ARTICLES

nature genetics

ITPKC functional polymorphism associated with Kawasaki disease susceptibility and formation of coronary artery aneurysms

Yoshihiro Onouchi[1], Tomohiko Gunji[1,2], Jane C Burns[3], Chisato Shimizu[3], Jane W Newburger[4], Mayumi Yashiro[5], Yoshikazu Nakamura[5], Hiroshi Yanagawa[6], Keiko Wakui[7], Yoshimitsu Fukushima[7], Fumio Kishi[8], Kunihiro Hamamoto[9], Masaru Terai[10], Yoshitake Sato[11], Kazunobu Ouchi[12], Tsutomu Saji[13], Akiyoshi Nariai[14], Yoichi Kaburagi[14,26], Tetsushi Yoshikawa[15], Kyoko Suzuki[16], Takeo Tanaka[17], Toshiro Nagai[18], Hideo Cho[19], Akihiro Fujino[20], Akihiro Sekine[21], Reiichiro Nakamichi[22], Tatsuhiko Tsunoda[22], Tomisaku Kawasaki[23], Yusuke Nakamura[24,25] & Akira Hata[1]

Kawasaki disease is a pediatric systemic vasculitis of unknown etiology for which a genetic influence is suspected. We identified a functional SNP (itpkc_3) in the inositol 1,4,5-trisphosphate 3-kinase C (*ITPKC*) gene on chromosome 19q13.2 that is significantly associated with Kawasaki disease susceptibility and also with an increased risk of coronary artery lesions in both Japanese and US children. Transfection experiments showed that the C allele of itpkc_3 reduces splicing efficiency of the *ITPKC* mRNA. ITPKC acts as a negative regulator of T-cell activation through the Ca^{2+}/NFAT signaling pathway, and the C allele may contribute to immune hyper-reactivity in Kawasaki disease. This finding provides new insights into the mechanisms of immune activation in Kawasaki disease and emphasizes the importance of activated T cells in the pathogenesis of this vasculitis.

© 2008 Nature Publishing Group http://www.nature.com/naturegenetics

Kawasaki disease (OMIM 300530) is an acute, self-limited vasculitis of infants and children characterized by prolonged fever unresponsive to antibiotics, polymorphous skin rash, erythema of the oral mucosa, lips and tongue, erythema of the palms and soles, bilateral conjunctival injection and cervical lymphadenopathy[1]. Coronary artery aneurysms develop in 15–25% of those left untreated[2], making Kawasaki disease the leading cause of acquired heart disease among children in developed countries. Treatment with intravenous immunoglobulin (IVIG) abrogates the inflammation in approximately 80% of affected individuals and reduces the aneurysm rate to less than 5%. Cardiac sequelae of the aneurysms include ischemic heart disease, myocardial infarction and sudden death[3]. Epidemiological features such as seasonality and clustering of cases suggest an infectious trigger, although no pathogen has been isolated and the etiology remains unknown.

Several lines of evidence suggest the importance of genetic factors in disease susceptibility and outcome. First, the incidence of Kawasaki disease is 10–20 times higher in Japan than in Western countries[4]. Second, the risk of Kawasaki disease in siblings of affected children is 10 times higher than that in the general population ($\lambda_s = 10$), and the incidence of Kawasaki disease in children born to parents with a history of Kawasaki disease is twice as high as that in the general

[1]Laboratory for Gastrointestinal Diseases, SNP Research Center, RIKEN, Yokohama, Kanagawa, 230-0045, Japan. [2]Department of Hard Tissue Engineering, Graduate School Tokyo Medical and Dental University, Tokyo 113-8549, Japan. [3]Department of Pediatrics, University of California San Diego, School of Medicine, La Jolla, California and Rady Children's Hospital San Diego, California 92093, USA. [4]Department of Cardiology, Boston Children's Hospital, Boston, Massachusetts 02115, USA. [5]Department of Public Health, Jichi Medical School, Minamikawachi, Tochigi 329-0498, Japan. [6]Saitama Prefectural University, Koshigaya, Saitama 343-8540, Japan. [7]Department of Preventive Medicine, Shinshu University School of Medicine, Matsumoto 390-8621, Japan. [8]Department of Molecular Genetics, Kawasaki Medical School, Kurashiki, Okayama 701-0192, Japan. [9]Department of Pediatrics, Fukuoka University School of Medicine, Fukuoka, Fukuoka 831-8501, Japan. [10]Department of Pediatrics, Tokyo Women's Medical University Yachiyo Medical Center, Yachiyo, Chiba 276-8524, Japan. [11]Department of Pediatrics, Fuji Heavy Industries Ltd. Health Insurance Society General Ohta Hospital, Ohta, Gunma 373-8585, Japan. [12]Department of Pediatrics, Kawasaki Medical School, Kurashiki, Okayama 701-0192, Japan. [13]Department of Pediatrics, Toho University School of Medicine, Tokyo 143-8541, Japan. [14]Department of Pediatrics, Yokohama Minami Kyousai Hospital, Yokohama, 236-0037, Kanagawa, Japan. [15]Department of Pediatrics, Fujita Health University, Toyoake, Aichi 470-1192, Japan. [16]Department of Pediatrics, Toyokawa Citizen's Hospital, Toyokawa, Aichi 442-8561, Japan. [17]Department of Pediatrics, National Hospital Organization Kure Medical Center, Kure, Okayama 737-0023, Japan. [18]Department of Pediatrics, Dokkyo Medical University, Koshigaya Hospital, Koshigaya, Saitama 343-8555, Japan. [19]Department of Pediatrics, Kawasaki Municipal Hospital, Kawasaki 210-0013, Japan. [20]Department of Surgery, Keio University School of Medicine, Tokyo 160-8582, Japan. [21]Laboratory for Genotyping and [22]Laboratory for Medical Informatics, SNP Research Center, RIKEN, Yokohama, Kanagawa 230-0045, Japan. [23]Japan Kawasaki Disease Research Center, Tokyo 111-0031, Japan. [24]Laboratory for Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan. [25]SNP Research Center, RIKEN, Yokohama, Kanagawa 230-0045, Japan. [26]Present address: Department of Pediatrics, National Hospital Organization Yokohama Medical Center, Yokohama, Kanagawa 245-8575, Japan. Correspondence should be addressed to Y.O. (onouchi@src.riken.jp).

Received 13 April; accepted 2 October; published online 16 December 2007; doi:10.1038/ng.2007.59

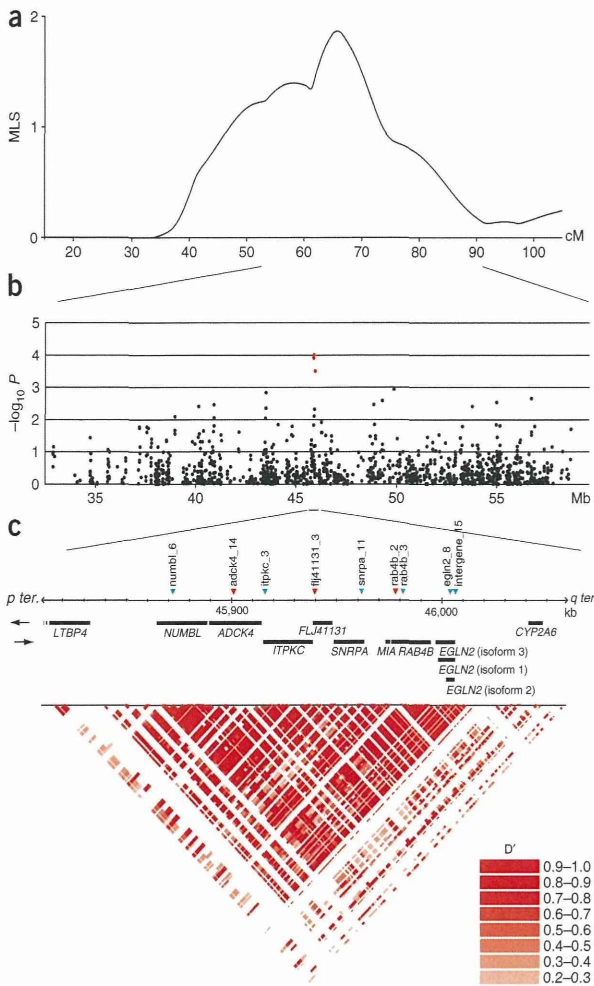

Figure 1 Results of SNP screening of chromosome 19 and structure of the linkage disequilibrium (LD) block in Japanese individuals showing SNPs significantly associated with Kawasaki disease. (**a**) Maximum lod score plot of affected sib-pair analysis conducted on 78 Japanese families. MLS, maximum lod score. (**b**) Case-control association analysis of 1,222 SNPs in 94 individuals with Kawasaki disease and 564 controls. *x* and *y* axes indicate the position from the *p* terminus of the chromosome and –log of *P* value for allele frequency comparison, respectively. The three most significant SNPs are marked by red dots. (**c**) Genes oriented *q* terminus to *p* terminus are in upper row, with genes in the opposite orientation shown below. Arrowheads indicate the position of SNPs significantly associated with Kawasaki disease: red arrowheads indicate the original three SNPs found by association studies, and blue arrowheads indicate the six SNPs from resequencing that were in LD with original three SNPs.

© 2008 Nature Publishing Group http://www.nature.com/naturegenetics

population[5,6]. Familial aggregation of the disease has also been observed[7]. Although association studies have identified candidate genes that may influence Kawasaki disease susceptibility, a systematic genetic approach has not been previously applied to study this disease.

Recently, we conducted affected sib-pair analysis of Kawasaki disease[8] that demonstrated linkage to several chromosomal regions, including chromosome 19. Here we show the results of linkage disequilibrium (LD) mapping carried out on 19q13.2, through which we identified a functional SNP in intron 1 of *ITPKC* that is significantly associated with risk of Kawasaki disease and with formation of coronary artery aneurysms. We also characterized ITPKC as a negative regulator of the Ca^{2+}/NFAT signaling pathway in T cells.

RESULTS

Linkage disequilibrium mapping

Through linkage analysis of 78 Japanese sib pairs concordant for Kawasaki disease, we identified a peak in the maximum lod-score plot at 19q13.2–13.3, located about 65.4 cM (48 Mb) from the *p* terminus of the chromosome[8] (**Fig. 1a**). An initial screening of 1,222 SNPs in 94 individuals with Kawasaki disease and 564 controls (see **Supplementary Methods** online) identified 131 candidates ($P < 0.05$; **Supplementary Table 1** online). Through association analysis of these 131 SNPs in an independent cohort of 276 Japanese individuals with Kawasaki disease and 282 controls, we found a cluster of three SNPs that were highly significant ($P < 0.01$; **Fig. 1b** and **Table 1**). The three SNPs (adck4_14, flj41131_3 and rab4b_2) were in strong linkage disequilibrium ($r^2 > 0.85$) within a single LD block identified by the HapMap database (**Fig. 1c**). In this LD block spanning about 150 kb, eight genes had been mapped: Numb (*Drosophila*) homolog like (*NUMBL*), aarF domain containing kinase 4 (*ADCK4*), *ITPKC*, hypothetical protein LOC284325 (*FLJ41131*), small nuclear ribonucleoprotein polypeptide A (*SNRPA*), melanoma inhibitory activity

Table 1 Results of association analyses between three independent sets of Japanese Kawasaki disease and control subjects

				94 KD vs. 564 controls[b]		276 KD vs. 282 controls		267 KD vs. 752 controls	
				Allele 1 vs. Allele 2		Allele 1 vs. Allele 2		Allele 1 vs. Allele 2	
SNPs	dbSNP ID	Allele 1/2	Chromosome position[a]	χ^2	P	χ^2	P	χ^2	P
numbl_6	–	C/T	45872187	–	–	3.9	0.049	13.7	0.00022
adck4_14	rs2288450	C/T	45901017	14.7	0.00012	5.0	0.026	15.7	7.4×10^{-5}
itpkc_3	rs28493229	G/C	45916044	–	–	5.0	0.026	16.3	5.4×10^{-5}
flj41131_3	rs3745213	C/T	45939849	15.1	0.00010	7.6	0.0060	17.7	2.6×10^{-5}
snrpa_11	rs17713068	T/G	45961895	–	–	7.3	0.0068	21.1	4.4×10^{-6}
rab4b_2	rs2287691	C/G	45978003	12.9	0.00032	7.3	0.0068	17.8	2.5×10^{-5}
rab4b_3	rs2287692	G/A	45981596	–	–	7.9	0.0050	19.1	1.2×10^{-5}
egln2_8	rs10416308	G/A	46004101	–	–	9.7	0.0019	13.0	0.00031
intergene_15	rs10405596	C/T	46006560	–	–	9.6	0.0020	13.2	0.00028

SNPs in bold are those identified in initial screening.
[a]Based on Build 36 NCBI reference sequence. [b]Genotype data for the 564 controls were available only for the three SNPs in bold.

