Title:

Genetic variation in the SLC8A1 calcium signaling pathway is associated with susceptibility to Kawasaki disease and coronary artery abnormalities

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Running title: Genetic variation in SLC8A1 in Kawasaki disease

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Abstract

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 (GWAS) 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 GWAS dataset. Pathway analysis using European GWAS data identified 100 significantly associated pathways ($p< 5 \times 10^{-4}$). Gene stability selection identified 116 single nucleotide polymorphisms (SNPs) 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 SNPs in solute carrier family 8 member 1 (*SLC8A1*), a sodium/calcium exchanger encoding NCX1, were validated in an independent Japanese GWAS dataset (metaanalysis 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.

Conclusion

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.

Keywords:

Kawasaki disease, aneurysm, pathway analysis, calcium signaling pathway, sodium calcium exchanger

Introduction

Kawasaki disease (KD) is an acute, self-limited vasculitis of young children that results in coronary artery aneurysms (CAA) in 20% of untreated patients and ~5% of patients treated with intravenous immunoglobulin (IVIG)[1]. Although the etiology of KD remains unknown, the current paradigm is that host genetics play an important role in susceptibility and disease outcome with a 10-50-fold higher incidence in children of Asian descent compared to those of European descent[2]. Although the association of six single nucleotide polymorphisms (SNPs) with KD susceptibility has been discovered by genome-wide association studies (GWAS) and 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 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 dataset. 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 CA status are described in the Supplemental methods. 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 EBV transformed B-cell lines were obtained from a healthy adult population-based cohort from The Centre for Applied Genomics (TCAG) 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 one investigator (JCP, an experienced pediatric electrophysiologist), blinded to allele status of the subject. Details are described in supplemental methods..

Imputation and meta-analysis using imputed GWAS data

GWAS data imputation was performed in three steps: quality control (QC), pre-phasing, and imputation[7]. Details are described in supplemental methods.

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 6,252 controls of European ancestry[5], we employed a pathway-based analysis using our previously reported cumulative trend test statistic[8] 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} as 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 (MSigDB) 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 dataset with 428 cases and 3379 controls[9].

Immunohistochemical (IHC) staining of tissue

IHC 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 (FACS)

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

Expression quantitative trait loci (eQTL) analysis

Systematic eQTL analyses using previously published transcriptome data[11] was assessed on the discovered SNPs by grouping subjects into three groups (x-axis) using their genotypes followed by plotting the corresponding gene expression levels (y-axis).

One-way ANOVA 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 non-parametric tests due to 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 more than three 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 dataset (405 KD subjects, 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 $p < 5x10^{-4}$ (Figure 1, Supplemental Table S1). 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 arthritis[4] identified 26 genes with 116 SNPs responsible for driving the pathway association (Supplemental Table S2). In order to characterize the function of these 26 genes, we performed a gene ontology (GO) functional enrichment analysis (https://david.ncifcrf.gov/home.jsp). This analysis revealed significant enrichment in six functional GO terms with $p<5x10^{-4}$ with calcium ion transport (GO:0006816, $p=1.05x10^{-4}$) at the top (Supplemental Table S3). Six GO terms included five 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, beta 2 subunit (*CACNB2*), Solute Carrier Family 8, Member 1 (*SLC8A1*), and ryanodine receptor 2 (*RYR2*) (Supplemental Table S3 and Supplemental Figure S1 and Figure 2). The top three pathways (acetylcholine pathway, endothelial release factors, and nitric oxide (NO) pathways) included 218, 301, and 78 genes, respectively. The genes responsible for driving the association of the pathways with KD susceptibility comprised seven of the 26 genes that passed gene stability selection. Of these seven genes, four were calcium

channel genes: *CACNA1C, CACNB2, SLC8A1* and *RYR2* (Table 1). Four of these calcium channel genes were in the top three pathways compared to only three of 18 non-calcium channel genes (p=0.01). Therefore, the top ranked three pathways were enriched for calcium channel genes and this pointed to genetic variants in calcium channel genes as important in KD susceptibility.

Since 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 seven genes in the top three pathways was tested in an imputed Japanese GWAS dataset[9]. Three SNPs from *SLC8A1* were associated with KD susceptibility in the Japanese dataset (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 (Supplemental Table S1) including the top three pathways. The three validated *SLC8A1* SNPs (rs10490051, rs13017968 and rs12989852) were in the same LD block with the cluster of associated SNPs identified by the European descent GWAS (Figure 2). The validated SNPs were located 80kb downstream from the splice donor site of the first exon that was shared by all of the15 transcript variants of SLC8A1 (Supplemental Figure S2).

