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Significance of Ara h 2 in clinical reactivity and effect of cooking methods on allergenicity

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ABSTRACT

Background: The prevalence and clinical severity of peanut allergy vary between Western countries and Asia. It has been suggested that cooking methods are responsible for this discrepancy.

Objectives: To evaluate the specific IgE responses to major peanut allergens in peanut allergic Korean children and to examine the influence of different cooking methods on peanut proteins.

Methods: Raw peanut protein extracts were immunolabeled with serum samples from 42 children with a level of peanut specific IgE of 15 kUA/L or higher to detect specific binding to Ara h 1, Ara h 2, and Ara h 3. Clinical severity scores were assessed on a scale of 0 to 5. Protein extracts from boiled, roasted, fried, and pickled peanuts were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting with pooled serum samples from 7 patients.

Results: Most patients' serum samples reacted with Ara h 1 (76.2%) and Ara h 3 (78.6%) from raw peanuts, whereas only 53.0% of patients had specific IgE against Ara h 2. IgE binding to Ara h 2 was more prevalent in patients with more severe reaction than in those with mild reactions. IgE binding to Ara h 2 was increased by roasting, but there was significantly less IgE binding after vinegar treatment.

Conclusion: Our results suggest that Ara h 2 is an important allergen to predict clinical symptoms but less prevalent in Korean children than in Western children. This finding may be attributed in part to different cooking methods and dietary habits among regions.

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Introduction

The peanut, *Arachis hypogaea*, has attracted a great deal of attention in Western countries because peanut allergy is relatively common, is often severe and has a tendency to persist throughout adulthood.¹ Nine allergens have been reported in peanuts, and Ara h 1, Ara h 2, and Ara h 3 are well defined as major allergens. Ara h 1 migrates as a single protein band at 63 kDa, whereas Ara h 2 migrates as a doublet at 17 to 20 kDa.^{2–6} Ara h 3 is composed of a set of protein bands with molecular weights ranging from 14 to 45 kDa.^{2–6} Among them, Ara h 2, which remains stable in response to heat treatment and digestion,^{7,8} is the most frequently recognized major peanut allergen in Western children.

The prevalence of peanut allergy in children in the United Kingdom and North America has doubled in the last 10 years and is now approximately 1.8% and 1.2%, respectively.^{9,10} Food

anaphylaxis fatality registries have implicated peanuts as a trigger in 59% of 63 deaths in the United States¹¹ and in 19% of 48 fatalities in the United Kingdom.¹² In Korea, the prevalence of peanut allergy was 0.68% in infants¹³ and 0.14% (36 of 27,425) and 0.10% (14 of 14,777) in elementary schools and middle schools, respectively, according to the International Study of Asthma and Allergies in Childhood survey in 2000,¹⁴ indicating that peanut allergy was less common than in Western countries. In terms of the clinical severity of peanut allergy, reports of severe anaphylactic reactions and fatalities from peanuts in Asia are relatively rare,¹⁵ and it appears that there are still differences in the prevalence and severity of peanut allergy in different parts of the world.^{15–17}

Many genetic and environmental factors could be responsible for the epidemiologic characteristics of peanut allergy.¹⁸ It is generally agreed that the discrepancy in peanut allergy between Western and Asian countries stems from the effects of different cooking methods on the allergenicity of peanuts.^{19,20} A previous study²⁰ suggested that roasting at higher temperatures increases the allergenic properties of peanut proteins and may help explain the difference in the prevalence of peanut allergy observed between the United States and China.

Peanuts have not attracted the attention of clinicians and scientists in Korea because the prevalence of peanut allergy appears

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to be low; therefore, peanut allergens relevant in Korean peanut allergic patients have not been characterized to date. This study was conducted to determine what peanut allergens were involved in allergic reaction in Koreans and to investigate whether the cooking method used in Korea influences the reactivity of peanut protein to peanut specific IgE (sIgE).