Table 2 Results of association analysis with combined Japanese Kawasaki disease and control samples and TDT[a] analysis of US samples

			Japanese (case-control association analysis)[b]										United States (TDT)				
			Genotype			Allele 1 vs. Allele 2		Genotype 11 vs. 12 + 22									
SNPs	Allele 1/2	Subjects	11	12	22	χ^2	*P*	χ^2	*P*	OR	95% CI		T:U[c]	χ^2	*P*	OR	95% CI
numbl_6	C/T	KD	378	235	23	27.4	1.6×10^{-7}	30.5	3.3×10^{-8}	1.80	1.46–2.22		64:31	11.5	0.00071	2.06	1.34–3.17
		Control	748	259	25												
adck4_14	C/T	KD	374	235	26	31.7	1.8×10^{-8}	34.6	4.0×10^{-9}	1.87	1.52–2.30		64:31	11.5	0.00071	2.06	1.34–3.17
		Control	752	254	27												
itpkc_3	G/C	KD	376	234	27	32.4	1.2×10^{-8}	35.8	2.2×10^{-9}	1.89	1.53–2.33		64:30	12.3	0.00045	2.13	1.38–3.29
		Control	756	249	29												
flj41131_3	C/T	KD	372	237	28	37.8	7.8×10^{-10}	39.9	2.7×10^{-10}	1.95	1.58–2.41		66:32	11.8	0.00059	2.06	1.35–3.15
		Control	757	250	26												
snrpa_11	T/G	KD	375	235	27	40.8	1.7×10^{-10}	45.1	1.9×10^{-11}	2.05	1.66–2.53		55:40	2.4	0.12	1.38	0.91–2.07
		Control	771	235	28												
rab4b_2	C/G	KD	376	235	26	37.3	1.0×10^{-9}	41.7	1.1×10^{-10}	1.99	1.61–2.46		66:50	2.2	0.14	1.32	0.91–1.91
		Control	766	239	28												
rab4b_3	G/A	KD	374	235	27	39.5	3.2×10^{-10}	44.3	2.9×10^{-11}	2.04	1.65–2.51		57:39	3.4	0.066	1.46	0.97–2.20
		Control	767	235	29												
egln2_8	G/A	KD	374	232	27	36.7	1.4×10^{-9}	39.6	3.2×10^{-10}	1.96	1.59–2.42		68:51	2.4	0.12	1.33	0.93–1.92
		Control	763	243	27												
intergene_15	C/T	KD	375	237	25	36.5	1.6×10^{-9}	41.0	1.5×10^{-10}	1.98	1.60–2.44		63:49	1.8	0.19	1.29	0.89–1.87
		Control	764	243	27												

[a]Transmission disequilibrium test. $n = 209$. [b]637 KD (94 + 276 + 267) and 1,034 controls (282 + 752). [c]'T' and 'U' indicate transmitted and untransmitted allele 2 of each SNP, respectively.

© 2008 Nature Publishing Group http://www.nature.com/naturegenetics

(*MIA*), Ras-related GTP-binding protein 4b (*RAB4B*) and EGL nine (*C. elegans*) homolog 2 (*EGLN2*). Resequencing the 150-kb region from 12 Japanese individuals with Kawasaki disease and 12 healthy controls, we identified 109 SNPs and four deletion polymorphisms (**Supplementary Table 2** online). We discovered one previously unknown and five known SNPs that were in the same LD group ($r^2 > 0.80$) with the initial three SNPs (**Table 1**). We confirmed the association of these nine SNPs with Kawasaki disease in an independent case-control set (267 individuals with Kawasaki disease and 752 healthy controls; **Tables 1** and **2**). The association of these SNPs remained significant after Bonferroni correction for multiple testing ($n = 1{,}222$, $P < 0.001$). Meta-analysis of these two independent sets by the Mantel-Haenszel method confirmed significance (**Supplementary Fig. 1** online).

Transmission disequilibrium test (TDT) analysis of 209 US multiethnic trios showed asymmetric transmission of four of the nine SNPs (numbl_6, adck4_14, itpkc_3 and flj41131_3; **Table 2**). Of the 209 US trios, 106 were European Americans, and asymmetric transmission of these same four SNPs was again observed in this subgroup (data not shown). The results of a combined analysis of Japanese case-control and US TDT studies are summarized in **Supplementary Figure 1**. The significance of these SNPs in two different ethnic populations provided further evidence that genetic variation at this locus influences Kawasaki disease susceptibility.

LD analysis of the European American subgroup ($n = 106$) showed that the 150-kb region containing the nine SNPs was separated into three LD blocks: the four significant SNPs on the *p*-terminal side, the three in the middle and the other two on the *q*-terminal side (**Supplementary Fig. 2** online). Hence, the difference in haplotype structure in the European American and Japanese populations

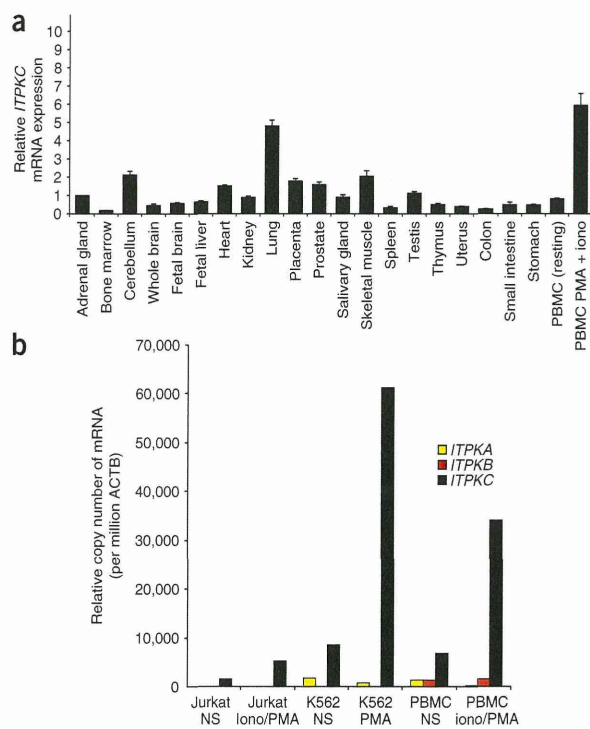

Figure 2 Comparison of relative mRNA expression of *ITPKC* in different tissues and cell lines. (**a**) Quantitative RT-PCR was carried out on RNA extracted from different human tissues, and the results were normalized to β-actin transcripts. RNA from both resting PBMCs and PBMCs stimulated with ionomycin (iono) and PMA was also analyzed. Results are mean ± s.d. of triplicate assays. (**b**) Expression pattern of ITPK isoforms in leukemic cell lines and PBMCs. Bars indicate relative mRNA copy number of *ITPKA* (yellow), *ITPKB* (red) and *ITPKC* (black), respectively. Expression was evaluated both in resting state and activated state. NS, no stimulation.

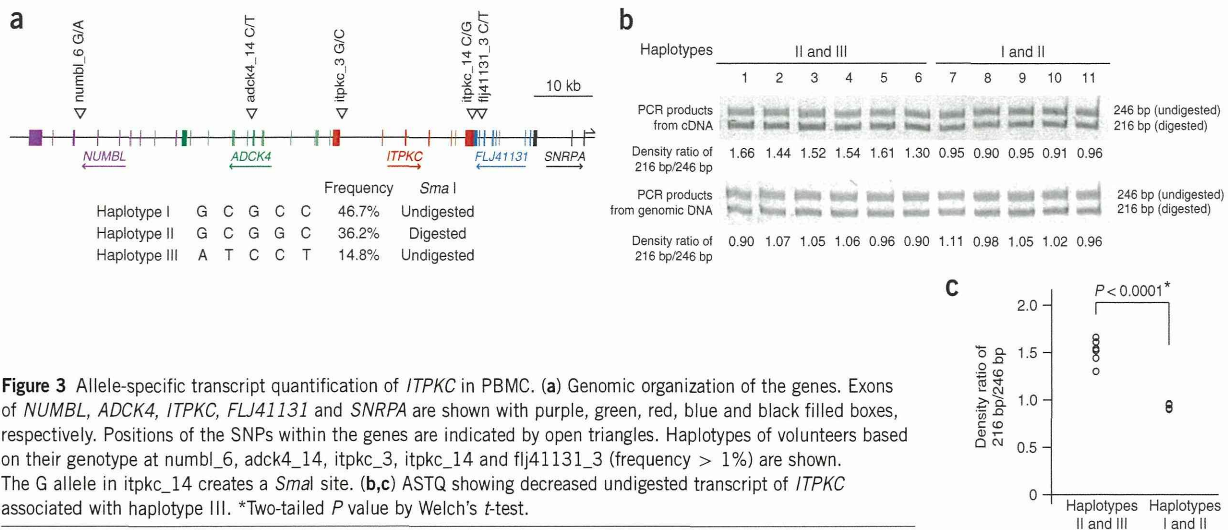

Figure 3 Allele-specific transcript quantification of *ITPKC* in PBMC. (**a**) Genomic organization of the genes. Exons of *NUMBL, ADCK4, ITPKC, FLJ41131* and *SNRPA* are shown with purple, green, red, blue and black filled boxes, respectively. Positions of the SNPs within the genes are indicated by open triangles. Haplotypes of volunteers based on their genotype at numbl_6, adck4_14, itpkc_3, itpkc_14 and flj41131_3 (frequency > 1%) are shown. The G allele in itpkc_14 creates a *Sma*I site. (**b**,**c**) ASTQ showing decreased undigested transcript of *ITPKC* associated with haplotype III. *Two-tailed *P* value by Welch's *t*-test.

© 2008 Nature Publishing Group http://www.nature.com/naturegenetics

suggested that these four SNPs were the likely candidates influencing Kawasaki disease susceptibility. The SNPs were located within introns of *NUMBL, ADCK4, ITPKC* and *FLJ41131*, respectively (**Fig. 1c** and **Supplementary Table 2**).

Identification of *ITPKC* as the most plausible candidate gene

To determine the most likely candidate gene out of the four, we first carried out multivariate analysis of the four SNPs to assess whether a single causal SNP or some synergistic interaction of the SNPs within the locus conferred the disease risk. However, the likelihood ratio test applied to each single SNP showed a similar trend of association in simple contingency table analyses ($P = 0.00027$, 0.000061, 0.000081 and 0.000067 for numbl_6, adck4_14, itpkc_3 and flj41131_3, respectively). Moreover, no epistasis worthy of note was shown between any two of the four SNPs or in any combination of each significant SNP and the other SNPs of the same gene region that were not independently associated with Kawasaki disease (data not shown). It seemed likely that the strong LD of the locus made the association of these SNPs equivalent. Thus, we considered that further biological evidence would be needed to identify the causal SNP and the gene responsible for the association.

We then reviewed the function of the four positional candidate genes. Although none of these had been previously recognized to have a role in immune activation, we postulated that *ITPKC* was the most likely candidate for such a role. ITPKC is one of the three isoenzymes of inositol 1,4,5-trisphosphate 3-kinase (ITPK) that phosphorylate inositol 1,4,5-trisphosphate (IP3), a key second messenger in many cell types. ITPK has been postulated to have a critical role in T-cell receptor (TCR) signaling, as IP3 kinase activity in Jurkat cells is rapidly upregulated after TCR stimulation[9], although the relative importance of the three known isoenzymes, ITPKA, ITPKB and ITPKC, has not yet been determined. Because individuals with Kawasaki disease have marked activation of the immune system, we hypothesized that the ITPKC might have a role in regulation of the immune response.

To study the role of ITPKC, we first analyzed the tissue distribution of *ITPKC* expression by RT-PCR. We detected expression in all tissues sampled, with the highest constitutive expression in cerebellum, lung and skeletal muscle. In the latter two tissues, high expression had been shown by RNA blot analysis in a previous report[10] (**Fig. 2a**). Low expression was detected in immune-related organs such as bone marrow, spleen, thymus and resting peripheral blood mononuclear cells (PBMCs). However, expression was notably induced in PBMCs when stimulated with phorbol 12-myristate 13-acetate (PMA) and the Ca^{2+} ionophore ionomycin (**Fig. 2a**). We compared the mRNA expression of the three isoenzymes in PBMCs and two leukemic cell lines (Jurkat and K562). Before stimulation, the expression of all three isoenzymes was low; after stimulation, only the expression of the *ITPKC* isoenzyme was induced (3- to 7-fold increase; **Fig. 2b**). This result prompted us to pursue *ITPKC* as the most likely candidate gene in the associated haplotype block.

