

Genetic Variation in the SLC8A1 Calcium Signaling Pathway Is Associated With Susceptibility to Kawasaki Disease and Coronary Artery Abnormalities

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Background—Kawasaki disease (KD) is an acute pediatric vasculitis in which host genetics influence both susceptibility to KD and the formation of coronary artery aneurysms. Variants discovered by genome-wide association studies and linkage studies only partially explain the influence of genetics on KD susceptibility.

Methods and Results—To search for additional functional genetic variation, we performed pathway and gene stability analysis on a genome-wide association study data set. Pathway analysis using European genome-wide association study data identified 100 significantly associated pathways ($P < 5 \times 10^{-4}$). Gene stability selection identified 116 single nucleotide polymorphisms in 26 genes that were responsible for driving the pathway associations, and gene ontology analysis demonstrated enrichment for calcium transport ($P = 1.05 \times 10^{-4}$). Three single nucleotide polymorphisms in solute carrier family 8, member 1 (*SLC8A1*), a sodium/calcium exchanger encoding NCX1, were validated in an independent Japanese genome-wide association study data set (meta-analysis $P = 0.0001$). Patients homozygous for the A (risk) allele of rs13017968 had higher rates of coronary artery abnormalities ($P = 0.029$). NCX1, the protein encoded by *SLC8A1*, was expressed in spindle-shaped and inflammatory cells in the aneurysm wall. Increased intracellular calcium mobilization was observed in B cell lines from healthy controls carrying the risk allele.

Conclusions—Pathway-based association analysis followed by gene stability selection proved to be a valuable tool for identifying risk alleles in a rare disease with complex genetics. The role of *SLC8A1* polymorphisms in altering calcium flux in cells that mediate coronary artery damage in KD suggests that this pathway may be a therapeutic target and supports the study of calcineurin inhibitors in acute KD. (*Circ Cardiovasc Genet.* 2016;9:559-568. DOI: 10.1161/CIRCGENETICS.116.001533.)

Key Words: aneurysm ■ calcium channel ■ coronary artery ■ Kawasaki disease ■ quantitative trait loci ■ sodium–calcium exchanger

Kawasaki disease (KD) is an acute, self-limited vasculitis of young children that results in coronary artery aneurysms (CAAs) in 20% of untreated patients and in ~5% of patients treated with intravenous immunoglobulin.¹ Although the pathogenesis of KD remains unknown, the current paradigm is that host genetics play an important role in susceptibility and disease outcome, with a 10- to 50-fold higher incidence in children of Asian descent compared with those of European descent.² Although the association of 6 single nucleotide polymorphisms (SNPs) with KD susceptibility has been discovered by genome-wide association studies (GWAS) and

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linkage studies and validated in different ethnic groups, these variants explain only a fraction of disease risk.³ GWAS is best suited to the discovery of strongly associated SNPs. However, in a complex genetic disease, such as KD, susceptibility may be influenced by variation in several weakly associated genes in the same biological pathway. To discover additional variants implicated in disease susceptibility and aneurysm formation, we performed a pathway-based analysis followed by gene stability selection to find the genes responsible for driving the

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pathway association (Figure 1). This approach had previously been used to identify novel susceptibility genes in rheumatoid arthritis, another inflammatory disorder with complex genetics.⁴ The top variants were further tested for association in a Japanese KD GWAS data set. Three SNPs in solute carrier family 8, member 1 (*SLC8A1*) were validated and their protein product, NCX1, studied for genotype–phenotype associations, expression in KD autopsy tissues, and patterns of gene expression in whole blood.

Materials and Methods

Subjects

The recruitment of KD patients and the details of their clinical presentation and diagnosis have been previously described.^{5,6}

The classifications of coronary artery (CA) status are described in methods in the [Data Supplement](#). The Institutional Review Boards of the participating centers reviewed and approved this study, and parental consent and assent as appropriate were obtained from parents and participants.

Control DNA and Epstein–Barr virus (EBV)-transformed B cell lines were obtained from a healthy adult population-based cohort from The Center for Applied Genomics at The Hospital For Sick Children, Toronto, Canada.

ECG Analysis

Fifty-eight ECGs were available during acute phase (illness day ≤ 16 ; AA, $n=16$; and CC, $n=42$). Fifteen lead ECGs were interpreted by 1 investigator (Dr Perry, an experienced pediatric electrophysiologist) blinded to allele status of the subject. Details are described in methods in the [Data Supplement](#).

