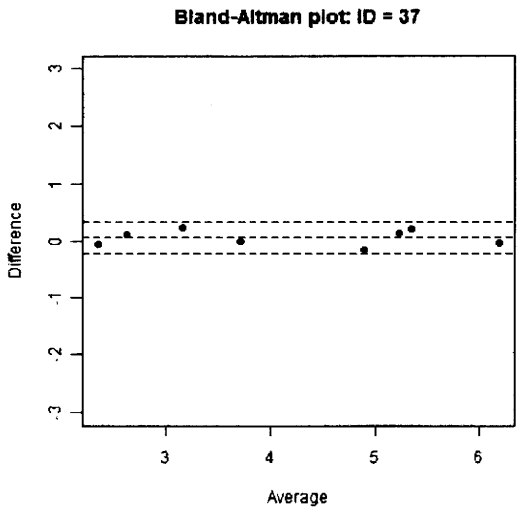
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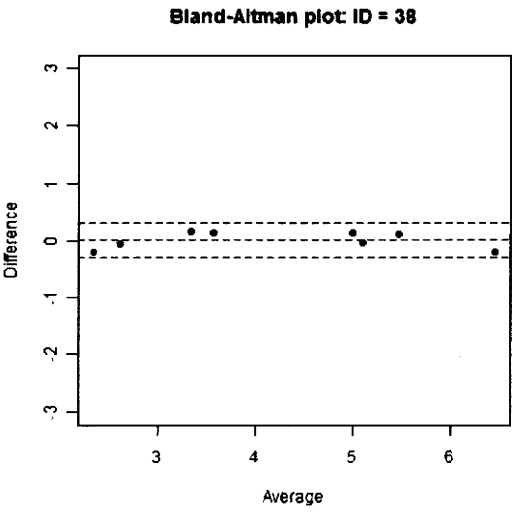
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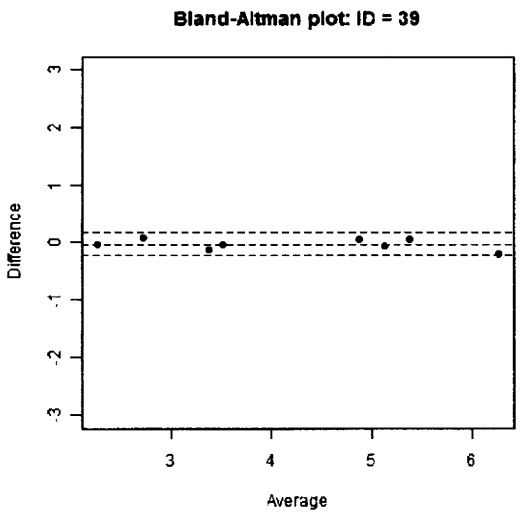
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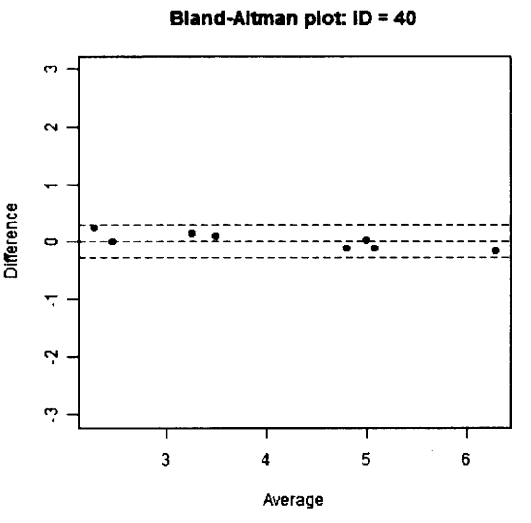
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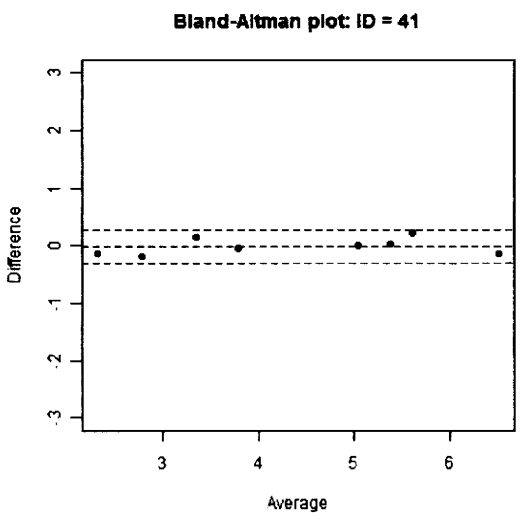
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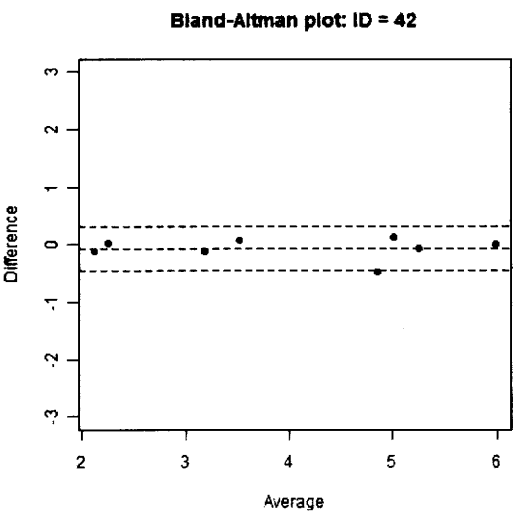
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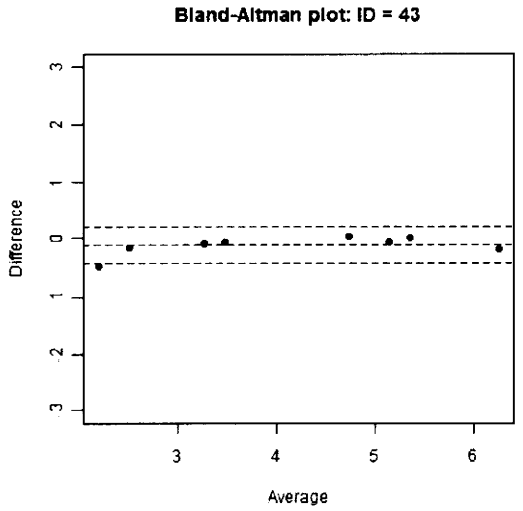
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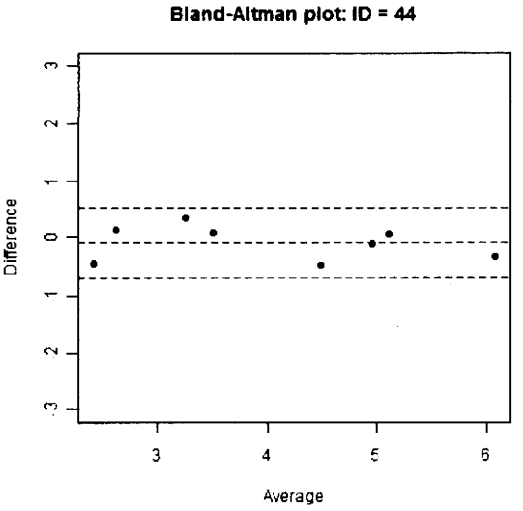
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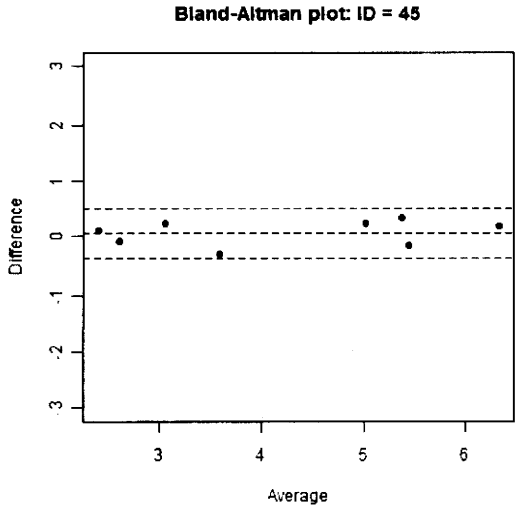
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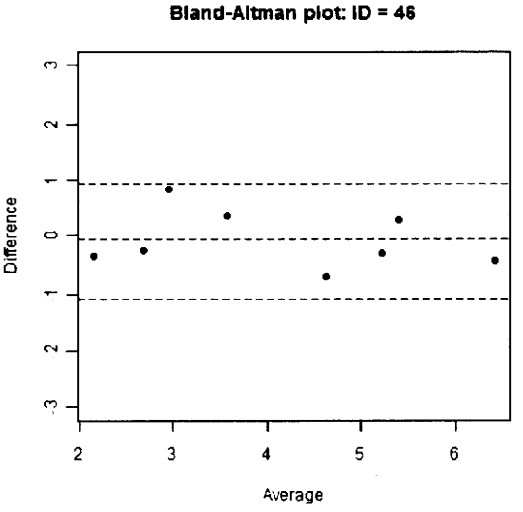
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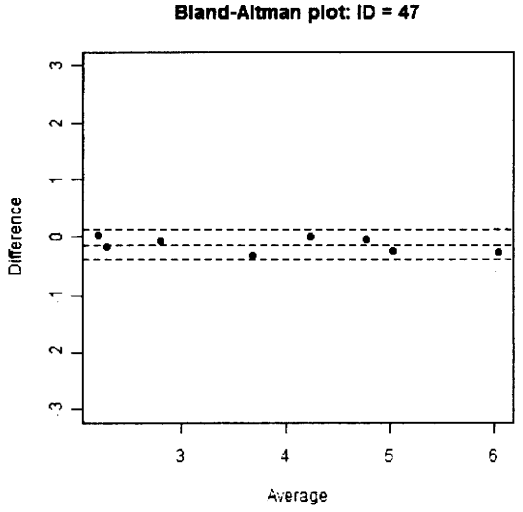
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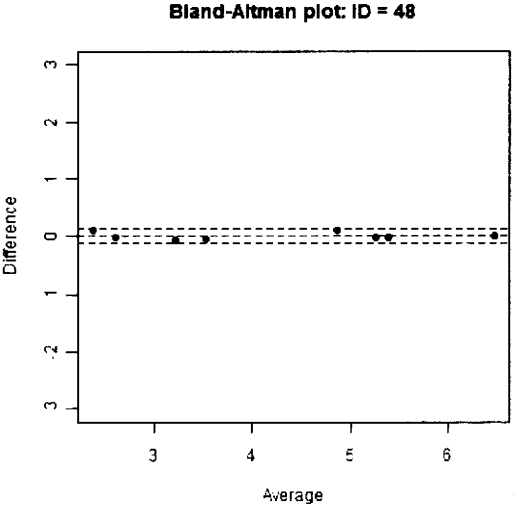
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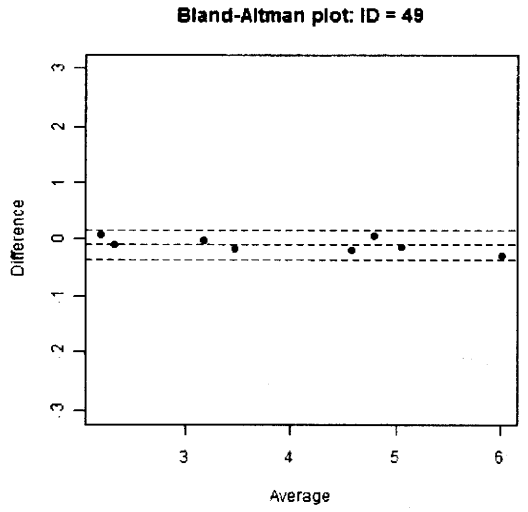
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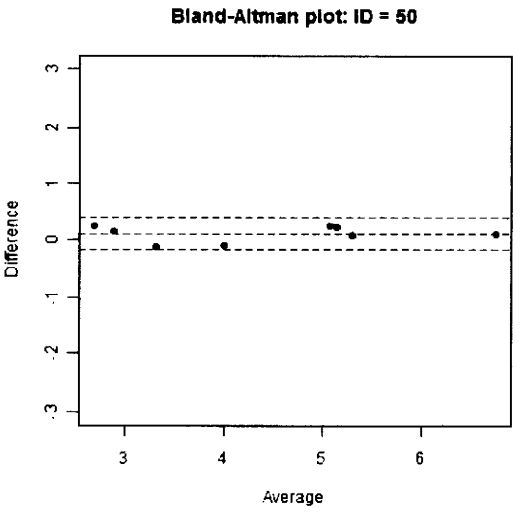
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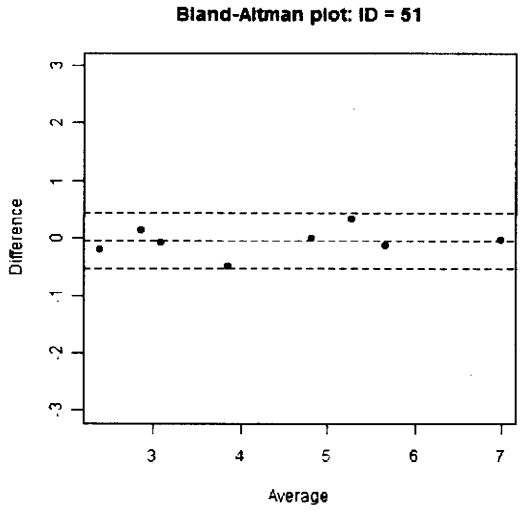
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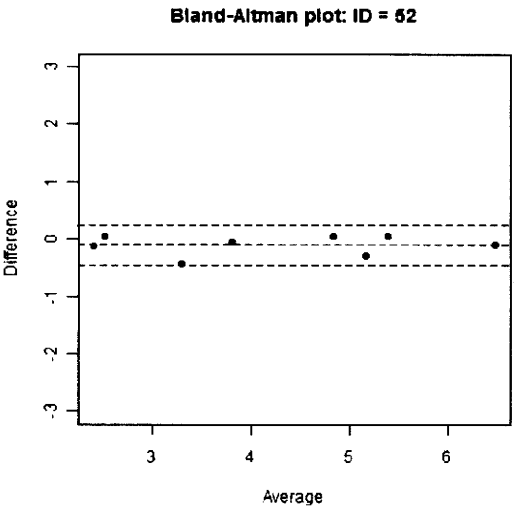
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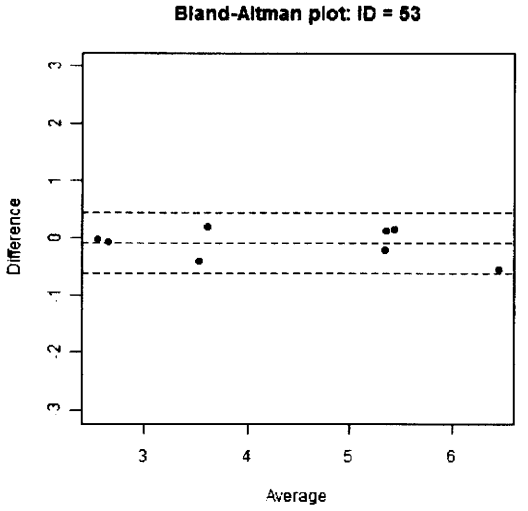
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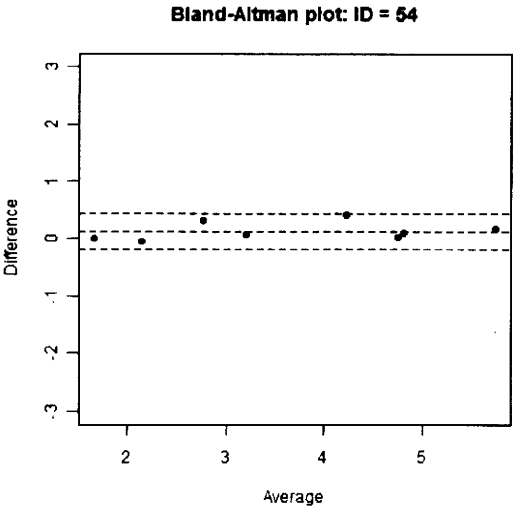
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Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