Methods

Patient Characteristics

Forty-two patients with suspected peanut allergy (10 girls and 32 boys; median age, 22.5 months; age range, 5–67 months) at the Allergy Clinic of Samsung Medical Center were included in the study to evaluate the sensitization of each allergen of peanut. Suspected peanut allergy was defined as a level of peanut sIgE of 15 kUA/L or higher according to the cutoff level with a positive predictive value greater than 95%.²¹ Peanut sIgE levels were measured using the Phadia UniCAP system (Uppsala, Sweden). We evaluated a history of allergic symptoms after eating peanuts to investigate the significance of Ara h 2 in severe allergic reactions. Twenty of 42 patients had a history of unintentional ingestion of peanuts. Table 1 gives their IgE levels, symptoms, and clinical severity scores, which were assessed on a scale of 0 to 5 with a grading system reported previously.²² Serum samples were pooled from 7 patients to determine if the allergenicity of peanuts was altered by cooking method. Serum samples from healthy and nonatopic individuals who had never had symptoms after the ingestion of peanuts were used as negative controls. After written informed consent was received, blood samples were obtained and the serum samples were frozen at -80°C until use. This study was approved by the Samsung Medical Center Institutional Review Board.

Preparation of Peanut Extracts

In general, there are 4 types of peanuts, Virginia, Runner, Spanish, and Valencia. Modified Virginia-type peanuts, which are

Table 1
Characteristics of patients with a history of unintentional ingestion of peanut

Patient No.	Sex	Age, mo	Total IgE, kUA/L	Peanut specific IgE, kUA/L	Symptoms	Grade
1	M	23	836	80.2	Nonlaryngeal angioedema	2
2	M	48	563	61	Generalized urticaria, rhinorrhea, and cough	3
3	M	62	763	101	Cough and severe dyspnea	4
4	M	24	70.1	19.2	Hoarseness, breathing difficulty, and stridor	4
5	F	24	1,580	83	Nonlaryngeal angioedema and asthma requiring treatment	4
6	M	48	1,547	34	Generalized urticaria and asthma requiring treatment	4
7	M	6	18.6	27.4	Generalized urticaria and vomiting	3
8	F	36	2,059	101	Cough, vomiting, urticaria, respiratory distress, stridor, cyanosis	4
9	M	23	NC	26.1	Generalized urticaria and nonlaryngeal angioedema	2
10	F	34	1,141	101	Generalized urticaria and dyspnea	4
11	M	13	2,803	101	Rash	1
12	M	8	2,426	47	No symptoms	0
13	M	33	691	18.1	No symptoms	0
14	M	6	2,343	76.2	Rash	1
15	M	22	12,820	101	No symptoms	0
16	M	23	1,171	17.4	Rash	1
17	M	13	2,834	22.2	No symptoms	0
18	F	23	1,249	23	No symptoms	0
19	M	20	1,070	68	No symptoms	0
20	M	62	4,869	53.7	No symptoms	0

Abbreviation: NC, not checked.

widely consumed in Korea, were obtained from the Korean Rural Developmental Administration.⁶ The peanut kernels were boiled in water, roasted at 170°C , or fried in pure soybean oil for 10 minutes. Some boiled peanuts were put in table vinegar overnight.

The peanuts were then ground using a mortar and pestle until a smooth paste was achieved. The paste was subsequently defatted by washing with n-hexane, after which it was dried overnight at room temperature. Next, the protein was extracted from the dried powder by agitating with phosphate-buffered saline for 2 hours at 4°C . After centrifugation at $3,000g$ for 20 minutes at 4°C , the supernatant was collected, filtered ($0.45\text{-}\mu\text{m}$ pore size), and lyophilized. The protein concentrations were then determined by a Bradford Assay (Bio-Rad, Hercules, California) using a microplate reader (Bio-Rad). All extracts were stored at -80°C until use.

Electrophoresis, Western Blotting, and Analysis of the sIgE Responses

The protein extracts and purified allergens were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli.²³ The samples were then reduced by heating with 4 times SDS-PAGE gel loading buffer (LDS Sample Buffer, catalog No. NP0007; mixed 1:10 with reducing agent, catalog No. NP0004) and loaded into each well of a gel (4%–12% Bis-Tris Aminomethane Gel, NuPAGE, Invitrogen, Carlsbad, California). Precision plus protein standards (product No. 161-0374, Bio-Rad) were used as molecular weight markers to estimate the protein size.

