5. リウマチ・膠原病

川崎病
Kawasaki disease

東京女子医科大学八千代医療センター　寺井　勝（てらい　まさる）

わが国では年間1万人の患者発症がみられる．標準治療は経口アスピリンに加えた大量免疫グロブリンの静注療法であるが，約10～20%に治療不応例がみられる．川崎病関連遺伝子の解明が進んでおり，難治例に対する新たな治療の展開が期待される．

診断のポイント

厚生労働省川崎病研究班作成の川崎病診断の手引き（表）があり，2002年2月に改訂5版が作成された．

初期につきやすい病名に，麻疹，溶連菌感染症，尿路感染症，髄膜炎，薬疹，その他の発疹性ウイルス感染症がある．頸部リンパ節腫脹は，年長児では発熱に先行することもあり，通常，片側で大きく発赤を伴う．痛みのため患児は首を動かすことができない．溶連菌迅速抗原が陽性の場合，抗菌薬を投与して解熱しない場合は川崎病[1]を疑う．

市中感染症であるアデノウイルス，RSウイルス，インフルエンザウイルスの流行期には，ウイルス抗原が陽性となる川崎病もある．その他，参考となる所見として胆嚢腫大，BCG部位の発赤・痂皮形成，爪の変化などがある．6カ月未満の乳児や年長児では主要症状が揃わず診断に苦慮する場合があり，急性熱性疾患では川崎病を念頭において鑑別すべきである．

重症度評価

川崎病の治療目標は，炎症を早期に鎮静化し冠動脈病変などの致死的な心合併症を防ぐことにある．免疫グロブリン治療に反応する場合とそうでない場合では，治療後の血清アルブミン値が大きく異なる．治療不応例では血清アルブミン値が低く，浮腫が強く発熱が持続する．この結果，冠動脈壁の浮腫と虚血を生じ，冠動脈瘤に発展するリスクが高くなる（図）．

したがって，免疫グロブリン治療前に前もってハイリスクの患者を選別できれば，重点的な治療を提供することも可能となる．多くの臨床医がこの課題に取り組んでいる．これまでに，久留米スコア，群馬スコアなどが報告されている．ちなみに群馬スコアでは，①NA ≤ 133，②治療開始病日4日以内，③AST ≥ 100，④好中球（%）≥ 70%，の4項目をそれぞれ2点，①CRP ≥ 10 mg/dL，②月齢12カ月以下，③血小板数 ≤ 30万/mm^3，の3項目をそれぞれ1点として計算した結果，4点以上をハイリスク群としている．

基本病態

四肢末端の浮腫に代表される浮腫病変が，初期より全身にみられる．病変の局在は初期には毛細血管や微小動静脈に限局しているが，やがて，中型筋型動脈，中型静脈に炎症の場が拡大していく．炎症現場の血管周囲には，好中球，マクロファージ，リンパ球の浸潤がみられ，とくに外膜側の炎症細胞の浸潤が顕著である．四肢にみられる硬性浮腫は心臓性浮腫と異なり，圧痕を残さないことが特徴である．一般に心臓性浮腫は，静水圧上昇による血管外への水分の漏出によっておこる．これに対し川崎病の浮腫は，血管透過性の亢進によるvascular leakageによりおこると考えられ，アルブミンを主とする血漿蛋白の血管外への漏出により血管外浮腫が増強する非心臓性浮腫である[2][3]．

本症にみられる浮腫病変の特徴をまとめると，以下の点をあげることができる．

①川崎病では病初期より浮腫病変が全身にみられる．

②臓器の浮腫には毛細血管や微小血管レベルでのvascular leakageが大きな役割をはたしている．

③種々の炎症性サイトカインが炎症局所で産生されている．

④川崎病の浮腫は心不全や腎不全の浮腫とはメカニズムが異なる．

⑤時に，心嚢液貯留もみられる．

治療の実際

川崎病急性期には，免疫グロブリンの大量静注（2 g/kg）に経口アスピリン（30～50 mg/kg）を組み合せた治療が国際的に標準治療として定着している．川崎病における免疫グロブリンの作用機序はいまだ

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表　川崎病（MCLS, 小児急性熱性皮膚粘膜リンパ節症候群）診断の手引き（文献7）より引用）

本症は，主として4歳以下の乳幼児に好発する原因不明の疾患で，その症候は以下の主要症状と参考条項とに分けられる

A．主要症状
1．5日以上続く発熱（ただし，治療により5日未満で解熱した場合も含む）
2．両側眼球結膜の充血
3．口唇，口腔所見：口唇の紅潮，苺舌，口腔咽頭粘膜のびまん性発赤
4．不定形発疹
5．四肢末端の変化　①急性期：手足の硬性浮腫，掌蹠ないしは指趾先端の紅斑
　②回復期：指趾の先からの膜様落屑
6．急性期における非化膿性頸部リンパ節腫脹

六つの主要症状のうち五つ以上の症状を伴うものを本症とする
ただし，上記6主要症状のうち，四つの症状しか認められなくても，経過中に断層心エコー法もしくは心血管造影法で，冠動脈瘤（いわゆる拡大を含む）が確認され，他の疾患が除外されれば本症とする

B．参考条項
以下の症候および所見は，本症の臨床上，留意すべきものである
1．心血管：聴診所見（心雑音，奔馬調律，微弱心音），心電図の変化（PR・QTの延長，異常Q波，低電位差，ST-Tの変化，不整脈），胸部X線所見（心陰影拡大），断層心エコー図所見（心膜液貯留，冠動脈瘤），狭心症状，末梢動脈瘤（腋窩など）
2．消化器：下痢，嘔吐，腹痛，胆嚢腫大，麻痺性イレウス，軽度の黄疸，血清トランスアミナーゼ値上昇
3．血液：核左方移動を伴う白血球増多，血小板増多，赤沈値の促進，CRP陽性，低アルブミン血症，α_2グロブリンの増加，軽度の貧血
4．尿：蛋白尿，沈渣の白血球増多
5．皮膚：BCG接種部位の発赤・痂皮形成，小膿疱，爪の横溝
6．呼吸器：咳嗽，鼻汁，肺野の異常陰影
7．関節：疼痛，腫脹
8．神経：髄液の単核球増多，けいれん，意識障害，顔面神経麻痺，四肢麻痺

備考　1．主要症状Aの5は，回復期所見が重要視される
2．急性期における非化膿性頸部リンパ節腫脹は他の主要症状に比べて発現頻度が低い（65%）
3．本症の性比は，1.3〜1.5：1で男児に多く，年齢分布では4歳以下が80〜85%を占め，致命率は0.1%前後である
4．再発例は2〜3%に，同胞例は1〜2%にみられる
5．主要症状を満たさなくても，他の疾患が否定され，本症が疑われる容疑例が約10%存在する．この中には冠動脈瘤（いわゆる拡大を含む）が確認される例がある

完全には解明されていない．

治療例の約85%において，治療開始後24〜48時間で解熱が得られる．しかしながら，残りの約15%では免疫グロブリン治療後も解熱が得られず，このような治療不応例では冠動脈瘤の発生リスクが高まることが知られている．

これら免疫グロブリン治療不応例に対する治療は確立されていない．これまでに，ステロイドパルス療法，好中球エラスターゼ阻害薬のウリナスタチンやシベレスタット，シクロスポリンによる免疫抑制薬，抗腫瘍壊死因子抗体，血漿交換療法が試みられている．

最新ガイドライン/エビデンス

日本小児循環器学会が急性期治療ガイドライン[4]を出している．とくに，初回免疫グロブリン治療不応の治療選択として，免疫グロブリンの追加治療，ステロイド療法（パルス療法ないしプレドニゾロンの静注または経口投与），ウリナスタチン静注療法，血漿交換療法を提唱している．しかしながら近年，抗ヒト腫瘍壊死因子αモノクローナル抗体製剤や免疫抑制薬の使用経験が蓄積されており，急性期治療ガイドラインは改訂される予定である．

前述したように，抗ヒト腫瘍壊死因子αモノクローナル抗体製剤（インフリキシマブ）の使用経験が国内外で報告されている．免疫グロブリン治療不応例で，免疫グロブリン再投与無効例あるいはメチルプレドニゾロン追加無効例に対して，体重あたり5 mgのインフリキシマブを1回投与したところ臨床症状の改善がみられたとの報告が出された[5]．米国ではインフリキシマブの有効性に関する多施設トライアルが行われている．

私の治療方針

初期治療のアスピリン量は 50 mg/kg を使用している．たとえ肝のトランスアミナーゼの上昇があったとしても，原則，アスピリンを使用することとしている．川崎病の肝機能異常は胆嚢腫大の閉塞機転によるもので，免疫グロブリン治療により胆嚢の浮腫が軽快すると肝機能も改善する．また，追加免疫グロブリンの投与時期であるが，初回免疫グロブリン治療開始後 48 時間の時点で 37.5℃以上の発熱があれば，迷うことなく追加治療を行っている．

千葉大学大学院羽田明氏，和歌山県立医科大学鈴木啓之氏らとの共同研究として，追加免疫グロブリン治療に反応しない場合に，免疫抑制薬であるシクロスポリン投与を 2 週間行っている．対象は 4 カ月齢以上で，1 日量 4 mg/kg を経口的に投与する．この場合，トラフ値が 60〜200 ng/dL となるように投与量を調節している．大部分の例で，シクロスポリン投与翌日には解熱が得られる．