＜雑誌＞

分担研究者：中村好一

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Ritei Uehara, hiroshi Igarashi, Mayumi Yashiro, Yosikazu Nakmura, Hiroshi Yanagawa	Kawasaki Disease Patients with Redness or Crust Formation at the Bacille Calmette-Guerin Inoculation Site	The Pediatric Infectious Disease Journal	29(5)	430-433	2010
Yoshihiro Onouchi, Kouichi Ozaki, Jane C. Buns, Chisato Shimizu, Hiromichi Hamada, Takafumi Honda, Masaru Terai, Akihito Honda, Takashi Takeuchi, Shoichi Shibuta, Tomohiro Suenaga, Hiroyuki Suzuki, Kouji Higashi, Kumi Yasukawa, Yoichi Suzuki, Kumiko Sasago, Yasushi Kemmmotsu, Shinichi Takatsuki, tsutomu Saji, Tetsushi Yoshikawa, Toshiro Nagai, kunihiro Hmamamoto, Fumio Kishi, Kazunobu Ouchi, YOshitake Sato, Jane W.Newbuger, Annette L.Baker, Stanford T. Shulman, Anne H. Rowley, Mayumi Yashiro, yoshikazu Nakamura, Keiko wakui, Yoshimitsu Fukushima, Akihiro Fujino, Tatsuhiko Tsunoda, Tomisaku Kawasaki, Akira Hata, Yusuke Nakamura, Toshihiro Tanaka	Common variants in *CASP3* confer susceptibility to Kawasaki disease	Human Molecular Genetics	19(14)	2898-2906	2010

