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

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Abstract

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.

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 lymphadenopathy1. Coronary artery aneurysms develop in 15–25% of those left untreated2, 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 death3. 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 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 (*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 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].

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 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 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 tools28^{-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 described39.

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

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 1967;16:178–222. English translation by. Shike, H.; Burns, JC.; Shimizu, C., translators. Pediatr Infect Dis J. Vol. 21. 2002. p. 993-995.
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 1975;86:892–898. [PubMed: 236368]
3. Kato H, et al. Long-term consequences of Kawasaki disease A 10- to 21-year follow-up study of 594 patients. Circulation 1996;94:1379–1385. [PubMed: 8822996]
4. Cook DH, et al. Results from an international survey of Kawasaki disease in 1979–82. Can J Cardiol 1989;5:389–394. [PubMed: 2605549]
5. Fujita Y, et al. Kawasaki disease in families. Pediatrics 1989;84:666–669. [PubMed: 2780128]
6. Uehara R, Yashiro M, Nakamura Y, Yanagawa H. Kawasaki disease in parents and children. Acta Paediatr 2003;92:694–697. [PubMed: 12856980]
7. Dergun M, et al. Familial occurrence of Kawasaki syndrome in North America. Arch Pediatr Adolesc Med 2005;159:876–881. [PubMed: 16143748]
8. Onouchi Y, et al. A genome-wide linkage analysis for Kawasaki disease: evidence for linkage to chromosome 12. J Hum Genet 2007;52:179–190. [PubMed: 17160344]
9. Imboden JB, Pattison G. Regulation of inositol 1,4,5-trisphosphate kinase activity after stimulation of human T cell antigen receptor. J Clin Invest 1987;79:1538–1541. [PubMed: 3494750]
10. Dewaste V, et al. Cloning and expression of a cDNA encoding human inositol 1,4,5-trisphosphate 3-kinase C. Biochem J 2000;352:343–351. [PubMed: 11085927]
11. Berridge MJ, Irvine RF. Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 1984;312:315–321. [PubMed: 6095092]
12. Harnick DJ, et al. The human type 1 inositol 1,4,5-trisphosphate receptor from T lymphocytes Structure, localization, and tyrosine phosphorylation. J Biol Chem 1995;270:2833–2840. [PubMed: 7852357]
13. Weiss A, Littman DR. Signal transduction by lymphocyte antigen receptors. Cell 1994;76:263–274. [PubMed: 8293463]
14. Macian F. NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol 2005;5:472–484. [PubMed: 15928679]

15. Cogan JD. A novel mechanism of aberrant pre-mRNA splicing in humans. Hum Mol Genet 1997;6:909–912. [PubMed: 9175738]

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 2004;103:586–593. [PubMed: 14504098]

17. Morisaki H, Morisaki T, Newby LK, Holmes EW. Alternative splicing: a mechanism for phenotypic rescue of a common inherited defect. J Clin Invest 1993;91:2275–2280. [PubMed: 8486786]

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 2007;28:150–158. [PubMed: 17001642]

19. Krawczak M, Reiss J, Cooper DN. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. Hum Genet 1992;90:41–54. [PubMed: 1427786]

20. Majewski J, Ott J. Distribution and characterization of regulatory elements in the human genome. Genome Res 2002;12:1827–1836. [PubMed: 12466286]

21. Yeo G, Hoon S, Venkatesh B, Burge CB. Variation in sequence and organization of splicing regulatory elements in vertebrate genes. Proc Natl Acad Sci USA 2004;101:15700–15705. [PubMed: 15505203]

22. Louie E, Ott J, Majewski J. Nucleotide frequency variation across human genes. Genome Res 2003;13:2594–2601. [PubMed: 14613976]

23. McCullough AJ, Berget SM. G triplets located throughout a class of small vertebrate introns enforce intron borders and regulate splice site selection. Mol Cell Biol 1997;17:4562–4571. [PubMed: 9234714]

