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

INTRODUCTION

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

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

RESULTS

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

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

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

Screening of functionally significant variants

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

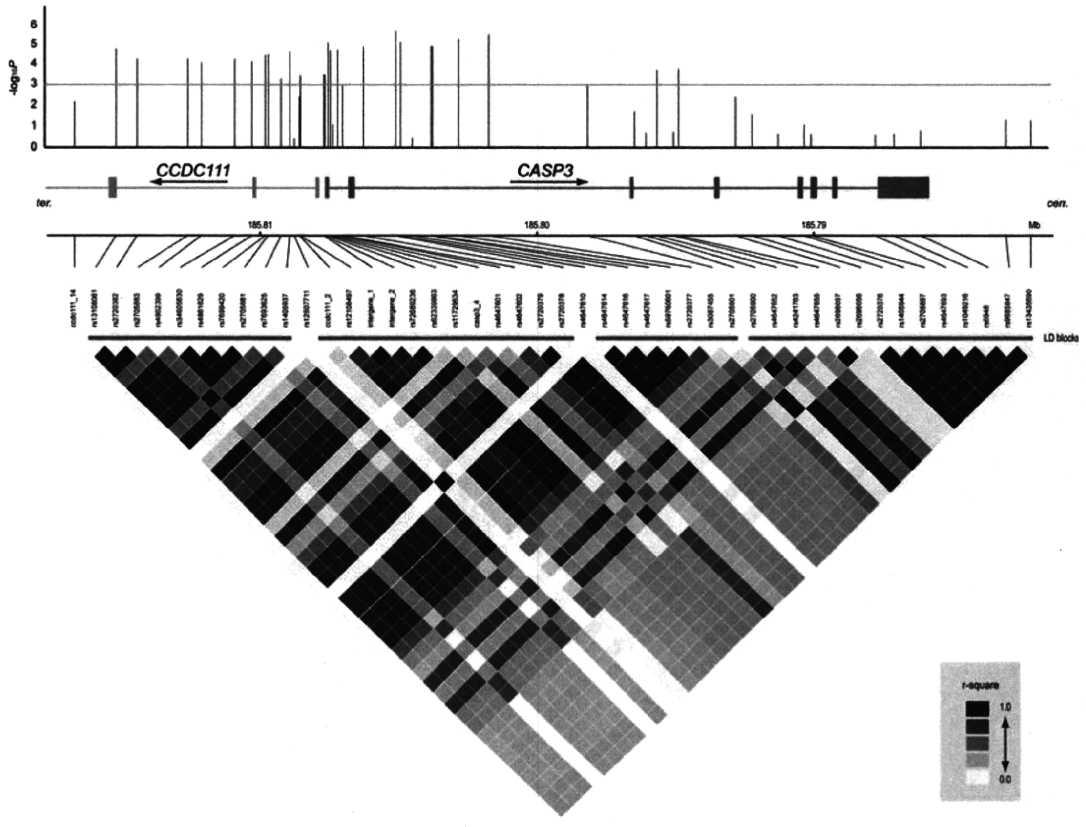


Figure 1. Linkage disequilibrium (LD) structure of the *CASP3* locus and association of the variants with KD in Japanese subjects. Pairwise LD plots with 46 variants distributed across the 36 kb region in and surrounding *CASP3* are illustrated using Haploview software. Values for r^2 were calculated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

