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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

### 書籍

	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
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#### IV. 研究成果の刊行物・別刷

1

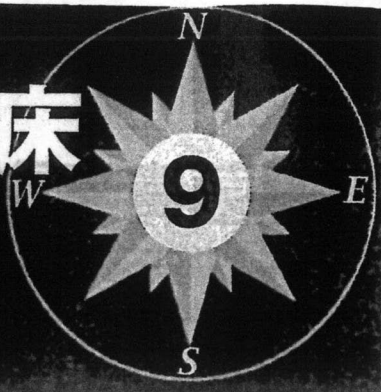
## 病因・溶連菌毒素 説

鈴木 啓之

川崎病のすべて 2009 pp34-35

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# 川崎病のすべて

総編集◎五十嵐 隆 東京大学  
専門編集◎石井 正浩 北里大学

患児の長い人生の要点となる  
“急性期診療”に重きを置いた道標!

患児の80%が4歳未満、病因不明で発症メカニズムも解明されていない  
“川崎病”は近年増加しています。その全貌を明らかにすべく、  
疫学・基礎医学・臨床医学・社会医学などすべての分野からアプローチ!  
病因諸説、有効な治療法、急性期・遠隔期別の心血管後遺症解説など  
実地診療に役立つ最先端知識を集結しました。



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## 病因

## 溶連菌毒素説

鈴木啓之

溶連菌：溶血性レンサ球菌  
(*streptococcus hemolyticus*)

SAg：superantigen

SPE：streptococcal pyrogenic  
exotoxin (レンサ球菌発熱性毒  
素)

TSST：toxic shock syndrome  
toxin (毒素性ショック症候群毒  
素)

TCR：T cell receptor

\*1  
しかし、これらの事実は、SPE-C  
が川崎病患児に残した生体反応  
であり、SPE-C が関与した可能  
性を間接的に証明したものにす  
ぎない。

\*2  
理由として、①菌量が少ない、②  
培養が困難、③細菌自体が川崎  
病発症時にすでに死滅している、  
などが考えられ、分離が困難であ  
ることが予想される。

\*3  
上咽頭から下部消化管に存在す  
る細菌は、たとえ少数の菌や培養  
の困難な菌、また死滅した菌でも  
すべて便に排出されると想定さ  
れる。

## 病因究明の歴史

川崎先生が本疾患を発表された1967年以来40年余りが経過したが、前半の約20年間においては、溶連菌説<sup>1,2)</sup>をはじめリケッチア説、ダニ説など多彩な説が展開され、またそれらの説に対する反論<sup>3)</sup>もあり、結論が得られない状況が続いた。

1990年代に入りスーパー抗原(SAg)が原因の一つとして取り上げられて以来<sup>4)</sup>、後半の約20年間は、SAgと通常の感染因子(conventional antigen)の2つを中心に展開してきた。

SAgは溶連菌やブドウ球菌が産生するSPE-C、SPE-AやTSST-1がその中心であり、通常の感染因子は病原菌の種類は不明であるが、川崎病患児組織中に存在するとされる封入体がその中心である。

この流れのなかで、1990年代中ごろから筆者らはとくに溶連菌のSAgを中心に研究してきた。溶連菌を第一ターゲットにした理由は、川崎病診断の基準となる6つの主要症状と溶連菌感染症との強い類似性にある。

## SAg説研究の推移

SAg説を追求する研究は、個々のSAgの免疫賦活化の機構であるT細胞レセプター(TCR)レパートリーの活性化や偏り、SAgに対する抗体反応を中心に行われてきた。しかし、TCRレパートリーの解析結果は報告者によって異なり、SAgと川崎病の関係を明確にできない状況が続いた。

筆者らは川崎病急性期の末梢血TCRレパートリーを解析した結果、Vβ2とVβ6.5のポリクローナルな選択性を見出し、また急性期のSPE-Cに対する抗体陽性反応を明らかにし、川崎病急性期患児がSPE-Cに曝露され、川崎病発症にSPE-Cが関与する可能性を報告した<sup>5)\*1</sup>。

川崎病発症にSPE-CやSPE-C産生菌が関与することを確実にするためには、抗原因子、つまりSPE-Cそのもの、あるいはSPE-C産生菌をとらえる必要がある。しかし、SAgは極微量でも強い炎症反応を惹起できるため、逆に検出することは困難が予想され、実際にSAg説のなかでもSAgを分離したとの報告はない。