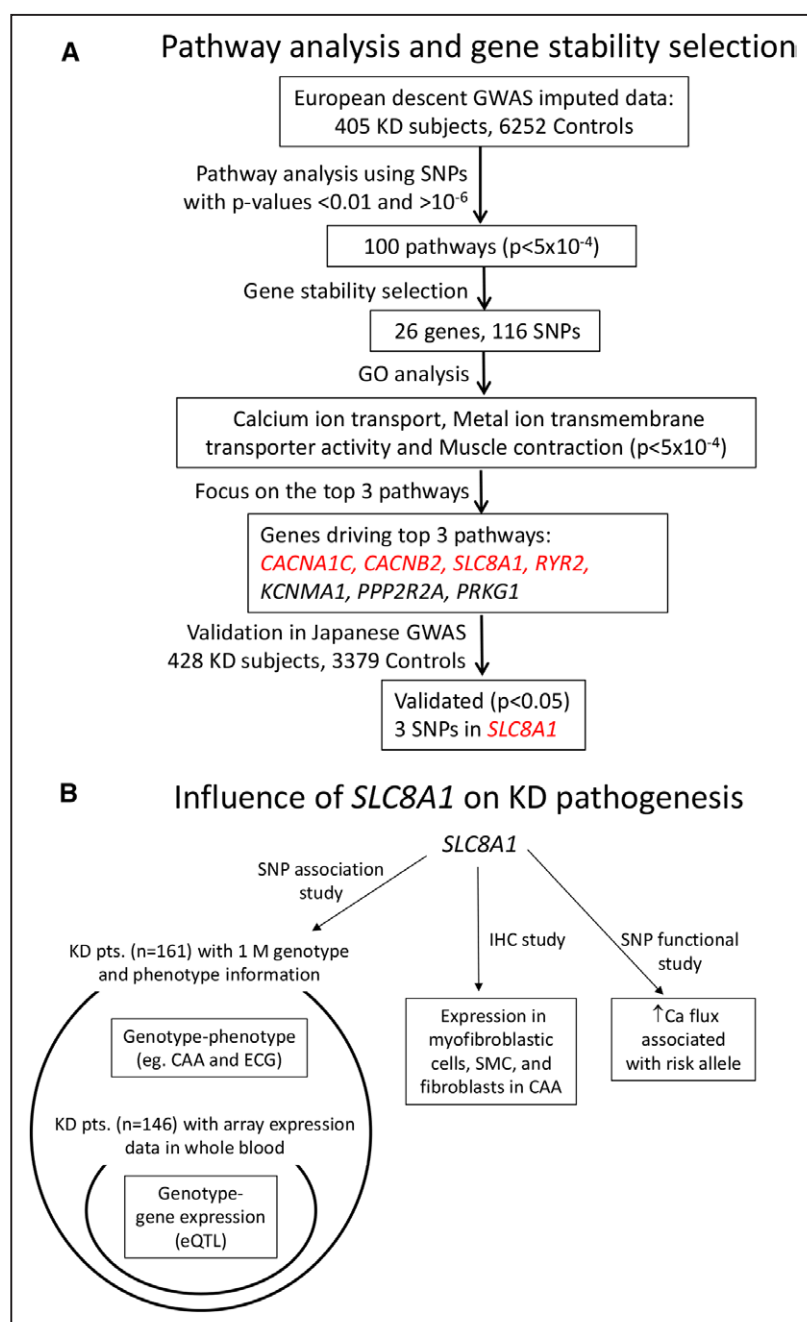


Figure 1. Workflow of study. **A**, Pathway analysis and gene stability selection. **B**, Influence of *SLC8A1* on Kawasaki disease (KD) pathogenesis. CAA indicates coronary arterial aneurysms; *CACNA1C*, calcium channel, voltage-dependent, L-type, $\alpha 1C$ subunit; *CACNA2D3*, calcium channel, voltage-dependent, $\alpha 2/\delta$ subunit 3; *CACNB2*, calcium channel, voltage-dependent, $\beta 2$ subunit; ECG, electrocardiogram; eQTL, expression quantitative trait loci; GO, gene ontology; GWAS, genome-wide association studies; IHC, immunohistochemical; *KCNMA1*, potassium channel, calcium activated large conductance subfamily M α , member 1; *PPP2R2A*, protein phosphatase 2 regulatory subunit B, α ; *PRKG1*, protein kinase, cGMP-dependent, type I; *RYR2*, ryanodine receptor 2; *SLC8A1*, solute carrier family 8, member 1; SMC, smooth muscle cells; and SNP, single nucleotide polymorphism. Font in red indicates calcium signaling pathway genes.

Imputation and Meta-Analysis Using Imputed GWAS Data

GWAS data imputation was performed in 3 steps: quality control, prephasing, and imputation.⁷ Details are described in methods in the [Data Supplement](#).

Pathway and Gene Stability Analysis

Details of the analytic strategy have been previously published.⁴ In brief, using genotype data from a GWAS comprising 405 KD patients and 6252 controls of European ancestry,⁵ we used a pathway-based analysis using our previously reported cumulative trend test statistic⁸ to assess the association between KD and the cumulative genetic variation in biological pathways, before taking the genes in the top 100 significantly associated pathways forward to a gene-based stability selection,⁴ to identify the genes driving the pathway association. We included only SNPs with a *P* value between 10^{-2} and 10^{-6} because previous experience has demonstrated that including more significant pathways leads to a greater false discovery rate. Pathways ($n=2341$, containing ≈ 9000 genes) were assembled using the Molecular Signatures Database Pathway Commons database (University of Toronto, MSKCC–Computational Biology Center), as well as custom pathways based on literature searches and Ingenuity Pathways Analysis (<http://www.ingenuity.com/>). Association between KD susceptibility and variants found by this approach were validated in a Japanese GWAS data set with 428 cases and 3379 controls.⁹

Immunohistochemical Staining of Tissue

Immunohistochemical was performed as previously described.¹⁰ Anti-human NCX1 mouse monoclonal antibody (1:100 dilution, ab2869; Abcam) or rabbit IgG (negative control) was used to stain the tissues.¹⁰

Calcium Flux Analysis by Fluorescence-Activated Cell Sorting

Intracellular calcium $[Ca^{2+}]_i$ mobilization in EBV-infected B cells was acquired using Fluo-4/AM and Fura Red (Life Technologies) after ionomycin (1 μ M) addition. Details are described in methods in the [Data Supplement](#).

Expression Quantitative Trait Loci Analysis

Systematic expression quantitative trait loci analyses using previously published transcriptome data¹¹ was assessed on the discovered SNPs by grouping subjects into 3 groups (*x* axis) using their genotypes followed by plotting the corresponding gene expression levels (*y* axis). One-way analysis of variance and *t* test were performed to test for differential expression among/between genotype groups.

Statistical Analysis

Associations between genetic variants and the Z-worst for the coronary arteries were performed using nonparametric tests because of the non-normality of the Z score. *P* values were calculated by Mann–Whitney *U* test for continuous variables and chi test or Fisher's exact *t* test for categorical variables. For the comparison of >3 variants, the Kruskal–Wallis test was used.

Results

Pathway Analysis and Gene Stability Selection

Association of the pathways with KD susceptibility was calculated using our European descent GWAS data set (405 KD subjects and 6252 controls; Figure 1).⁵ The SNPs with *P* values <0.01 and $>10^{-6}$ were included in the pathway analysis,⁴ which identified 100 pathways significantly associated with KD susceptibility with $P<5\times 10^{-4}$ (Figure 1; Table I in the [Data](#)

[Supplement](#)). This *P* value was chosen to give a good balance between truly associated pathways and false positives.⁴ Gene stability selection that was applied in an analysis of rheumatoid arthritis⁴ identified 26 genes with 116 SNPs responsible for driving the pathway association (Table II in the [Data Supplement](#)). To characterize the function of these 26 genes, we performed a gene ontology functional enrichment analysis (<https://david.ncicrf.gov/home.jsp>). This analysis revealed significant enrichment in 6 functional gene ontology terms with $P<5\times 10^{-4}$ with calcium ion transport (GO:0006816; $P=1.05\times 10^{-4}$) at the top (Table III in the [Data Supplement](#)). Six gene ontology terms included 5 calcium channel genes: calcium channel, voltage-dependent, L-type, alpha 1C subunit (*CACNA1C*), calcium channel, voltage-dependent, alpha 2/delta subunit 3 (*CACNA2D3*), calcium channel, voltage-dependent, β 2 subunit (*CACNB2*), solute carrier family 8, member 1 (*SLC8A1*), and ryanodine receptor 2 (*RYR2*) (Table III and Figure I in the [Data Supplement](#); Figure 2). The top 3 pathways (acetylcholine pathway, endothelial release factors, and nitric oxide pathways) included 218, 301, and 78 genes, respectively. The genes responsible for driving the association of the pathways with KD susceptibility comprised 7 of the 26 genes that passed gene stability selection. Of these 7 genes, 4 were calcium channel genes: *CACNA1C*, *CACNB2*, *SLC8A1*, and *RYR2* (Table 1). Four of these calcium channel genes were in the top 3 pathways compared with only 3 of 18 noncalcium channel genes ($P=0.01$). Therefore, the top-ranked 3 pathways were enriched for calcium channel genes, and this pointed to genetic variants in calcium channel genes as important in KD susceptibility.