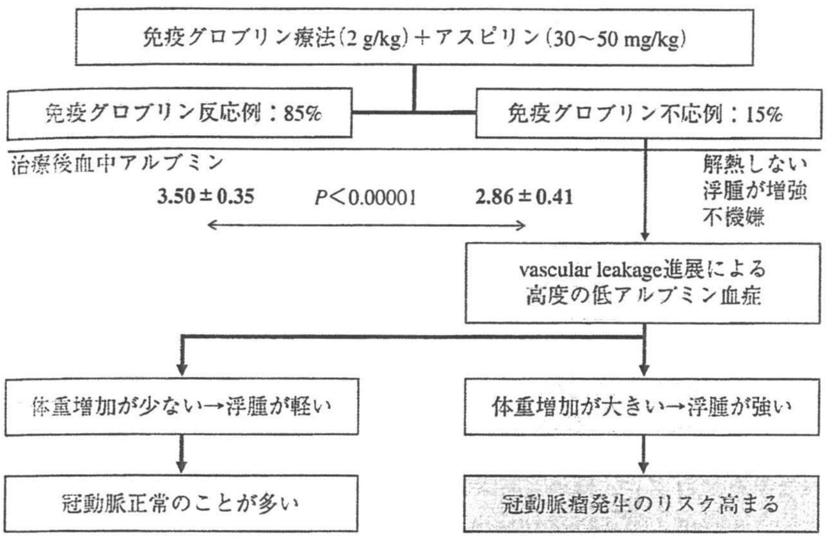

図　免疫グロブリン治療の効果と病態生理

治療不応例では血管透過性が進展し，高度の低アルブミン血症と血管外浮腫が強くなり，冠動脈瘤発生のリスクが高まる

ピットフォールと対策

初回免疫グロブリン治療不応例への追加治療として，ステロイドは病期によっては瘤形成を助長するため好ましくないという考えかたが支配的である．とくに，9 病日を超えて投与することには慎重な意見が多い．川崎病では血管透過性が亢進しており，重症例では硬性浮腫が顕著である．血清の低アルブミンに代表されるように，血漿蛋白質が血管外に漏出している．そのため，過剰な水分投与は血管外浮腫を助長し，冠動脈壁の虚血をもたらすと考えられる．

近年のトピックス

川崎病を惹起しうる外的因子として，感染性微生物，毒素，ストレス，環境因子などの単一あるいは複合要因が考えられるが，いまだに結論が出ていない．一方，個体側の遺伝的因子が明らかにされつつある．Onouchi らにより，川崎病発症に関連する遺伝子が特定された[6]．T 細胞の活性化にかかわると考えられるイノシトール三リン酸酵素（ITPKC）の遺伝子多型である．免疫グロブリン治療との反応性との関連も指摘されている．今後，さらなる個体側因子の解明により，炎症性サイトカイン産生のメカニズム，新たな治療法の開発に繋がることが大いに期待される．

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著者連絡先

〒276-8524　千葉県八千代市大和田新田 477-96
東京女子医科大学八千代医療センター
寺井　勝

14

Detection of multiple superantigen genes in stools of patients with Kawasaki disease

Suenaga T, Suzuki H, Shibuta S, Takeuchi T, Yoshikawa N

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Detection of Multiple Superantigen Genes in Stools of Patients with Kawasaki Disease

Tomohiro Suenaga, MD, Hiroyuki Suzuki, MD, PhD, Shoichi Shibuta, MD, Takashi Takeuchi, MD, and Norishige Yoshikawa, MD, PhD

Objectives To investigate whether superantigens (SAgs) are involved in the development of Kawasaki disease (KD) by examining SAg genes in the stool of patients with KD.

Study design Stool specimens were obtained from 60 patients with KD and 62 age-matched children (36 children with acute illness and 26 healthy children). Total DNA was extracted from these stool samples. Using polymerase chain reaction, we examined genes of 5 SAgs: streptococcal pyrogenic exotoxin-A (SPE-A), SPE-C, SPE-G, SPE-J, and toxic shock syndrome toxin-1.

Results At least 1 of the 5 SAg genes was detected in 42 (70%) specimens from patients with KD, 14 (38.9%) from the febrile group, and 7 (26.9%) from the healthy group. The detection rate between subjects with and without KD was of at least 1 of the 5 SAg genes ($P < .001$), and more than 2 SAg genes were significantly different ($P = .002$).

Conclusions SAg may be involved in the development of KD; data suggest that multiple SAgs may trigger KD. *(J Pediatr 2009;155:266-70).*

Kawasaki disease (KD) is an acute systemic vasculitis occurring in infants and children younger than 4 years of age.[1] KD is also the most common cause of acquired heart disease in children, resulting in coronary arterial lesions (CAL) in approximately 25% of untreated patients. Although the etiology of KD is unknown, epidemiologic data suggest that an infectious agent may play a role in triggering the development of KD.[2] However, KD does not appear to be easily transmittable. Therefore, some genetic factors also may play an important role in the development of KD. Indeed, Onouchi et al[3] have reported that in functional polymorphism, inositol 1,4,5-trisphosphate 3-kinase C (ITPKC) is associated with susceptibility to KD and formation of CAL.

Several studies have examined the relationship between the onset of KD and superantigens (SAgs), which are powerful mitogenic proteins produced by microbes.[4-13] Small amounts of SAg proteins can stimulate the production of a large amount of proinflammatory cytokines such as interleukin (IL)-1, IL-2, and IL-6 through T-lymphocytes. Such responses are consistent with the acute phase of KD. We have reported that streptococcal pyrogenic exotoxin type C (SPE-C), one of the SAgs produced by *Streptococcus pyogenes*, might be associated with KD, based on analysis of T-lymphocyte receptor (TCR) repertoire and titer of antibody against SPE-C.[7] Previous studies of the relationship between KD and SAg have been limited to examination of the TCR repertoire and antigen-antibody reaction.[4-13] These studies have demonstrated only indirectly that SAg may be related to the development of KD. Isolation of SAg-producing bacteria from patients with KD could confirm association; however, this may be difficult because bacteria may be present in low density, may not be cultivatable easily, or may be nonviable at the time of onset of KD.

We therefore examined SAg genes in stools of patients with KD, which might include SAg genes derived from any bacteria present from the upper respiratory tract or the upper or lower gastrointestinal tract, to assess the relationship of SAgs to the development of KD.

Methods

Patients and Stool Samples

The study was approved by the Ethics Committee of Wakayama Medical University (Wakayama, Japan). Informed consent was obtained from the parents of the patients. Sixty patients who fulfilled the diagnostic criteria for KD[14] and

CAL	Coronary artery lesions
IL	Interleukin
KD	Kawasaki disease
PCR	Polymerase chain reaction
SAg	Superantigen
SPE	Streptococcal pyrogenic exotoxin
TCR	T-lymphocyte receptor
TSST	Toxic shock syndrome toxin

From the Department of Pediatrics, Wakayama Medical University, Wakayama, Japan

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were admitted to our hospital between June 2004 and June 2007 were enrolled consecutively.

Stool specimens were obtained from the 60 patients with KD at the acute stage of the disease (KD group) and from 62 age-matched children without KD: 36 with acute illnesses (febrile group) and 26 healthy children. The first stools were collected from patients with KD upon admission. In addition, cultures were performed at the hospital laboratory using samples taken from the pharynx and stool of patients with KD to examine the presence of *S pyogenes* and *Staphylococcus aureus*. The 36 febrile children included 9 with respiratory syncytial viral infection, 6 with viral enteritis due to norovirus or rotavirus, 3 with influenza virus infection, 3 with mycoplasma pneumonia, and 15 with other infectious diseases. Children with known streptococcal and staphylococcal infections were excluded from among these febrile children. Stool samples from the healthy control children were collected when they visited our center for vaccinations.

DNA Extraction

Stools samples were kept at −20°C until extraction and purification of total DNA using a stool DNA isolation Kit (QIAGEN, DNA Stool Mini Kit; QIAGEN GmbH, Hilden, Germany); concentration of the extracted DNA was measured using a spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, Delaware).

Polymerase Chain Reaction and Primer Design

Using total DNA extracted from stools as a template, polymerase chain reaction (PCR) was performed for 5 SAg genes– SPE-A, SPE-C, SPE-G, SPE-J, and toxic shock syndrome toxin-1 (TSST-1)– which are sensitive to TCRVβ2 and/or have been reported to be related to KD.[4-13,15] Extracted DNA from *S pyogenes* (JCM No.5674), purchased from the Microbe Division of Riken BioResource Center (Wako, Saitama, Japan), was used as a positive PCR control for SPE-A, SPE-G, and SPE-J. In addition, extracted DNAs from *S pyogenes* (ATCC No.700294) and *S aureus* (ATCC No.51651), purchased from ATCC (Manassas, Virginia), were used as positive PCR controls for SPE-C and TSST-1, respectively.

Both the sense and anti-sense specific primers for each SAg gene were designed to include 20 base pairs to obtain PCR products of around 200 base pairs, using Primer3 (Whitehead Institute for Biomedical Research, Cambridge, Massachusetts). The sequences of the specific primers are as follows: SPE-A; SPE-A-fw (GGTGACCCTGGTTACT CACG) and SPE-A-rev (CCCTCCGTAGAGACATGCAC), SPE-C; SPE-C-fw (AGGAATTACGCCTGCTCAAA) and SPE-C-rev (AATTTCGATTCTGCCGCTTA), SPE-G; SP E-G-fw (ACCCCATGCGATTATGAAAA) and SPE-G-rev (GGGAGACCAAAAACATCGAC), SPE-J; SPE-J-fw (CTTT CATGGGTACGGAAGTG) and SPE-J-rev (GCTCTCGA CCTCAGAATCAA), TSST-1; TSST-1-fw (GTAAGCCCT TTGTTGCTTGC) and TSST-1-rev (CTGATGCTGCCATCT GTGTT). Sizes of each PCR product (bp) of the 5 SAg genes are 229, 217, 170, 196, and 215, respectively.