またSAg産生菌の分離も、溶連菌やブドウ球菌に関してのこれまでの研究からは明確な結果が得られていない<sup>\*2</sup>。

一方、本教室の坊岡らは、胃壁内に存在するが培養が容易でない*Helicobacter pylori*菌のクラリスロマイシン耐性株の存在を、患児の便<sup>\*3</sup>から全DNAを抽出する方法で証明した<sup>6)</sup>。

筆者らは川崎病急性期患児(入院時)の便から全DNAを抽出し、溶連菌

やブドウ球菌が産生する SAg の遺伝子断片の存在の有無を検討した。

## SAg 遺伝子検出の研究

- 対象と方法を ① に記す。

### 結果

- 5種の SAg 遺伝子のうち少なくとも1つの SAg が検出されたのは、KD 群 60 例中 42 例 (70.0%)、発熱群 36 例中 14 例 (38.9%)、健常群 26 例中 7 例 (26.9%) で、KD 群は発熱群 ( $p=0.005$ ) ととも健常群 ( $p<0.001$ ) ととも有意差を認めた。
- 発熱群と健常群を合わせて非 KD 群 (62 例) とし、KD 群 (60 例) と比較するとその差はより明瞭となり、KD 群の便からは検討した 5 つの SAg 遺伝子のうち少なくとも1つの SAg が有意に多く検出された ( $p<0.001$ )。
- 個々の SAg で両群 (KD 群：非 KD 群) 間に有意差を認めたのは SPE-A ( $p=0.031$ )、SPE-G ( $p=0.001$ )、SPE-J ( $p=0.029$ ) で、SPE-C も有意差を認めなかったものの  $p=0.07$  であり、KD 群に多くみられた。一方、ブドウ球菌由来の TSST-1 は  $p=0.6$  と差を認めなかった。

### 考察

- これらの事実は、SAg が KD 発症に関与している可能性を示唆し、さらに複数の SAg が独立して KD 発症に関与している可能性も示唆している。
- 溶連菌の 4 つの SAg は、各々が黄色ブドウ球菌の TSST-1 よりも高頻度に検出され、溶連菌の SAg のほうが川崎病発症により強く関与する結果であったが、TSST-1 を含めた複数の SAg が別々に川崎病発症の引き金になりえる可能性を示唆している\*4。

## 今後の問題点

- 今回の研究でも KD 患児の咽頭・便から *S. pyogenes* は分離されていない\*5。そこで、① いかなる菌種の細菌が、この SAg 遺伝子を所有しているのか、② 上気道から下部消化管のどこにその細菌はいるのか、③ 実際にその細菌は SAg 遺伝子断片に相当する SAg を産生しているのか、などが今後、解決しなければならない問題点である。

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### ① 研究の対象と方法

- 川崎病 (KD) 患児 60 例を対象とし、川崎病以外の急性熱性疾患患児 36 例を発熱群、健康小児 26 例を健常群とした。対象および対照から便を採取し、市販のキットを用いて全 DNA を抽出した
- 検討した SAg 遺伝子は、今まで TCR レポートリー分析のなかで比較的意見の一致を認めている Vβ2 に親和性のある、あるいは病因 SAg として報告された溶連菌が産生する SPE-A, SPE-C, SPE-G, SPE-J とブドウ球菌が産生する TSST-1 の 5 つである
- 便由来の全 DNA をテンプレートとして PCR 法を用いて遺伝子増幅を行った

KD : Kawasaki disease

\*4  
溶連菌の複数の SAg が独自に有意差をもって検出されていること、さらに SAg を産生するとされるマイコプラズマ、エルシニア、EB ウイルスなどが KD 様症状を生じること、今まで KD 患児の TCR レポートリー分析では活性化されるレポートリーが報告者によって異なっていたことも、逆に、KD 発症への SAg の関与が溶連菌由来 SAg を中心として複数である可能性を示唆している。

EB : Epstein-Barr

*S. pyogenes* : *Streptococcus pyogenes*

\*5  
この事実は、便から溶連菌 SAg 遺伝子が検出されることをどのように説明するかが大きな問題点であるが、最近、溶連菌の SAg 遺伝子が *Streptococci* 間で gene transfer を生じるとの報告があり興味深い<sup>7)</sup>。

Common variants in CASP3 confer susceptibility to  
Kawasaki disease.