To determine whether any of the four SNPs in *ITPKC* or adjacent loci affected transcript abundance of *ITPKC in vivo*, we carried out allele-specific transcript quantification (ASTQ; **Fig. 3**). The RT-PCR product from mRNA isolated from PBMCs of individuals with haplotype II (G allele in itpkc_14), but not haplotypes I and III, could be digested with *Sma*I (**Fig. 3a**). The *Sma*I-treated RT-PCR product from six individuals with haplotypes II and III had a higher ratio of digested to undigested forms, suggesting lower transcript abundance from haplotype III (containing alleles associated with Kawasaki disease susceptibility; **Fig. 3b**, lanes 1–6). Five individuals with haplotypes I and II (containing alleles not associated with Kawasaki disease susceptibility and the C- or G-allele at itpkc_14, respectively; **Fig. 3b**, lanes 7–11) had an equal ratio of digested to undigested PCR product, suggesting that the difference between haplotypes II and III was due to the SNPs. The mean ratio was 1.51 for the former group and 0.93 for the latter ($P < 0.0001$; **Fig. 3c**). This finding further encouraged us to consider *ITPKC* as the most plausible candidate gene in the locus.

Regulatory role of ITPKC in T-cell activation

The increase in *ITPKC* expression after cell stimulation prompted us to study the role of IPTKC in immune activation (**Fig. 4**). IP3 is generated by the hydrolysis of phosphatidylinositol 4,5-biphosphate by phospholipase C when activated by various external stimuli[11]. In T cells, IP3, released by stimulation of the TCR complex, increases intracellular Ca^{2+} through IP3 receptors (IP3Rs) expressed on endoplasmic reticulum[12]. Subsequent Ca^{2+} influx across the plasma membrane leads to nuclear translocation of nuclear factor of activated T cells (NFAT) and activates transcription of interleukin-2 (*IL2*) and other cytokines[13,14].

© 2008 Nature Publishing Group http://www.nature.com/naturegenetics

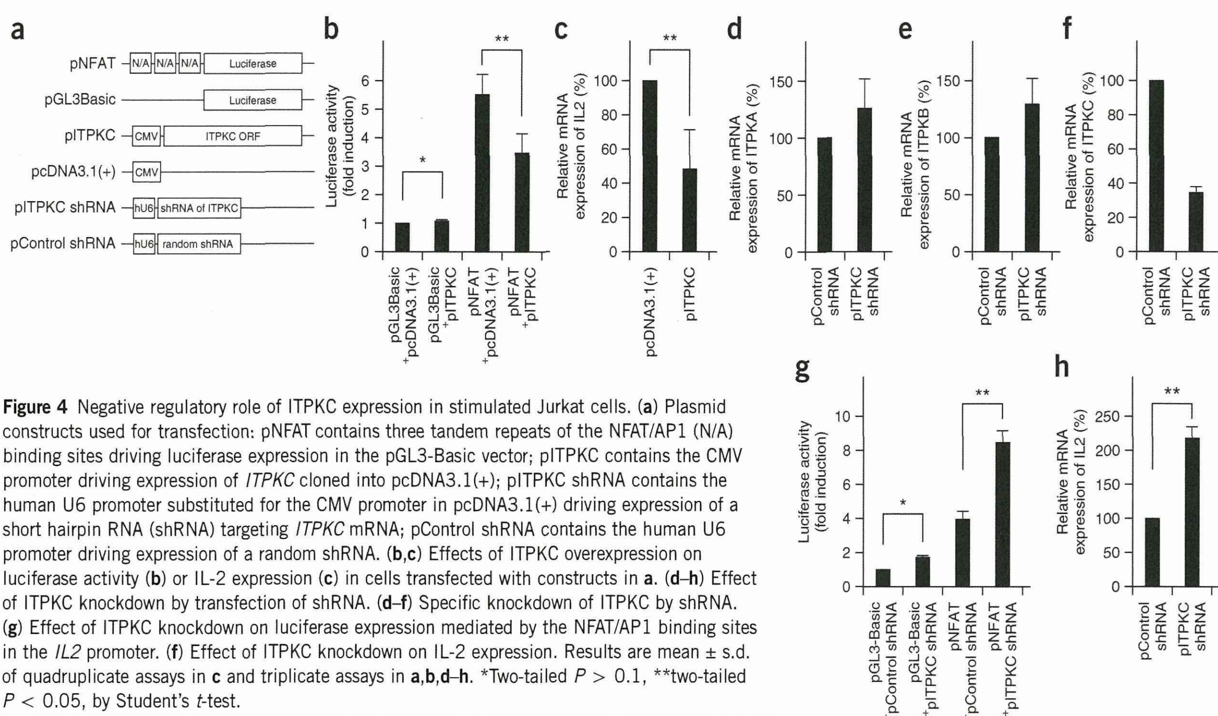

Figure 4 Negative regulatory role of ITPKC expression in stimulated Jurkat cells. (**a**) Plasmid constructs used for transfection: pNFAT contains three tandem repeats of the NFAT/AP1 (N/A) binding sites driving luciferase expression in the pGL3-Basic vector; pITPKC contains the CMV promoter driving expression of *ITPKC* cloned into pcDNA3.1(+); pITPKC shRNA contains the human U6 promoter substituted for the CMV promoter in pcDNA3.1(+) driving expression of a short hairpin RNA (shRNA) targeting *ITPKC* mRNA; pControl shRNA contains the human U6 promoter driving expression of a random shRNA. (**b**,**c**) Effects of ITPKC overexpression on luciferase activity (**b**) or IL-2 expression (**c**) in cells transfected with constructs in **a**. (**d**–**h**) Effect of ITPKC knockdown by transfection of shRNA. (**d**–**f**) Specific knockdown of ITPKC by shRNA. (**g**) Effect of ITPKC knockdown on luciferase expression mediated by the NFAT/AP1 binding sites in the *IL2* promoter. (**f**) Effect of ITPKC knockdown on IL-2 expression. Results are mean ± s.d. of quadruplicate assays in **c** and triplicate assays in **a**,**b**,**d**–**h**. *Two-tailed $P > 0.1$, **two-tailed $P < 0.05$, by Student's *t*-test.

We postulated that ITPKC regulates NFAT by modulating the abundance of IP3. When *ITPKC* was overexpressed in Jurkat cells, NFAT-mediated activation after stimulation with phytohemagglutinin (PHA) and PMA was significantly reduced (**Fig. 4b**). Next, we assessed NFAT-mediated activation when expression of *ITPKC* was decreased. In contrast to overexpression, knockdown of *ITPKC* using plasmids expressing short hairpin RNA (shRNA) resulted in enhanced NFAT-mediated activation in response to the same stimulation (**Fig. 4f,g**). ITPKA and ITPKB also catalyze phosphorylation of IP3, and their expression was observed in PBMCs, even though the expression was much lower than that of *ITPKC* (**Fig. 2b**). Thus, we assessed mRNA expression of these two genes to exclude the possibility that the shRNA designed for *ITPKC* also silenced *ITPKA* and *ITPKB*, thereby accounting for the effect on NFAT activation. We observed no suppression and actually saw a slight increase in transcript concentrations for both genes (**Fig. 4d,e**). Consistent with these results, *IL2* transcription in stimulated Jurkat cells decreased in response to *ITPKC* overexpression and increased following *ITPKC* knockdown (**Fig. 4c,h**). Given that NFAT mediates the expression of many proteins beside IL-2 that have important roles in T-cell regulation, ITPKC, and not ITPKA or ITPKB, may act as a key negative regulator of T-cell function.

Functional significance of itpkc_3

As none of the four significant SNPs was located in a protein coding region of *ITPKC* (**Fig. 3a**), we investigated the role of these SNPs in

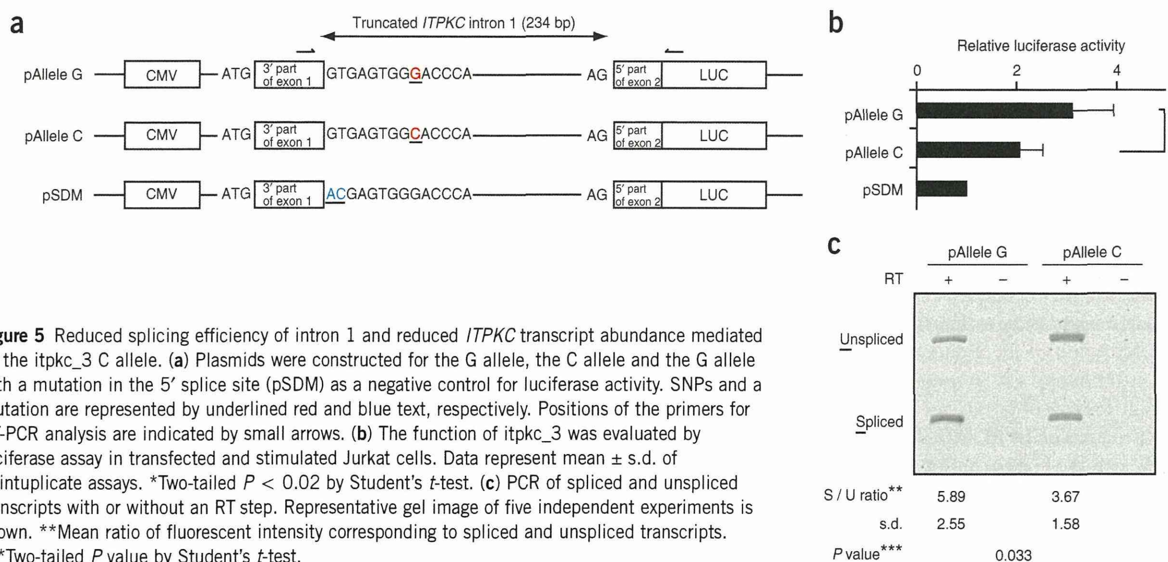

Figure 5 Reduced splicing efficiency of intron 1 and reduced *ITPKC* transcript abundance mediated by the itpkc_3 C allele. (**a**) Plasmids were constructed for the G allele, the C allele and the G allele with a mutation in the 5′ splice site (pSDM) as a negative control for luciferase activity. SNPs and a mutation are represented by underlined red and blue text, respectively. Positions of the primers for RT-PCR analysis are indicated by small arrows. (**b**) The function of itpkc_3 was evaluated by luciferase assay in transfected and stimulated Jurkat cells. Data represent mean ± s.d. of quintuplicate assays. *Two-tailed $P < 0.02$ by Student's *t*-test. (**c**) PCR of spliced and unspliced transcripts with or without an RT step. Representative gel image of five independent experiments is shown. **Mean ratio of fluorescent intensity corresponding to spliced and unspliced transcripts. ***Two-tailed *P* value by Student's *t*-test.

Table 3 Association analysis of itpkc_3 with stratified samples

	Japanese (case-control association analysis)[d]							United States (TDT)					
	Genotype												
Samples	GG	GC	CC	χ^2	*P*	OR	95% CI	n^e	T:U[f]	χ^2	*P*	OR	95% CI
KD linked to 19q13.2[a]	21	18	1	8.2	0.0042	2.46	1.30–4.65	–	–	–	–	–	–
KD with family history[b]	53	44	4	19.1	0.000012	2.46	1.63–3.73	–	–	–	–	–	–
KD with CALs[c]	61	44	2	12.4	0.00044	2.05	1.37–3.08	108	37:11	14.1	0.00018	3.36	1.72–6.59
KD without CALs	172	94	12	13.4	0.00025	1.68	1.27–2.21	100	27:18	1.8	0.18	1.50	0.63–2.72
Control	756	249	29										

[a]Probands of 78 sib pairs in previous linkage analysis whose IBD allele number were estimated to be >1.0 at itpkc_3 (63cM). [b]Probands of 93 affected sib pairs, 4 parent-child pairs, 1 monozygotic twin, 1 dizygotic twin, 1 first cousin pair and 1 second cousin pair. [c]KD cases having coronary dilatation or aneurysms during the acute phase. [d]Genotype frequency comparison in dominant model of inheritance. [e]Number of affected individuals. [f]'T' and 'U' indicate transmitted and untransmitted C allele of itpkc_3, respectively.

transcriptional regulation. Using the TFSEARCH program (see URLs section in Methods), we predicted binding of the AP-1 transcription factors to the sequence containing flj41131_3 and lowered the score with a nucleotide substitution at the SNP (from 91.8 to 79.4; **Supplementary Fig. 3a** online). The SNP is located within intron 7 of *FLJ41131*, but because of close tail-to-tail gene arrangement (**Fig. 1c**), the distance between the SNP and the 3′ end of the *ITPKC* gene is only 1.2 kb. We tested the hypothesis that flj41131_3 affects the expression of *ITPKC* by altering activity of an enhancer element outside the gene. However, we observed no significant difference in luciferase assays using constructs corresponding to the two alleles of flj41131_3 (**Supplementary Fig. 3b**). Moreover, we did not observe higher concentrations of the digested transcripts in ASTQ analysis of an individual who was heterozygous at flj41131_3 and homozygous for major alleles at itpkc_3, adck4_14 and numbl_6 (data not shown). These findings led us to examine the functional significance of SNPs other than flj41131_3. No transcription factor was clearly predicted to bind to any alleles of numbl_6, adck4_14 and itpkc_3, and luciferase assays with constructs for these SNPs showed no functional effects (**Supplementary Fig. 3c**). Thus, we explored other possible mechanisms by which these SNPs might alter *ITPKC* expression.