Because there was no independent European descent cohort with which to validate the SNPs discovered by the gene stability analysis, the association of the 40 SNPs in 7 genes in the top 3 pathways was tested in an imputed Japanese GWAS data set.⁹ Three SNPs from *SLC8A1* were associated with KD susceptibility in the Japanese data set (nominal *P*: 0.005–0.0006 in European and 0.048–0.006 in Japanese; Table 2). *SLC8A1* was a member of 18 different pathways (Table I in the [Data Supplement](#)) including the top 3 pathways. The 3 validated *SLC8A1* SNPs (rs10490051, rs13017968, and rs12989852) were in the same linkage disequilibrium (LD) block, with the cluster of associated SNPs identified by the European descent GWAS (Figure 2). The validated SNPs were located 80 kb downstream from the splice donor site of the first exon that was shared by all of the 15 transcript variants of *SLC8A1* (Figure II in the [Data Supplement](#)).

Characteristics of KD Patients by Genotype

To determine if risk alleles in *SLC8A1* influenced clinical parameters and disease outcome in KD patients, as a function of genotype, we compared demographic, clinical, and laboratory data from an independent cohort of 161 well-phenotyped KD patients who were also genotyped using the Illumina 1 million SNP chip (Figure 1B). The characteristics of 161 patients grouped by *SLC8A1* rs13017968 genotype are summarized in Table 3. Subjects who were homozygous for the risk allele were also more likely to develop aneurysms/dilation (14 of 25 [56%] homozygotes for the risk allele [A] versus 40 of 136 [29%] with the AC+CC genotype; $P=0.018$) and have a higher maximum Z

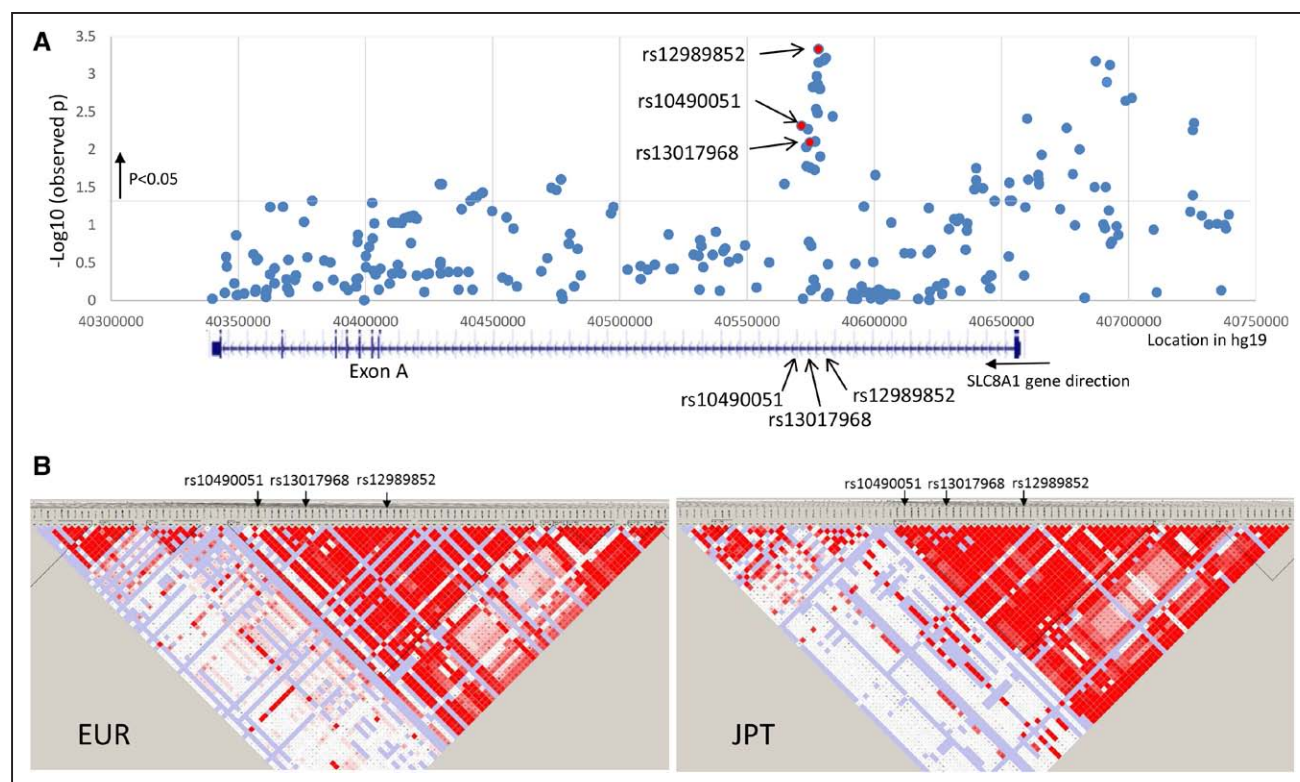


Figure 2. Association with Kawasaki disease (KD) susceptibility was validated for 3 single nucleotide polymorphism (SNPs) in *SLC8A1* in the same linkage disequilibrium (LD) block. **A**, Association results using imputed genome-wide association studies (GWAS) data were plotted against chromosome location. Red dots showed the validated SNPs (rs10490051, rs13017968, and rs12989852). *SLC8A1* is encoded on the negative strand, so the gene structure is shown 3' to 5'. **B**, Location of the 3 validated SNPs and their LD block in European decent (EUR) and in Japanese (JPT). *SLC8A1* indicates solute carrier family 8, member 1.