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

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

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

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

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

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

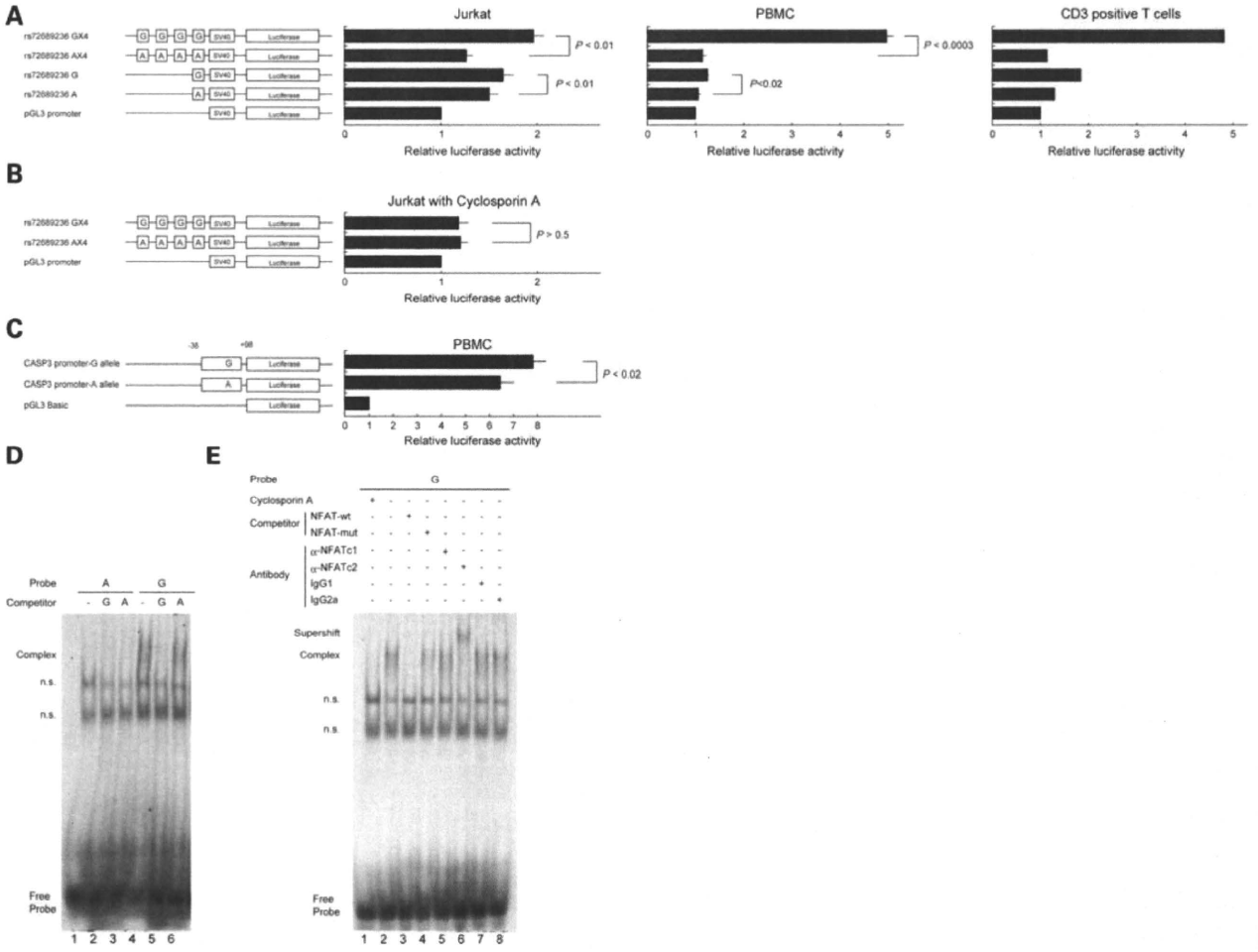


Figure 2. Functional analyses of the G and A alleles of rs72689236. (**A**) Single or four tandem copies of oligonucleotides for the G allele and A allele of rs72689236 were cloned upstream of the SV40 promoter in the PGL3 luciferase reporter vector and transfected into Jurkat cells (left), PBMCs (middle)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