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Daisuke Sudo, Yoshiro Monobe, Mayumi Yashiro, Atsuko Sadakane, Ritei Uehara, Yoshikazu Nakamura	Case-control study of giant coronary aneurysms due to Kawasaki	Pediatrics International	52	790-794	2010
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鈴木啓之、萩野廣太郎、中村好一、上原里程、屋代真弓、柳川洋	川崎病急性期にステロイド投与を受けた症例の冠動脈障害発生の分析	日本小児科学会雑誌	114(5)	853-857	2010
河合邦夫、屋代真弓、中村好一、柳川洋	出生年コホート別にみた川崎病心後遺症の種類別の累積罹患率	小児保健研究	69(3)	380-386	2010

分担研究者：小川俊一

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小川俊一	川崎病後冠動脈狭窄のカテーテル治療	小児科	51	403-410	2010

分担研究者：服部成介

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HIdetake Kurihatra, Yutaka Harita, Kichiro Ichimura, Seisuke Hattori, Tatsuo Sakai	SIRP-α-CD47 system functions as an intercellular signal in the renal glomerulus	Am J Physiol Renal Physiol	299	F517-F527	2010
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Fuse S, Kobayashi T, Arakaki Y, Ogawa S, Katoh H, Sakamoto N, Hamaoka K, Saji T	Standard method for ultrasound imaging of coronary artery in children.	Ped Int.	52	876-882	2010
JCS Working Group	Guidelines for Diagnosis and management of Cardiovascular Sequelaein Kawasaki Disease (JCS 2008)	Circ J	74(9)	1989-2020	2010
佐地勉、高月晋一	川崎病の心血管障害、小児科診療	小児の治療指針	73(suppl.)	364-347	2010
小林徹、佐地勉	川崎病（心合併症を含む）	小児臨床	63	618-622	2010
佐地勉	Question12 子どもの病気 川崎病 冠動脈拡張改善のアスピリンはいつまで服用？再発は？	暮らしと健康	3	84	2011

分担研究者：賀藤均

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Fuse S, Kobayashi T, Arakaki Y, Ogawa S, Katoh H, Sakamoto N, Hamaoka K, Saji T	Standard method for ultrasound imaging of coronary artery in children	Ped Int.	52	876-882	2010

分担研究者：坂本なほ子

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Fuse S, Kobayashi T, Arakaki Y, Ogawa S, Katoh H, Sakamoto N, Hamaoka, Saji T.	Standard method for ultrasound imaging of coronary artery in children	Pediatr Int	52	876-882	2010

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著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
阿部淳	川崎病	五十嵐隆	総合小児科診療のための小児科学レビュー2010	総合医学社	東京	2010	226-232
佐地勉	急性期川崎病への抗サイトカイン療法（抗TNFα製剤Infliximab）		Annual Review 循環器2011	中外医学社	東京	2011	331-336

Ⅳ. 研究成果の刊行物・別冊（主なもの）

Kawasaki Disease Patients With Redness or Crust Formation at the Bacille Calmette-Guérin Inoculation Site

Ritei Uehara, MD, Hiroshi Igarashi, MD,†‡ Mayumi Yashiro, BA,* Yosikazu Nakamura, MD, MPH,* and Hiroshi Yanagawa, MD**

Background: A specific diagnostic test for Kawasaki disease (KD) is currently unavailable. Redness or crust formation at the Bacille Calmette-Guérin (BCG) inoculation site is listed as a positive sign in the diagnostic guidelines of KD. The purpose of this study was to investigate the epidemiologic features of KD patients with such changes at the BCG inoculation site and to evaluate the specificity of this sign in KD diagnosis.

Methods: Data on KD patients who received BCG vaccination were analyzed from a Japanese nationwide epidemiologic survey on KD conducted in 2007. Patients who had 5 or 6 principal signs (complete cases) with redness or crust formation at the BCG inoculation site were compared by sex, year of hospital visit, day of first hospital visit, recurrent status, and presence of KD in siblings. To evaluate the specificity of the sign for KD diagnosis, patients aged 2 years or younger who were diagnosed as having respiratory syncytial virus or rotavirus infection using a commercial rapid test and who required hospitalization were observed.

Results: Of the 15,524 KD patients with a history of BCG vaccination, 7745 (49.9%) had redness or crust formation at the BCG inoculation site. This was observed in more than 70% of complete KD patients aged 3 to 20 months. Of these patients, the proportion with this sign in the group whose first day of hospital visit was within 1 to 4 days from the onset was significantly larger than that of the other patients groups (5–9 or 10+ days) (52.1%, $P < 0.001$). Among the patients with respiratory syncytial virus or rotavirus infection, none showed these changes at BCG inoculation site.

Conclusions: Redness or crust formation at the BCG inoculation site is a useful diagnostic sign for KD among children aged 3 to 20 months in countries with a BCG vaccination program. Even if patients have 4 or fewer signs of the clinical criteria for KD, physicians should consider that patients with redness or crust formation at the BCG inoculation site could suffer from KD.

Key Words: Kawasaki disease, BCG vaccine, diagnosis, epidemiology

(*Pediatr Infect Dis J* 2010;29: 430–433)

Kawasaki disease (KD) is a systemic vasculitis with unknown etiology mostly affecting children aged 5 years or younger. As no specific diagnostic test is currently available, diagnosis is based on clinical signs and exclusion of other diseases. KD is defined as an illness in patients with at least 5 of the following 6 principal clinical signs: (1) fever persisting for 5 days or more (inclusive of patients whose fever subsided before the fifth day in response to therapy), (2) bilateral conjunctival injection, (3) changes to the lips and oral cavity (eg, reddening of the lips, strawberry tongue), (4) polymorphous exanthema, (5) changes to peripheral extremities (eg, reddening of the palms and soles, edema, desquamation), and (6) cervical lymphadenopathy.[1]

Bacille Calmette-Guérin (BCG) vaccine is used to prevent meningitis and disseminated tuberculosis in children,[2] and about 100 million children receive this vaccine each year. Japan has been conducting universal BCG vaccination of infants using a multiple puncture technique since 1951[3] and the vaccination policy regarding BCG for infants was changed in 2005. Since then, it has been recommended that all children should receive the BCG vaccine by 6 months of age. According to a BCG vaccination survey in Japan in 2006, 97% of children received the BCG vaccine by that time.[4]

Redness or crust formation at the BCG inoculation site is listed as a symptom or finding both on the fifth revised edition of the diagnostic guidelines of KD in Japan[1] and on the American Heart Association scientific statement.[5] Japanese pediatricians previously reported findings of erythema at the BCG inoculation site in 281 KD patients who visited their hospital between 1976 and 1980.[6] In this report, erythema was observed at the BCG site in more than 50% of KD patients 1 to 12 months after inoculation. Several KD cases with BCG reactivation, inflammation, or induration were also reported from other countries.[7–10] According to an investigation of skin biopsy specimens from the BCG inoculation site in KD patients, extensive edema in the papillary dermis with marked dilation of the capillaries was found.[11] In addition, raised levels of cytokines such as interleukin-1 alpha and tumor necrosis factor alpha were detected at the site. Redness or crust formation at the BCG inoculation site in KD patients was hypothetically ascribed to cross-reactivity between mycobacterial heat shock protein 65 and human homolog HSP63.[12,13]

The epidemiology of KD patients with redness or crust formation at the BCG inoculation site is poorly understood. In this study, we investigated the epidemiologic characteristics of KD patients with these changes using data from a large-scale nationwide survey of the disease in Japan, and evaluated the specificity of this sign for KD diagnosis.

METHODS

Epidemiologic Characteristics of KD Patients With Redness or Crust Formation at the BCG Inoculation Site

Nationwide epidemiologic surveys on KD have been conducted approximately every 2 years in Japan since 1970. The 19th survey on KD was conducted in January 2007 and included patients who visited hospitals from January 1, 2005 to December 31, 2006. All pediatric hospitals and other general hospitals with a pediatric department and 100 or more beds were included in the nationwide survey. Pediatricians were asked to complete a questionnaire for all KD cases they had diagnosed over the 2-year

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Address for correspondence: Ritei Uehara, MD, Department of Public Health, Jichi Medical University, 3311–1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan. E-mail: u-ritei@jichi.ac.jp.
Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.pidj.com).

