Occupational respiratory allergy to lettuce in lettuce farmers

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Abstract

Background: Lettuce-associated respiratory allergy has never been reported before. The aim of this study was to clarify the clinical condition of lettuce-associated respiratory allergy and to identify the lettuce antigen which induces allergic symptoms.

Methods: We distributed questionnaires to 1,168 lettuce farmers and performed medical examinations in those who exhibited respiratory symptoms related to occupational exposure to lettuce. We analyzed specific IgE-binding proteins in the sera of patients through immunoblotting analysis and determined molecular characterization of the IgE-binding bands using liquid chromatography–mass spectrometry.

Results: A total of 932 farmers(80%) responded to the questionnaire. Of those, 7% exhibited lettuce-associated respiratory symptoms, during harvesting and packaging. Thirteen patients were diagnosed with allergy to lettuce and agreed to undergo further examinations. The percentage of activated basophils in these patients was significantly higher compared with that reported in negative controls ($P < 0.05$). Lettuce-specific IgE (ImmunoCAP®) and skin prick testing was positive in 46% and 62% of patients, respectively. Notably, occupational lettuce-allergic asthma was detected in one patient through specific bronchial provocation testing. The IgE-binding bands recognized in the sera of >50% of patients were identified as epidermis-specific secreted glycoprotein EP1-like (51 kDa).

Conclusion: The present analysis identified a novel lettuce allergen. This allergen may have clinically useful applications, such as specific IgE testing and allergen specific immunotherapy.

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1.Introduction

Exposures to allergens increases the risk of occupational respiratory conditions, including asthma and asthma-like syndromes(i.e., eosinophilic bronchitis, aluminum pot room asthma, and irritable

larynx syndrome)(1-3). Occupational asthma can be classified into two types: immunologically induced asthma, and irritant-induced asthma. The former type involves allergens that can be divided into two groups, according to their molecular weight. The high molecular weight agents (>10 kDa) include animal or plant allergens, and induce the production of specific IgE antibodies and typical allergic responses. In contrast, the low molecular weight agents include occupational chemicals that induce asthma through unknown mechanisms(3).

Although the means by which farmers become sensitized is unknown, grains and cereals,(e.g., wheat, rye, and barley), are established plant-derived antigens(3). Although lettuce (*Lactuca sativa*) is extensively cultivated globally, occupational lettuce-induced respiratory allergy is rarely reported. Only a limited number of case reports have specifically reported respiratory symptoms in lettuce farmers in Japan(4). In contrast, both contact and food allergy to lettuce have recently been described(5-10). Indeed, previous studies using IgE antibodies identified several proteins as possible allergens in the sera of patients with lettuce anaphylaxis or oral allergy syndrome (OAS)(7-12). Therefore, the aim of this study was to: [1] to clarify the clinical condition of lettuce-induced respiratory allergy, and [2] to identify new major allergens recognized by IgEs in the sera of patients with lettuce-induced respiratory symptoms.

2.Material and methods

2-1 QUESTIONNAIRE

In 2013, we distributed a questionnaire to all 1,168 lettuce farmers in the Hyogo prefecture, Japan (Supplementary Table 1). In 2017, this questionnaire was also distributed to lettuce farmers in the Kagawa prefecture, Japan.

2-2 PATIENTS AND CLINICAL EXAMINATION

A total of 13 lettuce farmers with a clinical history of lettuce-induced respiratory symptoms during harvesting and packaging were enrolled into the study from the outpatient clinic of two general hospitals: Hyogo prefectural Awaji Medical Center and Kobe University Hospital, Japan. The patients underwent examinations in either of the two general hospitals. All patients provided written informed consent prior to their participation. Protocol approval was obtained from the institutional review board of both of the hospitals (permission number #280007). The general parameters of this study are openly available in UMIN at https://www.umin.ac.jp/, reference number UMIN000027002.

Following a detailed medical interview, skin prick tests (SPTs) were performed in all patients using lettuce leaves extracts, stem extracts and latex with applied water (0.1mg/mL). The skin was pricked with a drop of sample and the responses were read after 15 min. Ten mg/mL histamine dihydrochloride (Torii, Tokyo, Japan) and PBS served as positive and negative controls, respectively. A positive reaction was defined as a wheal diameter of ≥ 3 mm in the absence of a reaction to the PBS and in the presence of a positive reaction to histamine dihydrochloride. Determination of the total IgE and lettuce-specific IgE were performed using the CAP System® (Thermo-Fisher, Uppsala, Sweden). Determination of the other allergen specific IgE were performed using the CAP System® and fluorescence immunoassay(SRL, Tokyo, Japan). Basophil activation tests (BATs) were performed in all patients through the BAT system(BML,