Onouchi Y, Ozaki K, Buns JC, Shimizu C, Hamada H, Honda T, Terai M, Honda A, Takeuchi T, Shibuta S, Suenaga T, Suzuki H, Higashi K, Yasukawa K, Suzuki Y, Sasago K, Kemmotsu Y, Takatsuki S, Saji T, Yoshikawa T, Nagai T, Hamamoto K, Kishi F, Ouchi K, Sato Y, Newburger JW, Baker AL, Shulman ST, Rowley AH, Yashiro M, Nakamura Y, Wakui K, Fukushima Y, Fujino A, Tsunoda T, Kawasaki T, Hata A, Nakamura Y, Tanaka

Hum Mol Genet. May 10. 2010

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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 effector 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-suppurative 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

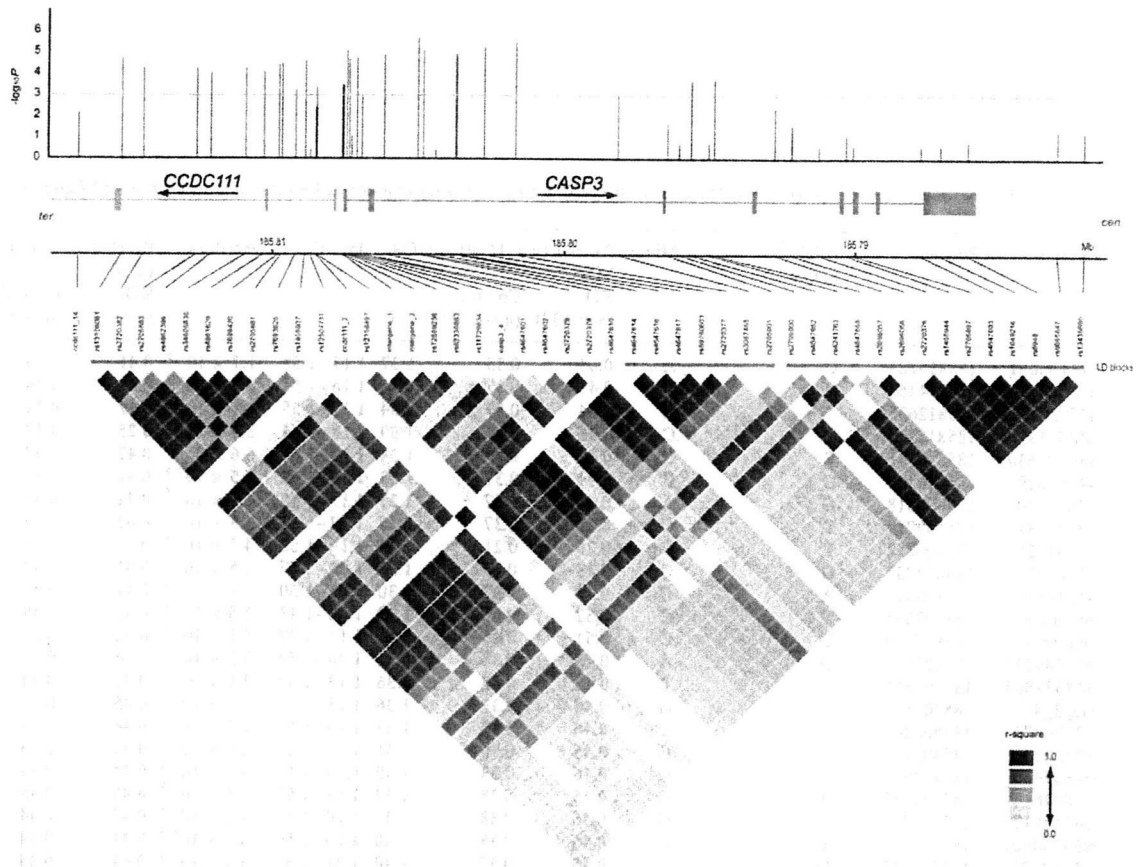
The candidate region on 4q35 was attractive because several immune genes have been mapped around the peak of linkage, including the interferon regulatory factor 2 gene (*IRF2*), *CASP3* and toll-like receptor 3 gene (*TLR3*), which all lie within 1.7 Mb of the linkage peak. Previous reports describing 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-trisphosphate

receptor, Type 1 (ITPR1) in apoptotic T cells. ITPR1 is a receptor for inositol 1,4,5-trisphosphate (IP<sub>3</sub>), 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 untranslated or untranscribed 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<sup>+</sup> 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 cyclosporin 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