PCR was performed in a total volume of 30 μL, which included template DNA (100 ng), 10 × PCR buffer containing 15 mmol/L $MgCl_2$ (3 μL), 2 mmol/L of each deoxyribonucleotide triphosphate (3 μL), 15 pmol of paired primers (1.5 μL), 1.0 U Taq polymerase (0.2 μL; AmpliTaq Gold; Applied Biosystems, Branchburg, New Jersey), and 3 μg bovine serum albumin, using a GeneAmp PCR System 9700 (Applied Biosystems).

Amplifications were performed as follows: first, 1 cycle for 10 minutes at 95°C; second, 40 cycles for 1 minute at 94°C, 1 minute at 57°C, followed by 1 minute at 72°C; and finally, 1 cycle for 7 minutes at 72°C. The products were analyzed by electrophoresis on a 2 % agarose gel stained with ethidium bromide. If the PCR products were found at the same position as each positive control, the sequences of the products were examined using a 310 Genetic Analyzer (Applied Biosystems) and were confirmed for each of the 5 SAg sequences.

Statistical Analysis

Statistical analysis was performed using SPSS version 11.0.1 J (SPSS Inc., Chicago, Illinois). Associations between categorical variables were examined by unpaired *t* test, 1-way analysis of variance, and Fisher exact test. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. Differences at a 2-tailed *P* value of <.05 were considered statistically significant.

Results

Patients with KD, febrile control children, and healthy control children had similar sex ratios (male:female) (34:26, 18:18, and 13:13) and mean ages (24.6, 25.9, and 25.5 months, respectively). The stools were collected from the patients with KD at a mean of 5.7 days from disease onset. Although not significantly different, the concentrations of DNA extracted from stools of healthy control children (m = 59.7 ng/μL) were slightly higher than those of patients with KD (m = 37.8 ng/μL) and febrile control children (m = 38.5 ng/μL). These differences may have been due to the consistency of the stools, because many of the stools from the patients with KD and febrile control children were watery and fatty.

S pyogenes was not isolated from any throat specimens, but *S aureus* was isolated from throat specimens from 2 patients with KD. *TSST-1* gene was not detected in the stools of these 2 patients with KD. *S pyogenes* was not isolated from any stool specimens, but *S aureus* was isolated from stool samples from 4 patients with KD (one patient whose throat culture was also positive). TSST-1 gene was detected in only 1 of these patients with KD.

The results of PCR for the 5 SAg genes in the 3 groups are summarized in Tables I, II, III, and IV. Cases in which PCR revealed positivity for SPE-C and SPE-G are shown in the Figure. At least 1 of 5 SAg genes was detected in 42 specimens (70%) from the KD group, 14 specimens (38.9%) from the febrile group, and 7 specimens (26.9%) from the healthy group (Table I). Differences in the rate of detection of at least

Table I. Numbers of patients in whom each of the 5 superantigen genes were detected in stools

	KD group (n = 60)	Febrile group (n = 36)	Healthy group (n = 26)
SPE-A	7 (1)	0	1 (1)
SPE-C	16 (3)	6 (3)	2 (1)
SPE-G	26 (12)	7 (4)	2 (2)
SPE-J	9 (7)	2 (0)	0
TSST-1	9 (1)	4 (3)	3 (2)
SPE-A + SPE-G	2		
SPE-C + SPE-G	7	1	
SPE-G + SPE-J	1	1	
SPE-A + TSST-1	1		
SPE-C + TSST-1	1	1	1
SPE-G + TSST-1	1		
SPE-C + SPE-G + SPE-J		1	
SPE-A + SPE-C + TSST-1	1		
SPE-C + SPE-G + TSST-1	1		
SPE-C + SPE-J + TSST-1	1		
SPE-A + SPE-C + SPE-G + TSST-1	2		
At least 1 of 5 SAgs	42 (70%)	14 (38.9%)	7 (26.9%)
Multiple SAgs	18 (30%)	4 (11.1%)	1 (3.8%)

Upper column: Values are numbers of patients in whom at least 1 SAg gene was detected, including the indicated SAg. (Values) are numbers of patients in whom only the indicated SAg gene was detected.

1 SAg gene between KD:febrile and KD:healthy (KD:febrile, $P = .005$; KD:healthy, $P < .001$; Fisher exact test) (Table II, III). Additionally, detection of at least 1 SAg gene became clearer when children with and without KD (both febrile and healthy) were compared (significant difference, $P < .001$, Table IV). There were significant differences in the rate of detection of the SPE-G gene between KD and each of the other 2 groups (KD:febrile, $P = .026$; KD:healthy, $P = .001$) and for the SPE-A gene between the KD group (11.7%) and the febrile group (0%) ($P = .043$). Comparing KD and the combined non-KD group, significant differences were found in the rates of detection of SPE-A ($P = .031$), SPE-G ($P = .001$), and SPE-J ($P = .029$) (Table IV).

Three or more of 5 SAg genes were detected in 18 specimens from the KD group (30%), 4 specimens from the febrile group (11.1%), and 1 specimen from the healthy group (3.8%) (Tables I through (IV); differences were significant between KD and each of the other 2 groups (KD:febrile, $P = .044$; KD:healthy, $P = .009$, Fisher exact test). There was also a significant difference in the rate of detection of ≥3 SAgs between KD and non-KD groups ($P = .002$, Fisher exact test, Table IV).

There were no significant differences in sex ratio, age (month), or the incidence of resistance to immunoglobulin therapy or CAL between patients with (42 cases) and without (18 cases) KD SAg genes detected.

Discussion

It is still controversial whether the etiology of KD may involve a conventional antigen or a SAg.[16] We have investigated SAgs derived from *S pyogenes* as causative agents of KD because the main symptoms of KD are similar to those of streptococcal infection. Although investigations have indirectly linked SAgs with KD, few articles have detected either SAgs themselves or SAg-producing bacteria in patients with KD).[11] We sought to detect SAg genes as antigenic agents in stool specimens because all bacteria present in the upper respiratory and entire gastrointestinal tracts are thought to be finally eliminated into stools. Thus, it is likely that all gene fragments derived from these bacteria will be contained in stools.[17] Even if bacteria are nonviable at the onset of KD and/or nonculturable, it is likely that gene fragments from these bacteria are contained in stools in the early acute phase of KD. We selected 4 SAgs sensitive to TCRVβ2: SPE-C, SPEG, SPE-J, and TSST-1. Although there has been considerable disagreement in previous studies about which elements of the TCR repertoire are activated in patients with KD, most have suggested that TCRVβ2 may be activated.[4,7-11] In addition, we also selected SPE-A, a candidate SAg with etiologic relevance by analysis of antibody responses.[12]

Our results revealed that all of the 5 selected SAg genes were detected more frequently in the stools of children with KD than in those without KD, suggesting that these bacteria are harbored in the upper airway or gastrointestinal tract more frequently in patients with KD. Moreover, the SPE-A, SPE-G, and SPE-J genes, in particular, were detected more frequently in the stools of patients with KD. These results suggest that single or multiple SAgs may each be related to the development of KD, although we examined only 5 of

Table II. Rate of detection of superantigens (SAgs) genes in patients with KD and with the febrile group

	KD group (n=60)	Febrile group (n=36)	OR	95%CI	p-value#
SPE-A	7 (11.7)	0 (0)	-	-	0.043
SPE-C	16 (26.7)	6 (16.7)	1.82	0.64-5.18	0.321
SPE-G	26 (43.3)	7 (19.4)	3.17	1.20-8.36	0.026
SPE-J	9 (15.0)	2 (5.6)	3.00	0.61-14.7	0.200
TSST-1	9 (15.0)	4 (11.1)	1.41	0.40-4.97	0.761
At least one of five SAgs	42 (70.0)	14 (38.9)	3.67	1.54-8.74	0.005
MultipleSAgs	18 (30.0)	4 (11.1)	3.43	1.06-11.1	0.004

SPE: Streptococcal pyrogenic exotoxin
TSST: Toxic Shock Syndrome Toxin (values):%
#: Fisher's exact test

Table III. Rate of detection of superantigens (SAgs) genes in patients with KD and in the healthy group

	KD group (n=60)	Febrile group (n=26)	OR	95%CI	p-value#
SPE-A	7 (11.7)	1 (3.8)	3.30	0.39-28.3	0.426
SPE-C	16 (26.7)	2 (7.7)	4.36	0.92-20.6	0.081
SPE-G	26 (43.3)	2 (7.7)	9.17	1.99-42.4	0.001
SPE-J	9 (15.0)	0 (0)	-	-	0.052
TSST-1	9 (15.0)	3 (11.5)	1.35	0.33-5.46	1.000
At least one of five SAgs	42 (70.0)	7 (26.9)	6.33	2.27-17.7	<0.001
MultipleSAgs	18 (30.0)	1 (3.8)	10.71	1.35-85.2	0.009

SPE: Streptococcal pyrogenic exotoxin
TSST: Toxic Shock Syndrome Toxin (values):%
#: Fisher's exact test

Table IV. Rate of detection of superantigens (SAgs) genes in patients with and without KD

	KD group (n=60)	non-KD* group(n=62)	OR	95%CI	p-value#
SPE-A	7 (11.7)	1 (1.6)	8.06	0.96-67.6	0.031
SPE-C	16 (26.7)	8 (12.9)	2.45	0.96-6.27	0.070
SPE-G	26 (43.3)	9 (14.5)	4.50	1.88-10.7	0.001
SPE-J	9 (15.0)	2 (3.2)	5.29	1.09-25.6	0.029
TSST-1	9 (15.0)	7 (11.3)	1.39	0.48-4.00	0.600
At least one of five SAgs	42 (70.0)	21 (33.9)	4.56	2.13-9.77	<0.001
MultipleSAgs	18 (30.0)	5 (8.1)	4.89	1.68-14.2	0.002

SPE: Streptococcal pyrogenic exotoxin
TSST: Toxic Shock Syndrome Tox (values):%
*: non-KD:both febrile and healthy children
#: Fisher's exact test