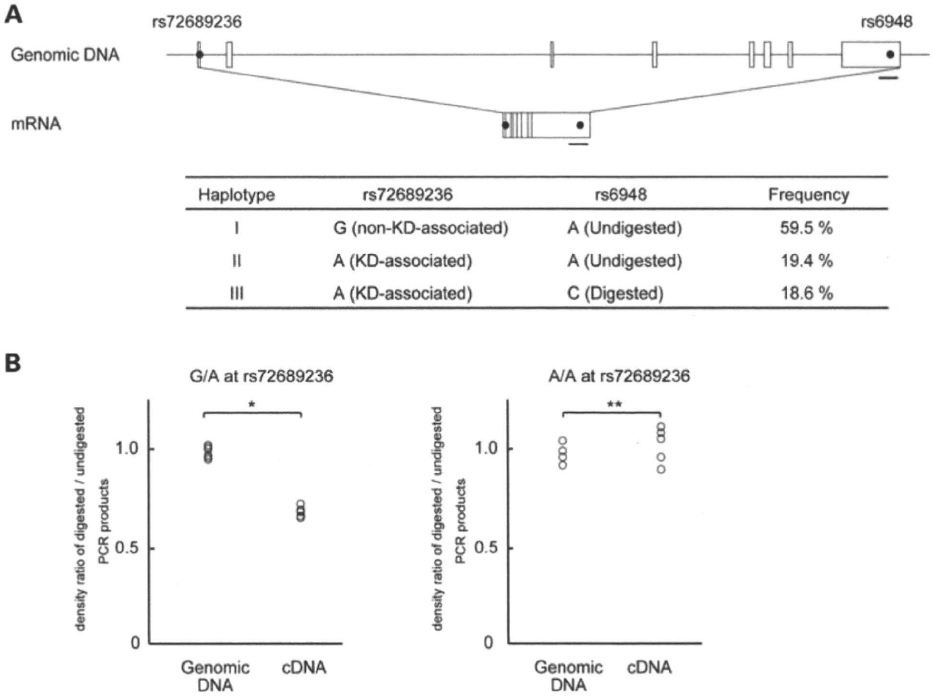
from the *Interleukin-2* (*IL-2*) promoter. And finally the complex was supershifted by a monoclonal antibody against NFATc2 (Fig. 2E).

Allele specific expression of *CASP3*

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

Association study in US KD families of European ancestry

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



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

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

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

DISCUSSION

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

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

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

Variants	TDT Risk allele	RAF[a]	T:U[b]	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^{-3}
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
rs7699420	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^{-3}
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^{-3}
rs62339863	T	0.71	122:78	1.56	1.18–2.08	1.9×10^{-3}
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^{-3}
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^{-3}
rs4647652	C	0.87	58:50	1.16	0.79–1.69	0.44
rs4647655	del (TTCAG GATTT)	0.87	59:50	1.18	0.81–1.72	0.39

[a]Risk allele frequency.
[b]'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^{2+}/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^{2+}/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/). 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 = $-2 \times$ (the maximized value of the conditional log-likelihood) $+ 2 \times$ (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). $CD3^+$ 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 phRGTK 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 $CD3^+$ T cells was conducted with U-014 program. Twenty-four hours after transfection, Jurkat cells, PBMCs and $CD3^+$ 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.

Electorophoretic 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 $MgCl_2$, 2% glycerol and protease inhibitor cocktail]. Eighteen base pairs of double-stranded oligonucleotides corresponding to G and A alleles of rs72689236 were labeled with digoxygenin -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-lysin 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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Conflict of Interest statement. None declared.

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Original Article:

Title: Cyclosporin A treatment for refractory Kawasaki disease

－A pilot study of 28 patients with Kawasaki disease resistant to both initial and additional IVIG－

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

Abbreviated title: CyA treatment for refractory Kawasaki disease

Running head title: CyA treatment for refractory KD

Abstract

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

Methods: We prospectively collected clinical data of CyA treatment (4-8mg/kg/day, oral administration) for refractory KD patients using a same protocol among several hospitals. Refractory KD is defined as the persistence or recurrence of fever (37.5 ℃ or more of an axillary temperature) at least 24hrs after second intravenous immunoglobulin (IVIG) (2g/kg) following the initial one.

Results: Subjects were enrolled out of 329 KD patients who were admitted to our eight hospitals between January 2008 and June 2010. Among a total of twenty-eight patients of refractory KD treated with CyA, 18 (64.3%) responded promptly to be afebrile within three days and decreased C-reactive protein levels, the other 4 became afebrile within 4-5 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 3-7 days after the start of CyA treatment, there were no serious adverse effects such as arrhythmias. Four patients (1.2%), two before and the other two 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.