Differences in splicing efficiency associated with nucleotide changes within introns have previously been observed[15,16]. Of the four significant SNPs, only itpkc_3 was located in an intron of *ITPKC* (**Fig. 3a**). Its location near the 5′ splice site further encouraged us to investigate the role of this SNP in regulating splicing. We constructed a minigene containing a truncated intron 1 with portions of exons 1 and 2 at either end and the luciferase gene fused in-frame downstream of exon 2 (**Fig. 5a**). When transfected into Jurkat cells, the plasmid containing the C allele had significantly lower luciferase activity compared to the plasmid containing the G allele (**Fig. 5b**). RT-PCR with primers designed to amplify cDNAs generated from transcripts of these plasmids yielded two bands. The lower and upper bands corresponded to spliced and unspliced transcripts, respectively. As expected, we observed a lower spliced/unspliced ratio of the transcripts for the C allele (**Fig. 5c**). Because no amplification was observed from the templates without a reverse transcriptase step, a possible plasmid DNA contamination in the cDNA templates as the source of the 'unspliced' bands was excluded. To our knowledge, no splice variants of this gene using a different 5′ splice site, which could rescue splicing inefficiency[17], have been reported in the literature or public databases. Furthermore, RT-PCR of the transcripts in PBMCs from individuals with the C allele did not detect such variants (data not shown). Therefore, we speculate that reduced splicing associated with the C allele could result in lower *ITPKC* transcript concentrations that might, in turn, lead to increased T-cell activation.

Association analysis with stratified samples

To further explore the effects of the proposed risk allele, we stratified the samples by the following two factors: family history of Kawasaki disease and presence of coronary artery lesions (CALs). Among the 78 Japanese affected sib pairs, 40 pairs shared more than one allele near itpkc_3. In this subset, the itpkc_3 C allele was over-represented compared to controls (n = 40, odds ratio (OR) = 2.46, 95% confidence interval (CI) = 1.30–4.65; **Table 3**). We observed the same trend in Japanese probands with a positive family history of Kawasaki disease (n = 101, OR = 2.46, 95% CI = 1.63–3.73; **Table 3**). These data strongly corroborate the association between itpkc_3 and Kawasaki disease. This allele also seemed to confer an increased risk of developing CALs (Japanese individuals with Kawasaki disease: n = 106, OR = 2.05, 95% CI = 1.37–3.08; US individuals with Kawasaki disease: n = 108 OR = 3.36, 95% CI = 1.72–4.96; **Table 3**).

DISCUSSION

We identified a SNP that contributes to Kawasaki disease susceptibility and disease outcome, starting from an LD mapping strategy for the chromosome 19q13.2–13.3 region for which evidence of linkage was observed in a previous sib-pair analysis[8]. We showed for the first time that ITPKC in humans is inducible in PBMCs and modulates NFAT activation. We further defined a role of ITPKC as a negative regulator of T-cell activation by showing that the itpkc_3 C allele results in increased *IL2* transcript abundance.

To our knowledge, alteration of splicing efficiency as a result of a single base substitution at nine nucleotides from the 5′ splice site has been rarely observed[18]. The SNP position was outside the limit of the consensus donor site sequence (+6)[19], and no cryptic splice site was generated by the nucleotide change. One possible explanation for this finding could be that a GGG motif might act as an intronic splicing control element, and the alteration of the motif to GGC reduced this activity. In an analysis of mammalian genomes, G nucleotides and G triplets were over-represented at the ends of introns[20,21]. Cumulative evidence suggests that these G-rich sequence elements have an important role in pre-mRNA splicing[15,16,22,23]. Change in the secondary structure of the pre-mRNA by a nucleotide substitution outside the consensus sequence[24,25] is another possible mechanism that could influence splicing. When the structure of pre-mRNAs in this region was predicted using the Mfold program (see URLs section in

© 2008 Nature Publishing Group http://www.nature.com/naturegenetics

Methods), the C-allele transcript was found to be likely to form a more stable stem-loop structure than the G-allele transcript (**Supplementary Fig. 4a** online).

An electrophoresis mobility shift assay (EMSA) using RNA oligonucleotides including itpkc_3 and nuclear extracts from either HeLa or Jurkat cells showed specific binding of an unknown protein to the G allele (**Supplementary Fig. 4b**). Identification of the RNA-binding nuclear factor may reveal the precise mechanism through which this SNP alters transcript abundance.

The biological impact of this SNP in Kawasaki disease pathogenesis requires further study. The weaker negative regulatory effect of itpkc_3 C allele on *IL2* is consistent with the significant elevation of IL-2 in acute Kawasaki disease compared to other febrile illnesses[26]. Autopsy studies in children who die during the acute phase of Kawasaki disease show infiltration of T cells, particularly $CD8^+$ cytotoxic T cells (CTL), into the coronary artery wall[27]. This suggests that T-cell activation and infiltration into selected compartments are critically involved in the pathogenesis of Kawasaki disease. Increased activation of T cells influenced by the *ITPKC* polymorphism may be responsible for a greater and more prolonged expansion of pro-inflammatory T cells during the acute phase, thus affecting Kawasaki disease susceptibility and leading to greater disease severity.

The association of the itpkc_3 C allele with Kawasaki disease may have direct clinical implications. In both Japan and the United States, approximately 10–20% of individuals with Kawasaki disease are resistant to IVIG therapy, and these individuals are at highest risk of developing CALs. Although the sample size was limited, the C allele also conferred an increased risk of IVIG resistance in the US cohort for which information regarding IVIG response was available ($n = 37$, OR = 4.67, 95% CI = 1.34–16.24; **Supplementary Table 3** online). Clinical scoring systems have been devised to identify this subgroup, but there is room for improvement in sensitivity and specificity to make them truly useful clinical tools[28–31]. Identifying a genetic signature for the subgroup of IVIG-resistant individuals would permit the use of more intensified therapy (for example, anti-cytokine therapy or plasmapheresis) to prevent the development of CALs. Cyclosporin A (CsA) mediates immunosuppression through blocking calcineurin, which is an important downstream molecule in the Ca^{2+}/NFAT signaling pathway[32]. A single case report describes the successful use of CsA in an individual with Kawasaki disease resistant to IVIG[33]. If further study confirms the importance of the Ca^{2+}/NFAT pathway in T-cell activation in acute Kawasaki disease, then a clinical trial of CsA in IVIG-resistant individuals may be warranted.

Because IP3 also acts as a second messenger in B cells, macrophages and neutrophils[11,34], the function of this SNP should be examined in these effector cells in Kawasaki disease. ITPKC is also expressed in the myocardium. The potential importance of IP3 and Ca^{+2} influx in the myocardium is also relevant to individuals with Kawasaki disease in whom subclinical myocarditis is a common feature of the acute illness. The potential role of this SNP in other inflammatory disorders of the vascular wall and myocardium, including other forms of systemic vasculitis, myocarditis and atherosclerosis, should also be considered.

METHODS

Subjects. The 564 control samples in the initial screening were members of the general Japanese population with various common diseases of adulthood unrelated to Kawasaki disease. Genotype data relating to 1,222 SNPs for this population was obtained from a database at our institute. We recruited 637 Japanese individuals with Kawasaki disease and 1,034 healthy control subjects from several medical institutes in Japan. The ethical committee of RIKEN approved the study, and all the parents of the patients gave written informed consent. All Japanese individuals with Kawasaki disease (male/female ratio = 384:253) were diagnosed by pediatricians based on the Japanese criteria for the disease[35]. Mean age of disease onset was 29.3 months (range 2–127 months).

Trios of Kawasaki disease–affected children and their biological parents ($n = 209$) were recruited at Rady Children's Hospital San Diego and at Boston Children's Hospital. Details regarding this cohort of US individuals with Kawasaki disease have been previously described[36]. Genomic DNA from whole blood, lymphoblastoid cell lines or mouth wash samples was extracted according to standard procedures.

SNP genotyping. We genotyped SNPs using the Invader and TaqMan assays as described previously[37].

Statistical analysis. The case-control association study was analyzed using a χ^2 test. We carried out a multivariate logistic regression analysis for the association between Kawasaki disease and multiple SNPs using forward-backward stepwise procedures to select SNPs and their interactions. For each step of the forward or backward process, we carried out a log likelihood test (0.05 significance threshold) to change the set of SNPs or their interactions. Meta-analysis of data from different case-control sets was conducted by Mantel-Haenszel methodology. The transmission disequilibrium test was performed using TDT software[38] integrated in Haploview version 3.32 (see URLs section below). Integration of the case-control and TDT data was conducted as previously described[39].

RNA extraction and quantitative RT-PCR. Total RNA from normal human tissues (except PBMCs) was purchased from Clontech. We isolated PBMCs from healthy human volunteers from venous blood using the Lymphoprep reagent (Axis-Shields). To assess the induction of *ITPKC* in stimulated white blood cells, we treated Jurkat cells and PBMCs with ionomycin (1 mg ml^{-1}) and PMA (50 ng ml^{-1}) for 8 h. K562 cells were treated only with PMA (50 ng ml^{-1}) for 8 h. We extracted total RNA from cell lines and PBMCs using the NucleoSpin RNA II kit (Macherey-Nagel). 1 μg of each RNA was reverse transcribed with Superscript III reverse transcriptase and oligo dT primers (Invitrogen). We quantified transcripts for *ITPKA*, *ITPKB*, *ITPKC* and *IL2* with TaqMan probe and primers (Hs00176658_m1, Hs00176666_m1 and Hs00363893_m1 for *ITPKA*, *ITPKB* and *ITPKC*, respectively, and Hs00174114_m1 for *IL2*). Amplification and detection were done using a Mx3000P thermal cycler (Stratagene). Results were normalized to the transcript levels of β-actin.

Allele-specific transcript quantification (ASTQ). ASTQ was carried out as described previously[40]. Genomic DNAs and cDNAs were amplified for 31 cycles with these primers. At the last cycle, we added forward primer labeled with Alexa Fluor 488 at the 5′ end. Amplicons were digested with *Sma*I according to manufacturer's instructions. Separation was conducted on 12% polyacrylamide gels in 25 mM Tris and 250 mM glycine. Quantification was carried out by using FLA-7000 analyzer (Fujifilm).

URLs. JSNPs, http://snp.ims.u-tokyo.ac.jp/index_ja.html; TFSEARCH, http://mbs.cbrc.jp/research/db/TFSEARCH.html; Mfold, http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi; International HapMap Project, http://www.hapmap.org/cgi-perl/gbrowse/hapmap_B36/; Haploview version 3.32, http://www.broad.mit.edu/mpg/haploview/.

GenBank accession number. Inositol 1,4,5-trisphosphate 3-kinase C (*ITPKC*) mRNA, NM_025194.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS
We thank the individuals with Kawasaki disease and their families who participated in this project. We are grateful to H. Aotsuka, H. Nakajima, F. Kudo, M. Miura, K. Akagi, T. Matsubara, M. Nishibatake, S. Oku, K. Sameshima, Y. Tanaka, Y. Nomura, S. Ogita, M. Sakauchi, T. Isobe, T. Sano, T. Matsushita and other physicians who contributed DNA samples. We thank J. Pancheri and A. Baker for collection of DNA samples and D. Scherrer, H. Sugiyama and M. Saito for technical assistance. This work was supported by grants from

© 2008 Nature Publishing Group http://www.nature.com/naturegenetics

the Japanese Millennium Project, from the Japan Society for the Promotion of Science (16591069 to Y.O.) and from the National Heart, Lung, Blood Institute of the National Institutes of Health (HL69413 to J.C.B.).