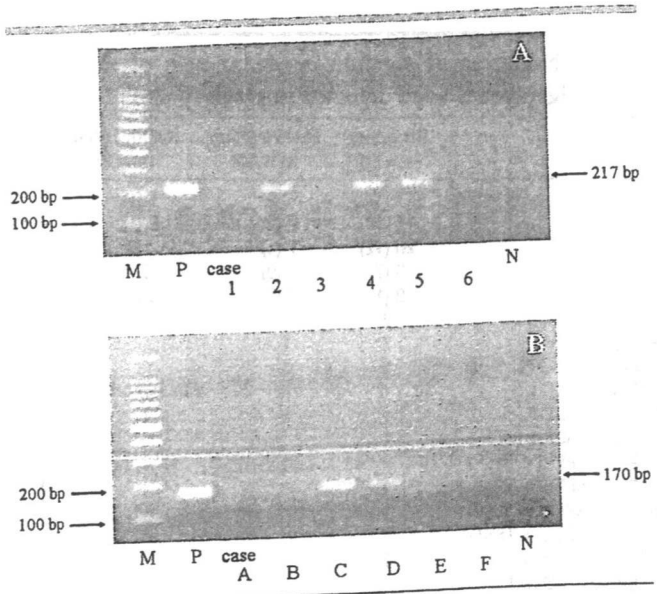

Figure. Electrophoresis samples after PCR for SPE-C (upper) and SPE-G (bottom). **A,** PCR results for SPE-C in patients 1 through 6. PCR product is 217 bp. M, marker; P, positive control; N, negative control. Cases 2, 4, and 5 show positive results. **B,** PCR results for SPE-G in patients A through F. PCR product is 170 bp. M, marker; P, positive control; N, negative control. Cases C and D show positive results.

the more than 30 SAgs. We hypothesize that multiple factors can trigger KD and that even 1 can cause disease. Several phenomena appear to support our hypothesis. First, microorganisms producing SAgs such as *S pyogenes, S aureus, Yersinia pseudotuberculosis,*[18] *Mycoplasma,*[19] and Epstein-Barr virus[20] can produce conditions that mimic KD. Second, previous studies have revealed conflicting variations in the TCR repertoire in KD. Third, epidemiological studies have shown that KD can recur.[21]

In the present study, the 5 SAg genes investigated were detected slightly more frequently in the stools of patients in the febrile group than in those of healthy children. Although we did not examine the use of antibiotics in the febrile group, it is likely that many more DNA fragments would be eliminated in the stools of febrile children as a result of bacteria killed by antibiotic therapy. We also did not collect information on antibiotic use in patients with KD. However, as the incidence of SAg genes in the KD group was significantly higher than that in the febrile group, we think that the use of antibiotics did not our results.

There were several limitations to our study. First, we did not determine where the bacteria containing these SAg genes exist in patients with KD. Second, the bacteria containing these SAg genes were not clarified. Third, it was unclear whether bacteria containing SAg genes actually produced the SAgs examined. Fourth, we did not examine the antibody response to each SAg gene detected in stools. Recently, however, Vojtek et al[22] reported that a SAg gene of group A *Streptococcus* might be transferred among streptococci. Thus, *Streptococcus* species such as α-hemolytic streptococci in the throat of patients with KD may be a candidate likely to receive such group A *Streptococcus* SAg genes, although Hirota et al[23] have reported that bacteria in the throat flora of patients during the acute phase of KD show no significant mitogenic activity such as SAg production.

Many epidemiological analyses have revealed that host factors such as genes and race might be related to the development of KD. Although SAg genes were detected less frequently in the stools of children without KD, such children may not develop KD because they lack critical host factors. A recent study of susceptibility to KD has shown that polymorphism with functional loss of ITPKC, which is a negative regulator of activated T-lymphocytes, induces persistent T-lymphocyte activation, leading to massive production of proinflammatory cytokines. The fact that these pathways of T-cell activation may be related to KD susceptibility appears to support our hypothesis of multiple SAgs triggering the onset of KD, as SAgs are powerful T-lymphocyte activators. Recent studies also have suggested that HLA class II polymorphism may determine responses to bacterial SAgs.[24-27] HLA class II molecules play a very important role in T-lymphocyte activation by SAgs via the TCR. Moreover, it is possible that many other genes may play a role in host determinants of KD. ■

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Reprint requests: Dr Hiroyuki Suzuki, Department of Pediatrics, Wakayama Medical University, 811-1 Kimiidera, Wakayama, 641-0012, Japan. E-mail: hsuzuki@wakayama-med.ac.jp.

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15

頚部リンパ節腫脹のフォロー中に発熱と冠動脈変化を認め、川崎病不全型と考えられた１例

津野嘉伸、南　孝臣、比嘉明日美、津田祐子、坊岡美奈、
泉　鉉吉、宮脇正和、渋田昌一、武内　崇、　鈴木啓之、吉川徳茂

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川崎病—第33回近畿川崎病研究会—


津野　嘉伸

頸部リンパ節腫脹のフォロー中に発熱と冠動脈変化を認め，川崎病不全型と考えられた1例

Tsuno Yoshinobu 津野　嘉伸
Minami Takaomi 南　孝臣
Higa Asumi 比嘉明日美
Tsuda Yuko 津田　祐子
Booka Mina 坊岡　美奈
Izumi Genkichi 泉　鉉吉
Miyawaki Masakazu 宮脇　正和[1)]
Shibuta Shoichi 渋田　昌一
Takeuchi Takashi 武内　崇
Suzuki Hiroyuki 鈴木　啓之
Yoshikawa Norishige 吉川　徳茂[2)]

[1)]社会保険紀南病院小児科　[2)]和歌山県立医科大学小児科

はじめに

川崎病は症候群であり，現在も原因不明のため，主要症状の揃わない川崎病不全型(容疑例および主要症状4以下例)の診断は特に難しい．しかし，不全型においても，冠動脈障害(CAL)の出現頻度は完全型と変わらず，CALを認めた症例の約5％を主要症状3項目以下の不全型が占めており，冠動脈障害を減らすためにも，完全型に準じた速やかなガンマグロブリン大量療法(IVIG)が必要とされている．

年長児では，川崎病主要症状が揃いにくいこともあり，頸部リンパ節腫脹を認めた場合，川崎病不全型を常に鑑別診断の1つに置きながら診療に当たることが重要である．

今回，2年間にわたる慢性頸部リンパ節腫脹の経過観察中に発症した川崎病不全型と考えられる1例を経験したので報告する．

症例呈示

症　例：6歳，女児．

主　訴：発熱，右頸部リンパ節腫脹．

家族歴：同胞2名中第2子，特記すべきことなし．

既往歴：2006年7月，右頸部リンパ節腫脹(最大φ5cm)，抗生物質にて縮小傾向となった．細胞診にてclass Ⅱであった．2007年2月，左右頸部リンパ節および腋窩リンパ節腫脹(最大φ5cm)．抗生物質投与後，徐々に縮小傾向となった．2008年1月，左右頸部リンパ節腫脹(最大φ3cm)を認めた．EBウイルスおよびサイトメガロウイルスは未感染，猫引っかき病否定のために提出したバルトネラ抗体は陰性，結核菌QFTも陰性であった．自己免疫疾患の可能性も考慮したが，抗核抗体が160倍と上昇を認めた以外は，すべて陰性であった．その他，各腫瘍マーカーも正常範囲であり，CD4/CD8比も1.44と異常は認めなかった．リンパ節生検を施行するも病理学的には悪性を疑う所見なく，非特異性のリンパ節炎であった(図1)．以後，徐々に縮小傾向となった．

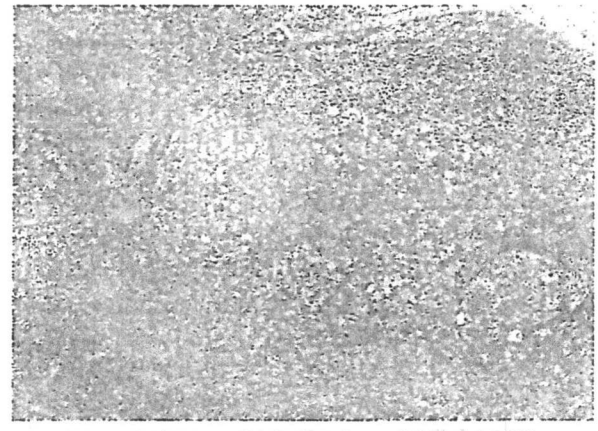
図1　頸部リンパ節組織像(2008年1月に生検)
濾胞構造は保たれており，小型で異型性の乏しいリンパ球が浸潤．

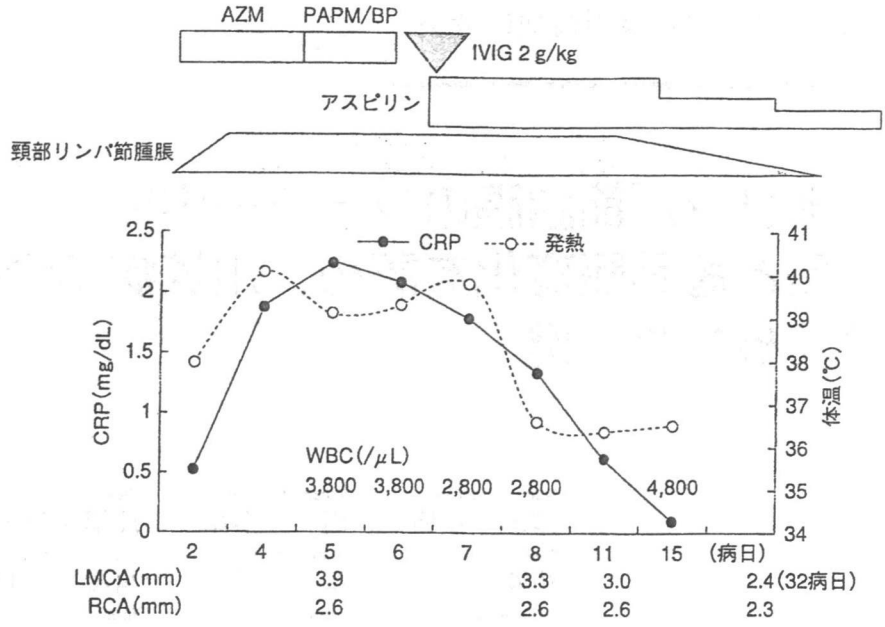

図2　症例の経過

表1　入院時検査所見(第5病日)