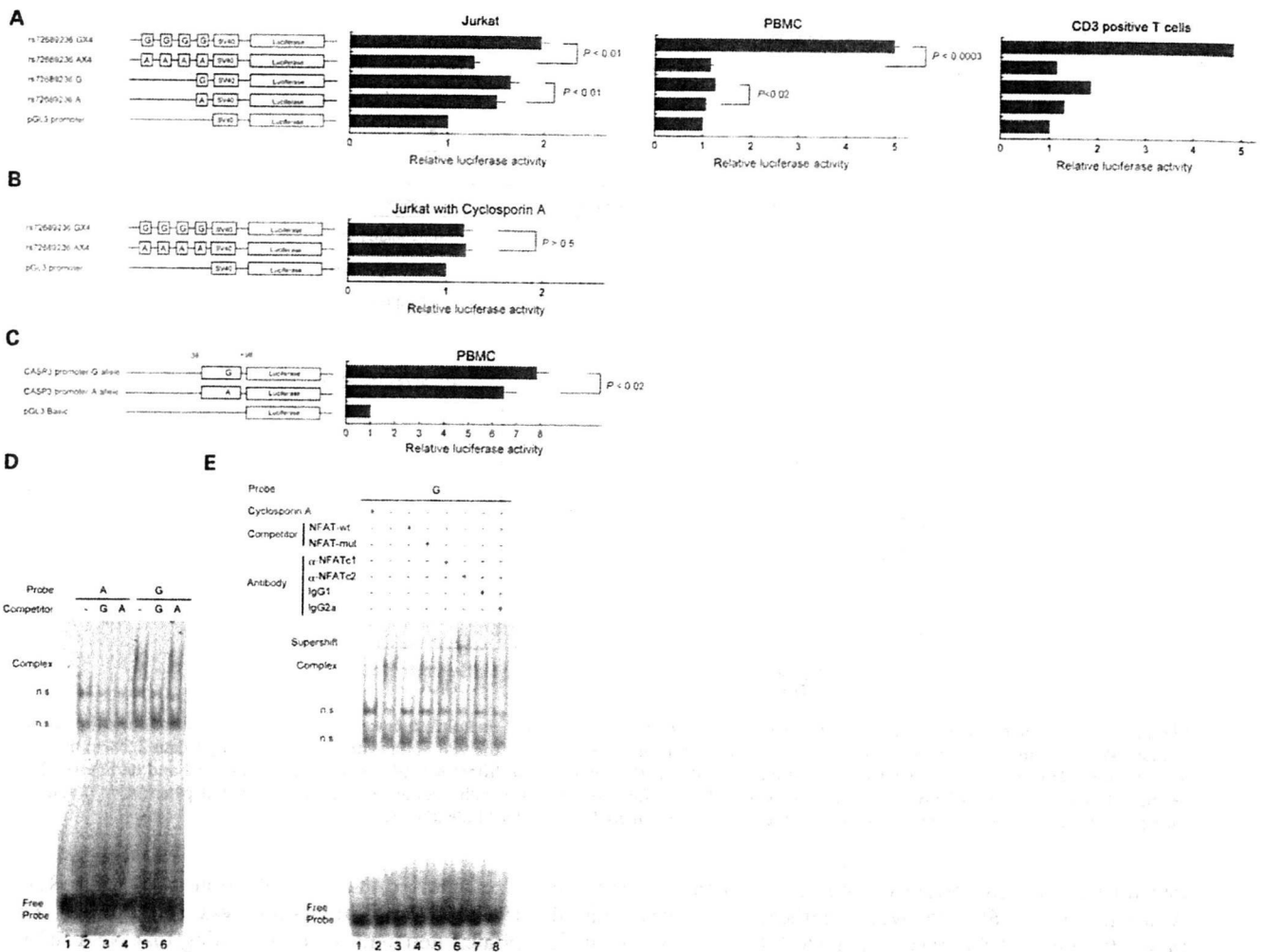
Table 1. Association of genetic variants in the region of *CASP3* and Kawasaki disease in two independent panels of Japanese subjects