Tokyo, Japan)using lettuce stem extracts, as described in the Appendix S1. Three patients were excluded as non-responder, with the result of no difference of positive control(1mg/ml anti-IgE antibody, Beckman Coulter) and negative control(PBS). The sera of seven non-allergic individuals were used as negative control. Specific inhalation challenges (SICs) were performed using lettuce stem extracts in the 4 patients who provided written informed consent. Placebo-controlled SICs were performed using normal saline 30 minutes prior to exposure to the lettuce stem extracts. Inhalation tests were performed with 5ml lettuce stem extract (0.04mg/ml, 0.4mg/ml) and 5ml PBS by ultrasonic nebulizer (Omron,Kyoto,Japan).–Bronchial reaction was monitored using FEV1(Forced expiratory volume in the first second). A positive SIC result was defined as ≧15% decrease of FEV1 versus the pre-challenge value (13).

2-3 PREPARATION OF THE LETTUCE EXTRACT

Fresh lettuce (*Lactuca sativa. var. capitate,* Supplementary Fig.2A) leaves and stem were grated using a commercial food grater. The grated lettuce was filtered through three layers of gauze.

The fresh lettuce stem was cut using a sharp scalpel, and the white juice(latex) spread on the section was collected(Supplementary Fig.2B). This lettuce latex was dissolved into distilled water to prevent hardening.

2-4 ELISA

ELISAs were used to detect the binding of patients and control serum IgE to the lettuce latex, lettuce stem and lettuce leaves extract. Fifty microliters of lettuce latex, lettuce stem extract, and lettuce leaves extract (50 μg/mL) in phosphate buffered saline (PBS) were added to each well of the ELISA plates (Asahi Glass, Tokyo, Japan) and allowed to coat overnight at 4^{o}C. After blocking the plates with 1% BSA in 10 mM PBS containing 0.1% Tween 20 (PBS-T) for 1 hour at room temperature and 3 washes with PBST, 1:50 dilutions of patient or control sera in Can get Signal® Immunoreaction Enhancer Solution1 (Toyobo Co.,LTD, Osaka, Japan) were added to the

wells and incubated for 1 hour at 37°C. After washing the wells with PBS-T 5 times, 1:3000 dilutions of HRP conjugated affinity-purified goat anti-human IgE antibody (Kirkegaard&perry Laboratories, Gaithersburg,MD, USA) in Can get Signal® Immunoreaction Enhancer Solution2 were added to the wells and incubated for 1 hour at 37°C. After 5 washes with PBST, the bound secondary antibodies were detected by reactions with 50 μL of a TMB peroxidase substrate (Kirkegaard&perry Laboratories). The reactions were stopped by the addition of 50 μL of 1 M phosphoric acid, which also amplified the signal. The absorbance of each well was measured at 450 nm using a plate reader (Wallac ARVO SX 1420 multi-label counter, PerkinElmer, Waltham, MA, USA).

2-5 SDS-PAGE

The lettuce protein (approximately 6-15 μg protein/lane) was separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE)(14). Proteins on the gel were stained with Coomassie Brilliant Blue R-350(GE Healthcare, Chicago, USA) or Silver Staining kit ProteoSliver (Sigma-Aldrich, St.Louis, USA) to detect the total protein patterns.

2-6 Immunoblot Analysis

The immunoblotting analysis was conducted by transferring the SDS-PAGE gel onto an Immobilon-P™ polyvinylidene difluoride membrane (Millipore, Burlington, USA) using a semi-dry blotting method(15). This method has been described in detail elsewhere(16). The sera of two non-allergic individuals were used as negative controls. Following an incubation in PBST containing 5% skim milk for blocking, the membrane was incubated overnight with human sera (1:20 dilution) at 4°C. After 3 washes with PBST for 10 minutes, the membranes were incubated with a HRP-conjugated goat anti-human IgE antibody (1:3000 dilution) for 1 hour, after which the membranes were washed 4 times with PBST for 10 minutes. The bound secondary antibodies were detected using an Enhanced chemiluminescence western blotting substrate (GE Healthcare,

Chalfont St. Giles, UK). The resultant chemiluminescent signals were detected on X-ray films (GE Healthcare).

2-7 Inhibition experiment

Fraction B10 (by a gel filtration column chromatography) was used for inhibition experiment. The fraction B10 was separated through SDS-PAGE and transferred onto PVDF membranes. Lettuce latex (0.1mg/mL) or distilled water were preincubated for 1hr at 4℃ with a patient 003's sera (diluted 1:1), before exposure to membranes. After preincubation, patient 003's sera were incubated with the membranes. Bound IgE was detected by using ECL Plus.

2-8 Purification of lettuce latex allergen

Allergen was purified from lettuce latex by ion-exchange chromatography and gel-filtration chromatography using high-performance liquid chromatography, as described in the Appendix S1. After selecting the appropriate fractions, the effluents were studied through SDS-PAGE and immunoblotting.