<末梢血>		GPT	13 IU/L
WBC	3,800/μL	TG	34 mg/dL
Neutro	59.7%	HDL-C	36 mg/dL
Lymph	34.1%	Na	136 mEq/L
Mono	5.9%	K	4.2 mEq/L
Eosin	0.0%	Cl	100 mEq/L
Baso	0.3%	UA	0.44 mg/dL
RBC	441万/μL	BUN	10.6 mg/dL
Hb	12.0 g/dL	Glu	93 mg/dL
Plt	17.8万/μL	T-Bil	0.3 mg/dL
		CRP	2.23 mg/dL
<生化学>			
TP	6.7 g/dL	<尿化学>	
Alb	3.6 g/dL	β_2-MG	733 μg/dL
LDH	403 IU/L		
CK	50 IU/L	<咽頭培養>	
GOT	34 IU/L	MSSA	(2+)

現病歴：2008年10月下旬に右頸部リンパ節腫脹が出現．その3日後に38℃の発熱出現．第2病日に38℃の発熱持続し，当科受診，右頸部リンパ節φ3×2.5 cm触知した．

血液検査で，WBC 4,200/μL，CRP 0.52 mg/dL．咽頭炎の診断でAZM処方にて帰宅．第3病日に40.0℃の発熱，頸部リンパ節の自発痛あり，首を回すのも困難な状況であった．第4病日に40.0℃の発熱持続し，当院救急外来受診．右頸部リンパ節φ4×3 cmとさらに腫大．WBC 4,200/μL，CRP 1.89 mg/dL．第5病日に当科再診，39.1℃の発熱持続．WBC 3,800/μL，CRP 2.23 mg/dL，発熱5日目であり，精査加療目的で入院した(表1)．

入院時現症：体温39.1℃，体重19 kg，咽頭軽度発赤，呼吸音清，心音純・心雑音なし，右頸部リンパ節腫大φ4×3 cm，圧痛あり，腹部平坦軟，肝脾触れず，眼球結膜充血なし，全身に発疹なし，口唇紅潮なし，四肢の浮腫なし，BCG発赤なし．

臨床経過(図2)：入院後(第5病日)，感染性のリンパ節炎も否定できないため，PAPM/BP点滴するも解熱を得られず，第6病日に5日間以上の発熱，頸部リンパ節腫脹，口唇発赤から川崎病不全型を疑った．頸部痛が強く，首の回旋も困難であった．心エコーで右冠動脈起始部は2.6 mmであったが，左主幹動脈が3.9 mmと軽度拡張し，エコー輝度の上昇を認めた(図3)．

通常，川崎病の冠動脈拡張は第8病日頃から起こるが，早期に冠動脈拡張を認める例も報告されている．頸部エコー上，患児の右頸部のリンパ節は，1つの大きなものではなく，最大18 mmのリンパ節が集まった形で，川崎病におけるリンパ節腫脹のエコー所見とも合致していた[1]．また，血流はリンパ節門から流入し，血流は豊富であった(図4)ことから，化膿性壊死性リンパ節炎の所見とは異なっていた．

以上を総合的に判断し，川崎病不全型として，第6病日よりアスピリン(30 mg/kg/day)およびIVIG(2

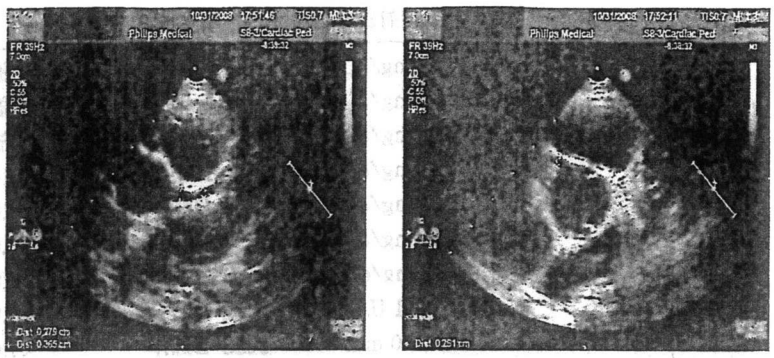

図3　冠動脈エコー所見(第5病日)
左冠動脈の軽度拡張および輝度上昇を認めた.

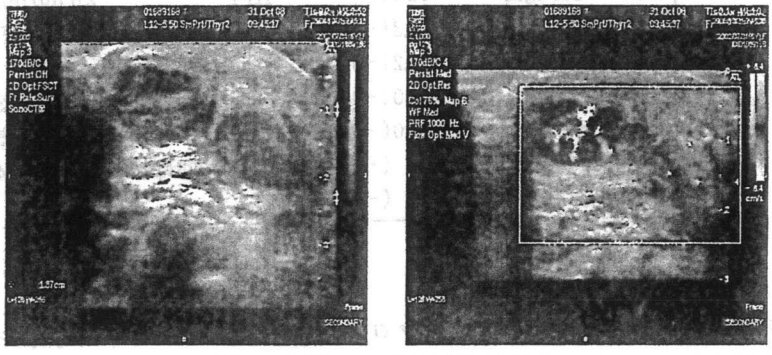

図4　頸部エコー所見(第5病日)
右頸部に最大18 mmのリンパ節が多房性に散在.リンパ節門から流入する血流は豊富.

g/kg)点滴を開始した.第8病日に解熱を認め,CRPは1.35 mg/dLと低下傾向を示した.頸部リンパ節の圧痛は消失し,首の回旋も可能になったが,リンパ節径は4×2 cm大が持続した.第11病日よりアスピリン20 mg/kg/day,第16病日より10 mg/kg/dayに減量した.左冠動脈は第12病日に3.0 mm,第29病日には2.5 mmと径は縮小しており,CALは認められなかった.また,経過とともにエコー上の冠動脈輝度は改善していった.右冠動脈の拡張や輝度上昇は認められず,頸部リンパ節はようやく2 cm大まで縮小した.

考　　察

第17回の全国調査成績[2]によると,全報告数のうち13.2%の川崎病不全型が存在し,CALを形成する例も多数報告されており,不全型も典型例に準じた治療戦略による対処が必要と考えられている[3-7].川崎病不全型と診断する際には,様々な感染を否定するなど,慎重な鑑別診断が重要である.

頸部リンパ節腫脹を来す代表的疾患は,感染症では溶連菌感染症,EBウイルス感染症,結核,猫引っかき病など,悪性腫瘍では白血病や悪性リンパ腫,自己免疫疾患では川崎病のほかに亜急性壊死性リンパ節炎,若年性特発性関節炎(JIA),全身性エリテマトーデス(SLE)などが挙げられる[8].川崎病不全型では,頸部リンパ節腫脹の出現頻度が約35%と低くなっている[9].

本症例は炎症反応が軽度であったが,抗生物質は無効で,川崎病主要症状は3/6を満たすにとどまっていた.ウイルス感染やPFAPA(periodic fever, aphthous stomatitis, pharyngitis, and adenopathy)症候群などの可能性も考慮され,右頸部リンパ節腫脹と周囲の発赤,および首の回旋ができないほどの頸部痛を認めたが,扁桃の白苔はなかった.心エコーで,左冠動脈の拡張および輝度上昇を認めた.また,頸部エコーで血流豊富なリンパ節が多房性に散在しており,川崎病症例のエコー所見と一致していた.亜急性壊死性リンパ節炎との鑑別は重要で,亜急性壊死性リンパ節炎[10]は感冒様症状・扁桃腫脹後の頸部リンパ節腫脹(55.5～97.5%),または全身リンパ節腫脹(1.3～22.2%)と発熱(30.2～50%)を特徴とし,腫脹したリンパ節は半数以上の症例において有痛性であるとされている.検査所見では白血球減少(25～58.3%),CD4/CD8比の低下が

表2　入院前までに施行した検査所見

IgG	1,202 mg/dL	抗核抗体	160倍
IgA	95 mg/dL	Homogeneous	160倍
IgM	120 mg/dL	Speckled	160倍
IgE	606 mg/dL	抗ss-DNA IgG抗体	<10
C3	161 mg/dL	抗ds-DNA IgG抗体	<10
C4	46 mg/dL	抗RNP抗体	<7.0
CH_{50}	55.3 mg/dL	PR3-ANCA	<10
sIL-2R	1,021 U/L	抗SS-A抗体	<7.0
血沈1時間値	7.0 mm	抗SS-B抗体	<7.0
血沈2時間値	28.0 mm	抗好中球細胞質抗体	<10
マイコプラズマ抗体	(−)	CEA	1.3 ng/mL
EBV-VCA IgM	<2.0(−)	AFP	2.0 ng/mL
EBV-VCA IgG	0.3(−)	NSE	17.4 ng/mL
EBV-EBNA IgG	0.2(−)		
CMV-IgM	0.60(−)	CD4	43.8
CMV-IgG	<2.0(−)	CD8	30.5
パルトネラ抗体	(−)	CD4/CD8比	1.44
結核菌QFT	(−)		