AUTHOR CONTRIBUTIONS

Y.O., A.H. and Yusuke N. designed the study. Y.O., J.C.B., C.S., J.W.N., F.K., K.H., M.T., Y.S., K.O., T.S., A.N., Y.K., T.Y., K.S., Takeo T., T.N., H.C. and A.F. collected most of the samples. M.Y., Yoshikazu N., H.Y. and T.K. provided information regarding the Japanese nation-wide survey of Kawasaki disease. K.W. and Y.F. established lymphoblastoid cell lines of individuals with Kawasaki disease. Tatsuhiko T. and A.S. supported the initial SNP screening by providing genotyping data for the control population. Y.O. performed genotyping and statistical analyses. R.N. and Tatsuhiko T. performed logistic regression analyses. Y.O. and T.G. performed functional assays. Y.O., J.C.B., C.S., J.W.N. and A.H. wrote the paper.

Published online at http://www.nature.com/naturegenetics
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

© 2008 Nature Publishing Group http://www.nature.com/naturegenetics

1. Kawasaki, T. Acute febrile mucocutaneous syndrome with lymphoid involvement with specific desquamation of the fingers and toes: my clinical observation (in Japanese). *Jpn. J. Allergy* **16**, 178–222 (1967). English translation by Shike, H., Burns, J.C. & Shimizu, C. *Pediatr. Infect. Dis. J.* **21**, 993–995 (2002).
2. Kato, H., Koike, S., Yamamoto, M., Ito, Y. & Yano, E. Coronary aneurysms in infants and young children with acute febrile mucocutaneous lymph node syndrome. *J. Pediatr.* **86**, 892–898 (1975).
3. Kato, H. *et al.* Long-term consequences of Kawasaki disease. A 10- to 21-year follow-up study of 594 patients. *Circulation* **94**, 1379–1385 (1996).
4. Cook, D.H. *et al.* Results from an international survey of Kawasaki disease in 1979–82. *Can. J. Cardiol.* **5**, 389–394 (1989).
5. Fujita, Y. *et al.* Kawasaki disease in families. *Pediatrics* **84**, 666–669 (1989).
6. Uehara, R., Yashiro, M., Nakamura, Y. & Yanagawa, H. Kawasaki disease in parents and children. *Acta Paediatr.* **92**, 694–697 (2003).
7. Dergun, M. *et al.* Familial occurrence of Kawasaki syndrome in North America. *Arch. Pediatr. Adolesc. Med.* **159**, 876–881 (2005).
8. Onouchi, Y. *et al.* A genome-wide linkage analysis for Kawasaki disease: evidence for linkage to chromosome 12. *J. Hum. Genet.* **52**, 179–190 (2007).
9. Imboden, J.B. & Pattison, G. Regulation of inositol 1,4,5-trisphosphate kinase activity after stimulation of human T cell antigen receptor. *J. Clin. Invest.* **79**, 1538–1541 (1987).
10. Dewaste, V. *et al.* Cloning and expression of a cDNA encoding human inositol 1,4,5-trisphosphate 3-kinase C. *Biochem. J.* **352**, 343–351 (2000).
11. Berridge, M.J. & Irvine, R.F. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**, 315–321 (1984).
12. Harnick, D.J. *et al.* The human type 1 inositol 1,4,5-trisphosphate receptor from T lymphocytes. Structure, localization, and tyrosine phosphorylation. *J. Biol. Chem.* **270**, 2833–2840 (1995).
13. Weiss, A. & Littman, D.R. Signal transduction by lymphocyte antigen receptors. *Cell* **76**, 263–274 (1994).
14. Macian, F. NFAT proteins: key regulators of T-cell development and function. *Nat. Rev. Immunol.* **5**, 472–484 (2005).
15. Cogan, J.D. *et al.* A novel mechanism of aberrant pre-mRNA splicing in humans. *Hum. Mol. Genet.* **6**, 909–912 (1997).
16. von Ahsen, N. & Oellerich, M. The intronic prothrombin 19911A>G polymorphism influences splicing efficiency and modulates effects of the 20210G>A polymorphism on mRNA amount and expression in a stable reporter gene assay system. *Blood* **103**, 586–593 (2004).
17. Morisaki, H., Morisaki, T., Newby, L.K. & Holmes, E.W. Alternative splicing: a mechanism for phenotypic rescue of a common inherited defect. *J. Clin. Invest.* **91**, 2275–2280 (1993).
18. Krawczak, M. *et al.* Single base-pair substitutions in exon-intron junctions of human genes: nature, distribution, and consequences for mRNA splicing. *Hum. Mutat.* **28**, 150–158 (2007).
19. Krawczak, M., Reiss, J. & Cooper, D.N. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Hum. Genet.* **90**, 41–54 (1992).
20. Majewski, J. & Ott, J. Distribution and characterization of regulatory elements in the human genome. *Genome Res.* **12**, 1827–1836 (2002).
21. Yeo, G., Hoon, S., Venkatesh, B. & Burge, C.B. Variation in sequence and organization of splicing regulatory elements in vertebrate genes. *Proc. Natl. Acad. Sci. USA* **101**, 15700–15705 (2004).
22. Louie, E., Ott, J. & Majewski, J. Nucleotide frequency variation across human genes. *Genome Res.* **13**, 2594–2601 (2003).
23. McCullough, A.J. & Berget, S.M. G triplets located throughout a class of small vertebrate introns enforce intron borders and regulate splice site selection. *Mol. Cell. Biol.* **17**, 4562–4571 (1997).
24. Buratti, E. & Baralle, F.E. Influence of RNA secondary structure on the pre-mRNA splicing process. *Mol. Cell. Biol.* **24**, 10505–10514 (2004).
25. Varani, L. *et al.* Structure of tau exon 10 splicing regulatory element RNA and destabilization by mutations of frontotemporal dementia and parkinsonism linked to chromosome 17. *Proc. Natl. Acad. Sci. USA* **96**, 8229–8234 (1999).
26. Yoshioka, T. *et al.* Polyclonal expansion of TCRBV2- and TCRBV6-bearing T cells in patients with Kawasaki disease. *Immunology* **96**, 465–472 (1999).
27. Brown, T.J. *et al.* CD8 T lymphocytes and macrophages infiltrate coronary artery aneurysms in acute Kawasaki disease. *J. Infect. Dis.* **184**, 940–943 (2001).
28. Fukunishi, M. *et al.* Prediction of non-responsiveness to intravenous high-dose gamma-globulin therapy in patients with Kawasaki disease at onset. *J. Pediatr.* **137**, 172–176 (2000).
29. Egami, K. *et al.* Prediction of resistance to intravenous immunoglobulin treatment in patients with Kawasaki disease. *J. Pediatr.* **149**, 237–240 (2006).
30. Sano, T. *et al.* Prediction of non-responsiveness to standard high-dose gamma-globulin therapy in patients with acute Kawasaki disease before starting initial treatment. *Eur. J. Pediatr.* **166**, 131–137 (2007).
31. Kobayashi, T. *et al.* Prediction of intravenous immunoglobulin unresponsiveness in patients with Kawasaki disease. *Circulation* **113**, 2606–2612 (2006).
32. Ho, S. *et al.* The mechanism of action of cyclosporin A and FK506. *Clin. Immunol. Immunopathol.* **80**, S40–S45 (1996).
33. Raman, V., Kim, J., Sharkey, A. & Chatila, T. Response of refractory Kawasaki disease to pulse steroid and cyclosporin A therapy. *Pediatr. Infect. Dis. J.* **20**, 635–637 (2001).
34. Lewis, R. Calcium signaling mechanisms in T lymphocytes. *Annu. Rev. Immunol.* **19**, 497–521 (2001).
35. Ayusawa, M. *et al.* Revision of diagnostic guidelines for Kawasaki disease (the 5th revised edition). *Pediatr. Int.* **47**, 232–234 (2005).
36. Burns, J.C. *et al.* Family-based association analysis implicates IL-4 in susceptibility to Kawasaki disease. *Genes Immun.* **6**, 438–444 (2005).
37. Suzuki, A. *et al.* Functional haplotypes of *PADI4*, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat. Genet.* **34**, 395–402 (2003).
38. Spielman, R.S., McGinnis, R.E. & Ewens, W.J. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am. J. Hum. Genet.* **52**, 506–516 (1993).
39. Kazeem, G.R. & Farrall, M. Integrating case-control and TDT studies. *Ann. Hum. Genet.* **69**, 329–335 (2005).
40. Uejima, H., Lee, M.P., Cui, H. & Feinberg, A.P. Hot-stop PCR: a simple and general assay for linear quantitation of allele ratios. *Nat. Genet.* **25**, 375–376 (2000).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ORIGINAL STUDIES

Cyclosporin A Treatment for Kawasaki Disease Refractory to Initial and Additional Intravenous Immunoglobulin

Hiroyuki Suzuki, MD, PhD, Masaru Terai, MD, PhD,† Hiromichi Hamada, MD, PhD,† Takafumi Honda, MD,† Tomohiro Suenaga, MD,* Takashi Takeuchi, MD,* Norishige Yoshikawa, MD, PhD,* Shoichi Shibuta, MD,‡ Masakazu Miyawaki, MD, PhD,‡ Ko Oishi, MD,§ Hironobu Yamaga, MD,¶ Noriyuki Aoyagi, MD,|| Seiji Iwahashi, MD,** Ritsuko Miyashita, MD,†† Yoshihiro Onouchi, MD, PhD,‡‡ Kumiko Sasago, PhD,§§ Yoichi Suzuki, MD, PhD,§§ and Akira Hata, MD, PhD§§*

Background: There are still no definite treatments for refractory Kawasaki disease (KD). In this pilot study, we evaluated the use of cyclosporin A (CyA) treatment in patients with refractory KD.

Methods: We prospectively collected clinical data of CyA treatment (4–8 mg/kg/d, oral administration) for refractory KD patients using the same protocol among several hospitals. Refractory KD is defined as the persistence or recurrence of fever (37.5°C or more of an axillary temperature) at the end of the second intravenous immunoglobulin (2 g/kg) following the initial one.

Results: Subjects were enrolled out of 329 KD patients who were admitted to our 8 hospitals between January 2008 and June 2010. Among a total of 28 patients of refractory KD treated with CyA, 18 (64.3%) responded promptly to be afebrile within 3 days and had decreased C-reactive protein levels, the other 4 became afebrile within 4 to 5 days. However, 6 patients (21.4%) failed to become afebrile within 5 days after the start of CyA and/or high fever returned after becoming afebrile within 5 days. Although hyperkalemia developed in 9 patients at 3 to 7 days after the start of CyA treatment, there were no serious adverse effects such as arrhythmias. Four patients (1.2%), 2 before and the other 2 after the start of CyA treatment, developed coronary arterial lesions.

Conclusion: CyA treatment is considered safe and well tolerated, and a promising option for patients with refractory KD. Further investigations will be needed to clarify optimal dose, safety, and timing of CyA treatment.

Key Words: Kawasaki disease, intravenous immunoglobulin-resistant (IVIG-resistant), cyclosporin A, T-cell activation

(*Pediatr Infect Dis J* 2011;30: 871–876)

Accepted for publication April 20, 2011.

From the *Department of Pediatrics, Wakayama Medical University, Wakayama, Japan; †Department of Pediatrics, Tokyo Women's Medical University Yachio Medical Center, Yachio, Japan; ‡Department of Pediatrics, Social Insurance Kinan Hospital, Tanabe, Japan; §Department of Pediatrics, Hashimoto Municipal Hospital, Hashimoto, Japan; ¶Department of Pediatrics, Naga Hospital, Kinokawa, Japan; ||Department of Pediatrics, Wakayama Rosai Hospital, Wakayama, Japan; **Department of Pediatrics, Hidaka General Hospital, Gobo, Japan; ††Department of Pediatrics, Izumiotsu Municipal Hospital, Izumiotsu, Japan; ‡‡Laboratory of Cardiovascular Diseases, Center for Genomic Medicine, RIKEN, Yokohama, Japan; and §§Department of Public Health, Graduate School of Medicine, Chiba University, Chiba, Japan.

Supported by the Ministry of Health, Labour and Welfare, Japan (2008–2010) through a Grant-in-Aid.