Variants	Position <sup>a</sup>	Alleles <sup>b</sup> Major	Minor	Panel 1 (n = 1669)		OR	95% CI	P-values	Panel 2 (n = 660)		OR	95% CI	P-value
				MAF KD (n = 638)	Control (n = 1031)				MAF KD (n = 282)	Control (n = 378)			
rs13108061	185815223	C	A	0.43	0.35	1.37	1.19-1.58	$1.7 \times 10^{-5}$	0.41	0.34	1.36	1.09-1.71	$6.9 \times 10^{-5}$
rs2720382	185814464	A	T	0.44	0.37	1.34	1.16-1.54	$5.8 \times 10^{-5}$	0.42	0.34	1.41	1.13-1.77	$2.5 \times 10^{-5}$
rs2705883	185812635	T	C	0.44	0.37	1.34	1.17-1.55	$4.7 \times 10^{-5}$	0.42	0.34	1.41	1.13-1.77	$2.5 \times 10^{-5}$
rs4862399	185812136	T	C	0.26	0.20	1.39	1.18-1.64	$8.4 \times 10^{-5}$	0.25	0.19	1.38	1.06-1.80	0.016
rs34605630	185810931	T	C	0.44	0.37	1.34	1.16-1.54	$6.6 \times 10^{-5}$	0.42	0.34	1.41	1.12-1.76	$2.8 \times 10^{-5}$
rs4861629	185810306	G	C	0.44	0.37	1.33	1.16-1.54	$7.5 \times 10^{-5}$	0.42	0.34	1.41	1.12-1.76	$2.9 \times 10^{-5}$
rs7699420	185809818	G	A	0.44	0.37	1.35	1.17-1.55	$4.3 \times 10^{-5}$	0.42	0.34	1.43	1.14-1.79	$1.8 \times 10^{-5}$
rs2705881	185809719	T	C	0.44	0.37	1.35	1.17-1.56	$3.3 \times 10^{-5}$	0.42	0.34	1.41	1.12-1.76	$3.1 \times 10^{-5}$
rs7693625	185809252	T	C	0.26	0.21	1.34	1.14-1.58	$4.7 \times 10^{-4}$	0.25	0.21	1.27	0.98-1.65	0.070
rs1405937	185808932	G	C	0.43	0.36	1.36	1.18-1.57	$2.5 \times 10^{-5}$	0.41	0.34	1.37	1.10-1.72	$5.7 \times 10^{-5}$
rs12108497	185808551	A	G	0.43	0.37	1.30	1.13-1.50	$2.9 \times 10^{-4}$	0.41	0.33	1.42	1.13-1.78	$2.4 \times 10^{-5}$
Intergene_1	185807690	T	C	0.52	0.45	1.29	1.12-1.48	$3.6 \times 10^{-4}$	0.52	0.39	1.69	1.36-2.11	$2.7 \times 10^{-5}$
Intergene_2	185807669	G	C	0.51	0.45	1.28	1.11-1.47	$5.1 \times 10^{-4}$	0.52	0.39	1.66	1.33-2.07	$6.3 \times 10^{-5}$
rs72689236	185807547	G	A	0.46	0.38	1.38	1.20-1.59	$7.2 \times 10^{-6}$	0.44	0.35	1.43	1.15-1.79	$1.6 \times 10^{-5}$
rs62339863	185807461	G	T	0.45	0.38	1.36	1.18-1.57	$2.1 \times 10^{-5}$	0.44	0.34	1.53	1.22-1.92	$2.0 \times 10^{-5}$
casp3_4	185807195	-	G	0.46	0.38	1.36	1.18-1.57	$2.1 \times 10^{-5}$	0.45	0.35	1.53	1.22-1.91	$1.9 \times 10^{-5}$
rs2720379	185806266	T	C	0.46	0.38	1.37	1.19-1.58	$1.2 \times 10^{-5}$	0.44	0.34	1.53	1.22-1.91	$2.0 \times 10^{-5}$
rs2720378	185805107	C	G	0.45	0.36	1.41	1.22-1.63	$2.0 \times 10^{-6}$	0.43	0.34	1.50	1.20-1.88	$4.1 \times 10^{-5}$
rs4647610	185804925	A	G	0.46	0.38	1.39	1.20-1.60	$6.1 \times 10^{-6}$	0.44	0.34	1.53	1.22-1.91	0.00021
rs4647616	185803825	A	G	0.45	0.38	1.37	1.19-1.57	$1.5 \times 10^{-5}$	0.45	0.35	1.51	1.21-1.89	0.00028
rs4647617	185803775	A	G	0.46	0.38	1.37	1.19-1.58	$1.3 \times 10^{-5}$	0.45	0.34	1.53	1.22-1.91	0.00021
rs59760601	185802837	T	C	0.46	0.38	1.38	1.20-1.59	$8.7 \times 10^{-6}$	0.44	0.34	1.53	1.22-1.91	0.00021
rs2720377	185801740	G	A	0.45	0.37	1.40	1.21-1.61	$3.5 \times 10^{-6}$	0.43	0.34	1.48	1.18-1.85	0.00061
rs4647652	185795678	T	C	0.43	0.37	1.31	1.14-1.51	$2.1 \times 10^{-4}$	0.43	0.33	1.51	1.20-1.89	0.00036
rs4647655	185794892	TTCAGGATT	-	0.43	0.37	1.32	1.15-1.52	$1.3 \times 10^{-4}$	0.42	0.33	1.44	1.15-1.52	0.0015
	185794893												

<sup>a</sup>Positions of variants are based on Build 36.3 chromosome 4 reference sequence.