For immunoblotting, proteins from peanuts were transferred from the gel onto a polyvinylidene difluoride membrane using an iBlot Dry Blotting System (Invitrogen). The membrane was incubated with the patients' serum sample diluted 1:10. The membrane was blocked with 2% nonfat dried milk for 1 hour at room temperature, after which it was incubated with the serum samples diluted 1:10 in 2% nonfat dried milk overnight at room temperature. The membrane was then washed 3 times with phosphate-buffered saline with Tween 20 for 10 minutes each and incubated with biotin-labeled goat IgG anti-human IgE (KPL, Gaithersburg, Maryland) diluted 1:4,000 in 2% nonfat dried milk for 1 hour at room temperature. Next, the membrane was washed with phosphate-buffered saline with Tween 20 3 times as described, after which it was incubated with NeutrAvidin horseradish peroxidase (Pierce Chemical Co, Rockford, Illinois) for 30 minutes at room temperature. After 3 washes with phosphate-buffered saline with Tween 20, the membrane was reacted with Amersham ECL reagent (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for 2 minutes. The blotted membrane was then exposed to high-performance chemiluminescence film (GE Healthcare Limited, Buckinghamshire, United Kingdom), after which the film was developed. The optical density of SDS gels and immunoblots of proteins cooked in different ways were measured using a reflective densitometer (Bio-Rad), and the Quantity One software (Bio-Rad) and percentage of relative optical density of cooked peanuts with respect to that measured for the raw peanut were calculated.

Each allergen was confirmed on the basis of its size.^{2–6,24} The molecular weight of Ara h 1 is 63 kDa, which was also found as a single band in our study. Ara h 2 had a doublet of approximately 20 kDa. Ara h 3 is a complex that exists by proteolysis in a post-translational process. This protein consists of a triplet of approximately 42 to 45 kDa, a distinct band of approximately 25 kDa, and some less abundant peptide chains in the range of 12 to 18 kDa.^{25,26} In the present study, the proteins of approximately 25 to 45 kDa reacted with sIgE from the patients and were considered to be Ara h 3.

Results

IgE Reactivity of Each Peanut Allergen as Determined by Immunoblotting

Immunoblotting analysis was conducted to investigate the IgE reactivity toward Ara h 1, Ara h 2, and Ara h 3 of raw peanuts using serum samples from patients with suspected peanut allergy (Figure 1). The serum samples from most patients reacted with Ara h 1 (76.2%, 32 of 42) and Ara h 3 (78.6%, 33 of 42), whereas only 53.0% (22 of 42) of patients had reaction against Ara h 2. Fifteen of the 42 patients (36.0%) demonstrated a reaction to a protein of approximately 15 kDa (suggested to be Ara h 6) along with the major allergens. We compared the proportion of patients showing reactivity to each peanut allergen according to clinical severity after exposure to peanuts. As a result, IgE binding to Ara h 2 was demonstrated in 90% of patients with allergic reactions of grade 2 to 4 (1–10 lanes in Figure 1), which was higher than 30% in those with grade 0 to 1. IgE binding to Ara h 1 was found in 60% of patients with grade 2 to 4 and 60% of those with grade 0 to 1. In addition, IgE binding to Ara h 3 was revealed in 70% of patients with grade 2 to 4 and 80% of those with grade 0 to 1.

Comparison of Protein Fractions in Roasted, Fried, Boiled, and Pickled Peanuts by SDS-PAGE

Boiled, roasted, fried, and pickled peanut extracts were compared with that of raw peanut by means of SDS-PAGE. SDS-PAGE and percentage of relative optical density compared with raw peanut revealed that the patterns of each peanut protein differed among the cooking methods (Figure 2). Ara h 1 (molecular weight, 63 kDa) almost completely disappeared in response to frying or vinegar treatment and was reduced by boiling, whereas it was enhanced by roasting. The Ara h 1 trimer was not found in the roasted preparation. The intensity of Ara h 2 in boiled, roasted, and fried peanuts was enhanced by 67%, 81% and 23%, respectively, whereas it was reduced by 10% in response to vinegar treatment. Ara h 2 appeared as one band with weak intensity in the vinegar treatment. Ara h 3 was enhanced by all treatments, with especially thick and strong bands being observed in the vinegar treatment.