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DOI: 10.1097/INF.0b013e3181cacede

period.[14] This survey protocol and the questionnaire were reviewed and approved by the institutional review board at Jichi Medical University, Japan.

Two items related to BCG vaccination were included in the questionnaire of the 19th survey. One item asked whether patients had received the BCG vaccination and the other item asked whether redness or crust formation was observed at the BCG inoculation site. Patients who did not receive the BCG vaccination or whose history of BCG vaccination was unknown were excluded from the analysis.

The age-specific proportion of KD patients with redness or crust formation at the BCG inoculation site was observed. Clinical criteria were divided into 2 groups: 5 or more signs (complete KD) and 4 or fewer (incomplete KD). These patients were compared by sex, year of hospital visit, day of first hospital visit, recurrent status, and presence of KD in siblings. Day of first hospital visit was divided into 3 categories: 1 to 4 days, 5 to 9 days, and 10 days or more from the onset. The proportion of coronary artery abnormalities (CAA) among complete KD patients with redness or crust formation at the BCG site was compared with that of patients without such changes. The presence of CAA was also examined according to time period: <30 days or ≥30 days after KD onset. CAA was defined as a giant coronary aneurysm, coronary aneurysm, or coronary dilatation.[15]

Comparisons between redness or crust formation at the BCG inoculation site and categorical variables were made using χ^2 analysis or Fisher exact test; 95% confidence intervals were also calculated for these proportions. The significance level was $P < 0.05$. Statistical analyses were performed using the SAS 9.1 software program (SAS Institute Inc., Cary, NC).

Specificity of Redness or Crust Formation at the BCG Inoculation Site for Diagnosis of KD

To identify the prevalence of redness or crust formation at the BCG inoculation site in patients with febrile illness except for KD, we observed serial patients diagnosed with respiratory syncytial virus (RSV) or rotavirus infection and who required hospitalization at a general hospital (Oyama Municipal Hospital, Tochigi, Japan) between October 2008 and May 2009. The rapid diagnostic test for RSV infection (Check RSV, Alfresa Pharma Corp., Osaka, Japan) detects RSV antigen in respiratory tract specimen by using immunochromatography testing. Rapid diagnostic test for rotavirus infection (Rapid Testa Rota Adeno, Sekisui Medical Co. Ltd, Tokyo, Japan) detects rotavirus antigen in feces by using immunochromatography testing. Patients who were 2 years of age or younger and who had received BCG vaccination were included in this observation to compare with the prevalence of changes at the BCG inoculation site in KD patients.

RESULTS

Epidemiologic Characteristics of KD Patients With Redness or Crust Formation at the BCG Inoculation Site

Completed questionnaires were returned from 1543 (70.7%) of the 2183 hospitals contacted. A total of 20,475 patients diagnosed with KD by a physician were reported: 10,041 in 2005 and 10,434 in 2006. A total of 15,524 patients had a history of BCG vaccination. Of these, 7745 (49.9%) had redness or crust formation at the BCG inoculation site. The age-specific proportion of these patients is shown in Figure 1. Of those patients aged 3 to 20 months, more than 70% had redness or crust formation at the BCG inoculation site. The same finding was obtained in complete KD patients (n = 12,783).

Among all complete KD patients receiving the BCG vaccination, the proportion of male patients with redness or crust formation at the BCG site significantly larger than that of female patients with the same sign (52.2% vs. 46.4%, $P < 0.001$) (Table, Supplemental Digital Content 1, http://links.lww.com/INF/A353).

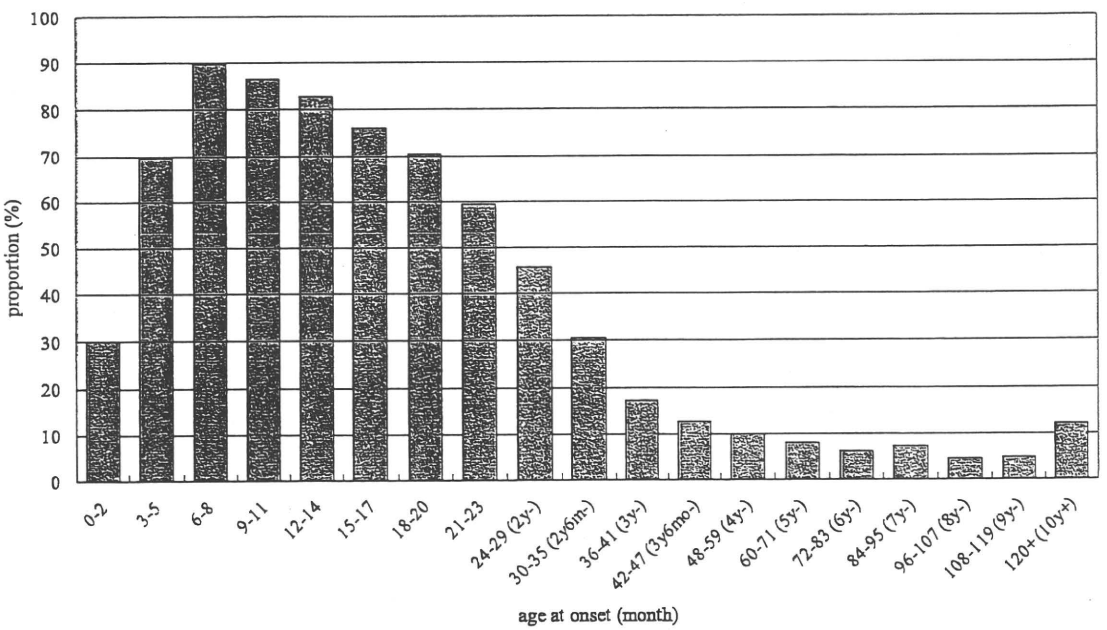

FIGURE 1. Age-specific proportion of Kawasaki disease patients who had redness or crust formation at the Bacille Calmette-Guérin inoculation site. Data of the nationwide survey of Kawasaki disease in Japan which was conducted in 2007 was used. This figure showed the proportion of 7745 patients with Kawasaki disease who had redness or crust formation at the Bacille Calmette-Guérin inoculation site by age at onset.

TABLE 1. Association Between Redness or Crust Formation at the Bacille Calmette-Guérin Inoculation Site and Coronary Artery Abnormality in Complete Kawasaki Disease

Redness or Crust Formation	CAA (<30 d of Illness)			CAA (≥30 d of Illness)		
	No. Patients	Proportion (95% CI)	*P*	No. Patients	Proportion (95% CI)	*P*
All patients (n = 12783)*						
(+)	623/6302	9.9 (9.2–10.7)	<0.001	196/6221	3.2 (2.7–3.6)	0.87
(−)	756/6400	11.8 (11.0–12.6)		196/6323	3.1 (2.7–3.6)	
3–20 mo of age (n = 5448)†						
(+)	451/4404	10.2 (9.4–11.2)	0.46	143/4358	3.3 (2.8–3.9)	0.91
(−)	95/1004	9.5 (7.7–11.4)		32/996	3.2 (2.2–4.5)	

* Number of missing denominator were 81 before 30 d of illness and 239 in 30 d of illness or after.
† Number of missing denominator were 40 before 30 d of illness and 94 in 30 d of illness or after.
CAA indicates coronary artery abnormality; CI, confidence interval.

The proportion of patients with the changes at the BCG inoculation site in the group whose first day of hospital visit was within 1 to 4 days from the onset was also significantly larger than that of the other patient groups (52.1%, $P < 0.001$). Patients with a recurrent KD status were significantly less likely to have redness or crust formation at the BCG inoculation site (24.0%, $P < 0.001$). When data from patients aged 3 to 20 months were analyzed, similar findings were obtained (Table, Supplemental Digital Content 1, http://links.lww.com/INF/A353). These findings except for sex were also observed among incomplete KD patients.

Comparing complete KD patients without changes at the BCG inoculation site, the proportion of CAA <30 days after disease onset was smaller in patients with changes at the BCG inoculation site (9.9% vs. 11.8%, $P < 0.001$) (Table 1). However, a similar association was not observed in patients aged 3 to 20 months. There was no association between redness or crust formation at the BCG inoculation site and CAA ≥30 days after the onset.

Specificity of Redness or Crust Formation at the BCG Inoculation Site for Diagnosis of KD

A total of 53 patients met the inclusion criteria for the identification of redness or crust formation at the BCG inoculation site among those with other febrile illnesses during the observation period. The mean age was 11.6 months (standard deviation: 5.5) and the range was 20 months (3–23 months). Forty-nine patients were diagnosed with RSV infection, and 4 with rotavirus infection. None of these patients had redness or crust formation at the BCG site.