24. Buratti E, Baralle FE. Influence of RNA secondary structure on the pre-mRNA splicing process. Mol Cell Biol 2004;24:10505–10514. [PubMed: 15572659]

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 1999;96:8229–8234. [PubMed: 10393977]

26. Yoshioka T, et al. Polyclonal expansion of TCRBV2- and TCRBV6-bearing T cells in patients with Kawasaki disease. Immunology 1999;96:465–472. [PubMed: 10233729]

27. Brown TJ. CD8 T lymphocytes and macrophages infiltrate coronary artery aneurysms in acute Kawasaki disease. J Infect Dis 2001;184:940–943. [PubMed: 11528596]

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 2000;137:172–176. [PubMed: 10931407]

29. Egami K, et al. Prediction of resistance to intravenous immunoglobulin treatment in patients with Kawasaki disease. J Pediatr 2006;149:237–240. [PubMed: 16887442]

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 2007;166:131–137. [PubMed: 16896641]

31. Kobayashi T, et al. Prediction of intravenous immunoglobulin unresponsiveness in patients with Kawasaki disease. Circulation 2006;113:2606–2612. [PubMed: 16735679]

32. Ho S, et al. The mechanism of action of cyclosporin A and FK506. Clin Immunol Immunopathol 1996;80:S40–S45. [PubMed: 8811062]

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 2001;20:635–637. [PubMed: 11419513]

34. Lewis R. Calcium signaling mechanisms in T lymphocytes. Annu Rev Immunol 2001;19:497–521. [PubMed: 11244045]

35. Ayusawa M, et al. Revision of diagnostic guidelines for Kawasaki disease (the 5th revised edition). Pediatr Int 2005;47:232–234. [PubMed: 15771703]

36. Burns JC, et al. Family-based association analysis implicates IL-4 in susceptibility to Kawasaki disease. Genes Immun 2005;6:438–444. [PubMed: 15889128]

37. Suzuki A, et al. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. Nat Genet 2003;34:395–402. [PubMed: 12833157]

38. Spielman R, McGinnis RE, Ewens WJ. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). Am J Hum Genet 1993;52:506–516. [PubMed: 8447318]

39. Kazeem GR, Farrall M. Integrating case-control and TDT studies. Ann Hum Genet 2005;69:329–335. [PubMed: 15845037]

40. Uejima H, Lee MP, Cui H, Feinberg AP. Hot-stop PCR: a simple and general assay for linear quantitation of allele ratios. Nat Genet 2000;25:375–376. [PubMed: 10932175]

