be critical for FDEIA in cases 6–8 of the OAS group. Similarly, some patients with OAS reacted to the 54-kDa protein in the immunoblotting performed by Crespo et al. FDEIA did not develop in these patients, which may be explained by insufficient intake.[23] However, although the pathophysiology of FDEIA remains unclear, various inducers, such as intake and exercise,[24] are involved in its development.[25] Further research is needed on the pathogenic mechanism of FDEIA.

Finally, as reported in Europe, the main allergen of OAS was a 14-kDa protein (Cit s 2), which has cross-reactivity with WBP and/or OGP in Japanese patients with orange allergy. The clinically relevant allergen in the development of FDEIA by orange was different from that of OAS and was suggested to be a 54-kDa protein, but it remains undefined. Further study is required.

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Conflict of interests

The authors declare no conflict of interest associated with this manuscript.

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