INTRODUCTION

Kawasaki disease (KD) [1] is an acute systemic vasculitis occurring in medium-sized arteries, especially coronary arteries. KD patients have been reported not only from East Asia including Japan but also USA 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 ten thousand children with KD were reported annually in 2005 and 2006,[6] and KD is now 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 3-4% by standard therapy with intravenous immunoglobulin (IVIG) and aspirin[7], 10-20% 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 of 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] Since 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 the NFAT pathway, may be a promising candidate 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.

Materials and Methods

Patients: We prospectively collected clinical data of CyA treatment for refractory KD patients using a same protocol among eight 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 Izumiotsu Municipal Hospital in Osaka prefecture. The study was approved by the ethics Committee of each institution. Study subjects aged 4 months or older were enrolled out of Japanese 329 patients who met the diagnostic criteria for KD[25] and were admitted to our eight hospitals between January 2008 and June 2010. Written Informed consent was obtained from the parents of the patients investigated.

Protocol (Figure 1)**:** All patients received initial IVIG infusion (2 g/kg for 24 hours) and aspirin (30-50 mg/kg/day) within 7 days after the onset of KD. The patients were considered afebrile when the axillary temperature remained below 37.5℃ for more than 24 hours. The aspirin dose was decreased to 5 mg/kg/day after 2-3 days without fever. We defined patients who remained febrile (37.5 ℃ 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 by oral administration (Neoral®, oral solution, Novartis Pharma Co. Ltd., Tokyo, Japan). The initial dose of CyA was 4mg/kg/day, and patients received oral CyA divided into two equal daily doses every 12 hours. The dose of CyA was adjusted to between 4 and 8 mg/kg/day to maintain a trough level of 60-200 ng/ml by 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 (<0.3mg/dL). In addition, the maximum duration of CyA treatment was decided on three weeks between January and December 2008, and on two 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 2-3 days until CyA treatment was discontinued. Laboratory tests included the concentrations of CRP, electrolyte (potassium), creatinine. In order 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, two-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 one 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 illness onset in patients who were judged to have CAL on the basis of 2DE, in order to confirm the presence of CAL.

Statisitical analysis

Statistical analysis was performed using Wilcoxon's signed rank test. Differences at a two-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 (Figure 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 two patients, who were less than four 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 3-6 (median; 4.5) and the additional IVIG on illness days 5-10 (median; 7). CyA treatment was initiated on days 7-12 of illness (median; 8). The dose of CyA was 4-8 mg/kg/day, and the duration of treatment was 5-50 (median; 14) days.

The duration until afebrile after the start of CyA treatment was 1-13 (median; 2) days. Eighteen of the 28 patients responded promptly to be afebrile within three days, and the other 4 became afebrile within 4-5 days after the start of CyA treatment. Axillary temperature (℃) 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 (nos. 3, 8, 10, 19, 26, and 28 in Table 1 and 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 (nos. 3, 10, 26 and 28 in Table 1 and 2) received a third IVIG infusion. Patient no. 3 received the third IVIG infusion on the 10th day after the start of CyA treatment. However, low-grade fever (37.5-38.5℃) continued, and therefore she received CyA for 50 days until she became completely afebrile. Patient no. 10 became afebrile on the 4th 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 patient no.26 and 28 became afebrile on the 7th after CyA treatment, but high fever returned after CyA treatment for 14 days. They received the third IVIG infusion on the next day or three days after CyA, and then they became afebrile. The other two patients (nos. 8, and 19) did not receive any other therapy, because high fever (more than 38.0℃) was absent, and they gradually became afebrile with continued CyA therapy.

Although 4 patients (nos. 3, 8, 10, and 19) developed CAL, the coronary arteries had already dilated (more than 4mm by 2DE) in 2 of them (no. 10 and 19) before CyA treatment. In addition, patient no. 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.