score (median 2.8; interquartile range 1.3–3.5 for AA genotype versus 1.7; interquartile range 1.1–2.6 for AC+CC; $P=0.049$) for the internal diameter of the right and left anterior descending coronary arteries, despite having a similar age distribution and median illness day at diagnosis compared with subjects without CAA. For a more robust analysis, we added additional patients with aneurysms ($n=91$) and normal coronary arteries ($n=92$) who were genotyped only for *SLC8A1* rs13017968 (Table IV in

the [Data Supplement](#)). Homozygotes for the risk allele ($n=55$) in this expanded cohort were more likely to develop CAAs/dilation (31/55 [56%] versus 24/55 [44%]; $P=0.029$; Table V in the [Data Supplement](#)). This suggests that *SLC8A1* rs13017968 may influence both KD susceptibility and the risk of aneurysm formation and dilatation. Although *SLC8A1* rs10490051 and rs12989852 showed a similar trend, homozygosity for these alleles was not significantly associated with aneurysm development ($P=0.08$ for rs10490051 and $P=0.2$ for rs12989852). Therefore, we focused on rs13017968 for the following genotype–phenotype analyses. A higher percentage of Asian subjects was homozygous for the risk allele compared with the other ethnic groups, which was consistent with the percentages in the 1000 Genomes database (Table VI in the [Data Supplement](#)), but no other unique characteristics were noted in subjects homozygous for the risk allele.

SLC8A1 SNPs and ECG

The association of genetic variants of *SLC8A1* with QT interval has been reported.¹² Therefore, we tested the potential association of *SLC8A1* genetic variants with ECG abnormalities in KD patients. There were no significant differences in PR, QRSd, QTc, QT dispersion, and Tp-e on the acute (pre-treatment) ECG by genotype (data not shown).

NCX1 in CAAs

Because patients homozygous for the *SLC8A1* risk allele had a higher rate of CAA formation, we explored the local

Table 1. Top 3 Significant Pathways and the Genes Responsible for Driving the Significance of the Pathway

Pathway	–log P	Total Number of Genes in the Pathway	Genes Responsible for Driving the Pathway Association
Acetylcholine pathway	11.0	218	<i>CACNA1C</i> , <i>CACNB2</i> , <i>SLC8A1</i> , <i>PPP2R2A</i> , <i>PRKG1</i> , <i>KCNMA1</i>
Endothelial release factors	9.8	301	<i>CACNA1C</i> , <i>CACNB2</i> , <i>SLC8A1</i> , <i>RYR2</i> , <i>PPP2R2A</i> , <i>PRKG1</i> , <i>KCNMA1</i>
Nitric oxide pathway	9.6	78	<i>CACNA1C</i> , <i>CACNB2</i> , <i>SLC8A1</i> , <i>PPP2R2A</i> , <i>PRKG1</i>

CACNA1C indicates calcium channel, voltage-dependent, L type, α 1C subunit; *CACNB2*, calcium channel, voltage-dependent, β 2 subunit; *KCNMA1*, potassium channel, calcium-activated large conductance subfamily M α , member 1; *PPP2R2A*, protein phosphatase 2, regulatory subunit B, α ; *PRKG1*, protein kinase, cGMP-dependent, type I; *RYR2*, ryanodine receptor 2; and *SLC8A1*, solute carrier family 8, member 1. Genes belonging to calcium ion transport (GO:0006816) are represented in bold.

Table 2. Genes and SNPs Discovered in the Gene Stability Analysis and Validated in a Japanese Cohort

Gene	Chr.	SNP	European Descent			Japanese			Combined P Value
			Risk Allele	OR	P Value	Risk Allele	OR	P Value	
<i>SLC8A1</i>	2	rs10490051	G	1.25	0.005	G	1.23	0.010	0.00015
		rs13017968	A	1.24	0.008	A	1.24	0.006	0.00014
		rs12989852	A	1.29	0.0006	A	1.18	0.048	0.00013

OR indicates odds ratio; and SNP, single nucleotide polymorphism.

expression of *SLC8A1* in KD autopsy tissues. *SLC8A1* encodes sodium calcium exchanger 1, NCX1, that is expressed on the cell membrane and functions as a bidirectional sodium/calcium channel. NCX1 is required to create a myofibroblast phenotype.¹³ We performed immunohistochemical staining on the coronary artery from a 3-month-old white male (CC wild-type homozygous at rs13017968) who died on illness day 12 with CAA. In these tissues, we previously demonstrated myofibroblast-like spindle-shaped cells.¹⁰ We also stained coronary artery tissue from a 3-year, 7-month-old white male (genotype unavailable because of unamplifiable DNA in formalin-fixed tissue) who died on illness day 7. NCX1 was expressed on spindle-shaped cells with a myofibroblast phenotype in the thickened intima (Figure 3A1 and 3C1), smooth muscle cells (Figure 3A2 and 3C2), and fibroblast-like, spindle-shaped cells in the adventitia (Figure 3A3 and 3C3). NCX1 expression was also detected in round, inflammatory cells infiltrating the arterial wall (Figure 3A4 and 3C4). The coronary arterial wall from a 9-month-old infant who died of acute pneumonia and, thus, served as a control showed no NCX1 staining in the intima, media, or adventitia (Figure 3E1 through 3E3) and no infiltration of inflammatory cells. Cardiomyocytes from KD autopsies, but not the control patient, also showed positive staining for NCX1 (Figure 3G through 3L).

Intracellular Calcium Mobilization as a Function of Genotype

To determine the role of NCX1 on intracellular calcium $[Ca^{2+}]_i$ levels and its mobilization, healthy adult controls from a population-based biorepository were genotyped at rs13017968, and EBV-transformed B cells from individuals with the AA (n=3), AC (n=2), and CC (n=3) genotypes were selected for functional assays. Cells were loaded with the ratiometrically opposite Ca^{2+} indicator dyes Fluo4AM and Fura3AM, and $[Ca^{2+}]_i$ levels were measured using flow cytometry. At the basal level, cells with the AA genotype showed increased $[Ca^{2+}]_i$ when compared with AC and CC genotypes (Figure 4), suggesting that the NCX1 AA genotype has a decreased ability to exclude Ca^{2+} from the intracellular compartment in the resting state in transformed B cell lines. Furthermore, when stimulated with ionomycin, the B cells with the AA genotype showed increased $[Ca^{2+}]_i$ flux when compared with cells with the AC and CC genotypes (Figure 4), suggesting an exaggerated response to stimuli in the AA genotype, resulting in a marked increase in $[Ca^{2+}]_i$ with stimulation. Thus, the polymorphism at rs13017968 is associated with a functional difference in regulation of $[Ca^{2+}]_i$ at rest and after stimulation.