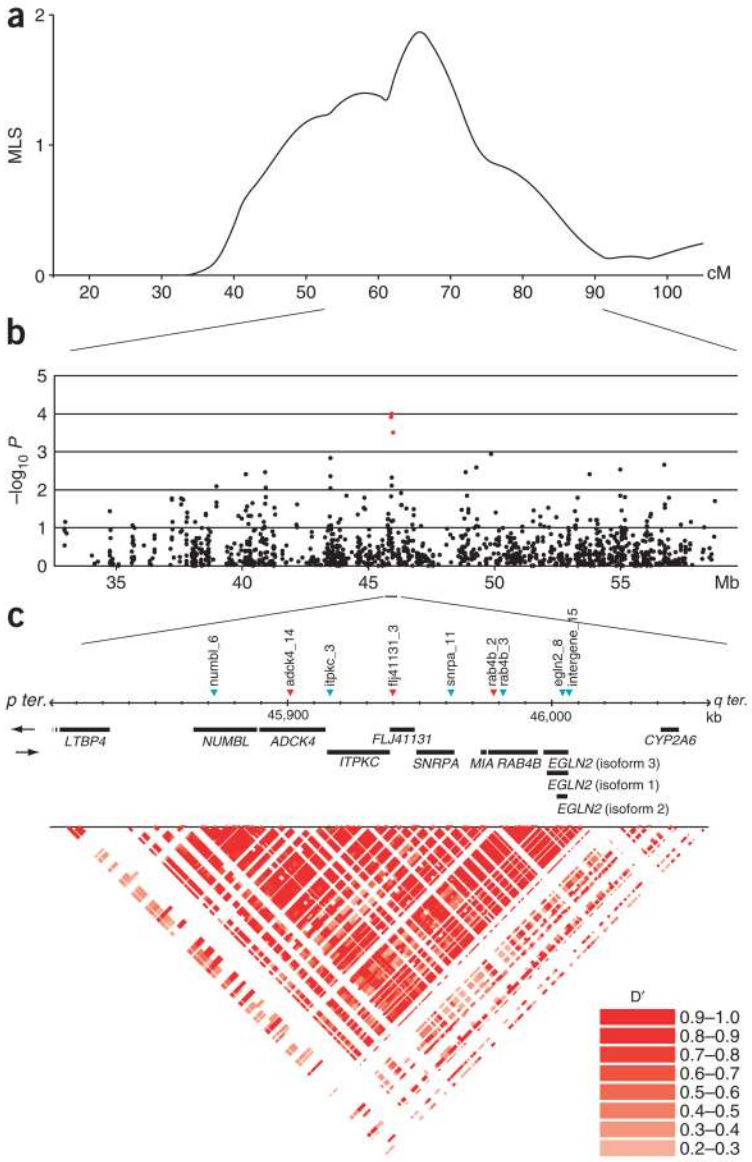

Figure 1.

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. × 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.