みられる．組織所見ではリンパ球を貪食したマクロファージの増生が認められる．今回は発熱，頸部リンパ節腫脹，白血球減少を認めた所見は合致するが，CD4/CD8比が正常範囲であったことや，頸部エコーでリンパ節の血流が豊富であったことから，亜急性壊死性リンパ節炎は否定的であった．

これらを総合的に判断し，川崎病不全型を否定できない以上，CALの予防を優先することとした．ガンマグロブリンについて十分に家族に説明し，同意を得た上で，川崎病不全型としてIVIGを行った．

今回の発症までの慢性的なリンパ節腫脹に関しては，結核性リンパ節炎，猫引っかき病などが鑑別疾患として挙げられるが，両者ともに入院前の検査で否定的であった(表2)．また，本症例は川崎病に合致しにくい点もいくつか認められた．1点目は，左冠動脈の一過性拡張が第5病日と早期に始まり，通常CALの形成が始まる第8病日頃から改善していったこと，2点目はIVIGにより速やかに解熱したが，リンパ節腫脹は縮小傾向を認めるものの遷延したことである．このことは，2年前からの経過と今回のエピソードが同一の疾患であり，川崎病でないのではないかという疑問を提示するが，今回は，上記のように原因不明の慢性リンパ節腫脹を来す疾患に川崎病を合併したと考えた．

現在も，小さいながら慢性のリンパ節腫脹が持続しており，今後も再腫脹や発熱を繰り返す可能性がある．その場合はやはり川崎病以外の疾患である可能性を考慮していかなければならない．本症例は抗核抗体が160倍と高値であることから，今後JIAやSLEなどの自己免疫疾患に発展していく可能性も考慮し，定期的にフォローしていく必要がある．また，遺伝性の周期性発熱を来す疾患も鑑別に挙げられる．次回，今回と同様の症状を認めた場合，診断を明らかにするよう全力で取り組むのはいうまでもないが，確定診断に至らない場合，疾患の可能性とリスクを家族に説明した上で，IVIG以外のステロイド投与などの治療法も検討しつつ，経過をみて遺伝子診断なども考慮していく必要があると考えられた．

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A Case of Incomplete Kawasaki Disease with Chronic Cervical Lymph Node Swelling

Yoshinobu Tsuno, Takaomi Minami, Asumi Higa,
Yuko Tsuda, Mina Booka, Genkichi Izumi,
Masakazu Miyawaki[1)], Shoichi Shibuta, Takashi Takeuchi,
Hiroyuki Suzuki and Norishige Yoshikawa[2)]

1) Department of Pediatrics, Social Insurance Kinan Hospital, Wakayama, Japan
2) Department of Pediatrics, Wakayama Medical University, Wakayama, Japan

A 6-year-old girl with chronic cervical lymph node (CLN) swelling had a high fever and the left main coronary dilatation. We diagnosed her as incomplete Kawasaki disease. We treated with aspirin (30 mg/kg/day) and single infusion of intravenous immunoglobulin (IVIG) (2 g/kg) on day 6, and her fever fell on day 8. The left main coronary dilatation also improved on day 29. But she still had chronic CLN swelling, we thought that we could not deny possibility that it develops autoimmune diseases, such as SLE, JIA, etc, or PFAPA syndrome in the future. So we need careful observation for her chronic CLN swelling.

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Associations of functional NLRP3 polymorphisms with susceptibility to food-induced anaphylaxis and aspirin-induced asthma.

Hitomi, Y., Ebisawa, M., Tomikawa, M., Imai, T., Komata, T., Hirota, T., Harada, M., Sakashita, M., Suzuki, Y., Shimojo, N ., Kohno, Y., Fujita, K., Miyatake, A., Doi, S., Enomoto, T., Taniguchi, M., Higashi, N., Nakamura, Y., Tamari, M.

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Associations of functional *NLRP3* polymorphisms with susceptibility to food-induced anaphylaxis and aspirin-induced asthma

Yuki Hitomi, PhD,[a] Motohiro Ebisawa, MD, PhD,[b] Morimitsu Tomikawa, MD,[b] Takanori Imai, MD, PhD,[b] Takatsugu Komata, MD,[b] Tomomitsu Hirota, DDS, PhD,[a] Michishige Harada, PhD,[a] Masafumi Sakashita, MD,[a] Yoichi Suzuki, MD, PhD,[c] Naoki Shimojo, MD, PhD,[d] Yoichi Kohno, MD, PhD,[d] Kimie Fujita, MD, PhD,[e] Akihiko Miyatake, MD, PhD,[f] Satoru Doi, MD, PhD,[g] Tadao Enomoto, MD, PhD,[h] Masami Taniguchi, MD, PhD,[b] Noritaka Higashi, MD, PhD,[b] Yusuke Nakamura, MD, PhD,[i] and Mayumi Tamari, MD, PhD[a] *Kanagawa, Chiba, Shiga, Osaka, Wakayama, and Tokyo, Japan*

Background: NLR family, pyrin domain containing 3 (NLRP3), controls the activity of inflammatory caspase-1 by forming inflammasomes, which leads to cleavage of the procytokines IL-1β and IL-18. Recent studies have shown associations of human *NLRP3* polymorphisms with susceptibility to various inflammatory diseases; however, the association with allergic diseases remains unclear.

Objective: We sought to examine whether *NLRP3* polymorphisms are associated with susceptibility to food allergy, food-induced anaphylaxis, and aspirin-induced asthma (AIA).

Methods: We selected 15 tag single nucleotide polymorphisms (SNPs) of *NLRP3* and conducted association analyses of *NLRP3* using 574 and 1279 samples for food allergy and AIA, respectively. We further performed functional analyses of the susceptible SNPs.

Results: Two *NLRP3* SNPs (rs4612666 and rs10754558) were significantly associated with susceptibility to food-induced anaphylaxis ($P = .00086$ and $P = .00068$, respectively). The *NLRP3* haplotype of the 2 SNPs also showed a significant association ($P = .000098$). We could confirm the association with susceptibility to another hypersensitivity phenotype, AIA (rs4612666, $P = .0096$). Functional analysis revealed that the risk alleles of rs4612666 and rs10754558 increased the enhancer activity of *NLRP3* expression and *NLRP3* mRNA stability, respectively.

Conclusion: Our results indicate that the *NLRP3* SNPs might play an important role in the development of food-induced anaphylaxis and AIA in a gain-of-function manner. Further research on the NLRP3 inflammasome will contribute to the development of novel diagnostic and therapeutic methods for food-induced anaphylaxis and AIA. (J Allergy Clin Immunol 2009;124:779-85.)

Key words: NLR family, pyrin domain containing 3, gene polymorphism, association study, food allergy, hypersensitivity, anaphylaxis, aspirin-induced asthma, enhancer activity, mRNA stability

Food allergy is defined as an adverse immune response to food proteins, and food-induced allergic reactions are responsible for a variety of symptoms involving the skin, gastrointestinal tract, and respiratory tract.[1] Food allergy has increased in the past 10 to 15 years, particularly in industrialized countries.[2] In Japan large-scale morbidity surveys demonstrated that food allergies occur in 5% to 10% of infants and preschool children, which is similar to the rate seen in other industrialized countries.[3-5] Although environmental factors could contribute to the recent increase in food allergies, several family studies have indicated that genetic factors also influence the risk of food allergies.[6,7] Little is known about the specific genes associated with susceptibility to food allergies, and recent studies have shown that polymorphisms of *CD14*[8]; signal transducer and activator of transcription 6 *(STAT6)*[9]; serine peptidase inhibitor, kazal type 5 *(SPINK5)*[10]; and *IL10*[11] are significantly associated with such susceptibility. Anaphylaxis is a life-threatening allergic reaction, and food is one of the most common responsible allergens.[12] In addition to the cutaneous, respiratory, and gastrointestinal symptoms, patients with anaphylaxis can experience cardiovascular symptoms, including hypotension, vascular collapse, and cardiac dysrhythmia, presumably because of massive mast cell mediator release.[13] Double-blind, placebo-controlled food challenge is the gold standard for the diagnosis of food allergy, and strict elimination of the allergenic food is the basic therapy for it.[13,14] Oral desensitization therapy is conducted in some cases to reduce the risk of a critical allergic reaction with accidental ingestion of allergenic food.[15] Although anaphylaxis is a severe side effect of both the food challenge test and oral desensitization therapy,[16] there are no completely reliable methods to estimate the risk for food-induced anaphylaxis.

From [a]the Laboratory for Respiratory Diseases, Center for Genomic Medicine, RIKEN, Kanagawa; [b]the Clinical Research Center for Allergy and Rheumatology, Sagamihara National Hospital, National Hospital Organization; the Departments of [c]Public Health and [d]Pediatrics, Graduate School of Medicine, Chiba University; [e]the School of Human Nursing, the University of Shiga Prefecture; [f]Miyatake Asthma Clinic, Osaka; [g]the Department of Pediatric Allergy, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases; [h]NPO Japan Health Promotion Supporting Network, Wakayama; and [i]the Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo.

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Reprint requests: Mayumi Tamari, MD, PhD, Laboratory for Respiratory Diseases, Center for Genomic Medicine, the Institute of Physical and Chemical Research (RIKEN), 1-7-22 Suehiro, Tsurumi-ku, Yokohama 230-0045 Kanagawa, Japan. E-mail: tamari@src.riken.jp.

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Abbreviations used
AIA: Aspirin-induced asthma
LD: Linkage disequilibrium
NLR: Nucleotide-binding domain, leucine-rich repeat–containing
NLRP3: NLR family, pyrin domain containing 3
OR: Odds ratio
SNP: Single nucleotide polymorphism

Aspirin-induced asthma (AIA) is a common clinical presentation of aspirin hypersensitivity, and this acute reaction is elicited through COX inhibition by nonsteroidal anti-inflammatory drugs.[17] AIA and autoimmune diseases partly share some clinical features and laboratory markers.[18] The natural course of AIA is similar to that of persistent viral infection of the respiratory system, and infectious factors have been shown to play a role in AIA.[17] However, the causative factors for the disease remain elusive.