Whole Blood Transcriptome Analysis in KD Patients

A subset of the 161 genotyped KD subjects (n=146) were also analyzed by microarray, which allowed expression quantitative trait loci analysis for the risk alleles in *SLC8A1* (Figure 1B). Patients homozygous for the risk allele had increased expression of solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3 (*SLC13A3*; $P=9.8 \times 10^{-5}$), gliomedin (*GLDN*; $P=0.0004$), claudin 8 (*CLDN8*; $P=0.0009$), and urotensin-2 (*UTS2*; $P=0.002$) and decreased expression of 2 pore segment channel 2 (*TPCN2*; $P=0.007$; Figure 5A through 5E). *SLC8A1* transcript levels in whole blood were not influenced by *SLC8A1* rs13017968 genotype, although we cannot rule out an effect of the risk allele on gene expression in other tissues (Figure 5F). No differential expression of *SLC8A1* by genotype was noted in the EBV-transformed B cells used for the calcium flux experiments, and the GTEx database (<http://www.gtexportal.org/home/>) showed no influence of the rs13017968 genotype on *SLC8A1* expression in different cell types listed in the database (data not shown). Although individuals who were homozygous for the *SLC8A1* risk allele were more likely to develop aneurysms and the risk allele influenced transcript levels of *SLC13A3*, *GLDN*, *CLDN8*, *UTS2*, and *TPCN2*, transcript levels for these genes were not correlated with aneurysm formation or treatment response (data not shown).

Computational Analysis of SNP Function

To search possible cell type-specific functions of the 3 SNPs of *SLC8A1*, we retrieved annotations from Encode and Roadmap using HaploReg (http://www.broadinstitute.org/mammals/haploreg/haploreg_v3.php). The 3 SNPs were predicted to lie in an enhancer region in mesenchymal cells, cardiomyocytes, and fibroblasts. However, the specific effect of the different alleles in these SNPs on enhancer function has not been determined (Table VII in the Data Supplement).

Discussion

Pathway analysis followed by gene stability selection suggested the importance of calcium channel genes in KD susceptibility and identified variants in *SLC8A1* that were independently replicated in a Japanese cohort and were associated with both susceptibility to KD and aneurysm formation. Individuals who were homozygous for the risk allele had a higher rate of CAA. The cluster of intronic SNPs were located in an enhancer region that is active in mesenchymal cells and cardiomyocytes. B cell lines homozygous for the risk allele had higher levels of calcium mobilization. NCX1, the gene

Table 3. Characteristics of Subjects (n=161) Stratified by Genotype of *SLC8A1* rs13017968

	<i>SLC8A1</i> rs13017968		P Value*
	AA, n=25	AC+CC, n=136	
Male, n (%)	14 (56)	82 (60)	NS
Age, y (IQR)	2.0 (0.8–4.2)	2.7 (1.5–4.1)	NS
Illness day, median (range)†	6 (3–10)	6 (2–10)	NS
Ethnicity, n (%)‡			
Asian	11 (44)	17 (13)	NA
Black	0 (0)	6 (4)	NA
White	3 (12)	39 (29)	NA
Hispanic	6 (24)	36 (26)	NA
More than one race	5 (20)	38 (28)	NA
Coronary artery status			
Aneurysms, n (%)	5 (20)§	13 (10)	0.018
Dilated, n (%)	9 (36)	27 (20)	
Normal, n (%)	11 (44)	96 (70)	
Z-worst, median (IQR)	2.8 (1.3–3.5)	1.7 (1.1–2.6)	0.0497
IVIg resistance, n (%)	3 (15)	32 (24)	NS
Pretreatment laboratory data, median (IQR)			
WBC, $\times 10^3/\text{mm}^3$	14 (12.3–18.7)	13.6 (10.7–18.6)	NS
Absolute lymph, $\times 10^3/\text{mm}^3$	3.2 (1.8–4.2)	2.8 (1.6–4.5)	NS
Absolute mono, $\times 10^3/\text{mm}^3$	0.8 (0.5–1.1)	0.6 (0.4–1.1)	NS
Platelet count, $\times 10^3/\text{mm}^3$	448 (284–578)	396 (313–467)	NS
ESR, mm/h	61 (48–90)	60 (44–79)	NS
CRP, mg/dL	12.5 (6.4–17.5)	8.0 (4.9–15.4)	NS

A is the risk allele. CRP indicates C-reactive protein; ESR, erythrocyte sedimentation rate; IVIg, intravenous immunoglobulin G therapy; NA, not applicable; NS, not significant; and WBC, white blood cell count.

*P values were calculated by Mann–Whitney U test for continuous variables and chi square test for categorical variables except ethnicity comparison.

†Illness day 1: first calendar day of fever.

‡The frequencies of individuals homozygous for the risk allele in the 1000 genome database were as follows: East Asians, 40.3%; European descent, 6.6%; and Hispanic, 10.4%.

§Ethnicities of 5 patients were 2 Asians, 2 Hispanics, and 1 mixture of Asian and white.

||Fisher's exact *t* test. Aneurysm+dilated compared with normal. Relative risk, 1.9; 95% confidence interval, 1.2–2.9.

product of *SLC8A1*, was expressed in inflammatory and myofibroblast-like cells in the arterial wall and in cardiomyocytes from KD autopsy tissues.

Pathway Analysis

Complex genetic diseases, such as KD, may be influenced by the cumulative effect of many variants in diverse biological pathways. These genetic variants, each with small effects, may not be identified by standard single SNP association analysis of GWAS data. In addition, different individuals may

be susceptible to a disease because of genetic variants in different genes in the same pathway that lead to a similar overall biological effect. To overcome this challenge, we performed a pathway analysis using the same methodology as previously described for rheumatoid arthritis.⁴ The KD pathway-based analysis with gene stability selection identified calcium channel genes in the top 3 pathways responsible for the association with KD susceptibility. This suggested that the calcium signaling pathway was a key player in influencing susceptibility to KD. Four of the 5 calcium signaling pathway genes (*CACNA1C*, *CACNB2*, *CACNA2D3*, and *RYR2*) were not validated in the Japanese GWAS data. This could be because of the marked differences in LD structure between subjects of European and Asian descent at these loci and to differences in allele frequencies between the populations tested (Figure III and Table VI in the [Data Supplement](#)). Further validation of these genetic variants in cohorts of European descent is required. Pathway-based analysis with gene stability selection may be a helpful tool to discover genetic variants in uncommon, complex genetic diseases, such as KD, which is rare enough that it is difficult to recruit cohorts of sufficient size for robust meta-analyses to identify risk alleles.