2-9 MASS SPECTROMETRY

Protein bands recognized in the sera of >50% of patients were selected for further study. The bands were extracted from the gel as previously described(16). LC-MS/MS analysis was performed a LCMS-IT-TOF instrument (Shimadzu, Kyoto, Japan) interfaced with a nano reverse-phase liquid chromatography system (Shimadzu). MS/MS data were analyzed using Mascot (Matrix Science, London, UK; version 2.3.01). The search parameters were as follows: enzyme, trypsin; variable modifications, carbamidomethyl (Cys), deamidated (Asn, Gln), and oxidation (Met); peptide mass tolerance, ±50 ppm; fragment mass tolerance, ±0.05 Da; max missed cleavages, 1.

2-10 STATISTICAL ANALYSIS

All statistical analyses were performed with EZR version 1.37 (Saitama Medical Center Jichi

Medical University; http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html; Kanda, 2018), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria, version 2.4.0).

Categorical data are presented as numbers and percentages. Numeric data presented as mean and standard deviation. Univariate analyses were performed using Fisher's exact test for categorical data. Parametric data analyzed using student t test, and Non-parametric data analyzed Mann-Whitney U test. Logistic regression models were used to analyze correlation associated with lettuce-induced respiratory symptoms. The results are expressed as odds ratios with 95% confidence intervals (CI). All p-values were two-sided and values <0.05 were considered statistically significant.

3.Results

3-1 QUESTIONNAIRE

A total of 932 lettuce farmers in the Hyogo prefecture responded to the questionnaire, representing an overall return rate of 80%. Table 1 shows the baseline characteristics of these lettuce farmers. Table 2 shows the point prevalence and characteristics of lettuce-associated symptoms. “Respiratory symptoms” means cough, sputum or dyspnea during any time of farming. “Respiratory symptoms associated with harvesting and packaging of lettuce” means cough, sputum or dyspnea only during harvesting and packaging lettuce. Among the responders, 8% reported specific lettuce-associated symptoms. Lettuce-associated respiratory symptoms were observed in 7% of the respondents. Forty nine farmers out of 63 farmers had respiratory symptoms during harvesting and packaging of lettuce. Of note, the mean time between the start of the occupation and onset of symptoms is 19 years, which seems to be relatively longer than other occupational allergic diseases(17). From Q6 of the questionnaire, only two lettuce farmers reported symptoms associated when eating lettuce. Multivariate analysis revealed that history of

bronchial asthma, allergic rhinitis, and pollinosis were significant factors related to lettuce-associated respiratory symptoms (P<0.05) (Supplementary Table 2). In 2017, the questionnaire was distributed to lettuce farmers in the Kagawa prefecture. Although the response rate in the Kagawa study was lower than that observed in the Hyogo study (34%), the analysis yielded almost identical results. Specifically, lettuce-associated respiratory symptoms were observed in 7% of respondents both in the Hyogo and Kagawa surveys, respectively (Table 2).

3-2 CLINICAL FEATURES OF PATIENTS WITH LETTUCE-INDUCED RESPIRATORY SYMPTOMS

The characteristics of the patients included in the study are shown in Table 3. The median age of the patients was 67 years with a sex distribution of five males and eight females. Eleven farmers were specialized on growing vegetables. Two farmers kept Japanese cattle (patient 3, patient 11) but did not have respiratory symptom during caring animals. All patients exhibited respiratory symptoms during the harvesting or packaging of lettuce. Detailed history examination suggested that patients developed respiratory symptom during cutting lettuce from stem to harvest, packaging of lettuce, and wiping lettuce stem juice(latex) in small poorly ventilated room. However, none of the patients exhibited symptoms after ingestion of lettuce leaves. Three patients exhibited skin symptoms, following accidental skin contact with the lettuce latex, while three patients had lettuce-associated nasal symptoms. The SPT and lettuce-specific IgE were positive in eight patients (62%), and six patients (46%), respectively. The median value of total IgE was 445.6. Sensitization to pollen or plant foods was observed in five patients (38%).

Allergy to molds was observed in four patients, and allergy to animals was observed in two patients(Supplementary table 4). The percentage of activated basophils in patients was significantly higher than that observed in the negative controls (P <0.05, Supplementary Fig.1). Moreover, when comparing the values of single patients with those of the negative controls, one

can conclude that 8 of 11 patients were positive. The specific bronchial provocation test was performed in four patients. Notably, this test was positive in only one patient (patient 3) (Supplementary table 3). These results indicate that patients with lettuce-induced respiratory symptoms were allergic to lettuce, and respiratory symptoms were associated with the development of asthma in at least one patient. Patient 3 is the most valuable patient in the whole study.