DISCUSSION

Redness or crust formation at the BCG inoculation site is a common finding among Japanese KD patients. More than 70% of complete KD patients aged 3 to 20 months had this finding. Cervical lymphadenopathy, which is one of the 6 principal signs of KD, was found in less than 60% of patients aged 2 years or younger.[16,17] Among the complete KD patients who were 2 years of age or younger, redness or crust formation at the BCG inoculation site was more prevalent than cervical lymphadenopathy. Although there was a high prevalence of redness or crust formation at the BCG inoculation site in complete KD patients, especially those aged 3 to 20 months, no patient with RSV or rotavirus infection showed the same changes at the BCG inoculation site. Even if patients have 4 or fewer signs of the clinical criteria for KD, physicians should consider that patients with redness or crust formation at the BCG inoculation site could have KD. Regarding observation of the prevalence of changes at the BCG inoculation site among patients with other febrile illness, most patients were diagnosed as having RSV or rotavirus infection during the observation period. Only one patient was diagnosed with influenza virus infection and required hospitalization. No patients with adenovirus infection or group A streptococcus infection met the inclusion criteria. One patient with human herpes virus 6 infection having erythema at the BCG inoculation site has been reported.[18] Further investigation of the prevalence of redness or crust formation at the BCG inoculation site in patients with other infectious diseases or febrile illnesses may be needed.

A higher prevalence of redness or crust formation at the BCG inoculation site was observed in complete KD patients who visited a hospital between 1 and 4 days after the onset of illness, suggesting that this sign appears during the early stages of the disease.[19] In addition, parents or guardians of children with changes at the BCG inoculation site are likely to take the child to hospital quickly if the symptoms are accompanied by fever or other principal signs of KD. Patients with recurrent KD status were less likely to have redness or crust formation at the BCG inoculation site; this could be because the age distribution of KD patients with changes at the BCG inoculation site was skewed toward old children. The proportion of KD patients aged 2 years or younger was only 17.0% among complete KD patients with recurrent KD status in the present survey. No association between redness or crust formation at the inoculation site and the development of CAA was found among patients aged 3 to 20 months, suggesting that these changes are not useful for predicting the presence of CAA.

Although the association between redness or crust formation at the BCG inoculation site and each principal sign is an important issue, we were unable to investigate this because information about principal signs was not collected in the 19th survey. Similarly, we were not able to assess the severity of inflammation in patients with changes at the inoculation site as laboratory data were not obtained in the survey. Regarding a BCG inoculation method, multiple puncture technique may be unique. Also in countries where an intradermal injection is used, similar investigation should be needed.

In conclusion, redness or crust formation at the BCG inoculation site is useful for the diagnosis of KD among children aged 3 to 20 months in countries with a BCG vaccination program. The prevalence of this sign among complete KD patients aged 3 to 20 months was higher than that of cervical lymphadenopathy. Even if such patients have 4 or fewer signs of the clinical criteria for KD, physicians should assess them for KD development.

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Common variants in *CASP3* confer susceptibility to Kawasaki disease

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Kawasaki disease (KD; OMIM 611775) is an acute vasculitis syndrome which predominantly affects small- and medium-sized arteries of infants and children. Epidemiological data suggest that host genetics underlie the disease pathogenesis. Here we report that multiple variants in the caspase-3 gene (*CASP3*) that are in linkage disequilibrium confer susceptibility to KD in both Japanese and US subjects of European ancestry. We found that a G to A substitution of one commonly associated SNP located in the 5′ untranslated region of *CASP3* (rs72689236; $P = 4.2 \times 10^{-8}$ in the Japanese and $P = 3.7 \times 10^{-3}$ in the European Americans) abolished

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binding of nuclear factor of activated T cells to the DNA sequence surrounding the SNP. Our findings suggest that altered *CASP3* expression in immune effecter cells influences susceptibility to KD.

INTRODUCTION

Kawasaki disease (KD) is characterized by high fever, polymorphous skin rash, injection of the conjunctiva, erythema of the palms and soles followed by desquamation, redness of oral mucosa and lips and non-supprative cervical lymphadenopathy (1,2). Despite clinical and epidemiological features suggesting an infectious trigger in the pathogenesis of KD, the etiology remains unknown. Marked activation of the immune system accompanied by infiltration of lymphocytes, macrophages and neutrophils into the vascular wall occurs during the acute phase of KD. The coronary arteries are selectively targeted and coronary artery lesions (CALs) develop in 20–25% of the patients without treatment (3). KD is now a leading cause of acquired cardiac disease in children in developed countries.

Previously, we performed an affected sibpair linkage study and identified several candidate regions (4q35, 5q31.4, 6q27, 7p15, 8q24, 12q24, 18q23, 19q13.2, Xp12 and Xq26) for KD susceptibility (4). Recently, we identified a functional SNP in *ITPKC*, encoding inositol 1,4,5-trisphosphate 3 kinase-C on 19q13.2, that confers both increased risk of KD and CAL formation (5). This effect is likely mediated through upregulating of the Ca^{2+}/NFAT pathway in T cells, thus increasing IL-2 production. These findings supported the hypothesis that genetically determined modulation of the immune response is fundamental to KD pathogenesis and suggested that genes with immune regulatory function located in chromosomal regions with positive linkage signals should be considered potential candidates for KD susceptibility. In an attempt to identify a novel susceptibility gene, we performed a positional candidate gene study for 4q35 region. We found that there is a set of common variants in *caspase-3* (*CASP3*) gene significantly associated with KD in both Japanese and European American subjects. We also demonstrate a functional significance of one commonly associated SNP which affects binding of nuclear factor of activated T cells (NFAT) to the 5′ untranslated region (UTR) of the gene.

RESULTS

Identification of the variants of *CASP3* gene associated with KD susceptibility

The candidate region on 4q35 was attractive because several immune genes have been mapped around the peak of linkage, including the interferon regulatory factor 2 gene (*IRF2*), *CASP3* and toll-like receptor 3 gene (*TLR3*), which all lie within 1.7 Mb of the linkage peak. Previous reports describing delayed apoptosis of peripheral blood lymphocytes (6) and neutrophils (7) from KD patients led us to focus on *CASP3*, which is located at 185.8 Mb on chromosome 4 close to the linkage peak (184.9 Mb). Caspase-3 is a key molecule of activation-induced cell death (AICD) (8) and it has also been reported to cleave the inositol 1,4,5-triphosphate receptor, Type 1 (ITPR1) in apoptotic T cells. ITPR1 is a receptor for inositol 1,4,5-trisphosphate (IP3), a substrate for ITPKC in T cells (9).

Based on linkage disequilibrium (LD) data at the web site of the International HapMap project, we selected 12 tagging SNPs with minor allele frequency (MAF) greater than 5% from the 36 kb region containing the *CASP3* gene flanked by 10 kb upstream and 5 kb downstream (Supplementary Material, Fig. S1). Using Haploview 4.1, the tagging SNPs were classified into four SNP groups at a threshold of $r^2 > 0.8$. Four tagging SNPs (rs4647693, rs2696057, rs2720378 and rs2705881) were selected as representatives of each group (Supplementary Material, Fig. S1). For the first stage of screening, the genotype at these four locations was determined for 638 Japanese KD patients and 1031 healthy Japanese controls. Three SNPs showed significant association with KD ($P < 0.05$ after Bonferroni correction for four tests; Supplementary Material, Fig. S1) when comparing allele frequencies between cases and controls. We then resequenced the 36 kb region in 24 Japanese subjects (12 KD subjects and 12 controls) and genotyped the first case–control panel for 34 additional variants and compared allele frequencies (Supplementary Material, Table S1). Twenty-five of the 46 variants (12 tagging SNPs + 34 additional variants) showed *P*-values < 0.001 ($P < 0.05$ after a conservative Bonferroni correction for 46 tests) and most were clustered in the 5′ region of *CASP3* (Fig. 1). To validate the association and identify of the causative variant, these 25 loci were further examined in an independent Japanese case–control panel with 282 KD patients and 378 controls. In this case–control panel, all of the 25 variants showed the same trend of association and rs2720378 was the most significant in a meta-analysis by the Mantel–Haenszel method [odds ratio (OR) = 1.44, 95% confidence interval (CI) 1.27–1.62; $P = 3.5 \times 10^{-9}$; Table 1]. Most of the 25 significant variants except for rs4862399 and rs7693625 were in high linkage disequilibrium with rs2720378 ($r^2 > 0.69$) and showed the same trend of association. No increase of association due to haplotypic effect was seen for the combination of rs2720378 and any other variations including rs4862399 and rs7693625 in a haplotype association study and logistic regression analysis (Supplementary Material, Tables S2 and S3).