NLR family, pyrin domain containing 3 (NLRP3; also known as NALP3, CIAS1, PYPAF1, or cryopyrin), is a member of the nucleotide-binding domain, leucine-rich repeat–containing (NLR) family of genes encoding proteins that comprise a nucleotide-binding domain and a leucine-rich repeat domain, and it controls the activity of inflammatory caspase-1 by forming complexes called inflammasomes.[19] Recent studies have shown that tight collaboration between pathogen-associated molecular patterns and damage-associated molecular patterns is needed to start an innate immune response to allergens,[20] and NLRP3 inflammasomes are activated by pathogen-associated molecular patterns, microbial toxins, live bacteria, viruses, and damage-associated molecular patterns.[21] However, the mechanism of activation of the NLRP3 inflammasome is not fully understood. After being activated, NALP3 recruits apoptosis-associated speck-like protein containing a card and procaspase-1, leading to autocatalytic processing and activation of caspase-1. Active caspase-1 catalyzes cleavage of the procytokines IL-1β and IL-18, which are both proinflammatory cytokines involved in the host response to infection and injury.[22] Excessive production of IL-1β and IL-18 is associated with septic shock and autoimmune disorders.[22]

The human *NLRP3* gene is located in 1q44. Previous studies have determined that the nonsynonymous coding substitution of *NLRP3* causes autoinflammatory diseases: V198M, L353P, A439V, and E627G are associated with familial cold autoinflammatory syndrome; R260W, A352V and G569R are associated with Muckle-Wells syndrome; and D303N, F309S and F537S are associated with chronic infantile neurological, cutaneous, and articular syndrome.[19] Furthermore, recent studies have shown that *NLRP3* polymorphisms are significantly associated with susceptibility to common inflammatory diseases, such as Crohn disease,[23] psoriatic juvenile idiopathic arthritis,[24] and essential hypertension.[25] However, the association of *NLRP3* polymorphisms with susceptibility to allergic diseases has not been reported.

To clarify the genetic factors that increase the risk of the hypersensitive phenotype of allergy, we conducted an association study of *NLRP3* polymorphisms with susceptibility to food allergy, food-induced anaphylaxis, and AIA in a Japanese population. Functional effects of the related *NLRP3* variants were also examined.

METHODS

Subjects

Three hundred twenty pediatric patients with food allergies and positive antigen-specific IgE results (CAP-RAST) were recruited at National Sagamihara Hospital, Kanagawa, Japan. We performed food challenge tests for 178 patients with food allergies. The diagnosis of food allergy was made either based on challenge tests or a definitive episode plus confirmation of antigen-specific IgE levels. Two hundred fifty-four nonatopic, nonasthmatic healthy unrelated control children were recruited from an elementary school affiliated with the Education Department of Chiba University, Chiba, Japan. Healthy subjects included in this group had no history of food allergies, asthma, or atopic dermatitis. Detailed information for patients with food allergy and healthy children is shown in Table I. Food-induced anaphylaxis was defined as symptoms evoked in multiple organs. Five hundred forty-nine adult asthmatic patients were recruited from Miyatake Asthma Clinic and the National Sagamihara Hospital.[26,27] Among the 549 patients, 79 were aspirin intolerant, and 470 were aspirin tolerant. All subjects with asthma were given diagnoses according to the criteria of the American Thoracic Society.[28] Detailed information on adult asthmatic patients is provided in Table E1 (available in this article's Online Repository at www.jacionline.org). All were unrelated Japanese subjects and provided written informed consent to participate in the study according to the rules of the Process Committee at the Center for Genomic Medicine, The Institute of Physical and Chemical Research (RIKEN).

Variation screening of *NLRP3*

We carried out screening of single nucleotide polymorphisms (SNPs) with genomic DNA from 12 healthy subjects. A total of 17 overlapping primer sets were designed on the basis of the *NLRP3* genomic sequence available from the National Center for Biotechnology Information (accession no. NM_001079821.1). The complete coding region of *NLRP3*, intron/exon boundaries, and 100 bases of the surrounding intronic sequence and approximately 3 kb of 5′ genomic DNA were resequenced. The PCR product was reacted with BigDye Terminator v3.1 (Applied Biosystems, Foster City, Calif). Sequences were assembled and polymorphisms were identified with the SEQUENCHER program (Gene Codes Corp, Ann Arbor, Mich).

Selection of *NLRP3* polymorphisms for genotyping

Genomic DNA was prepared from peripheral blood samples by using standard protocols. We selected tag SNPs of the exons and introns of *NLRP3* with a minor allele frequency of greater than 10% in the HapMap Japanese data set (http://www.hapmap.org/). Pairwise linkage disequilibrium (LD) was calculated as r^2 values by using the Haploview 4.1 program (http://www.broad.mit.edu/mpg/haploview/). Haplotype frequencies for the 2 most susceptible SNPs were also estimated with the Haploview 4.1 program. Genotyping of SNPs was performed by using the TaqMan allele-specific amplification method (Applied Biosystems).

Real-time quantitative RT-PCR

Total RNA from normal human tissues was purchased from Clontech (Mountain View, Calif), and total RNA from cell lines was extracted with a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). Each RNA was reverse transcribed with Superscript III reverse transcriptase and oligo dT primers (Invitrogen, Carlsbad, Calif). The expression of *NLRP3* transcripts was determined by using real-time quantitative RT-PCR with SYBR Premix Ex Taq (Takara, Shiga, Japan) and specific primers (5′-GGGGTCAT GATGTTCTGTGA-3′ and 5′-CAGGCTTTTCTTCTTGAAGTGTT-3′). In all experiments the amounts of cDNA were standardized by means of quantification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*.

Luciferase assay

Intron 7 and the last exon fragments of *NLRP3* from human genomic DNA were amplified by means of PCR by using specific primers (5′-GCCACTATG

TABLE I. Clinical information about patients with food allergy and control subjects

Characteristics	Patients with food allergy			Control subjects, n = 254 (%)
	Total, n = 320 (%)	Anaphylaxis (+), n = 98 (%)	Anaphylaxis (−), n = 222 (%)	
Age (y)	4.2 ± 3.3	5.4 ± 3.1	3.7 ± 3.3	9.0 ± 1.7
Sex				
Male	233 (72.8)	67 (68.4)	166 (74.8)	124 (48.8)
Female	87 (27.2)	31 (31.6)	56 (25.2)	130 (51.2)
Log serum total IgE (IU/mL)	2.6 ± 0.7	2.8 ± 0.4	2.6 ± 0.7	2.4 ± 1.3
Food allergy phenotype				
Infantile atopic dermatitis	268 (83.8)	76 (77.6)	192 (86.5)	—
Immediate type	294 (91.9)	96 (98.0)	198 (89.2)	—
OAS	12 (3.8)	4 (4.1)	8 (3.6)	—
FEIAn	6 (1.9)	5 (5.1)	1 (0.5)	—
Complications				
Atopic dermatitis	223 (69.7)	64 (65.3)	159 (71.6)	—
Bronchial asthma	108 (33.8)	42 (42.9)	66 (29.7)	—
Allergy rhinitis	47 (14.7)	17 (17.3)	30 (13.5)	—
Allergic conjunctivitis	39 (12.2)	15 (15.3)	24 (10.8)	—

OAS, Oral allergy syndrome; *FEIAn*, food-dependent exercise-induced anaphylaxis.

GAAAACAGCAC-3′ and 5′-AAGGAAGCACCCGTACCTGC-3′ and 5′-GTTGTCTGAAATGTATTTCAATT-3′ and 5′-TTTGAAAAATTTCTAGG TACTCT-3′, respectively). PCR products were subcloned into the reporter gene pGL3-promoter vector (Promega, Madison, Wis). Vector pRL-TK was used to normalize for variations in transfection efficiency. These plasmids were transfected into THP-1 cells by using FuGENE 6 (Roche, Basel, Switzerland). The luciferase activities were determined by using the Dual-Luciferase Reporter Assay system (Promega).

Allele-specific transcript quantification

PBMCs were isolated from 3 healthy donors with heterozygous *NLRP3* rs10754558 by means of density gradient centrifugation. Primary monocytes were sorted with human CD14 microbeads and an autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany), and genomic DNA and total RNA were isolated. We performed allelic expression analyses using the TaqMan assay with SNP genotyping probes. Genomic DNA was used as a control for equal biallelic representation. The ratio of 5-carboxyfluorescein (FAM) intensity to VIC intensity for *NLRP3* was plotted for mixtures of homozygous DNAs at 6 different ratios (3:1, 2:1, 3:2, 2:3, 1:2, and 1:3), with correction based on the signal intensities of heterozygote controls for a standard line. We then measured the allelic ratio for each cDNA and genomic DNA from each subject and calculated the allelic ratio of cDNA and genomic DNA based on the standard line.

Prediction of transcription factor binding sites

The TRANSFAC Professional 10.3 (http://www.biobase.de/pages/) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) Web sites were used to predict putative transcription factor binding sites. RNA secondary structures were predicted by using the mfold Web server (http://mfold.bioinfo.rpi.edu/) setting default parameters and a folding temperature of 37°C.

Electrophoretic mobility shift assays

Nuclear extracts were prepared from THP-1 cells. Extracts were quickly frozen and stored in aliquots at −80°C. Electrophoretic mobility shift assays was performed by using a 2nd Generation DIG Oligonucleotide 3′-end Labeling Kit and 2nd Generation DIG Gel Shift Kit (Roche), according to the manufacturer's instructions. We incubated the nuclear extract with 28-bp double-strand oligonucleotide probes for rs4612666 (C and T) for 30 minutes at room temperature. The oligonucleotide sequences were 5′-GGAGCTGGGAAGACGTAGTATTGGTGGG-3′ for the C allele and 5′-GGAGCTGGGAAGATGTAGTATTGGTGGG-3′ for the T allele, respectively. For the supershift experiments, a rabbit anti-human GATA-2 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) and GATA-2 consensus probe (5′-CACTTGATAACAGAAAGTGATAACTCT-3′) were used.