3-3 ELECTROPHORESIS, IMMUNOBLOTTING AND CHROMATOGRAPHY

Considering that some patients exhibited skin symptoms following the accidental contact of their skin with the lettuce latex, we hypothesized that this substance may contain allergens. Therefore, initially, we investigated the differences between the extracts from lettuce leaves and the lettuce latex. Fig. 1A shows the SDS-PAGE of the lettuce leave extracts and the lettuce latex. The latter showed multiple protein bands with an apparent molecular weight ranging from 10 to 100 kDa. These bands were partially different from those observed in the lettuce leave extract. In addition, immunoblotting analysis of lettuce leave extract and the lettuce latex with the with the pooled sera(Pt 001, 002, 003, 007, 013) showed marked differences in the IgE-binding bands (Fig. 1B). Several IgE-binding bands were observed in the lettuce latex with the sera of pooled sample, and main band was seen in 51kDa. In contrast, there were no bands observed in the lettuce leave extract with the sera of pooled two non-allergic individual's sample. The result of ELISA supported the tendency that patients' sera mark higher reactivity with lettuce latex than lettuce leaves extract, while negative control's sera react neither of them. (Fig. 1C). These findings suggested that allergens may be present only in the lettuce latex. Fig. 2A shows the binding band of specific IgE from the sera of 13 patients with lettuce associated respiratory symptoms. The most frequently recognized bands — detected in the sera of >50% of the patients — showed molecular

weights of approximately 51 kDa. As shown in Fig.2A, the sera of 11 patients (i.e., patients1-8,10,12, and 13) yielded a band at 51kDa. In contrast, there were no IgE-binding bands were seen in the sera of control individuals.

By first purification step with ion-exchange chromatography, 8 fractions of the lettuce latex was obtained(A1-A8, silver stain; Fig 3A, ELISA with pt 003's sera; Fig 3B). The result of immunoblot of fractions A5 and A6 contained protein band at 51kDa with pt 003's sera(Fig 3C). Peak fractions of the result of ELISA(A5, A6) were combined. Secondly purification step through a gel filtration column chromatography with HPLC system was performed with fractions A5 and A6.

Forty fractions were obtained by a gel filtration column chromatography. The result of ELISA using the 40 fractions with the sera of patient 003 showed a peak in fraction B10 as in Fig 4A. The result of silver staining analysis of fraction B10 showed protein band at 51kDa (Fig 4B, lane 1). The result of immunoblotting analysis with pt 002,012 and 003 proved specific IgE-binding band at 51kDa, compared with negative control (Fig 4B, lane2-5). Inhibition experiment also proved that 51kDa band disappears by inhibition with lettuce latex as in Fig 4B lane 6.

3-4 PROTEIN IDENTIFICATION AND CHARACTERIZATION

The 51kDa IgE binding bands from the gel filtration column chromatography fraction B10 were cut out from the gel and subjected to mass spectrometric analysis after in-gel tryptic digestion to determine the peptide sequences of the lettuce latex allergens. We performed nanoLC-MS/MS and obtained the sequence of several internal peptides (Table 4). Protein identification was performed through a search in the SwissProt database using the Mascot program (http://www.matrixscience.com). After excluding trypsin and keratin protein originates from experimenters, protein identification was reperformed through a search in the genome database of

Lactuca sativa (https://www.ncbi.nlm.nih.gov/genome?term=Lactuca%20sativa) using the Mascot program (Supplementary Table 4). This analysis led to the identification of the 51 kDa proteins as the epidermis-specific secreted glycoprotein EP1-like.

4. Discussion

This is the first study to identify the allergen of lettuce using the sera from patients with lettuce-induced respiratory allergy. Past reports have described the development of occupational dermatitis by exposure to lettuce, or the occurrence of systemic adverse reactions after ingestion of lettuce(5-10). Based on the reported lettuce-associated OAS and anaphylaxis cases, several proteins have been identified as allergens in previous studies. Lac s 1 (9 kDa, lipid transfer protein)(9-11), 16 kDa(8), 26 kDa (Thaumatin-like protein family), 35 and 45 kDa (asparatyl protease) (12) were identified in whole lettuce mainly leaves. However, a limited number of studies have investigated the respiratory symptoms induced by lettuce, and a causal allergen has not been identified thus far.

In the survey conducted in the Hyogo prefecture, 7% of the lettuce farmers reported the respiratory symptoms associated with lettuce farm work. The results of the questionnaire and patient' interviews suggested that most symptomatic farmers exhibited lettuce-associated respiratory symptoms during cutting of the lettuce stem, wiping the lettuce latex, and packaging lettuce in small poorly ventilated rooms. Of note, ingestion of lettuce leaves did not result in the occurrence of symptoms. These results suggested that the allergens are present in the lettuce stem, rather than the lettuce leaves.