Screening of functionally significant variants

We next assessed the functional significance of the variants in *CASP3*. Because all of the 25 variants were in untranscribed or untranslated of *CASP3*, we postulated that the variant(s) might influence expression of *CASP3*. We screened for possible enhancer activity around the associated variants by a reporter gene assay. To facilitate the screening, we cloned four tandem copies of oligonucleotides corresponding to both alleles of the variants upstream of the SV40 promoter in the luciferase reporter vector, pGL3, and transfected them into Jurkat cells.

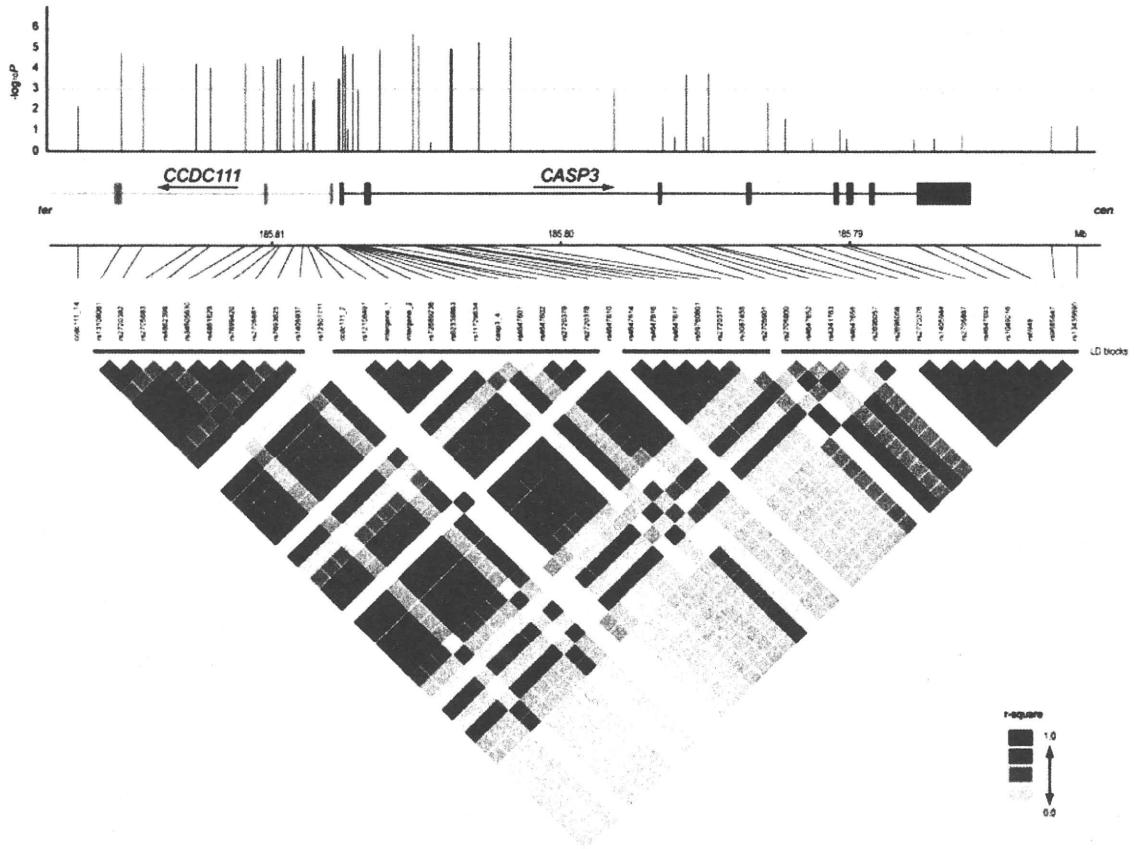


Figure 1. Linkage disequilibrium (LD) structure of the *CASP3* locus and association of the variants with KD in Japanese subjects. Pairwise LD plots with 46 variants distributed across the 36 kb region in and surrounding *CASP3* are illustrated using Haploview software. Values for r^2 were calculated using genotype data from Japanese control samples ($n = 1031$). Blue horizontal bars under SNP IDs represent LD blocks defined by Gabriel's rule. The genomic organization of *CASP3* and the coiled-coil domain containing 111 (*CCDC111*; only 5′ part is shown) is illustrated with blue and gray boxes representing the exons. Arrows under the gene names indicate the orientation of transcription. The position and the negative log of the *P*-values from the genetic association study (637 KD cases and 1031 controls; allelic frequency comparison) for each variant tested are shown by vertical bars in the upper panel. Threshold for statistical significance ($P = 0.001$) was indicated by a gray horizontal line in the upper panel.

In this screening, we found that the sequence surrounding rs72689236 located in the 5′-UTR of *CASP3* showed an enhancer activity which was significantly lower for the risk allele (A) compared with the protective allele (G) (Fig. 2A). We also found that the allelic difference was more prominent when these plasmids were transfected into peripheral blood mononuclear cells (PBMCs) or $CD3^+$ T cells. In contrast, the difference was modest when transfected into HeLa cells (data not shown). Enhancement of luciferase activity was also observed when the plasmids corresponding to intergene_1, rs62339863 and rs2720377 were transfected. However, there was no significant difference between either allele of these three SNPs. Neither enhancer function nor allelic difference was detected for rs2720378, rs4647610, rs4647616, rs4647617 and rs59760601 (Supplementary Material, Fig. S2).

Rs72689236 affects binding of NFAT to the 5′-UTR of *CASP3*

To elucidate the enhancer element that may lie near rs72689236 further, we conducted an electrophoretic mobility shift assay (EMSA) using nuclear extract from PBMCs and rs72689236 oligonucleotides as probes. As shown in Figure 2D, there was a band shift using the probe specific to the G allele. Although no binding sequence of known transcription factor was predicted near rs72689236, we focused on the GGAA sequence of which the first 'G' is changed to 'A' by the SNP. Sequence similarity to the consensus binding sequence of NFAT (GGAAAA) and a recent publication describing relationship between NFAT and *CASP3* expression (10) led us to postulate NFAT as a candidate transactivator for this site. We tested this hypothesis by conducting further luciferase assay and EMSA. In luciferase assay, both NFATc1 and NFATc2 overexpressed in HeLa cells, which have lower endogenous levels of NFATs (11), significantly enhanced the difference (Supplementary Material, Fig. S3). In contrast, cyclosporin A, a calcineurin inhibitor which suppresses NFAT signaling, minimized the difference observed in Jurkat cells (Fig. 2B). While in EMSA, formation of a DNA–protein complex was abolished by cyclosporine A added in the culture medium of PBMCs from which nuclear protein was extracted, and was competed by excess amount of unlabeled oligonucleotide with an NFAT binding sequence

Table 1. Association of genetic variants in the region of *CASP3* and Kawasaki disease in two independent panels of Japanese subjects