Comparison of IgE Binding to Peanuts Cooked in Different Ways

In some proteins, the IgE binding intensities were presented differently compared with SDS-PAGE (Figure 3). The IgE-binding intensities to Ara h 1 corresponded with the quantities of Ara h 1 proteins in SDS-PAGE, whereas IgE-binding intensities to Ara h 2 and Ara h 3 did not. The IgE-binding intensity to Ara h 2 only increased in roasted peanuts and showed no change in boiled and fried peanuts. However, the IgE reaction almost completely disappeared in pickled peanuts, even though only a slight reduction in quantity was observed on SDS-PAGE. There was increased or no change in the IgE-binding intensities of Ara h 3 in all treatments except for the vinegar treatment. Interestingly, Ara h 3 in the pickled peanuts was remarkably enhanced on SDS-PAGE but almost completely unreactive with IgE.

Discussion

Ara h 1, Ara h 2, and Ara h 3 are well-known major peanut allergens in Western countries, and sIgE against Ara h 2 has been found in more than 70% of peanut allergic patients in the United States and Europe.^{27–29} However, among studies of Asian populations, there has been only one report in the English-language literature reporting that 87.1% of peanut allergic patients in Singapore had sIgE to Ara h 2.³⁰ Interestingly, we found that the relevance of Ara h 1, Ara h 2, and Ara h 3 to peanut allergic patients in Korea was different from that in previous studies.^{27–30} IgE binding to Ara h 2 was observed less frequently than Ara h 1 and Ara h 3, although the reason for this is not yet clear. Our results suggest that this difference is likely due to the cooking method and characteristic of the study population. Increased allergenicity of Ara h 2 was induced by roasting in our study, which is similar to the results of previous studies,^{20,31} whereas other cooking methods produced no change or even reduced allergenicity of Ara h 2. The patients included in the present study were infants and young children, who do not eat roasted peanuts very often in Korea, which might explain why Ara h 2 is of relatively low clinical relevance in Korean children.

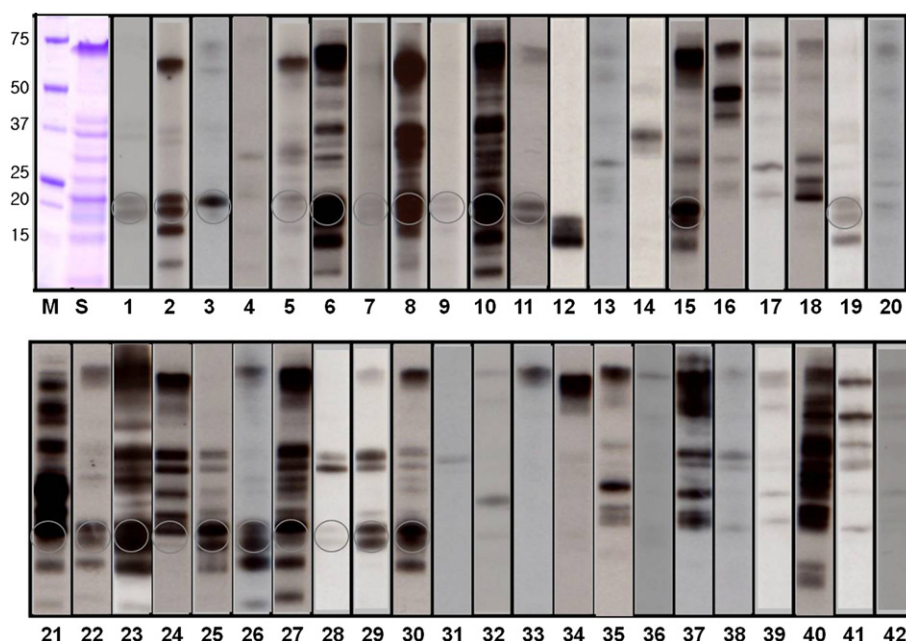


Figure 1. Immunoblotting of raw peanut protein with patients' serum samples. IgE reactivity to Ara h 2 is indicated by the solid circles. Lanes are as follows: M, molecular weight; S, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; 1–10, patients with allergic reactions of grade 2 to 4; 11–20, patients with allergic reactions of grade 0 to 1; 21 to 42, patients who had a peanut specific IgE level of 15 kUA/L or higher but had not eaten peanuts.