In the current study, we identified the following novel allergen: epidermis-specific secreted glycoprotein EP1-like (51 kDa). Studies have shown that EP1 proteins are involved in cell elongation(18). A higher level of EP1 is found in the basal parts of seedling hypocotyls and roots versus that reported in the apical parts. EP1 gene expression was detected in cells located on the

surface of the seedling, the epidermis of the root or root cap, and it is thought to be involved in the development of seeds(19).

Although we were unable to detect the specific band, Lac s1 (9 kDa) firstly identified in allergy to lettuce(10). The difference of respiratory versus ingestive allergic phenotypes causes the difference patterns of allergen recognition. The present allergens may be closely related to the development of respiratory symptoms (e.g., asthma) although the previously identified allergen was related to OAS or anaphylaxis(8-10,12). Moreover, we focused on the differences between parts of lettuce to gain further insight into the cause of these differences in allergen. Previous studies used whole lettuce, which was mainly composed of leaves. In contrast, in the current study, we used the lettuce latex instead of leaves. In 2017, Reyes-Chin-Wo S reported all the sequencing of the lettuce genome(20). Therefore we can more accurately examine the lettuce versus previous studies.

Several sensitization pathways may be related to allergic airway inflammation(21). In particular, two sensitization pathways are thought to be important in provoking lettuce-associated respiratory symptoms: trans-airway sensitization and transdermal sensitization. All three patients who suffered from respiratory symptom even with treatment exhibited atopic dermatitis and lettuce-associated dermatologic symptoms. Moreover, lettuce latex contains the sesquiterpene lactone, which proved to be sensitizers and often causes non IgE mediated Type Ⅳ allergic contact dermatitis(5). Therefore, we speculate that transdermal sensitization might be an important pathway involved in the development of lettuce-associated respiratory symptoms. These patients were ordinarily exposed to the lettuce latex and may have been sensitized through the skin. Indeed, atopic dermatitis can progress from a skin disease to asthma(22). The skin barrier in patients with atopic dermatitis has been linked to sensitization through aeroallergens(23). Oral sensitization is the most established sensitization pathway. However, the pathway is not applicable

to the present cases, because none of the patients exhibited OAS or anaphylaxis after ingestion of lettuce.

Although the result of ELISA and immunoblot of the patients' sera to lettuce latex supported that all the patients' sera contains much more lettuce specific IgE compared to negative control's sera, we could not deny the possibility that some of the patients had type IV allergy caused by sesquiterpene lactones, which are present in many plants from the Asteraceae family, including lettuce.

Patient 3 is the most valuable patient in the whole study, and patient 3 was allergic to mold. We can't deny that the inhalation provocation test was affected by mold, since we couldn't sterilize lettuce sample which denatures with heat. We think this is one of limitations of our study.

The epidermis-specific secreted glycoprotein is most likely glycosylated, as its name implies and as has been shown for related proteins from other species. Hence IgE binding to this protein may be caused by IgE specific to cross-reactive carbohydrate determinants (CCD). We think this is one of our limitations.

The results of the present study provide a basis for the development of *in vivo* and *in vitro* diagnostic tools, as well as allergen specific immunotherapy for patients with respiratory allergy to lettuce.

Conclusions

In conclusion, we surveyed lettuce-induced respiratory allergy, and identified a causative novel allergen in the lettuce latex: epidermis-specific secreted glycoprotein EP-like.

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Author' contributions: RS and TN conducted data collection, performed analysis, and wrote the manuscript. YN and HK conceived the research questions and designed the study. YK and KK helped with the writing of the manuscript. TK, HS, MK, KU, NK, SH, NHZ, and MY distributed the questionnaire to farmers. TM, EY, NHT, AF, and MS helped with data collection and contributed to statistical analyses. All authors contributed to data interpretation and critically revised the manuscript for important intellectual content. All authors approved the final version of the submitted manuscript.

Tables

Table 1. Baseline characteristics of lettuce farmers.

	Hyogo		Kagawa	
	N	**%**	**N**	**%**
Effective response rate	932/1168	80	368/1073	34
Mean age (years)*	65±10		65±12	
Sex (male/female)	455/477	49/51	225/143	61/39
Mean duration of farming (years)*	33±16		29±17	
Smoking status				
Current smoker	83	12	69	19
Ex-smoker	227	24	86	23
Never	622	64	213	58
Allergic history				
Pediatric asthma	6	0.6	5	1
Bronchial asthma	36	4	19	5
Allergic rhinitis	49	5	32	9
Pollinosis	79	9	54	15
Atopic dermatitis	8	0.9	11	3
Urticaria	29	3	27	7
Food allergy	14	2	12	3
Drug allergy	13	1	6	2

*Mean ± SD.

Table 2. Symptoms associated with Lettuce farm work.