<sup>b</sup>Nucleotides of reverse strand are shown.

<sup>c</sup>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<sup>+</sup> peripheral T cells (right; single assay). Data represent mean  $\pm$  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  $\pm$  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 ( $\times 100$ ) 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*

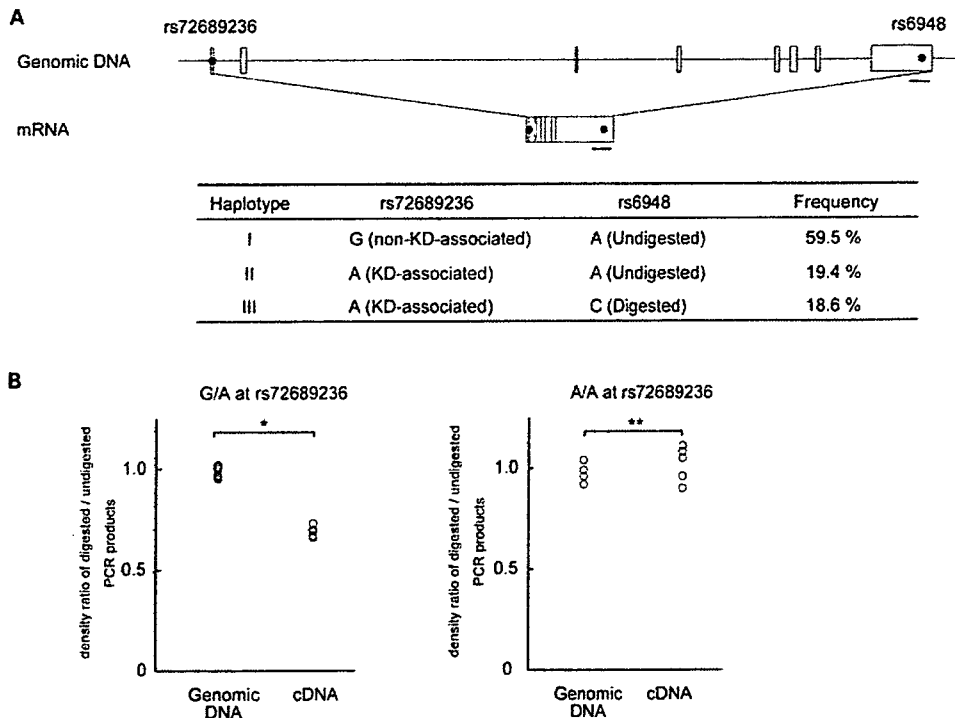
Next we compared levels of *CASP3* mRNA expressed from different alleles of rs72689236 in PBMCs by allele-specific transcript quantification (ASTQ) experiment. Primers for PCR were designed to encompass a SNP in the 3'-UTR of *CASP3* (rs6948) which was in LD with rs72689236. We examined eight healthy 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 absol-

utely 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





**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  $\text{Ca}^{2+}$ /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 effector 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.

**Table 2.** Transmission of genetic variants in *CASP3* from European American parents to their offspring with Kawasaki disease

Variants	TDT Risk allele	RAF <sup>a</sup>	T:U <sup>b</sup>	OR	95% CI	P-value
rs13108061	-	0.44	114:114	1.00	0.77-1.30	1.00
rs2720382	T	0.83	78:54	1.44	1.02-2.04	0.037
rs2705883	C	0.69	120:80	1.50	1.13-1.99	4.7 × 10 <sup>-3</sup>
rs4862399	T	0.88	50:49	1.02	0.69-1.51	0.92
rs34605630	C	0.68	122:85	1.44	1.09-1.89	0.010
rs4861629	C	0.81	82:58	1.41	1.01-1.98	0.043
rs769420	A	0.82	82:63	1.30	0.94-1.81	0.11
rs2705881	C	0.82	82:59	1.39	0.99-1.94	0.053
rs7693625	T	0.81	78:70	1.11	0.81-1.54	0.51
rs1405937	C	0.81	82:62	1.62	0.95-1.84	0.096
rs12108497	G	0.68	122:84	1.45	1.10-1.92	8.1 × 10 <sup>-3</sup>
Intergene_1	C	0.82	74:58	1.28	0.90-1.80	0.16
Intergene_2	C	0.72	112:78	1.44	1.08-1.92	0.014
rs72689236	A	0.70	120:79	1.54	1.16-2.05	3.7 × 10 <sup>-3</sup>
rs62339863	T	0.71	122:78	1.56	1.18-2.08	1.9 × 10 <sup>-3</sup>
casp3_4	G	0.66	134:95	1.41	1.08-1.83	0.010
rs2720379	C	0.68	121:82	1.48	1.11-1.95	6.2 × 10 <sup>-3</sup>
rs2720378	G	0.68	118:84	1.40	1.06-1.86	0.017
rs4647610	G	0.85	72:53	1.36	0.95-1.94	0.089
rs4647616	G	0.84	70:53	1.32	0.92-1.89	0.13
rs4647617	G	0.84	70:53	1.32	0.92-1.89	0.13
rs59760601	C	0.84	68:49	1.39	0.96-2.00	0.079
rs2720377	A	0.68	121:81	1.49	1.13-1.98	4.9 × 10 <sup>-3</sup>
rs4647652	C	0.87	58:50	1.16	0.79-1.69	0.44
rs4647655	del (TTCAG GATT)	0.87	59:50	1.18	0.81-1.72	0.39