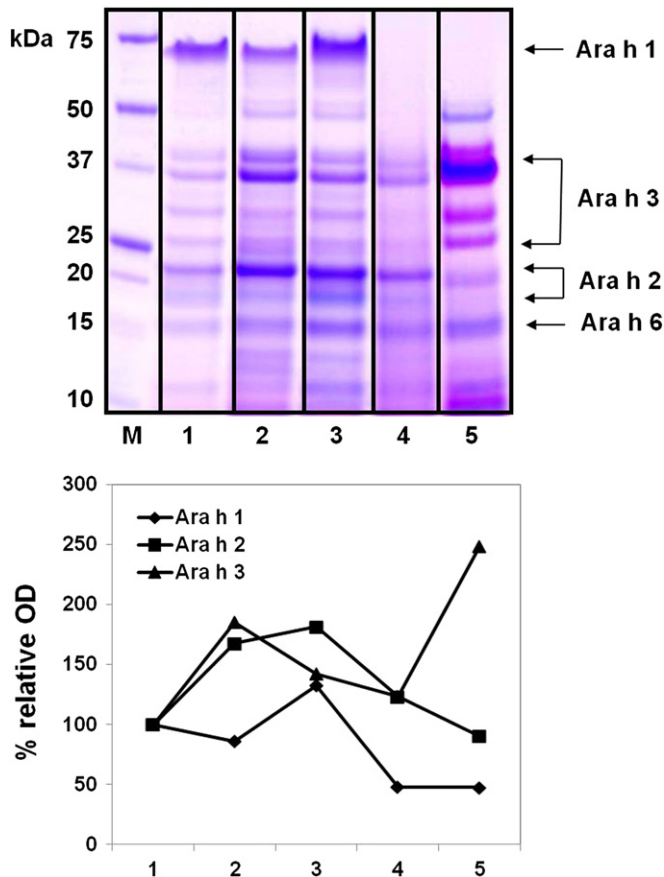


Figure 2. Electrophoretic maps of peanut protein extracts (top) and relative optical density compared with raw peanut of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (bottom). Lanes are as follows: M, molecular weight; 1, raw peanut; 2, boiled peanut; 3, roasted peanut; 4, fried peanut; and 5, pickled peanut.

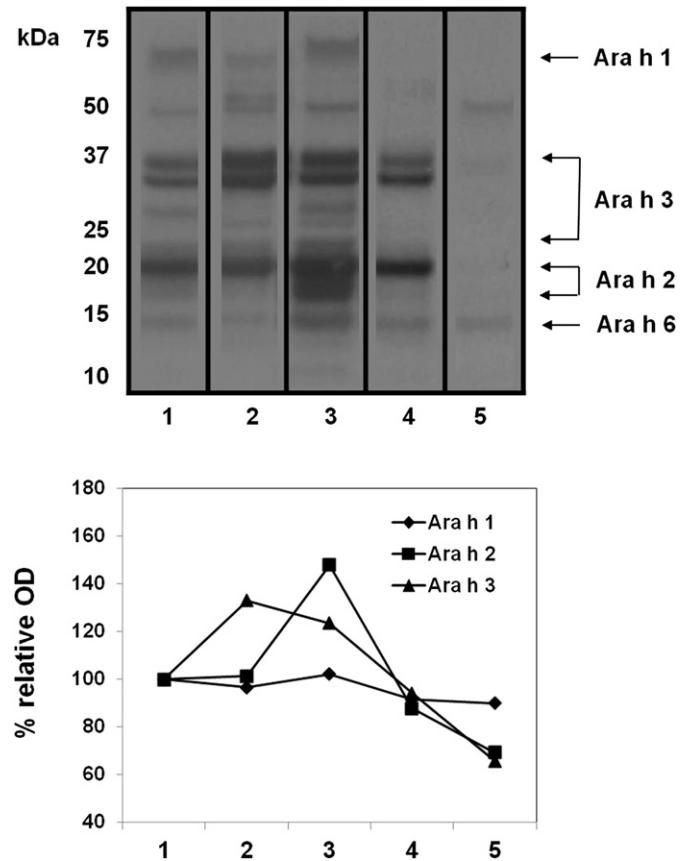


Figure 3. Immunoblotting analyses of pooled serum samples from peanut allergic patients (top) and relative optical density compared with raw peanut of immunoblots (bottom). Lanes are as follows: 1, raw peanut; 2, boiled peanut; 3, roasted peanut; 4, fried peanut; and 5, pickled peanut.