Variants	Position[a]	Alleles[b] Major	Minor	Panel 1 ($n = 1669$) MAF KD ($n = 638$)	Control ($n = 1031$)	OR	95% CI	*P*-values	Panel 2 ($n = 660$) MAF KD ($n = 282$)	Control ($n = 378$)	OR	95% CI	*P*-values	Combined[c] OR	95% CI	*P*-values	r^2 with rs2720378
rs13108061	185815223	C	A	0.43	0.35	1.37	1.19–1.58	1.7×10^{-5}	0.41	0.34	1.36	1.09–1.71	6.9×10^{-3}	1.37	1.21–1.54	3.8×10^{-7}	0.79
rs2720382	185814464	A	T	0.44	0.37	1.34	1.16–1.54	5.8×10^{-5}	0.42	0.34	1.41	1.13–1.77	2.5×10^{-3}	1.36	1.21–1.53	5.3×10^{-7}	0.75
rs2705883	185812635	T	C	0.44	0.37	1.34	1.17–1.55	4.7×10^{-5}	0.42	0.34	1.41	1.13–1.77	2.5×10^{-3}	1.36	1.21–1.54	4.3×10^{-7}	0.75
rs4862399	185812136	T	C	0.26	0.20	1.39	1.18–1.64	8.4×10^{-5}	0.25	0.19	1.38	1.06–1.80	0.016	1.39	1.21–1.59	4.0×10^{-6}	0.36
rs34605630	185810931	T	C	0.44	0.37	1.34	1.16–1.54	6.6×10^{-5}	0.42	0.34	1.41	1.12–1.76	2.8×10^{-3}	1.36	1.20–1.53	6.7×10^{-7}	0.74
rs4861629	185810306	G	C	0.44	0.37	1.33	1.16–1.54	7.5×10^{-5}	0.42	0.34	1.41	1.12–1.76	2.9×10^{-3}	1.35	1.20–1.53	8.0×10^{-7}	0.74
rs7699420	185809818	G	A	0.44	0.37	1.35	1.17–1.55	4.3×10^{-5}	0.42	0.34	1.43	1.14–1.79	1.8×10^{-3}	1.37	1.21–1.54	3.0×10^{-7}	0.74
rs2705881	185809719	T	C	0.44	0.37	1.35	1.17–1.56	3.3×10^{-5}	0.42	0.34	1.41	1.12–1.76	3.1×10^{-3}	1.37	1.21–1.54	3.6×10^{-7}	0.75
rs7693625	185809252	T	C	0.26	0.21	1.34	1.14–1.58	4.7×10^{-4}	0.25	0.21	1.27	0.98–1.65	0.070	1.32	1.15–1.51	8.7×10^{-5}	0.34
rs1405937	185808932	G	C	0.43	0.36	1.36	1.18–1.57	2.5×10^{-5}	0.41	0.34	1.37	1.10–1.72	5.7×10^{-3}	1.36	1.21–1.54	4.7×10^{-7}	0.79
rs12108497	185808551	A	G	0.43	0.37	1.30	1.13–1.50	2.9×10^{-4}	0.41	0.33	1.42	1.13–1.78	2.4×10^{-3}	1.33	1.18–1.51	2.7×10^{-6}	0.78
Intergene_1	185807690	T	C	0.52	0.45	1.29	1.12–1.48	3.6×10^{-4}	0.52	0.39	1.69	1.36–2.11	2.7×10^{-6}	1.39	1.24–1.57	4.0×10^{-8}	0.69
Intergene_2	185807669	G	C	0.51	0.45	1.28	1.11–1.47	5.1×10^{-4}	0.52	0.39	1.66	1.33–2.07	6.3×10^{-6}	1.38	1.23–1.55	8.5×10^{-8}	0.70
rs72689236	185807547	G	A	0.46	0.38	1.38	1.20–1.59	7.2×10^{-6}	0.44	0.35	1.43	1.15–1.79	1.6×10^{-3}	1.40	1.24–1.57	4.2×10^{-8}	0.88
rs62339863	185807461	G	T	0.45	0.38	1.36	1.18–1.57	2.1×10^{-5}	0.44	0.34	1.53	1.22–1.92	2.0×10^{-4}	1.41	1.25–1.58	2.4×10^{-8}	0.91
casp3_4	185807195	–	G	0.46	0.38	1.36	1.18–1.57	2.1×10^{-5}	0.45	0.35	1.53	1.22–1.91	1.9×10^{-4}	1.41	1.25–1.58	2.3×10^{-8}	0.90
rs2720379	185806266	T	C	0.46	0.38	1.37	1.19–1.58	1.2×10^{-5}	0.44	0.34	1.53	1.22–1.91	2.0×10^{-4}	1.41	1.26–1.59	1.2×10^{-8}	0.92
rs2720378	185805107	C	G	0.45	0.36	1.41	1.22–1.63	2.0×10^{-6}	0.43	0.34	1.50	1.20–1.88	4.1×10^{-4}	1.44	1.27–1.62	3.5×10^{-9}	1.0
rs4647610	185804925	A	G	0.46	0.38	1.39	1.20–1.60	6.1×10^{-6}	0.44	0.34	1.53	1.22–1.91	0.00021	1.42	1.26–1.61	6.6×10^{-9}	0.94
rs4647616	185803825	A	G	0.45	0.38	1.37	1.19–1.57	1.5×10^{-5}	0.45	0.35	1.51	1.21–1.89	0.00028	1.41	1.25–1.59	2.1×10^{-8}	0.94
rs4647617	185803775	A	G	0.46	0.38	1.37	1.19–1.58	1.3×10^{-5}	0.45	0.34	1.53	1.22–1.91	0.00020	1.41	1.25–1.59	1.4×10^{-8}	0.94
rs59760601	185802837	T	C	0.46	0.38	1.38	1.20–1.59	8.7×10^{-6}	0.44	0.34	1.53	1.22–1.91	0.00020	1.42	1.26–1.60	9.1×10^{-9}	0.94
rs2720377	185801740	G	A	0.45	0.37	1.40	1.21–1.61	3.5×10^{-6}	0.43	0.34	1.48	1.18–1.85	0.00060	1.42	1.26–1.60	8.7×10^{-9}	0.99
rs4647652	185795678	T	C	0.43	0.37	1.31	1.14–1.51	2.1×10^{-4}	0.43	0.33	1.51	1.20–1.89	0.00034	1.36	1.21–1.54	4.5×10^{-7}	0.75
rs4647655	185794892–185794893	TTCAGGATTT	–	0.43	0.37	1.32	1.15–1.52	1.3×10^{-4}	0.42	0.33	1.44	1.15–1.52	0.0015	1.35	1.20–1.53	7.9×10^{-7}	0.75

[a]Positions of variants are based on Build 36.3 chromosome 4 reference sequence.
[b]Nucleotides of reverse strand are shown.
[c]Combined data analysis was conducted with Mantel–Haenszel method.