Calcium Signaling Pathways in KD

Calcium signaling pathways affect diverse cellular processes in different cell types, including lymphocytes, macrophages, endothelial cells, fibroblasts, and vascular smooth muscle cells, all of which play important roles in KD pathogenesis.^{14,15} Each cell type uses a unique set of components from the calcium signaling toolbox to generate signals with different spatial and temporal properties.¹⁶ Importantly, polymorphisms in 3 calcium pathway genes, *ITPKC*, *ORAI1*, and *SLC8A1*, have now been validated to be associated with KD susceptibility and aneurysm formation.^{17,18} The *ITPKC* rs28493229 was excluded from the pathway analysis to avoid false-positive results because of the strong association of *ITPKC* with KD. We were unable to confirm an association with the *ORAI1* rs3741596 reported in Japanese cohorts because of the low (<1%) risk allele frequency of the *ORAI1* SNP in individuals of European and Hispanic descent.

SLC8A1 Polymorphisms

The 3 validated SNPs in *SLC8A1* are located 172 kb 5' upstream of exon A, 1 of 6 exons (A–F) that are differentially spliced to create 15 transcript variants (Figure II in the [Data Supplement](#)).¹⁹ Tissue-specific expression of these spliced variants has been reported.¹⁹ Of note, the validated genetic variants of *SLC8A1* are predicted to be in a regulatory region in smooth muscle cells and cardiomyocytes that use exon A. The risk allele frequency (A allele) of rs13017968 is 0.66 in Asians and 0.28 in European descendants. Although susceptibility to KD is 10- to 50-fold higher in Japanese compared with various European descent populations, the incidence of aneurysms is similar.²⁰ Combinations of different genetic variants in the same pathway may influence aneurysm formation in different ethnic and racial groups.

Gene Expression and Expression Quantitative Trait Loci

The *SLC8A1* variants reported here did not influence the gene's transcript levels in whole blood. However, *SLC8A1*