	Hyogo		Kagawa	
	N	**%**	**N**	**%**
Symptoms specifically associated with lettuce farm work	71	8	40	11
Respiratory symptoms	63	7	24	7
Rhinitis	39	4	23	6
Skin symptoms	11	1	13	4
Conjunctivitis	26	3	11	3
Gastro-intestinal tract symptoms	2	0.2	2	0.5
Symptoms associated with ingesting lettuce	0	0	2	0.5
Respiratory symptoms associated with harvesting and packing of lettuce	49	5	18	5
Mean years from start of harvesting lettuce to the onset of the symptoms	19±11		15±13	

*Mean ± SD.

Table 3. Clinical features of the patients who are allergic to lettuce.

Patient No.	Age	Sex	Past history	Smoking	Other allergy	Lettuce reaction	SPT (Leaves)	SPT (Stem)	SPT (Latex)	Total IgE (IU/mL)	ImmunoCAP Lettuce Specific IgE (IU/mL)	Uncontrollable symptom with Treatment
1	60	M	HL	-	Ce,Hi	R	ND	+	ND	413.4	<0.100	-
2	76	F	-	-	Ce	R	ND	+	ND	226.1	<0.100	-
3	73	M	AD	+	Mi	R,S	-	+	+	2784.3	5.65	+
4	70	F	HT,HL,GERD	-	-	R	ND	-	ND	46.8	<0.100	-
5	72	F	-	-	-	R	ND	±	ND	3.4	<0.100	-
6	60	M	-	+	-	R	ND	+	ND	126.3	0.439	-
7	81	M	HT	-	-	R,N	ND	-	ND	4.7	<0.100	-
8	63	F	HT,Psoriasis	-	Ce	R,N,E	ND	+	ND	28.6	<0.100	-
9	77	F	HT,HL	-	EA	R,S	ND	-	ND	120.8	<0.100	-
10	75	F	-	-	Mi,HD,Co,Sh,Mo	R	ND	-	ND	179.7	0.388	-
11	56	F	-	-	Ti,Or,Ce,Hi,Ja,Ca,Wh,So,	R,N	-	+	+	1384.3	2.3	-

					Pn,La,Se,Cr,Ki,Pc,To							
12	64	F	GIST	-	Ml,PM	R	ND	-	ND	200.2	<0.100	-
13	44	M	AD	-	Or,Ti,Ve	R,S	-	+	+	274.4	0.518	+

SPT, Skin prick test; BAT, basophil activation test; M, male; F, female; AD, Atopic dermatis; HL, hyperlipidaemia; HT, hypertension; GERD, gastro-oesophageal reflux disease; GIST, gastro-intestinal stromal tumour; Ce, ceder pollen; Hi, Hinoki cypress pollen; Mi, Mite; EA, egg albumin; HD, house dust; Co, cockroach; Sh, Shrimp; Mo, Moth; Ti, Timothy grass; Or, Orchard grass; Pc, peach; To, tomato; So, soybeans; La, latex; Ca, cat; Se, sesame; Ja, Japanese white birch; Wh, wheat; Cr, crab; Sb, buchwheats; Ap, apple; Ml, Milk; PM, pig meat; Ve, vernal grass; R, respiratory symptoms; N, nasal symptoms; S, skin symptoms; E, eyes symptoms.

Table 4. Protein identification and characterization

Estimated molecular weight by SDS-PAGE	51
Moleculer weight of identified protein	50971
% recognition of patient	84.6%(11/13)
Taxonomy	Lactuca sativa
SPROT ID	XP_023765238.1
Match score	195
Identification	Epidermis-specific secreted glycoprotein EP1-like
% of matched peptide	11%
Peptides(Matched peptides)	MTSPNSILVT ALLLLLSFQL LYTISEAIVP SADTFSYVNE GDFGEYIVEY DADYRTLPPF SNPFQLCFYN TTPNTFTLAL RMGTVRSESL MRWVWEANRG NPVGENATLT FGTDGNLVLA DSDGRIAWQT NTANKGVVGL QVLPTGNMVL HDGTGTFIWQ SFDSPTDTLL VGQSLRAGGA SNLVSRASAE NNIDGPYSLV MEPKRLALYY KSANSPYPML YWTSVEWFTV DVGSVTNGSL INLTLTSVPD TDEGFLYYLT FDYYITNPLS GWNRNMAFSR YNNTLSYLRL GIDGNLKFYT YNPNVQGVSW ELVYTFLDRN SIEGECQLPE RCEKFGLCED NQCVACPTPF GLSGWSKDCE ASKVTSCQAS DFGYFKLDGV DHFMTKYTTG DWVSNQWDCE SKCTKDCNCM GYFYHTADSR CWIAYDLKTL TRVGNSTHFA YIKAPYNLSL NGIRL

Taxonomy: Lactuca_sativa (45,242 sequences; 197,238,886 residues)