The authors do not have a commercial or other conflict of interest.

Address for correspondence: Hiroyuki Suzuki, MD, PhD, Department of Pediatrics, Wakayama Medical University, 811-1 Kimiidera, Wakayama, 641-0012, Japan. E-mail: hsuzuki@wakayama-med.ac.jp.

Copyright © 2011 by Lippincott Williams & Wilkins

ISSN: 0891-3668/11/3010-0871
DOI: 10.1097/INF.0b013e318220c3cf

Kawasaki disease (KD)[1] is an acute systemic vasculitis occurring in medium-sized arteries, especially coronary arteries. The reported patients with KD were not only from East Asia including Japan, but also United States and European countries.[2–5] Although many features of KD have been revealed by nationwide epidemiologic surveys in Japan, both its etiology and pathogenesis are still unclear. In the 19th nationwide survey conducted in Japan, more than 10 thousand children with KD were reported annually in 2005 and 2006,[6] and presently KD is a leading cause of acquired cardiac disease in childhood in the developed countries. Although the incidence of coronary arterial lesions (CAL) has been reduced to around 3% to 4% by standard therapy with intravenous immunoglobulin (IVIG) and aspirin,[7] 10% to 20% of patients with KD who fail to respond to IVIG, so-called refractory KD, show a high prevalence of CAL. There have been a significant number of reports on new therapeutic options for refractory KD, such as steroid,[8,9] steroid pulse,[10,11] infliximab,[12,13] plasma exchange,[14] and immunosuppressants.[15,16] Several recent reports have described the use of an intensified therapy comprising steroid or steroid pulse with initial IVIG in the early acute phase of KD.[17–20] However, the outcomes of such therapies are still controversial. With regard to infliximab, Burns et al[13] compared the outcome of second IVIG infusion (2 g/kg) and infliximab (5 mg/kg) after initial IVIG therapy. They concluded that infliximab was a potentially useful alternative to an additional IVIG infusion or intravenous pulse methylprednisolone for patients with IVIG-resistant KD, at least until best clinical practice had been established by future clinical trials. Currently, therefore, there are still no definite treatment recommendations for refractory KD and development of an optimal alternative for these patients is now an urgent matter.

We have reported that a functional polymorphism of *inositol 1,4,5-trisphosphate 3-kinase C* (*ITPKC*) is associated with susceptibility to KD and formation of CAL.[21] Because *ITPKC* acts as a negative regulator of T-cell activation by reducing amount of Ins(1,4,5)P3 (IP3), activated T cells may play a pivotal role in the pathogenesis of KD vasculitis. Several studies have already investigated the role of activated T cells in patients with KD.[22–24] From this viewpoint, cyclosporin A (CyA), which potently suppresses the activity of T cells by negative regulation of nuclear factor of activated T cells (NFAT) pathway, may be a promising option for the treatment of acute KD, especially in patients resistant to IVIG.

We, therefore, conducted the present pilot study to evaluate the use of CyA for patients with refractory KD who had shown resistance to both initial and additional IVIG therapy.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

MATERIALS AND METHODS

Patients

We prospectively collected clinical data of CyA treatment for refractory KD patients using a same protocol among 8 hospitals in Japan: Tokyo Women's Medical University Yachiyo Medical Center in Chiba prefecture, Wakayama Medical University, Hashimoto Municipal Hospital, Naga Hospital, Wakayama Rosai Hospital, Hidaka General Hospital, Social Insurance Kinan Hospital in Wakayama prefecture, and Izumi-otsu Municipal Hospital in Osaka prefecture. The study was approved by the ethics Committee of each institution. Of the 329 Japanese patients who met the diagnostic criteria for KD, study subjects aged 4 months or older resistant to both initial and additional IVIG were enrolled[25] and were admitted to our 8 hospitals between January 2008 and June 2010. Written informed consent was obtained from the parents of the patients enrolled.

Protocol

All patients received initial IVIG infusion (2 g/kg for 24 hours) and aspirin (30–50 mg/kg/d) within 7 days after the onset of KD (Fig. 1). The patients were considered afebrile when the axillary temperature remained below 37.5°C for more than 24 hours. The aspirin dose was decreased to 5 mg/kg/d after 2 to 3 days without fever. We defined patients who remained febrile (37.5°C or more of an axillary temperature) within 24 hours after completion of initial IVIG therapy as being resistant to initial IVIG, and additional IVIG (2 g/kg for 24 hours) was administered. We defined patients who remained febrile at the end of second IVIG infusion as resistant to additional IVIG. These patients resistant to both initial and additional IVIG were treated with CyA through oral administration (Neoral, oral solution, Novartis Pharma Co. Ltd., Tokyo, Japan). The initial dose of CyA was 4 mg/kg/d, and patients received oral CyA divided into 2 equal daily doses every 12 hours. The dose of CyA was adjusted to between 4 and 8 mg/kg/d to maintain a trough level of 60 to 200 ng/mL with reference to clinical and laboratory data such as body temperature and C-reactive protein (CRP) level. Serum samples for measuring the trough levels of CyA were obtained just before oral intake of CyA in the morning (about 12 hours after receiving oral CyA last night). The trough levels of CyA were examined more than twice a week during CyA treatment. CyA treatment was continued until the patients became afebrile and their CRP level decreased to a negative value (<0.3 mg/dL). In addition, the maximum duration of CyA treatment was decided as being 3 weeks between January and December 2008, and as being 2 weeks between January 2009 and June 2010. If patients remained febrile more than 5 days after the start of CyA treatment, or if fever returned after becoming afebrile within 5 days after the start of CyA treatment, CyA treatment was judged to be ineffective, and then the patients were given a third IVIG therapy. Patients resistant to both initial and additional IVIG, and who were less than 4 months of age, were also given a third of IVIG treatment. Laboratory assessments were performed before initial IVIG, before additional IVIG, before CyA, and sequentially thereafter every 2 to 3 days until CyA treatment was discontinued. Laboratory tests included the concentrations of CRP, electrolyte (potassium), and creatinine. To evaluate renal function, we calculated estimated glomerular filtration rate (eGFR) before and after CyA treatment as follows: 0.55× height (cm)/serum creatinine (enzyme method: mg/dL) + 0.2.

Evaluation of CAL

In all patients with KD, 2-dimensional echocardiography (2DE) was performed to evaluate CAL at least twice a week during hospitalization. Evaluation of the presence or absence of CAL was performed after 1 month of illness in accordance with the criteria of the Research Committee on Kawasaki disease.[26] In addition, we performed coronary angiography within 3 months after onset of illness in patients who were judged to have CAL on the basis of 2DE, to confirm the presence of CAL.

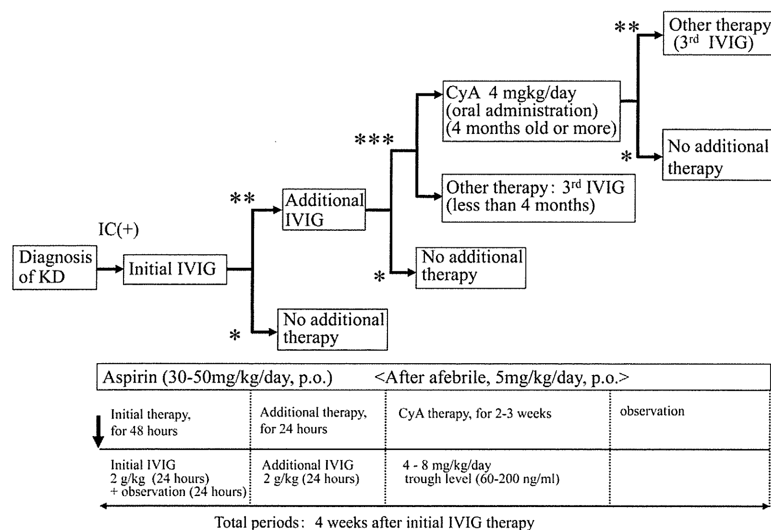

FIGURE 1. Study protocol. KD indicates Kawasaki disease; IVIG, intravenous immunoglobulin; CyA, cyclosporin A; IC, informed consent. *Responders to each treatment. **Patients resistant to initial IVIG or CyA. ***Patients resistant to additional IVIG. All patients with KD received initial IVIG infusion (2 g/kg) and aspirin (30–50 mg/kg/d). Patients resistant to initial IVIG received additional IVIG (2 g/kg). In addition, patients who were resistant to additional IVIG and were ≥4 months of age, were treated with CyA. Patients who were resistant to additional IVIG and aged less than 4 months were treated with a third course of IVIG.

© 2011 Lippincott Williams & Wilkins
Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

STATISTICAL ANALYSIS

Statistical analysis was performed using Wilcoxon signed rank test. Differences at a 2-tailed P value of <0.05 were considered statistically significant.

RESULTS

Of the 329 patients with KD, 245 (74.5%) became afebrile within 24 hours after completion of the initial IVIG therapy, and 84 (25.5%) continued to be febrile (Fig. 2). The latter 84 patients received additional IVIG, and 54 (64.3%) of them became afebrile before completion of the additional IVIG course. The other 30 (35.7%) failed to become afebrile, and 28 of them who were more than 4 months of age were treated with CyA. The remaining 2 patients, who were less than 4 months of age, received a third course of IVIG (2 g/kg), and subsequently became afebrile.

The characteristics of the 28 patients treated with CyA are summarized in Tables 1–3. They received the initial IVIG course on illness days 3 to 6 (median, 4.5) and the additional IVIG on illness days 5 to 10 (median; 7). CyA treatment was initiated on days 7 to 12 of illness (median; 8). The dose of CyA was 4 to 8 mg/kg/d, and the duration of treatment was 5 to 50 (median; 14) days.

The duration until patients became afebrile after the start of CyA treatment was 1 to 13 (median; 2) days. Of the 28 patients, 18 responded promptly to be afebrile within 3 days, and the other 4 became afebrile within 4 to 5 days after the start of CyA treatment. Axillary temperature (°C) of 2 days after the start of CyA treatment was significantly lower than that of the day of the start of CyA treatment ($P < 0.01$, Table 3). However, 6 patients (numbers 3, 8, 10, 19, 26, and 28 in Tables 1, 2) failed to become afebrile within 5 days after the start of CyA and/or high fever returned after becoming afebrile within 5 days. Four patients (numbers 3, 10, 26, and 28 in Tables 1, 2) received a third IVIG infusion. Patient 3 received the third IVIG infusion on the 10th day after the start of CyA treatment. However, low-grade fever (37.5°C–38.5°C) continued, and therefore she received CyA for 50 days until she became completely afebrile. Patient 10 became afebrile on the fourth day after CyA treatment, but high fever returned on the 13th day, despite CyA continuation. He received the third IVIG infusion on the 15th day after CyA, and then he became afebrile. Both patients, 26 and 28, became afebrile on the seventh day after CyA treatment, but high fever returned after CyA treatment for 14 days. They received the third IVIG infusion on the next day or 3 days after CyA, and then they became afebrile. The other 2 patients (numbers 8 and 19) did not receive any other therapy, because high fever (more than 38.0°C) was absent, and they gradually became afebrile with continued CyA therapy.

Although 4 patients (numbers 3, 8, 10, and 19) developed CAL, the coronary arteries had already dilated (>4 mm by 2DE) in 2 of them (numbers 10 and 19) before CyA treatment. In addition, patient 10 became afebrile within 24 hours after CyA treatment, but the diameter of the right coronary artery (C1–C2) was found to be increasing on a daily basis, reaching around 10 mm in diameter (giant coronary arterial aneurysm) in spite of continuation of CyA for 2 weeks.

Elevated serum CRP levels before CyA treatment decreased rapidly after CyA treatment. Thus, serum CRP levels before CyA treatment significantly decreased 2 days after CyA treatment ($P < 0.01$, Table 3).

After the start of CyA treatment, hyperkalemia developed (Table 3). The definition of hyperkalemia is ≧6 mEq/L in infants aged less than 12 months, and ≧5.5 mEq/L in children ≧12 months of age.[27] According to this definition, hyperkalemia developed in 1 of 4 infants and in 9 of 24 children. The peak values appeared 3 to 13 (median; 6.5) days after the start of CyA treatment. Although serum potassium levels increased after CyA treatment, both serum creatinine and eGFR did not change significantly (Table 3). In addition, no serious adverse effects such as ventricular arrhythmia occurred.