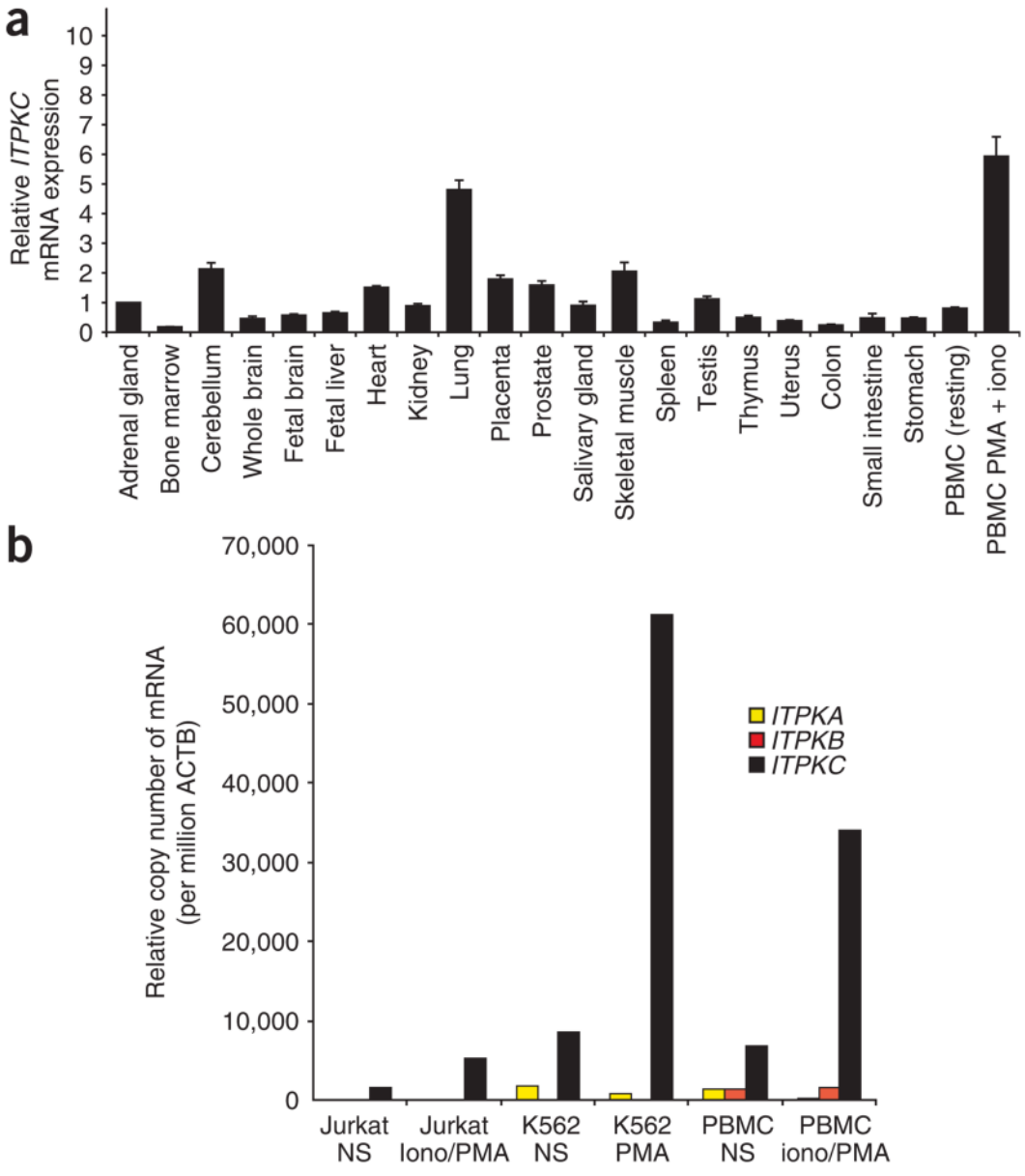

Figure 2.

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.

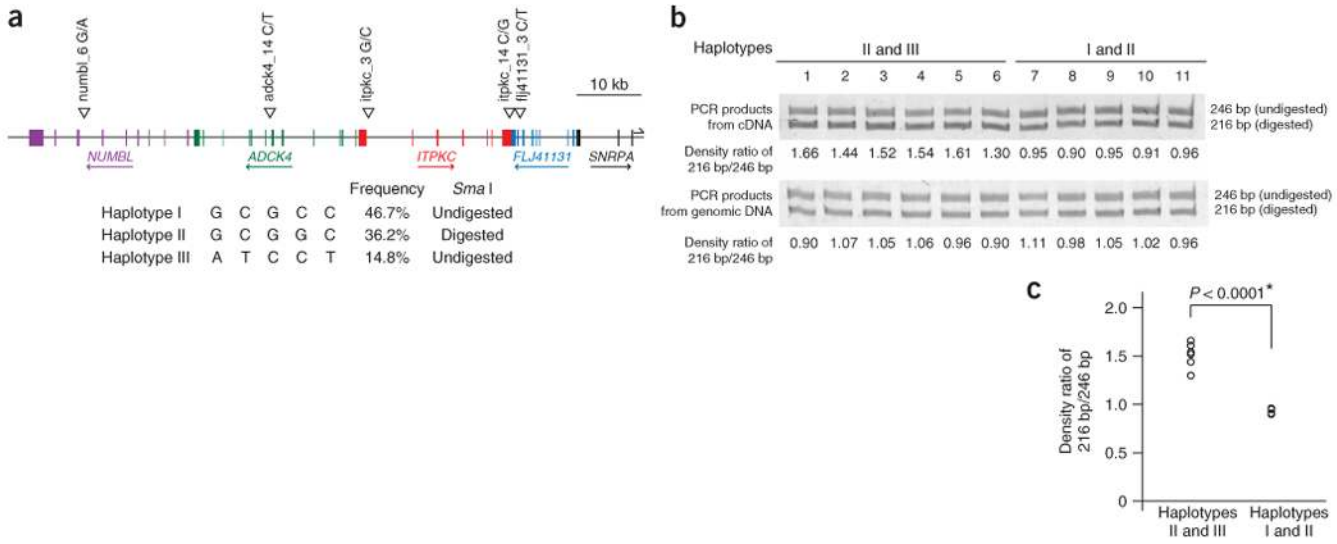

Figure 3.