Figure Legends;

Fig. 1. **Coomassie Brilliant Blue staining, immunoblotting analysis, and ELISA of the lettuce leave extract and the lettuce latex. A,** Coomassie Brilliant Blue-stained SDS-PAGE gel of the lettuce leave extract (LL) and the lettuce latex (La). Molecular weights are indicated on the left. MW, molecular weight (kDa). **B**, IgE-immunoblots of LL and La probed with the a pool of five patient's serum. **C,** ELISA analysis of negative controls of serum pool from two non-allergic individuals and 14 patients' serum with lettuce leaves extract(LL) and lettuce latex(La). Patients' sera shows higher absorbance when reacted with lettuce latex than lettuce leaves extract.

Fig. 2. IgE-binding bands in the lettuce latex. A, immunoblotting analysis shows IgE-binding bands. Lanes 1-13 represent the sera of the 13 patients. Lane C(Control) represents a negative control of serum pool from two non-allergic individuals. MW, molecular weight marker (kDa); CBB, Coomassie Brilliant Blue-stained SDS-PAGE gel of the lettuce latex.

Fig. 3. Silver staining, ELISA, and immunoblotting analysis of the fraction obtained through ion exchange chromatography.

A, Silver Staining of the 8 fractions obtained through ion exchange chromatography for the lettuce latex.

B, ELISA analysis of the 8 fractions obtained through ion exchange chromatography for the lettuce latex showed peak on fraction of A5 and A6 with pt 003's sera. Pt, patient 003; C, control

C, Immunoblotting analysis of the 8 fractions obtained through ion exchange chromatography for the lettuce latex with pt 003's sera. Fractions of A5 and A6 contain 51kDa band.

Fig. 4. ELISA, immunoblotting and inhibition analysis of the fraction obtained through gel filtration chromatography.

A, ELISA analysis of the 40 fractions obtained through gel filtration chromatography of the peak

fractions(A5+A6) of the ion exchange chromatography. Pt, patient 003; C, control

B, Immunoblotting analysis of specific IgE reactivity to the peak fraction(B10) obtained through gel filtration chromatography of the peak fractions(A5+A6) of the ion exchange chromatography. Lane 1 marked silver stain of fraction B10. Lane 2 marked pooled serum from healthy individuals as negative control reacted with the fraction B10. Lanes 3-5, representatives of sera from lettuce allergy patients(Pt012,002,003) reacted with the fraction B10, show single protein bands at 51kDa. Lane 6, the inhibition of the immunoblot with Patients 003's sera with lettuce latex.

Supplementary Fig.1 The results of Basophil activation testing. The percentage of activated basophils in the whole blood of patients was significantly higher than that reported in negative controls.

Supplementary Fig.2

A, Fresh lettuce (*Lactuca sativa. var. capitata*).

B, Lettuce latex. The fresh lettuce stem was cut using a sharp scalpel, and the white lettuce latex spread on the section is collected.