The importance of Ara h 2 in clinical peanut allergy has been suggested in other studies.^{2,22,32–37} A correlation between clinical severity and the recognition of Ara h 2 at low concentrations and Ara h 1 and 3 at higher concentrations was reported to indicate an apparent increased potency of Ara h 2.³² In addition, component-resolved diagnostics revealed Ara h 2 as the most important component for accurate discrimination of patients with peanut allergy and peanut-tolerant individuals.³⁷ Furthermore, Ara h 2 has been found to be a very potent allergen in *in vitro* assays of IgE cross-linking and cell activation.^{33–36} More children with moderate to severe reactions had IgE reactivity to Ara h 2 than those with mild reactions in our study, suggesting that Ara h 2 is related to the clinical severity of peanut allergy. In the present study, only 3 patients (patients 3, 5, and 24) appeared to bind almost exclusively to the higher molecular form in the doublet bands, suggesting individual differences in reactivity to isoforms of Ara h 2.

The allergenicity of peanut protein can be altered by different cooking methods.^{20,31} Various methods are used to cook peanuts in Korea, including roasting, boiling, frying, pickling, and salting. In this study, we found that vinegar treatment almost completely reduced the IgE reactivity toward peanut proteins, although there appeared to be a minimal decrease or even an increase in the intensity of Ara h 2 and Ara h 3, respectively, on SDS-PAGE analysis. These findings suggest that pH condition or charge of protein may be a very important factor influencing IgE binding. It has been reported that vinegar-processed lentil and chicken decreased wheal size on skin prick tests and that the charged group was less abundant in the protein-protein recognition site.^{38,39} Different cooking conditions, including temperature, solvent, and acidity,

denature proteins in different ways, resulting in the denatured proteins having varying properties. Because cooking methods and eating habits in each country are different, exposure to specific allergens in peanut proteins varies, leading to different clinical characteristics. Therefore, food allergy must be characterized in each geographic region.

The significance of cultural and dietary habits has been demonstrated in previous studies. For example, the prevalence of peanut allergy among Jewish children in the United Kingdom has been found to be 10-fold higher than that of Jewish children in Israel.⁴⁰ Israeli infants consume peanuts in higher quantities during the first year of life than UK infants; therefore, this difference in the prevalence of peanut allergy is not due to differences in atopy, social class, genetic background, or peanut allergenicity.⁴⁰ On the basis of their studies, the frequency, timing, or dose of allergen ingestion could be an important factor in the development of peanut allergy. Low doses of peanuts have previously been demonstrated to induce oral sensitization and increase the production of interleukin 4 and sIgE on challenge in animal models.⁴¹ In addition, recent findings suggest that infants who are exposed to food allergens early through the oral route are less likely to have food allergy than those without such exposure.^{42,43} However, this hypothesis is not applicable to Korea because peanut consumption by infants is rare in Korea. Therefore, it is necessary to conduct comparative studies of peanut allergy among countries with different food cultures.

This study has limitations, however, mostly stemming from its small sample size and the diagnostic methods of peanut allergy. We could not confirm the peanut allergy by the oral challenge test. It is

difficult to perform a challenge test with peanuts in this age group because parents do not want to take risks. Therefore, we selected patients with suspected peanut allergy with a level of peanut sIgE of 15 kUA/L or higher to predict clinical reactivity with greater than 95% certainty,²¹ although there is controversy regarding this cutoff level. The next logical step was division into 3 groups based on a history of peanut ingestion and clinical severity: no ingestion history, grade 0 to 1, and grade 2 to 4. These results can present the useful information for Ara h 2 and peanut allergy, although it is difficult to generalize from them.

Our results suggest that Ara h 2 may be an important allergen to predict severe clinical symptoms but less prevalent allergen in Korean children than in Western countries. This might be attributed, at least in part, to different cooking methods and dietary habits among regions. This difference might determine the clinical severity of peanut allergy.

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