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 *SmaI* site. (**b,c**) ASTQ showing decreased undigested transcript of *ITPKC* associated with haplotype III. *Two-tailed *P* value by Welch's *t*-test.

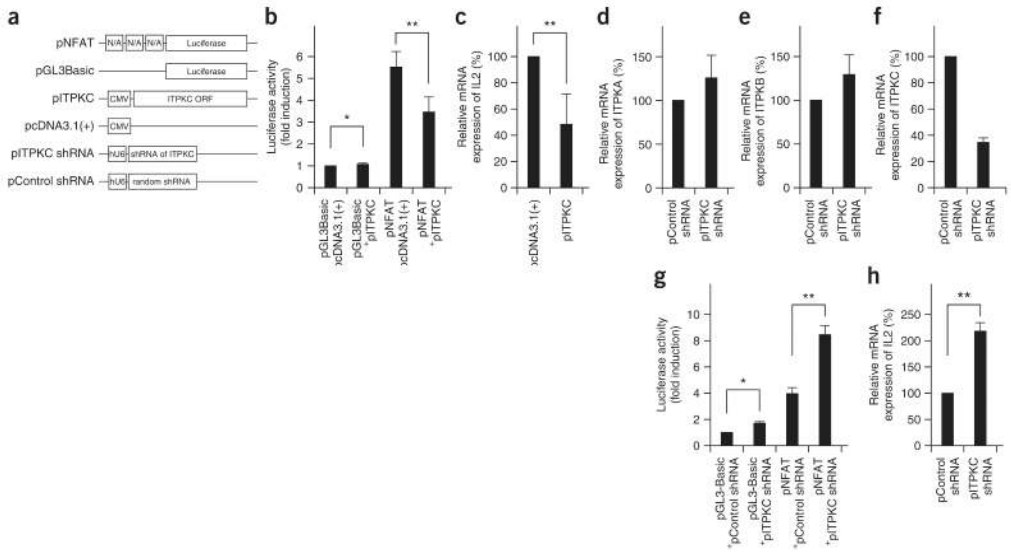

Figure 4.

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.

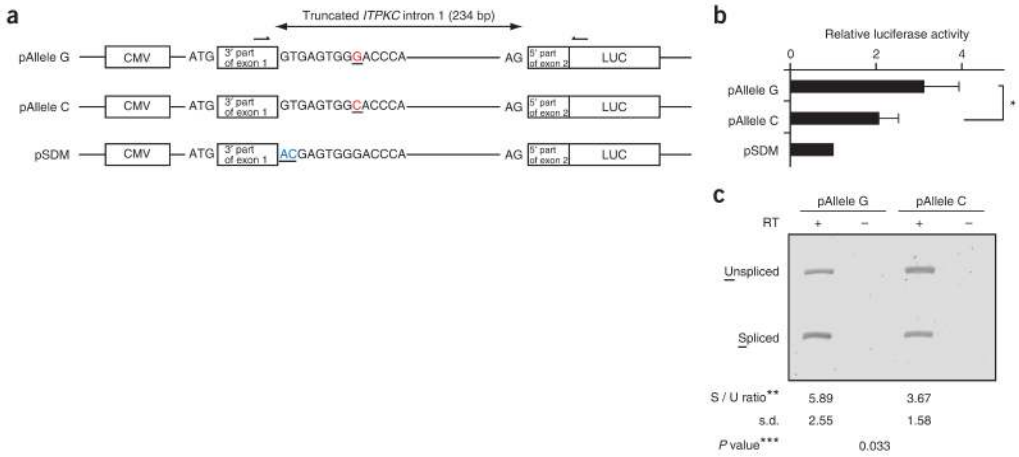

Figure 5.

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 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.

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.