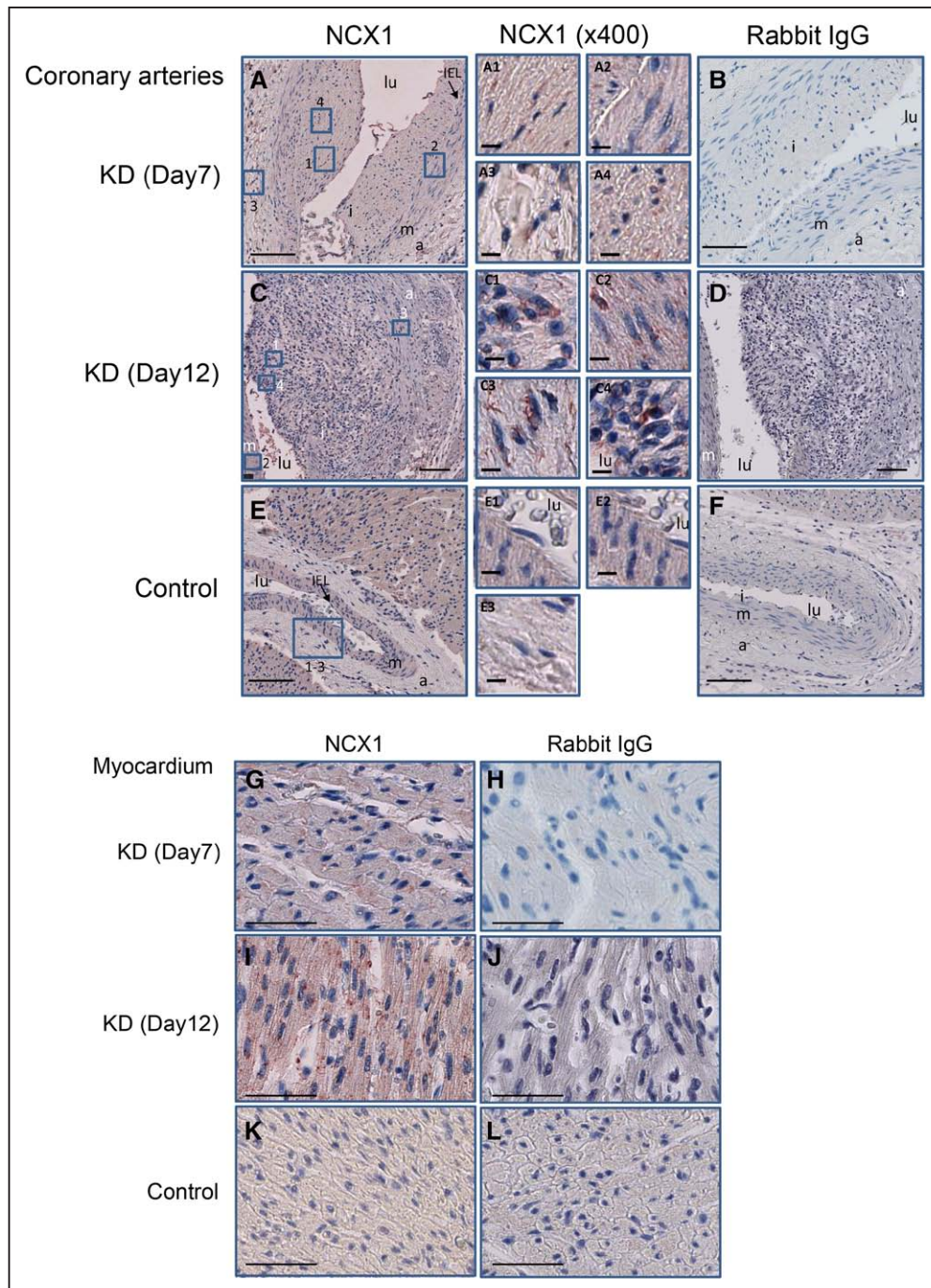


Figure 3. Immunohistochemical staining of aneurysmal wall from Kawasaki disease (KD) autopsy. Aneurysmal wall of coronary arteries (**A–D**) and myocardium (**G–J**) from KD autopsies of a 3-year, 7-month-old child who died on illness day 7 (**A**, **B**, **G**, and **H**) and a 3-month-old infant who died on illness day 12 (**C**, **D**, **I**, and **J**) and a control coronary artery (**E** and **F**) and myocardium (**K** and **L**) from a 9-month-old infant who died from pneumonia were stained with sodium calcium exchanger (NCX1) antibody (**A**, **C**, **E**, **G**, **I**, and **K**) and rabbit IgG for control (**B**, **D**, **F**, **H**, **J**, and **L**). Squares 1 to 4 in **A**, **C**, and **E** are magnified ($\times 400$) in **A1–A4**, **C1–C4**, and **E1–E3**. NCX1 was expressed in spindle-shaped cells in the intima (**A1** and **C1**), smooth muscle cells (**A2** and **C2**), fibroblasts (**A3** and **C3**), and small round inflammatory cells (**A4** and **C4**). Coronary artery from control patient showed no staining for NCX1 (**E1–E3**). Cardiomyocytes from KD autopsies showed positive staining of NCX1 but not from infant who died from pneumonia (**G**, **I**, and **K**). a indicates adventitia; i, intima; IEL, internal elastic lamina; lu, lumen; m, media. Black bars: 100 μ m for **A–F**, 10 μ m for magnified photos and 50 μ m for **G–L**.

SNP rs13017968 was a quantitative trait locus that correlated with transcript levels of 5 genes, *SLC13A3*, *GLDN*, *CLDN8*, *UTS2*, and *TPCN2*. Of these genes, only *UTS2* has a direct link to cardiovascular pathology. *UTS2*, the most potent vasoconstrictor in humans, influences myofibroblast

formation in rat ventricular fibroblasts, leading to fibrosis.^{21–23} *UTS2* also influences inflammation as a chemoattractant in CD14⁺ monocytes.²⁴ Therefore, increased *UTS2* levels in the patients with AA risk allele homozygous of rs13017968 may play a role in KD pathogenesis. Studies

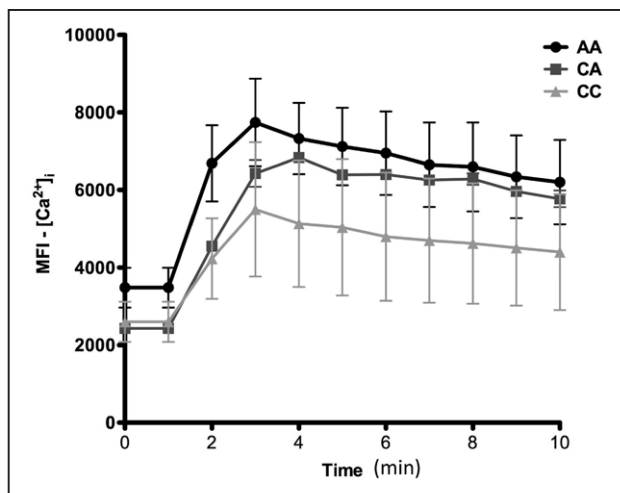


Figure 4. *SLC8A1* genotype is associated with differences in intracellular Ca^{2+} levels at rest and with stimulation. Mean fluorescence intensity (MFI) of Fluo-4 AM (y axis) acquired by fluorescence-activated cell sorting (FACS) of EBV transfected B cells from various *SLC8A1* genotypes (AA [n=3], CA [n=2], and CC [n=3]) plotted against time (x axis). Graph represents average MFI of 3 repeats from each *SLC8A1* genotypes. *SLC8A1* indicates solute carrier family 8, member 1.

are in progress to determine if *SLC8A1* risk allele carriers may have increased vascular inflammation mediated in part through *UTS2*.

Potential Role of NCX1 in KD Pathogenesis

NCX1, the gene product of *SLC8A1*, influences many cellular processes that are important in KD pathogenesis. KD subjects who were homozygous for the *SLC8A1* risk allele were more likely to develop CAAs. NCX1 localizes to the cell membrane where it functions as a bidirectional sodium/calcium exchanger. In primary human lung macrophages and circulating monocytes cultured in sodium-free medium, NCX1 mediates calcium influx and generation of tumor necrosis factor- α , a process that can be blocked by NCX1 inhibition.²⁵ Tumor necrosis factor- α is known to be an important proinflammatory cytokine in acute KD, and levels are highest in patients who develop CAA.²⁶ NCX1 is expressed on cells of mesenchymal origin, including fibroblasts, smooth muscle cells, and myofibroblasts, that all play key roles in aneurysm formation in KD patients.^{10,13,27–29} Transforming growth factor- β signaling leads to calcium flux in fibroblasts through NCX1 and results in increased expression of endothelial-mesenchymal transition (EMT)-related genes, including connective tissue growth factor and smooth muscle actin.¹³ We have previously reported that myofibroblast-like cells expressing connective tissue growth factor in the wall of CAA and NCX1 may influence this process. NCX1 also regulates cell motility, which was a key upregulated pathway in a transcriptomic analysis of acute and convalescent whole blood samples from KD patients.^{11,28,29}