<sup>a</sup>Risk allele frequency.

<sup>b</sup>'T' and 'U' indicate transmitted and untransmitted risk alleles of each variant.

Puga *et al.* (18) described that caspase-3 induced by Nfatc2 leads to T cell anergy by downregulating TCR signaling. The transient T cell anergy in KD patients in acute and convalescent phases which have been documented in several reports (19-21) might be, at least partly, related to induction of caspase-3 in T cells by activated NFATc2. No apparent gene-gene interaction between *ITPKC* and *CASP3* was detected in the logistic regression analysis of the SNPs (rs28493229 in *ITPKC* and rs2720378 or rs72689236 in this study, data not shown). However, it is of great interest that NFAT is involved in both pathways in which these SNPs have a functional role (Supplementary Material, Fig. S5). It has also been reported that Nfatc2 is a substrate for caspase-3 (22). It may be that the induction of caspase-3 acts as a negative feedback mechanism to regulate activation of the Ca<sup>2+</sup>/NFAT pathway. There are likely to be several molecular networks playing major roles in the pathogenesis of KD. Our present findings further highlight the Ca<sup>2+</sup>/NFAT pathway as a main axis in regulating these networks. Since many inhibitors of this pathway such as cyclosporine and tacrolimus are in clinical use, further elucidation of the role of caspase-3 in the pathophysiology of KD may lead to new preventive and therapeutic strategies for this vasculitis.

## MATERIALS AND METHODS

### DNA samples

We recruited 920 Japanese KD patients from several medical institutes in Japan. All Japanese KD patients (male:female:no

info = 554:365:1) were diagnosed by pediatricians according to the Japanese criteria (23). Median age of disease onset was 23.0 months (range 1-136). Healthy Japanese adults without a history of KD (*n* = 1409) were also recruited as controls from several medical institutes. DNA samples from 249 KD subjects of European descent (male:female = 163:86) and their biological parents were collected by several medical institutes participating in the US KD Genetics Consortium. The study was approved by the ethical committee of RIKEN and the institutional review board of all participating institutions. Written informed consent and assent as appropriate were obtained from subjects and their parents.

### Re-sequencing and genotyping

Data regarding tagging SNPs were obtained from the website of International HapMap Project ([http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap24\\_B36/](http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap24_B36/)). LD map in Figure 1 was created using Haploview 4.1 software (<http://www.broad.mit.edu/haploview/haploview>). For SNP discovery, we resequenced the genomic region (NT\_022792.17: from nt 17,956,305 to 17,992,719) using DNA from 12 KD patients and 12 controls. Repetitive sequences except for those in the region from the promoter to intron 1 of *CASP3* were excluded from the analysis. We genotyped SNPs and insertion/deletion polymorphisms using the Invader assay (24) and direct sequencing, respectively.

### Statistic analysis

Association of the SNPs was analyzed using a chi-square test. Meta-analysis of data from case-control sets was conducted by Mantel-Haenszel methodology. Transmission disequilibrium test was performed using TDT software (25) integrated in Haploview 4.1. Haplotype analysis was conducted by using the program THESIAS (26) (<http://genecanvas.ecgene.net/news.php>) and conditional log-likelihood with Akaike information criterion (AIC): AIC = -2 × (the maximized value of the conditional log-likelihood) + 2 × (the number of parameters). As the number of parameters, we used the number of alleles or haplotypes with frequencies >0.01 that were used for each model. In the logistic regression analysis of a SNP, we first applied a 1 degree-of-freedom (1 d.f.) likelihood ratio test to determine whether a 1 d.f. multiplicative allelic effects model or a 2 d.f. full genotype model was more appropriate (26). Because we did not find any significant difference from the full genotype model (*P* > 0.05), we assumed a multiplicative allelic effects mode. Next, we carried out a forward logistic regression analysis, where we started by assessing whether the most significant SNP was sufficient to model the association among the SNP set. For this, we used a 1 d.f. likelihood ratio test for adding each of the remaining SNPs to the model by assuming multiplicative allelic effects for the additional SNPs.

