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 ($P<5\times10^{-4}$). 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 ($P=1.05\times10^{-4}$). 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 $\approx 5\%$ of patients treated with intravenous immunoglobulin. 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. 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. 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
pathway association (Figure 1). This approach had previously been used to identify novel susceptibility genes in rheumatoid arthritis, another inflammatory disorder with complex genetics.1 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

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.
Imputation and Meta-Analysis Using Imputed GWAS Data

GWAS data imputation was performed in 3 steps: quality control, prephasing, and imputation. Details are described in methods in the Data Supplement.

Pathway and Gene Stability Analysis

Details of the analytic strategy have been previously published. In brief, using genotype data from a GWAS comprising 405 KD patients and 6252 controls of European ancestry, we used a pathway-based analysis using our previously reported cumulative trend test statistic 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, 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.

Immunohistochemical Staining of Tissue

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

Calcium Flux Analysis by Fluorescence-Activated Cell Sorting

Intracellular calcium [Ca^{2+}] mobilization in EBV-infected B cells was acquired using Fluo-4/AM and Fura Red (Life Technologies) after ionomycin (1 μM) 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 data 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). The SNPs with P values <0.01 and >10^-6 were included in the pathway analysis, which identified 100 pathways significantly associated with KD susceptibility with P<5x10^-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. Gene stability selection that was applied in an analysis of rheumatoid arthritis 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.ncifcrf.gov/home.jsp). This analysis revealed significant enrichment in 6 functional gene ontology terms with P<5x10^-4 with calcium ion transport (GO:0006816; P=1.05x10^-2) at the top (Table III 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
score (median 2.8; interquantile range 1.3–3.5 for AA genotype versus 1.7; interquantile 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.\[\text{SLC8A1 SNPs and ECG}\]

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 (pretreatment) ECG by genotype (data not shown).

\[\text{NCX1 in CAAs}\]

Because patients homozygous for the \( SLC8A1 \) risk allele had a higher rate of CAA formation, we explored the local...
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 rs1313017968) who died on illness day 12 with CAA. In these tissues, we previously demonstrated myofibroblast-like spindle-shaped cells.16 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 3C3). The coronary arterial wall from a 9-month-old infant who died of acute pneumonia and, thus, served as a control patient, also showed positive staining for NCX1 (Figure 3G and 3C4). NCX1 expression was also detected in round, inflammatory cells infiltrating the arterial wall (Figure 3A1 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 fluorescing dyes Fluo4AM and Fura3AM, and EBV-transformed B cells from individuals with the AA genotype showed increased $[Ca^{2+}]_{i}$ levels when compared with 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=9.8x10^{-12})*, glomulin (*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*). *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 2. Genes and SNPs Discovered in the Gene Stability Analysis and Validated in a Japanese Cohort

| Gene   | Chr. | SNP          | European Descent | Japanese | Combined |
|--------|------|--------------|------------------|----------|----------|
|        |      | Risk Allele | OR    | P Value | Risk Allele | OR    | P Value | OR    | P Value |
| SLC8A1 | 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.
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. 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. Each cell type uses a unique set of components from the calcium signaling toolbox to generate signals with different spatial and temporal properties. Importantly, polymorphisms in 3 calcium pathway genes, ITPKC, ORAI1, and SLC8A1, have now been validated to be associated with KD susceptibility and aneurysm formation. 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). Tissue-specific expression of these spliced variants has been reported. Of note, the validated genetic variants of SLC8A1 are predicted to be in a regulatory region in smooth muscle cells of different cell types, including lymphocytes, macrophages, 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. 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
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.\textsuperscript{21–23} UTS2 also influences inflammation as a chemoattractant in CD14\textsuperscript{+} monocytes.\textsuperscript{24} Therefore, increased UTS2 levels in the patients with AA risk allele homozygous of rs13017968 may play a role in KD pathogenesis. Studies
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 4.** SLC8A1 genotype is associated with differences in intracellular Ca²⁺ 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.

**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.34 Fluvasatin, an HMG-CoA reductase inhibitor (statin), decreased NCX1 mRNA and protein by inhibiting a small G protein, RhoB, in the cardiomyoblast cell line, H9c2.33 Conversely, lysophosphatidylcholine increased NCX1 mRNA and protein by activating RhoB.32 L-type calcium channel blockers, such as amiodarone 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 GWASs 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 for 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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