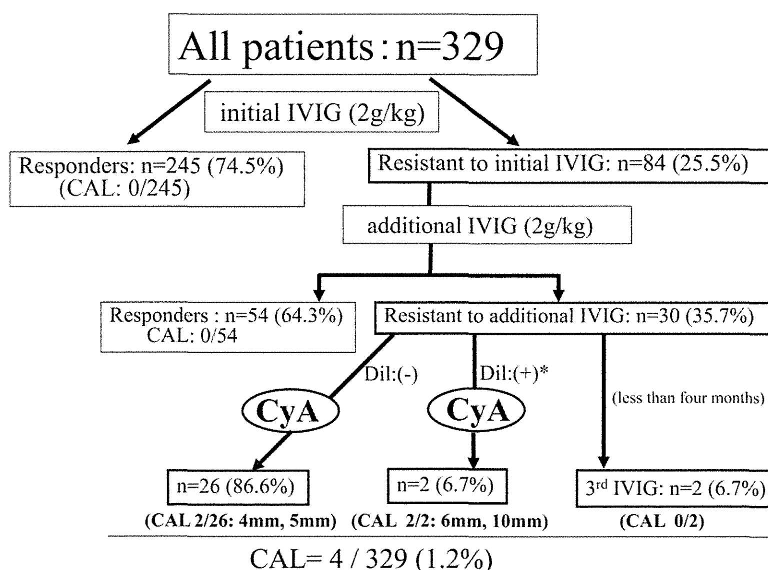

FIGURE 2. Protocol outcomes. KD indicates Kawasaki disease; IVIG, intravenous immunoglobulin; CyA, cyclosporin A; CAL, coronary arterial lesions. Of 329 patients with KD, 245 (74.5%) became afebrile after initial IVIG. A total of 84 patients resistant to initial IVIG received additional IVIG, of whom 30 failed to become afebrile within the treatment completion time. Among these 30 patients, 28 who were ≥4 months of age, were treated with CyA, and the other 2 patients, who were less than 4 months of age, received a third course of IVIG (2 g/kg). *CAL developed in these 2 patients (numbers 11 and 19 in Table 2) before CyA treatment (during additional IVIG).

© *2011 Lippincott Williams & Wilkins*

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

TABLE 1. Characteristics of 8 Patients Treated With CyA Between January and December 2008

Case No.	Age	Gender	Illness Days of IVIG		CyA			Duration (d) Until Afebrile After CyA	CAL
			Initial IVIG	Additional IVIG	Illness Days of CyA	Dose (mg/kg/d)	Duration (d)		
1	3 yr 3 mo	M	4	6	9	4	21	1	(-)
2	2 yr 0 mo	M	5	8	12	4	21	1	(-)
3	1 yr 11 mo	F	6	9	11	4–8	50	13 Third IVIG (21st ID)	An (5 mm)
4	1 yr 10 m	M	4	6	9	4	21	3	(-)
5	3 yr 0 mo	M	5	7	9	4	21	2	(-)
6	1 yr 3 mo	M	4	7	8	4–6	13	5	(-)
7	5 mo	F	6	8	9	4	21	3	(-)
8	1 yr 2 mo	M	3	5	7	4–8	21	13	An (4 mm)

These 8 patients ranged in age from 5 to 39 months. The male to female ratio was 6:2. CyA treatment was started in days 7 to 12 of illness, and the dose of CyA was 4 to 8 mg/kg/d. We increased and decreased the dose of CyA according to both clinical responses such as fever and the trough levels themselves. One patient (number 3) received a third IVIG infusion. Two patients (numbers 3 and 8) developed CAL.

CyA indicates cyclosporin A; IVIG, intravenous immunoglobulin; An, aneurysm; ID, illness day; CAL, coronary arterial lesions.

TABLE 2. Characteristics of 20 Patients Treated With CyA Between January 2009 and June 2010

Case No.	Age	Gender	Illness Days of IVIG		CyA			Duration (d) Until Afebrile After CyA	CAL
			Initial IVIG	Additional IVIG	Illness Days of CyA	Dose (mg/kg/d)	Duration (d)		
9	1 yr 5 mo	F	5	10	11	4–6	14	1	(-)
10	1 yr 4 mo	M	4	6	7	4–7	14	4 Third IVIG (22nd ID)	(-)
11	5 yr 3 mo	F	5	8	9	4	14	1	AnG (10 mm)
12	6 mo	M	4	6	8	4	14	2	(-)
13	4 yr 10 mo	M	4	7	8	4	14	1	(-)
14	1 yr 3 mo	M	4	6	8	4–6	13	5	(-)
15	2 yr 11 mo	M	5	7	8	4–6	14	4	(-)
16	4 yr 3 mo	M	4	7	8	4	10	1	(-)
17	2 yr 6 mo	F	5	7	8	4–6	11	1	(-)
18	1 yr 4 mo	M	3	6	7	4–5	13	5	(-)
19	5 yr 4 mo	M	5	7	8	4–5	14	6	An (6 mm)
20	2 yr 7 mo	M	5	7	8	4	14	3	(-)
21	1 yr 11 mo	F	4	7	8	4	5	1	(-)
22	4 mo	M	4	6	7	4	14	1	(-)
23	7 yr 9 mo	M	4	7	8	4	14	1	(-)
24	2 yr 6 mo	M	4	6	7	4	14	3	(-)
25	5 mo	F	5	8	9	4	14	1	(-)
26	1 yr 1 mo	F	4	6	8	4–8	14	7 Third IVIG (22nd ID)	(-)
27	1 yr 8 mo	M	5	7	8	4	8	1	(-)
28	1 yr 1 mo	M	5	7	9	4–8	14	7 Third IVIG (26th ID)	(-)

These 20 patients ranged in age from 4 to 93 months. The male to female ratio was 14:6. CyA treatment was started in days 7 to 11 of illness, and the dose of CyA was 4 to 8 mg/kg/d. In all, 4 patients (numbers 10, 19, 26, and 28) failed to become afebrile within 5 days after the start of CyA and/or high fever returned after becoming afebrile within 5 days. Three patients (numbers 10, 26, and 28) received a third course of IVIG infusion. Two patients (numbers 11 and 19) developed CAL before CyA treatment (during additional IVIG).

CyA indicates cyclosporin A; IVIG, intravenous immunoglobulin; An, aneurysm; AnG, giant Aneurysm; ID, illness day; CAL, coronary arterial lesions.

We examined the time course of the serum trough levels of CyA, which ranged between 60 to 200 ng/mL and were regarded as optimal. The level fluctuated in patients (numbers 3, 10, and 19 in Tables 1, 2) resistant to CyA therapy, because we increased and decreased the dose of CyA according to both clinical responses such as body temperature and the trough levels themselves.

DISCUSSION

IVIG plus aspirin is now an initial standard therapy for KD, and has actually reduced the incidences of CAL.[7] However, the precise mechanism by which IVIG suppresses KD vasculitis is still unclear. Furthermore, it has not been clarified why 10% to 20% of KD patients are resistant to IVIG treatment. Thus, it is important to develop additional strategies for such IVIG-resistant patients.

In this study, CyA appeared to exert both antifebrile and anti-inflammatory effects in patients with refractory KD. Its effect was particularly clear in 18 of the 28 patients with resistance, because they became afebrile within 3 days after the start of CyA treatment. Although it took 4 to 5 days for 4 of the 28 patients to become afebrile, their body temperature remained close to the definition level of "afebrile" within 2 days after the start of CyA treatment. In addition, symptoms such as rash and swelling of the cervical lymph nodes also improved. On the other hand, 6 of the 28 patients failed to become afebrile within 5 days after the start of

© 2011 Lippincott Williams & Wilkins
Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

TABLE 3. Summary of Clinical Parameters in 28 KD Patients Treated With CyA

		P
Age (mo)	4–93 (23)	
Male:female	20:8	
Illness d of initial IVIG	3–6 (4.5)	
Illness d of additional IVIG	5–10 (7)	
Illness d of the start of CyA treatment	7–12 (8)	
Duration (d) of CyA treatment	5–50 (14)	
Duration (d) until afebrile after CyA treatment	1–13 (2)	
Axillary temperature (centigrade)		
On the day of the start of CyA treatment	38.0–40.3 (38.9)	<0.01*
2 d after the start of CyA treatment	36.1–40.4 (37.6)	
CRP (mg/dL)		
On the day of the start of CyA treatment	1.2–16.8 (9.3)	<0.01*
2 d after the start of CyA treatment	0.4–16.1 (5.2)	
Serum potassium levels		
On the day of the start of CyA treatment	2.9–5.1 (4.1)	<0.01*
Maximum potassium levels	4.9–6.1 (5.4)	
Duration (d) until maximum potassium levels	3–13 (6.5)	
Serum creatinine levels		
On the day of the start of CyA treatment	0.1–0.33 (0.23)	0.156*
On the day of maximum potassium levels	0.12–0.33 (0.24)	
Estimated GFR		
On the day of the start of CyA treatment	85.5–152.3 (109.9)	0.123*
On the day of maximum potassium levels	83.4–135 (103.9)	

Values are ranges and (medians).
**P* values were calculated by Wilcoxon signed rank test.
CyA indicates cyclosporin A; IVIG, intravenous immunoglobulin.

CyA and/or high fever returned after becoming afebrile within 5 days, despite having an adequate trough level of CyA. These facts suggest that certain subgroups of patients with refractory KD may be resistant to CyA. Two possible reasons for this can be suggested. First, the timing of CyA treatment might be too late. We used CyA to selectively treat KD patients who had been resistant to 2 courses of IVIG therapy. Because CyA was a new option for refractory KD and we had no data about its safety and efficacy for patients with KD, parents and guardians were not expected to give their approval until the standard therapy for KD had proven ineffective. On the other hand, it is desirable to initiate any new treatment as rapidly as possible, as damage to the walls of coronary arteries might progress within the first 10 days of illness unless the process of acute inflammation can be suppressed. Therefore, we set up a protocol in which CyA treatment was started immediately after completing the additional IVIG therapy. Second, the suppressive effect of CyA on the calcineurin or NFAT pathway might be insufficient to control the severe vasculitis. Further analyses of both the timing of CyA treatment and genetic background factors including the NFAT and other pathways will be needed to clarify these issues.

We did not have any information about whether oral administration or intravenous infusion of CyA would be more effective. However, we selected oral administration, because the oral route is easier and more tolerable for young children and infants, who need to be treated for 2 or 3 weeks. The volume of CyA was small, and therefore the patients were able to take it without major problems. By examining the trough values, it became clear that oral administration of CyA allowed a sufficient high serum concentration (60–200 ng/mL) to be obtained. Further analyses of trough values in more patients with refractory KD will be needed to assess the best therapeutic levels of CyA in KD.

KD patients who were less than 4 months of age were excluded from the indication of CyA treatment, because there is insufficient information about the safety of CyA in infants and children with KD. However, CyA is known to be effective and safe for children with nephrotic syndrome resistant to steroid.[28,29] In the latter case, the dose of CyA was adjusted to maintain a trough level of 120 to 150 ng/mL during the initial 3 months of treatment, followed by 80 to 100 ng/mL during 4 to 12 months.[28] Adverse events in children with nephrotic syndrome comprised CyA-related nephrotoxicity (3.8%), bacterial infections (2/38 = 5.3%), and posterior reversible encephalopathy syndrome (1/38 = 2.6%). As the duration of CyA treatment for KD was much shorter than that for nephrotic syndrome, it was expected that CyA treatment might be associated with few adverse events in this series. Indeed, the increase of hepatic enzymes and hypertension in these 28 patients did not develop. However, many of our patients treated with CyA showed hyperkalemia, a feature that was absent in nephrotic syndrome. Although the definition of hyperkalemia in infants and children is controversial,[30] we classified these patients according to the criteria of Japan.[27] Fortunately, no harmful events such as ventricular arrhythmia occurred. The levels of serum potassium were not correlated with serum CyA trough levels, serum creatinine levels, and eGFR. In addition, there have been a report in which pseudohyperkalemia was observed in the sera of KD despite the normal plasma level.[31] This report indicates that pseudohyperkalemia should be considered in patients with KD whose platelet counts are markedly increased. Indeed, serum potassium levels were correlated closely with platelet counts in these 28 patients who were treated with CyA (data not shown). However, precise mechanism responsible for the hyperkalemia during CyA treatment in KD patients is still unclear. In this study, we did not examine the C2 levels of CyA, which might have been better correlated with the AUC_{0-4} (area under the curve) than the trough level, because of the difficulty involved in taking frequent blood samples from infants and small children. We think that analyses for both C2 levels of CyA and potassium clearance rate will be needed in the future studies.