Fig.1

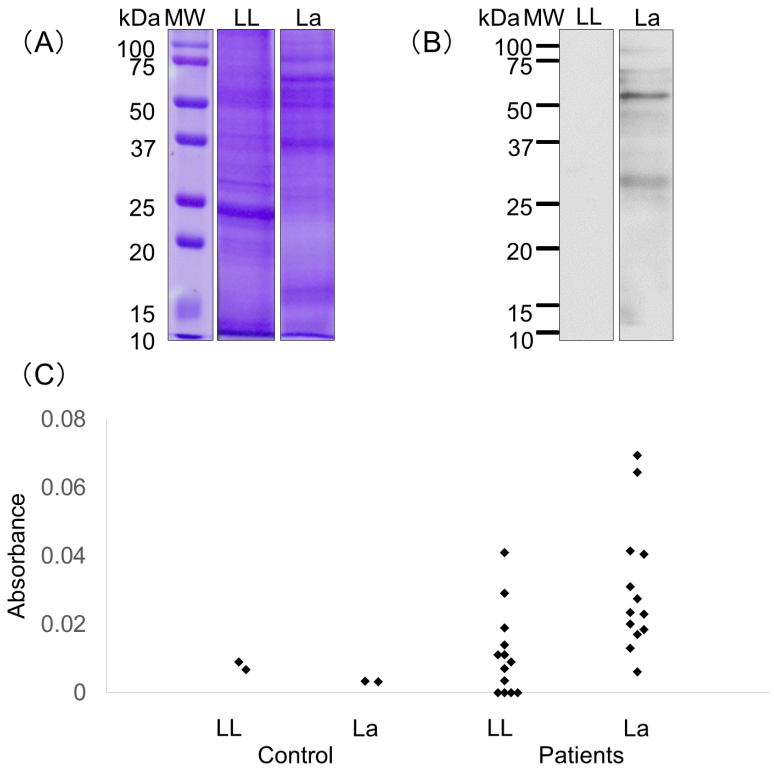

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Fig.2

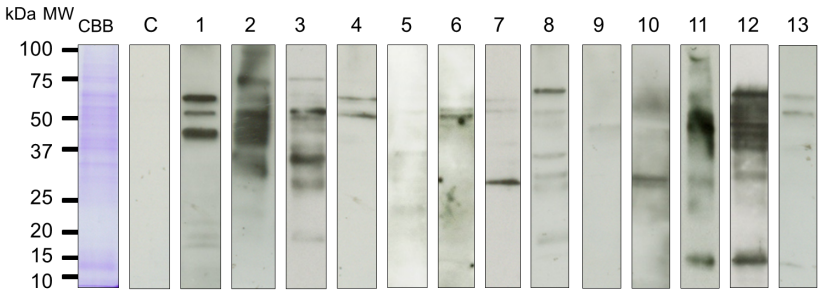

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Fig.3

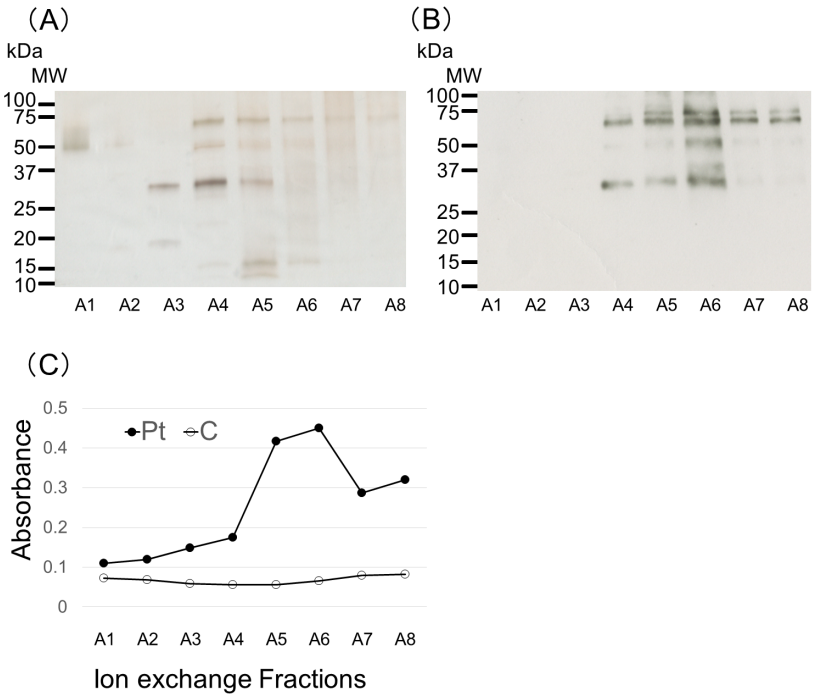

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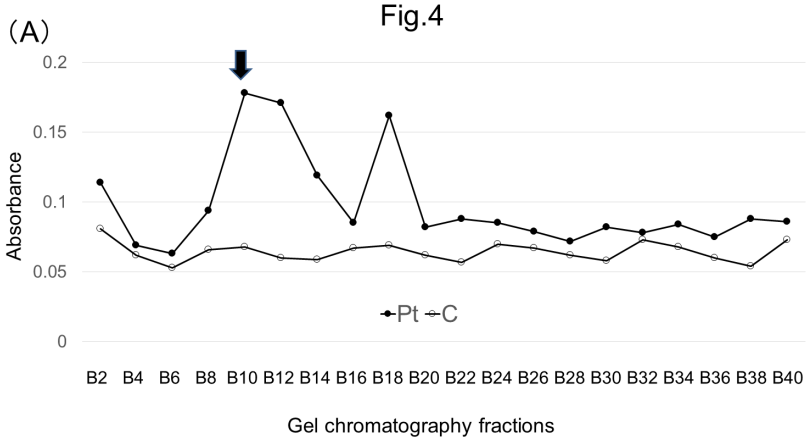
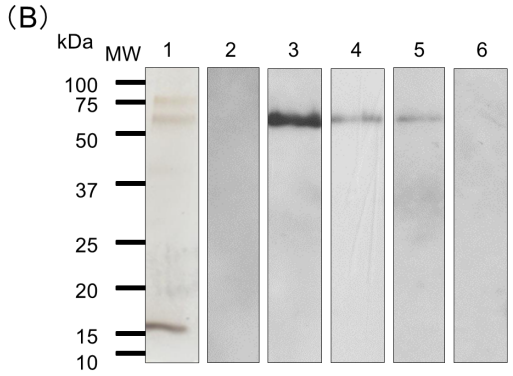
Fig.4





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