Statistical analysis

We calculated allele frequencies and tested agreement with Hardy-Weinberg Equilibrium using a χ^2 goodness-of-fit test at each locus. We then compared differences in allelic frequencies of the polymorphisms between cases and control subjects by using a 2×2 contingency χ^2 test with 1 *df* and calculated odds ratios (ORs) with 95% CIs. Logistic regression analysis was implemented for the susceptibility to anaphylaxis or AIA and genotype to assess the effects of sex (SPSS 14.0 J; SPSS, Inc, Chicago, Ill). The small sample size of this study decreased the ability to detect associations. Power in this study was estimated with the aid of Sample Power 2.0 (SPSS, Inc). If ORs of risk alleles with control group frequencies of 0.05, 0.1, 0.2, and 0.4 were greater than 3.41, 2.61, 2.16, and 1.98, respectively, power exceeded 80% (at $P = .05$) in allelic association tests of food allergy (222 subjects with food allergy without anaphylaxis [control] and 98 subjects with food-induced anaphylaxis). Similarly, in allelic association tests in patients with adult asthma (470 asthmatic patients without AIA [control] and 79 patients with AIA), a power of 80% was ensured if alleles with frequencies of 0.05, 0.1, 0.2, and 0.4 had ORs of greater than 3.41, 2.61, 2.16, and 1.98, respectively. A P value of less than .05 was considered statistically significant. Expression differences between genotypic groups were tested with the Student *t* test and Mann-Whitney *U* test.

RESULTS

Association of *NLRP3* SNPs with susceptibility to food-induced anaphylaxis

Thirty-nine polymorphisms with a frequency of greater than 10% in the *NLRP3* region were contained in the public databases at the National Center for Biotechnology Information dbSNP Web site (http://www.ncbi.nlm.nih.gov/SNP/). We selected 15 polymorphisms for association studies using tagger in the Haploview 4.1 program, and these 15 SNPs captured 39 of the 39 alleles with a mean r^2 value of 0.98 ($r^2 > 0.80$; Fig 1, *A*). We next carried out case-control association studies of the 15 SNPs. The control genotypes did not deviate from Hardy-Weinberg equilibrium. Although no significant association was observed between any SNP and food allergy, 7 SNPs showed significant associations with susceptibility to food-induced anaphylaxis (Table II and see Table E2 in this article's Online Repository at www.jacionline.org). Among

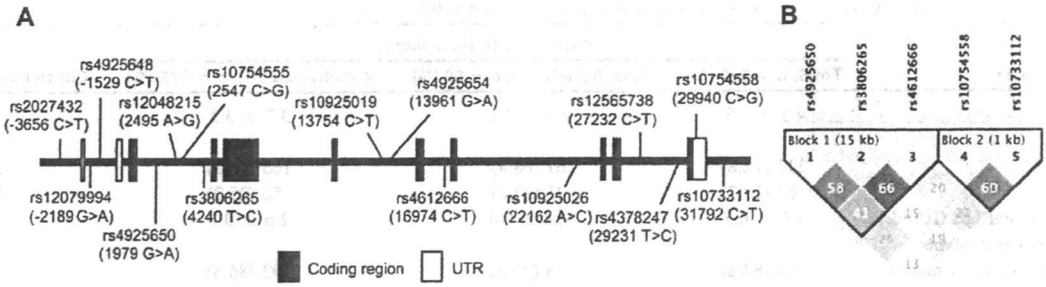

FIG 1. A, Exon–intron structure of the *NLRP3* gene and locations of genotyped tag SNPs. *UTR,* Untranslated region. **B,** LD structures of the 5 significantly associated *NLRP3* SNPs with susceptibility to food-induced anaphylaxis ($P < .005$). LD was calculated by using genotyping data. Pairwise r^2 values for all combinations of SNPs are shown in gray scale.

TABLE II. Association between *NLRP3* polymorphisms and susceptibility to food-induced anaphylaxis

			Anaphylaxis (+), n = 98 (%)			Anaphylaxis (−), n = 222 (%)			Frequency (allele 1)				
Allele 1/2	dbSNP ID	Location	1/1	1/2	2/2	1/1	1/2	2/2	(+)	(−)	*P* value	OR	95%CI
−3656 C/T	rs2027432	5′ Flanking	78 (80)	19 (19)	1 (1)	169 (76)	50 (23)	3 (1)	0.89	0.87	NS		
−2189 G/A	rs12079994	Intron 1	78 (80)	18 (18)	2 (2)	147 (67)	61 (28)	10 (5)	0.89	0.81	.021	1.81	1.09–2.99
−1529 C/T	rs4925648	Intron 1	81 (83)	17 (17)	0 (0)	166 (75)	54 (24)	2 (1)	0.91	0.87	NS		
1979 G/A	rs4925650	Intron 3	18 (18)	51 (52)	29 (30)	71 (32)	118 (53)	33 (15)	0.44	0.59	.00091	1.77	1.26–2.49
2495 A/G	rs12048215	Intron 3	58 (59)	39 (40)	1 (1)	123 (55)	85 (38)	14 (6)	0.79	0.75	NS		
2547 C/G	rs10754555	Intron 3	42 (43)	47 (49)	8 (8)	77 (35)	115 (52)	29 (13)	0.68	0.61	NS		
4240 T/C	rs3806265	Intron 3	43 (44)	44 (45)	10 (10)	62 (28)	114 (52)	43 (20)	0.67	0.54	.0029	1.71	1.20–2.43
13754 C/T	rs10925019	Intron 6	54 (55)	39 (40)	5 (5)	109 (49)	92 (41)	21 (10)	0.75	0.70	NS		
13961 G/A	rs4925654	Intron 6	67 (68)	25 (26)	6 (6)	144 (65)	71 (32)	7 (3)	0.81	0.81	NS		
16974 C/T	rs4612666	Intron 7	44 (45)	41 (42)	13 (13)	56 (26)	114 (52)	49 (22)	0.66	0.52	.00086	1.81	1.27–2.56
22162 A/C	rs10925026	Intron 8	27 (28)	49 (50)	22 (22)	83 (38)	111 (51)	26 (12)	0.53	0.63	.013	1.53	1.09–2.16
27232 C/T	rs12565738	Intron 10	81 (83)	16 (16)	1 (1)	176 (79)	43 (19)	3 (1)	0.91	0.89	NS		
29231 T/C	rs4378247	Intron 10	84 (86)	13 (13)	1 (1)	181 (82)	40 (18)	1 (1)	0.92	0.91	NS		
29940 C/G	rs10754558	Exon 11 (3′UTR)	25 (26)	46 (47)	27 (28)	83 (38)	109 (50)	25 (12)	0.49	0.63	.00068	1.80	1.28–2.54
31792 C/T	rs10733112	3′ Flanking	35 (36)	49 (50)	14 (14)	46 (21)	119 (54)	57 (26)	0.61	0.48	.0021	1.71	1.21–2.40

NS, Not significant; *UTR,* untranslated region.

5 food-induced anaphylaxis–susceptible SNPs for which the *P* value was less than .005, 3 (rs4925650, rs3806265, and rs4612666; $r^2 \geq 0.41$) and 2 (rs10754558 and rs10733112, $r^2 \geq 0.60$) were in moderate LD with each other (Fig 1, *B*). Because we could not find any SNP that was in strong LD with the 5 variants by resequencing and searching the dbSNP database, we considered the rs4612666 ($P = .00086$; OR, 1.81) and rs10754558 ($P = .00068$; OR, 1.80) variants that were the most susceptible SNPs in each LD block to be associated with the susceptibility to food-induced anaphylaxis. The results of stepwise logistic regression analysis for the susceptibility to anaphylaxis showed significant effects of the genotypes rs4612666 (Wald statistic = 13.38, $df = 1$, $P = .00025$) and rs10754558 (Wald statistic = 6.23, $df = 1$, $P = .013$). There was no significant effect of sex by means of logistic regression analysis. We further divided the subjects with food allergies into 2 groups, those with and without food challenge tests. We found significant associations between the 2 SNPs rs4612666 and rs10754558 and food-induced anaphylaxis in both groups, but a marked effect of food challenges was not observed (data not shown).

We next investigated the effects of *NLRP3* haplotypes with susceptibility to food-induced anaphylaxis. As shown in Table III, the frequency of combination of the most susceptible alleles between LD blocks (rs4612666-C and rs10754558-G) was significantly increased in patients with food-induced anaphylaxis (haplotype_1 vs haplotype_4; $P = .000098$; OR, 2.21).

An association study of *NLRP3* SNPs with susceptibility to AIA

Approximately 10% of adult asthmatic patients are affected by AIA. As well as food-induced anaphylaxis, patients with AIA show acute life-threatening hypersensitivity symptoms.[29] Therefore we performed an association study between the 5 food-induced anaphylaxis–susceptible *NLRP3* SNPs and susceptibility to AIA. rs4612666 showed a significant association with susceptibility to AIA, and the direction of association was similar to that of food-induced anaphylaxis (see Table E3 in this article's Online Repository at www.jacionline.org). The results of stepwise logistic regression analysis for the susceptibility to AIA showed significant effects of genotype rs4612666 (Wald statistic = 4.34, $df = 1$, $P = .037$). No significant effect of sex was found by means of logistic regression analysis. These results indicated that *NLRP3* SNPs were significantly associated with hypersensitivity, such as food-induced anaphylaxis and AIA.