It is difficult to evaluate whether CyA inhibits the development of CAL, which is the most serious outcome of KD. Hashino et al[10] reported that there were 17 patients (17/262 = 6.5%) resistant to both initial (2 g/kg) and additional (1 g/kg) IVIG therapy. They randomly divided these patients into 2 groups (third IVIG or steroid pulse therapy). After third additional option, there was no significant difference in the incidence of CAL between the 2 groups. In their study, 12 (70.6%) of 17 patients resistant to both initial (2 g/kg) and additional (1 g/kg) IVIG developed CAL. Thus, 12 (4.6%) of 262 patients developed CAL. Our present study was not a randomized control study but a pilot study, in which 4 (13.3%) of 30 patients resistant to both initial (2 g/kg) and additional (2 g/kg) IVIG therapy developed CAL. Thus, 4 (1.2%) of 329 patients developed CAL. All 4 patients were resistant to both initial and additional IVIG treatment, and were treated with CyA. However, coronary arterial dilatation occurred during additional IVIG (before CyA treatment) in 2 of the 4 patients, and CyA

© 2011 Lippincott Williams & Wilkins

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

did not inhibit the progression of CAL in these patients. In the other 2 patients, CAL developed after the start of CyA treatment.

There were several limitations to this study. First, the sample size was small. We were unable to analyze the factors affecting the response to CyA because only 6 cases were resistant to CyA. Second, this was a pilot study and not a randomized control clinical trial. Therefore, we were unable to conclude whether CyA exerts preventive effects against CAL in patients with refractory KD.

In summary, CyA treatment is considered well tolerated and a safe and promising option for patients with refractory KD. Oral administration of CyA showed good compliance with treatments and has both antifebrile and anti-inflammatory effects in KD patients who are resistant to IVIG. Further investigations will be needed to clarify the dose, safety, optimum timing, and duration of CyA treatment.

REFERENCES

1. Kawasaki T. Acute febrile mucocutaneous syndrome with lymphoid involvement with specific desquamation of fingers and toes in children: clinical observation of 50 cases. *Jpn J Allergol*. 1967;16:178–222.
2. Park YW, Han JW, Park IS, et al. Kawasaki disease in Korea, 2003–2005. *Pediatr Infect Dis J*. 2007;26:821–823.
3. Huang WC, Huang LM, Chang IS, et al. Epidemiologic features of Kawasaki disease in Taiwan, 2003–2006. *Pediatrics*. 2009;123:e401–e405.
4. Holman RC, Belay ED, Christensen KY, et al. Hospitalizations for Kawasaki syndrome among children in the United States, 1997–2007. *Pediatr Infect Dis J*. 2010;29:483–488.
5. Harnden A, Mayon-White R, Perera R, et al. Kawasaki disease in England: ethnicity, deprivation, and respiratory pathogens. *Pediatr Infect Dis J*. 2009;28:21–24.
6. Nakamura Y, Yashiro M, Uehara R, et al. Epidemiologic features of Kawasaki disease in Japan: results from the nationwide survey in 2005–2006. *J Epidemiol*. 2008;18:167–172.
7. Newburger JW, Takahashi M, Beiser AS, et al. A single intravenous infusion of gamma globulin as compared with four infusions in the treatment of acute Kawasaki syndrome. *N Engl J Med*. 1991;324:1633–1639.
8. Takeshita S, Kawamura Y, Nakatani K, et al. Standard-dose and short-term corticosteroid therapy in immunoglobulin-resistant Kawasaki disease. *Clin Pediatr (Phila)*. 2005;44:423–426.
9. Lang BA, Yeung ES, Oen KG, et al. Corticosteroid treatment of refractory Kawasaki disease. *J Rheumatol*. 2006;33:803–809.
10. Hashino K, Ishii M, Iemura M, et al. Re-treatment for immuno globulin-resistant Kawasaki disease: a comparative study of additional immune globulin and steroid pulse therapy. *Pediatr Int*. 2001;43:211–217.
11. Furukawa T, Kishiro M, Akimoto K, et al. Effects of steroid pulse therapy on immunoglobulin-resistant Kawasaki disease. *Arch Dis Child*. 2008;93:142–146.
12. Burns JC, Mason WH, Hauger SB, et al. Infliximab treatment for refractory Kawasaki syndrome. *J Pediatr*. 2005;146:662–667.
13. Burns JC, Best BM, Mejias A, et al. Infliximab treatment of intravenous immunoglobulin-resistant Kawasaki disease. *J Pediatr*. 2008;153:833–836.
14. Mori M, Imagawa T, Katakura S, et al. Efficacy of plasma exchange therapy for Kawasaki disease intractable to intravenous gamma-globulin. *Mod Rheumatol*. 2004;14:43–47.
15. Raman V, Kim J, Sharkey A, et al. Response of refractory Kawasaki disease to pulse steroid and cyclosporine A therapy. *Pediatr Infect Dis J*. 2001;20:635–637.
16. Lee TJ, Kim KH, Chun JK, et al. Low-dose methotrexate therapy for intravenous immunoglobulin-resistant Kawasaki disease. *Yonsei Med J*. 2008;49:714–718.
17. Inoue Y, Okada Y, Shinohara M, et al. A multicenter prospective randomized trial of corticosteroids in primary therapy for Kawasaki disease: clinical course and coronary artery outcome. *J Pediatr*. 2006;149:336–341.
18. Kobayashi T, Inoue Y, Otani Y, et al. Risk stratification in the decision to include prednisolone with intravenous immunoglobulin in primary therapy of Kawasaki disease. *Pediatr Infect Dis J*. 2009;28:496–502.
19. Newburger JW, Sleeper LA, McCrindle BW, et al. Randomized trial of pulsed corticosteroid therapy for primary treatment of Kawasaki disease. *N Engl J Med*. 2007;356:663–675.
20. Okada K, Hara J, Maki I, et al. Pulse methylprednisolone with gammaglobulin as an initial treatment for acute Kawasaki disease. *Eur J Pediatr*. 2009;168:181–185.
21. Onouchi Y, Gunji T, Burns JC, et al. ITPKC functional polymorphism associated with Kawasaki disease susceptibility and formation of coronary artery aneurysms. *Nat Genet*. 2008;40:35–42.
22. de Inocencio J, Hirsch R. The role of T cells in Kawasaki disease. *Crit Rev Immunol*. 1995;15:349–357.
23. Brogan PA, Shah V, Clarke LA, et al. T cell activation profiles in Kawasaki syndrome. *Clin Exp Immunol*. 2008;151:267–274.
24. Suzuki H, Suenaga T, Takeuchi T, et al. Marker of T-cell activation is elevated in refractory Kawasaki disease. *Pediatr Int*. 2010;52:785–789.
25. Japanese Kawasaki disease Research Committee. *Diagnostic Guidelines of Kawasaki Disease*. 5th ed. Tokyo, Japan: Japanese Kawasaki disease Research Committee; 2002.
26. Japan Circulation Society Joint Working Groups. Guideline for diagnosis and management of cardiovascular sequelae in Kawasaki disease (JCS2008).
27. Igarashi T. Hyperkalemia. In: Igarashi T, ed. *Clinical Pediatric Nephrology* [in Japanese]. 3rd ed. Tokyo, Japan: Shindan to Chiryo Sha Inc.; 2008:39–40.
28. Ishikura K, Ikeda M, Hattori S, et al. Effective and safe treatment with cyclosporine in nephrotic children: a prospective, randomized multicenter trial. *Kidney Int*. 2008;73:1167–1173.
29. Hamasaki Y, Yoshikawa N, Hattori S, et al. Cyclosporine and steroid therapy in children with steroid-resistant nephritic syndrome. *Pediatr Nephrol*. 2009;24:2177–2185.
30. Schwartz D, Conelly NR, Manikantan P, et al. Hyperkalemia and pyloric stenosis. *Anesth Analg*. 2003;97:355–357.
31. Shimizu T, Yamashiro Y, Yabuta K. Pseudohyperkalemia in Kawasaki disease. *Eur J Pediatr*. 1992;151:497–498.

© 2011 Lippincott Williams & Wilkins

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

急性脊髄損傷患者に対する顆粒球コロニー刺激因子を用いた ランダム化、実薬対照、並行群間比較試験

第Ⅲ相

実施計画書

臨床試験代表者
千葉大学医学部附属病院整形外科　教授　　高橋　和久

臨床試験責任医師
千葉大学医学部附属病院整形外科　講師　　大河　昭彦

臨床試験分担医師
千葉大学医学部附属病院整形外科　特任助教　　古矢　丈雄

作成日　　平成 24 年 10 月 29 日　　1.1 版

改訂履歴	作成日	改訂番号
	2012.8.8	初版 0.1
	2012.9.17	初版 0.2
	2012.9.24	初版 1.0
	2012.10.29	1.1 版

略号および用語の定義一覧

略号・用語	正式名称または内容
G-CSF	Granulocyte colony-stimulating factor（顆粒球コロニー刺激因子）
ASIA	American Spinal Injury Association（米国脊髄損傷学会）
AIS	ASIA Impairment Scale（米国脊髄損傷学会機能障害分類）
NASCIS-2	The second national acute spinal cord injury study（第 2 回米国急性脊髄損傷調査）
MMT	Manual muscle test（徒手筋力テスト）
CRF	Case report form

目次

1 背景 ... 6
2 疾患の背景 ... 6
3 疾患の標準治療 ... 6
4 本試験の経緯 ... 6
5 試験の目的と必要性 ... 8
6 試験薬の概要 ... 8
7 診断の基準 ... 8
8 対象患者 ... 9
8.1 選択基準 ... 9
8.2 除外基準 ... 9
9 被験者に説明し同意を得る方法 ... 10
10 試験の方法 ... 10
10.1 試験の種類・デザイン ... 10
10.2 試験のアウトライン ... 11
10.3 被験者の試験参加予定期間 ... 11
10.4 試験薬の用法･用量、投与期間 ... 11
10.5 試験薬の剤形・含有量、性状、包装、表示、貯法 ... 12
10.6 併用薬（療法）に関する規定 ... 13
10.7 休薬の方法 ... 13
10.8 試験薬の管理・交付手順 ... 13
10.9 症例登録、割付方法 ... 13
11 試験の評価項目 ... 14
11.1 主要評価項目 ... 14
11.2 副次的評価項目 ... 14
12 観察および検査項目と時期 ... 15
12.1 試験開始前 ... 15
12.2 試験薬投与期 ... 15
12.3 試験薬投与終了後 ... 16
12.4 試験終了時又は中止時 ... 16
13 中止基準 ... 17
14 有害事象発生時の取り扱い ... 17
14.1 有害事象発生時の被験者への対応 ... 17
14.2 重篤な有害事象の報告 ... 19
14.3 重篤な有害事象発生時の試験継続 ... 20
14.4 重要な有害事象の報告 ... 20
14.5 その他の有害事象 ... 20
15 実施計画書からの逸脱の報告 ... 20
16 試験の終了、中止、中断 ... 21

16.1 試験の終了 21
16.2 試験の中止、中断 21
17 試験実施期間 21
18 統計学的事項 21
18.1 主要評価項目 21
18.2 副次的評価項目 21
18.3 目標症例数と設定根拠 22
18.4 症例の取り扱い 22
18.5 データの取り扱い 22
18.6 解析対象集団 22
18.6.1 最大の解析対象集団 (full analysis set: FAS) 22
18.6.2 試験実施計画書に適合した対象集団 (per protocol set: PPS) 22
18.6.3 安全性解析対象集団 23
18.7 統計解析項目および解析計画 23
18.8 被験者背景 23
18.9 有効性の主たる解析 23
18.10 安全性の解析 23
18.11 中間解析 23
18.12 最終解析 24
19 被験者の人権および安全性・不利益に対する配慮ならびに個人情報保護 24
19.1 人権への配慮（プライバシーの保護） 24
19.2 安全性・不利益への配慮 24
19.3 個人情報保護 24
20 患者の費用負担 24
21 健康被害の補償および保険への加入 25
21.1 健康被害の補償 25
21.2 臨床研究保険（補償保険）への加入 25
21.3 賠償保険への加入 25
22 GCP およびヘルシンキ宣言への対応 25
23 記録の保存 25
24 症例報告書 25
24.1 データマネジメント 25
24.2 データの収集 25
24.3 症例報告書に直接記入され、かつ原資料(原データ)と解すべき資料の特定 25
25 モニタリング及び監査 26
26 研究結果の公表 26
27 研究組織 26
28 研究資金および利益の衝突 28
29 実施計画書等の変更 28

30　参考文献 28