### Luciferase assay

Jurkat E6-1 cells and HeLa cells were obtained from ATCC and the RIKEN Cell Bank, respectively. PBMCs from healthy volunteers were separated from venous blood using

Lymphoprep reagent (Axis-Shields). CD3<sup>+</sup> T cells were isolated using iMag system with a monoclonal antibody against human CD3 (clone HIT3a) conjugated with magnetic beads (BD Biosciences). We cloned single or four tandem copies of 31 nucleotides for each SNP region upstream of the SV40 promoter of the pGL3-promoter vector (Promega). The minimal promoter region of *CASP3* (nt -38 of 5' flanking to +17 of intron 1) was cloned into pGL3-basic vector. These reporter plasmids were co-transfected with pHRGK vector into the cells. C-016 and O-005 programs of the Nucleofector (Amaxa) were used for transfection into Jurkat E6-1 and HeLa, respectively. Transfection into PBMCs and CD3<sup>+</sup> T cells was conducted with U-014 program. Twenty-four hours after transfection, Jurkat cells, PBMCs and CD3<sup>+</sup> T cells were stimulated with 1 µg/ml of ionomycin (SIGMA) and 50 ng/ml of PMA (SIGMA) for 4 h and harvested. Suppression of NFAT activity was performed by adding 100 ng/ml of cyclosporin A (CALBIOCHEM) in the above-mentioned stimulation medium. Luciferase activity was measured with the Dual Luciferase Reporter Assay system (Promega). We also cloned cDNAs of NFATc1 (NM\_172390) and NFATc2 (NM\_173091) into pcDNA3.1(+) (Invitrogen) and co-transfected with reporter vectors for rs72689236 to test the effect of overexpression of these proteins on enhancer activity.

#### Electrophoretic mobility shift assay

PBMCs were incubated in RPMI 1640 medium supplemented with 10% of fetal bovine serum and stimulated with ionomycin (1 µg/ml) and PMA (50 ng/ml) for 2 h. Suppression of NFAT activity was achieved by adding 100 ng/ml of cyclosporin A to the stimulation medium. After lysing the cells with buffer A [10 mM HEPES-KOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 0.1% NP-40 and protease inhibitor cocktail], nuclear extracts were prepared using buffer C [50 mM HEPES-KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 5 mM MgCl<sub>2</sub>, 2% glycerol and protease inhibitor cocktail]. Eighteen base pairs of double-stranded oligonucleotides corresponding to G and A alleles of rs72689236 were labeled with digoxigenin -11-ddUTP using DIG Gel Shift Kit (Roche). Probes were incubated with 5 µg of nuclear extract pre-incubated with 0.2 µg of poly d(I-C), 1 µg of poly-L-lysine for 30 min in room temperature. For the supershift assay, nuclear extract and monoclonal antibodies (Santa Cruz) or isotype control IgGs (R&D SYSTEMS) were incubated for 1 h on ice prior to the binding reaction. Competition was conducted with 100× molar excess of unlabeled oligonucleotides. Sequences of the oligonucleotides are provided in Supplementary Material, Table S4. The binding reaction mixtures were separated on 5% non-denaturing polyacrylamide gel in 0.5 × TBE buffer, transferred onto a nylon membrane and detected with a chemiluminescent system (Roche).

#### Allele-specific transcript quantification

ASTQ was carried out as described previously (27). Sequences of primers for PCR were shown in Supplementary Material, Table S5. Total RNA was extracted from PBMCs after stimulation for 4 h with 1 µg/ml of ionomycin and 50 ng/ml of

PMA. Genomic DNAs and cDNAs were amplified for 36 cycles with the primers. At the last cycle, reverse primer labeled with Alexa Fluor 488 at the 5' was added. Amplicons were digested with *Ban*II (Takara) according to manufacturer's instruction. Separation was conducted on 12% non-denaturing polyacrylamide gels in 25 mM Tris and 250 mM glycine. Visualization and quantification of digested and undigested PCR products was carried out by using FLA-7000 analyzer and Multiguage software (Fujifilm).

#### Accession codes

Genbank: human chromosome 4 genomic DNA sequence, NT\_022792.17; mRNA sequences for human *CASP3*, NM\_004346.3 and NM\_032991.2.

#### SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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