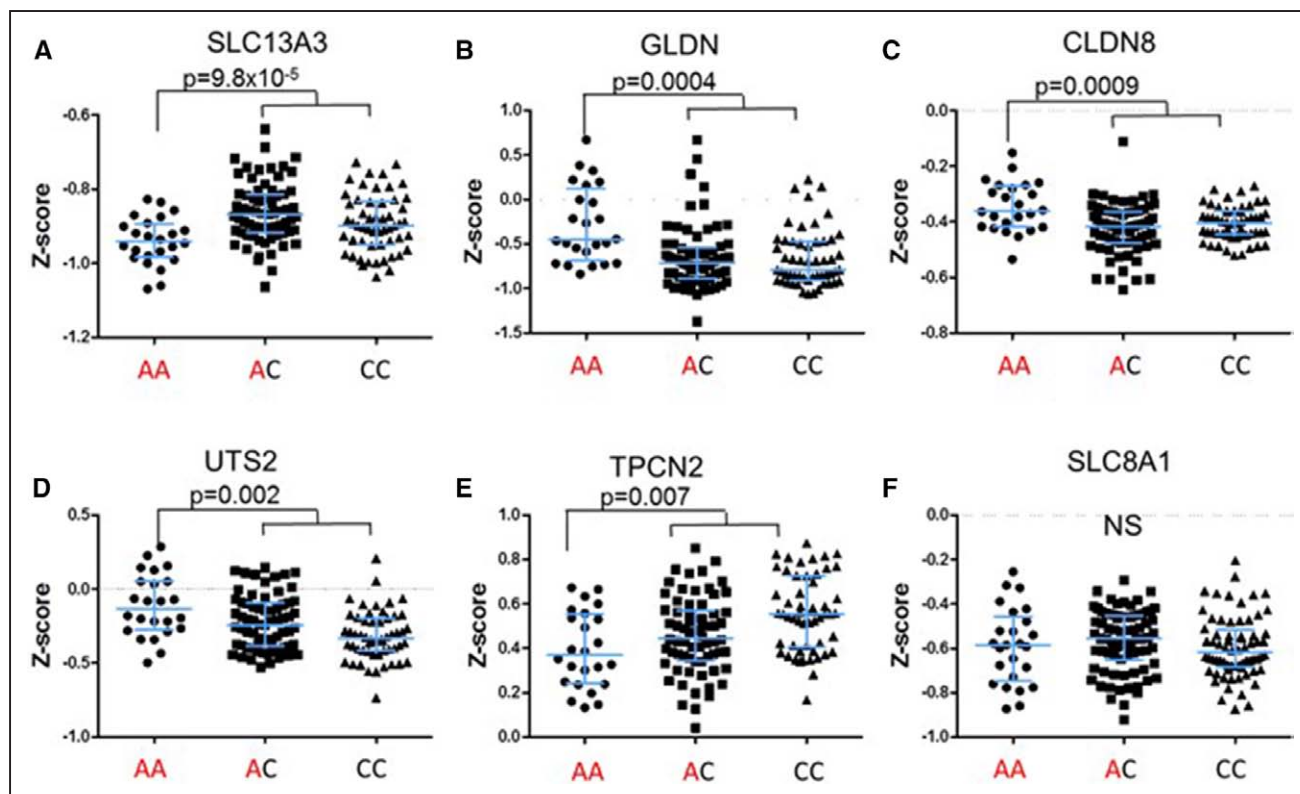


Figure 5. Transcript levels in whole blood. Transcript levels in whole blood during acute phase stratified by *SLC8A1* rs13017968 genotype (AA [n=24], AC [n=68], CC [n=54]) for *SLC13A3* (A), *GLDN* (B), *CLDN8* (C), *UTS2* (D), *TPCN2* (E), and *SLC8A1* (F). *P* values were calculated using Kruskal-Wallis test for A–F. The boxes go from the first quartiles to the third quartiles, the horizontal lines in the boxes are drawn at the median and the whiskers go from the minimum to the maximum. *SLC13A3*, located on the basolateral membrane of epithelial cells and transports dicarboxylates in a sodium-dependent manner. *GLDN*, an adhesion molecule that plays a central role in the formation of nodes of Ranvier and myelin sheath gaps. *CLDN8*, a component of tight junction strands. *TPCN2*, localizes to lysosomal membranes and enables nicotinic acid adenine dinucleotide phosphate (NAADP)-induced calcium ion release from lysosomal stores. See text for *UTS2* and *SLC8A1*.

Therapeutic Implications

Blocking the calcium signaling pathway may reduce acute inflammation in patients with KD. Cyclosporine inhibits not only calcineurin, thus, blocking the phosphorylation of the transcription factor nuclear factor of activated T cells (NFAT) but also directly inhibits NCX1 expression on the cell membrane by inhibiting protein folding by cyclophilin.³⁰ Fluvastatin, an HMG-CoA reductase inhibitor (statin), decreased NCX1 mRNA and protein by inhibiting a small G protein, RhoB, in the cardiomyoblast cell line, H9c2.³¹ Conversely, lysophosphatidylcholine increased NCX1 mRNA and protein by activating RhoB.³² L-type calcium channel blockers, such as amlodipine and verapamil, have anti-inflammatory effects, possibly mediated through blocking monocyte activation.³³ Clinical trials of both cyclosporine and atorvastatin (NCT01431105) are in progress in KD patients in Japan and in the United States.^{34–36} The potential for these agents to modulate inflammation in acute KD patients may be mediated, in part, through decreased expression of NCX1.

Strengths and Limitations

Pathway analysis allowed us to find genetic variants that were not discovered by GWAS in European descent and Japanese KD cohorts. Because there were no available European descent cohorts in which to perform validation, we used a Japanese cohort to test for association. Differences in LD structure between the 2 populations may have prevented us from validating additional variants identified through the pathway and gene stability analyses. The limited size of our cohort precluded multigene analyses or testing for SNP interactions within the calcium signaling pathway. In addition, it is likely that genetic variants in multiple members of calcium signaling pathways, such as ITPKC and ORA1, combine with SLC8A1 to influence KD susceptibility.³ Creation of genetic risk scores to encompass the contribution of multiple genetic variants will likely be a productive research avenue. To achieve these goals, we will need expanded cohorts and broad collaboration to permit robust validation of initial findings.

Conclusions

Pathway analysis with gene stability selection is a powerful tool to identify genes that influence susceptibility to complex genetic diseases. Variants in genes in the calcium signaling pathway are associated with both KD susceptibility and disease outcome. The association of SNPs in *SLC8A1* with KD susceptibility was confirmed in a Japanese cohort. The gene product of *SLC8A1*, NCX1, was expressed in spindle-shaped cells and smooth muscle cells in the vascular wall and myocardium from KD autopsies. This sodium/calcium channel protein is a therapeutic target for which candidate drugs are already under study. Translation of these findings into new therapies will be an important step toward improving outcomes for KD patients.

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Disclosures

None.

Appendix

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CLINICAL PERSPECTIVE

Genome-wide association studies can detect only strongly associated genetic variants. However, weakly associated genes may play an important role in a complex disease, such as Kawasaki disease. Pathway analysis with gene stability selection using existing genome-wide association study data is a powerful tool to identify weakly associated genes in the same biological pathway. Using this method, we found that the most important biological pathway responsible for Kawasaki disease susceptibility was the calcium signaling pathway. Association of single nucleotide polymorphisms in *SLC8A1*, the gene that encodes sodium calcium exchanger, with Kawasaki disease susceptibility was established in both European descent and Japanese cohorts. This single nucleotide polymorphism was also associated with coronary artery abnormality in the European descent cohorts. The *SLC8A1* calcium signaling pathway may be a therapeutic target and supports the study of calcineurin inhibitors in acute Kawasaki disease.