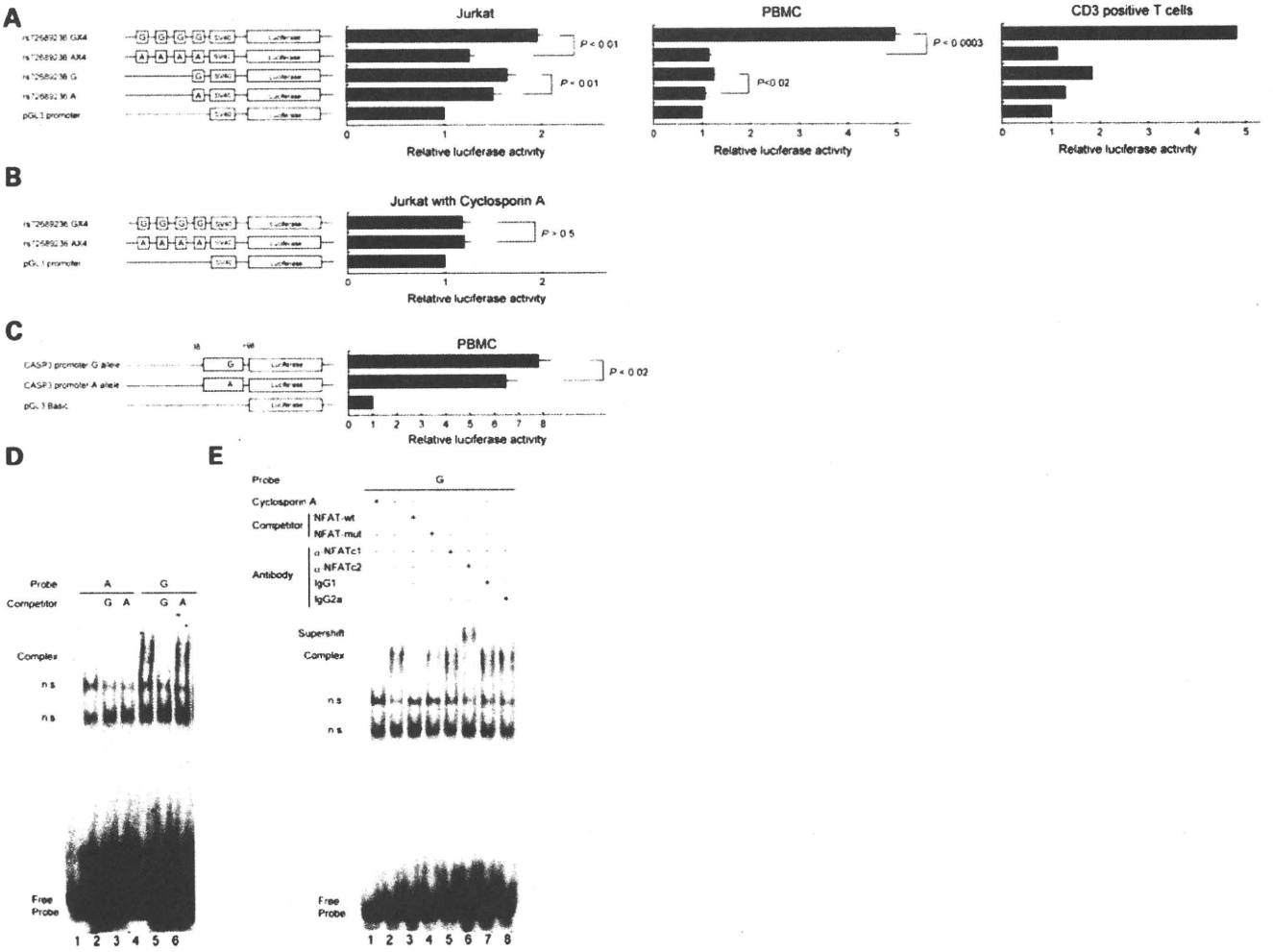


Figure 2. Functional analyses of the G and A alleles of rs72689236. (**A**) Single or four tandem copies of oligonucleotides for the G allele and A allele of rs72689236 were cloned upstream of the SV40 promoter in the PGL3 luciferase reporter vector and transfected into Jurkat cells (left), PBMCs (middle) and $CD3^+$ peripheral T cells (right; single assay). Data represent mean ± SEM of triplicate assays for Jurkat and PBMCs. (**B**) Effect of cyclosporine A on enhancer activity of rs72689236 G allele. (**C**) Transcriptional activity of *CASP3* promoter with different alleles of rs72689236. Data represent mean ± SEM of quintuplicate assays. (**D**) EMSA was performed using nuclear extracts from PBMCs stimulated with ionomycin and PMA. Oligonucleotides corresponding to the A allele (lanes 1–3) and to the G allele (lanes 4–6) were used as probes. Binding reaction was performed with no specific competitor and with excess amounts (×100) of either unlabelled G or A allele oligonucleotides. n.s., non specific bands. (**E**) Binding of NFATs to the rs72689236 G allele was assessed by EMSA using nuclear extracts from PBMCs treated with cyclosporine A in addition to ionomycin and PMA (lane 1), competition assay using oligonucleotides containing an NFAT binding sequence from the human *IL-2* promoter or its mutant (lanes 3 and 4) and a supershift assay with antibodies against NFATc1 (lane 5), NFATc2 (lane 6) and their isotype controls (lanes 7 and 8).

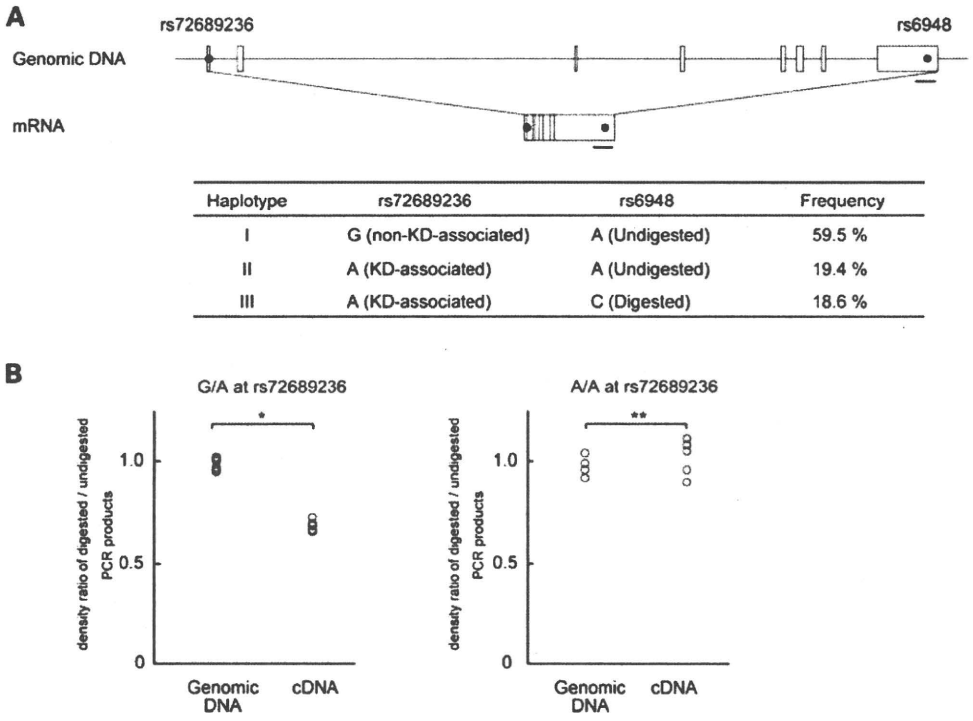
from the *Interleukin-2* (*IL-2*) promoter. And finally the complex was supershifted by a monoclonal antibody against NFATc2 (Fig. 2E).

Allele specific expression of *CASP3*

Next we compared levels of *CASP3* mRNA expressed from different alleles of rs72689236 in PBMCs by allele-specific transcript quantification (ASTQ) experiment. Primers for PCR were designed to encompass a SNP in the 3′-UTR of *CASP3* (rs6948) which was in LD with rs72689236. We examined eight healthy individuals who were heterozygous for both rs72689236 and rs6948, and therefore, inferred to have haplotypes I and III (Fig. 3A). In this haplotype combination, risk allele (A) and non-risk allele (G) of rs72689236 were absolutely linked to C and A allele of rs6948, respectively. The ratio of digested:undigested PCR products was approximately 0.7 for cDNAs and 1.0 for genomic DNA (Fig. 3B, left panel), indicating that the transcript abundance from haplotype III was lower compared with that from haplotype I. Such differences were not observed when the same experiment was conducted on PBMC from five other volunteers who were heterozygous at rs6948 but homozygous for the A allele at rs72689236 (Fig. 3B, right panel). These results suggest an effect of rs72689236 on mRNA expression levels of *CASP3*.

Association study in US KD families of European ancestry

Finally we investigated the association of the 25 variants and KD susceptibility in US subjects of European ancestry. In a



Haplotype	rs72689236	rs6948	Frequency
I	G (non-KD-associated)	A (Undigested)	59.5 %
II	A (KD-associated)	A (Undigested)	19.4 %
III	A (KD-associated)	C (Digested)	18.6 %

Figure 3. Allele-specific transcript quantification (ASTQ) of *CASP3* in PBMC. (**A**) Genomic structure of *CASP3* gene and location of the two SNPs (rs72689236 and rs6948). An amplicon of PCR was indicated by a horizontal bar. Haplotypes for the two SNPs with frequencies larger than 2.5% in the Japanese population were shown. (**B**) Comparison of relative expression level of *CASP3* mRNA from different haplotypes. Ratio of digested and undigested PCR products from genomic DNA and cDNA of PBMCs stimulated with PMA and ionomycin from healthy volunteers heterozygous (left panel; $n = 8$) and homozygous (right panel; $n = 5$) at rs72689236. *two-tailed $P = 3.0 \times 10^{-7}$, **two-tailed $P = 0.40$ by Student's *t*-test.

transmission disequilibrium test performed with US trios, the A allele of rs72689236 was significantly overtransmitted ($n = 249$, OR = 1.54, 95% CI 1.16–2.05, $P = 3.7 \times 10^{-3}$; Table 2). The association of the SNP was no more significant in the subgroup of patients who developed CALs or poorly responded to IVIG therapy in either the Japanese or US populations, indicating that the SNPs influence susceptibility but not disease outcome (data not shown). From both the association study and our functional analyses, we conclude that *CASP3* is a susceptibility gene for KD in Japanese and European American children.

DISCUSSION

KD is an immune-mediated vasculitis that is thought to result from an unknown infectious trigger in genetically susceptible hosts. Our previous findings that downregulation of ITPKC, which functions as a negative regulator of the Ca^{2+}/NFAT pathway in T cells, by an intronic SNP resulted in enhanced activation of the pathway highlighted the importance of regulation of T cell activation in the pathogenesis of KD (5). Caspase-3 is one of the effecter caspases that plays a central role in apoptosis. Peripheral T cells from caspase-3 deficient mice were less susceptible to AICD, a mechanism regulating the magnitude and duration of the T cell immune response (8). Furthermore, it was reported that *Casp3* transcription was selectively up-regulated after T cell receptor (TCR) ligation (12). NFATs are activated by a signal from the TCR and drive transcription of *IL-2* and other cytokines. It was also reported that the induction of *Casp3* mRNA in response to ionomycin stimulation was abolished in Th1 cells from Nfatc2 deficient mice, indicating that NFATc2 is a key transactivator for the gene in this cell type (10). Our data suggest that the sequence surrounding rs72689236 might be a binding site for NFATc2 and acts as an enhancer element in T cells activated in response to signals from TCR. Sequence comparison with chimpanzee indicates that the ancestral allele of rs72689236 is 'G' (data not shown). Interestingly, the GGAA sequence was seen in a similar position within the first non-coding exon of rodent *Casp3* genes (Supplementary Material, Fig. S4), suggesting that the enhancer element might be evolutionarily conserved. There remains a possibility that rs2720378 as well as the other associated variations also affect *CASP3* expression by other, unknown mechanisms. In addition, expression of caspase-3 is not restricted to T cells and a number of proteins are known as substrates for caspases (13). Caspase-3 is also known to play roles in cellular activities other than apoptosis (14–17). Further investigation is needed to understand the impact of reduced *CASP3* expression on the pathogenesis of KD.

We recognize some potential limitations to our study. It is possible that the observed association of the functional polymorphism in *CASP3* with KD susceptibility was somewhat inflated due to population structure. However, the positive linkage signal near *CASP3* in our previous sibpair study and the positive association in our present family-based one, neither of which are influenced by population stratification, suggest that the association has not been over-estimated.