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) vs. 40 of 136 (29%) with the AC+CC genotype) (p=0.018) and have a higher maximum Z score (median 2.8 (inter-quantile range (IQR) 1.3-3.5) for AA genotype vs. 1.7 (1.1-2.6) AC+CC, p=0.049) for the internal diameter of the right (RCA) and left anterior descending (LAD) coronary arteries despite having a similar age distribution and median illness day at diagnosis compared to 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 (Supplemental TableS4). Homozygotes for the risk allele (n=55) in this expanded cohort were more likely to develop coronary artery aneurysms/dilation (31/55 (56%) vs. 24/55 (44%) p=0.029) (Supplemental TableS5). This suggests that *SLC8A1* rs13017968 may influence both KD susceptibility as well as 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 to the other ethnic groups, which was consistent with the percentages in the 1,000 Genomes database (Supplemental Table S6), 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 electrocardiogram (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 coronary artery aneurysms

Since patients homozygous for the *SLC8A1* risk allele had a higher rate of coronary artery aneurysm 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 bi-directional 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 Caucasian 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[10]. We also stained coronary artery tissue from a 3y 7m-old Caucasian male (genotype unavailable due to 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 3 A1, C1), smooth muscle cells (Figure 3 A2, C2) and fibroblast-like, spindle-shaped cells in the adventitia (Figure 3 A3, C3). NCX1 expression was also detected in round, inflammatory cells infiltrating the arterial wall (Figure 3 A4, C4). 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 3 E1-3) and no infiltration of inflammatory cells. Cardiomyocytes from KD autopsies, but not the control patient, also showed positive staining for NCX1 (Figure 3 G-L).

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 to 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 rs1313017968 is associated with a functional difference in regulation of $[Ca^{2+}]_i$ at rest and following stimulation.

Whole blood transcriptome analysis in KD patients

A subset of the 161 genotyped KD subjects (n=146) were also analysed by microarray, which allowed eQTL 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*) ($p=9.8x10^{-5}$), gliomedin (*GLDN*) (p=0.0004), claudin 8 (*CLDN8*) (p=0.0009), urotensin-2 (*UTS2*) (p=0.002) and decreased expression of two pore segment channel 2 (*TPCN2*)

(p=0.007) (Figure 5A-E). *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 three SNPs of *SLC8A1*, we retrieved annotations from Encode and Roadmap using HaploReg (http://www.broadinstitute.org/mammals/haploreg/haploreg_v3.php).The three 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 (Supplemental TableS7).

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 associate 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 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 due to genetic variants in different genes in the same pathway that lead to a similar overall biologic 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 three 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 five calcium signaling pathway genes (*CACNA1C, CACNB2, CACNA2D3* and *RYR2*) were not validated in the Japanese GWAS data. This could be due to 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 (Supplemental Figure S3, Supplemental Table S6). 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 three calcium pathway genes, ITPKC, ORAI1, and SLC8A1, have now been validated to be associated with KD susceptibility and/or aneurysm formation[17, 18]. The ITPKC rs28493229 was excluded from the pathway analysis to avoid false positive results due to 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 very low (<1%) risk allele frequency of the ORAI1 SNP in individuals of European and Hispanic descent.

***SLC8A1* polymorphisms**

The three validated SNPs in *SLC8A1* are located 172Kb 5' upstream of Exon A, one of six exons (A-F) that are differentially spliced to create 15 transcript variants

(Supplementary Figure S2)[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 to 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 eQTL

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 five 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 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 coronary artery aneurysms. NCX1 localizes to the cell

membrane where it functions as a bi-directional sodium/calcium exchanger. In primary human lung macrophages and circulating monocytes cultured in sodium-free medium, NCX1 mediates calcium influx and generation of TNFα, a process that can be blocked by NCX1 inhibition[25]. TNFα is known to be an important pro-inflammatory 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]. TGFβ signaling leads to calcium flux in fibroblasts through NCX1 and results in increased expression of EMT-related genes including connective tissue growth factor (CTGF) and smooth muscle actin[13]. We have previously reported myofibroblast-like cells expressing CTGF 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].

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 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 (LPC) 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 the U.S.[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 two populations may have prevented us from validating additional variants identified through the pathway and gene stability analyses. The limited size of our cohort precluded multi-gene 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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Disclosure

None

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Table 1. Top three 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	***CACNA1C, CACNB2, SLC8A1****, PPP2R2A, PRKG1, KCNMA1*
Endothelial release factors	9.8	301	***CACNA1C, CACNB2, SLC8A1****,* ***RYR2,*** *PPP2R2A, PRKG1, KCNMA1*
Nitric Oxide pathway	9.6	78	***CACNA1C, CACNB2, SLC8A1****, PPP2R2A, PRKG1*

CACNA1C: calcium channel, voltage-dependent, L type, alpha 1C subunit, *CACNB2*: calcium channel, voltage-dependent, beta 2 subunit, *SLC8A1*: Solute Carrier Family 8, Member 1, *PPP2R2A*: protein phosphatase 2, regulatory

subunit B, alpha, *PRKG1*: protein kinase, cGMP-dependent, type I, *KCNMA1*: potassium channel, calcium activated large conductance subfamily M alpha, member 1, *RYR2*: ryanodine receptor 2, Genes belonging to calcium ion transport (GO:0006816) in bold.

Table 2. Genes and SNPs discovered in the gene stability analysis and validated in a Japanese cohort

			European descent			Japanese			
Gene	Chr.	SNP	Risk allele	OR	p	Risk allele	OR	p	Combined 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

Table 3. Characteristics of subjects (n=161) stratified by genotype of *SLC8A1* rs13017968. A is the risk allele.

	SLC8A1 rs13017968		
	AA	AC+CC	
	n=25	n=136	P*
Male, n (%)	14 (56)	82 (60)	NS
Age, years median (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
African-American	0 (0)	6 (4)	NA
Caucasian	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
Pre-treatment laboratory data, median (IQR)			

WBC, $\times 10^3/mm^3$	14 (12.3-18.7)	13.6 (10.7-18.6)	NS
Absolute Lymph, $\times 10^3/mm^3$	3.2 (1.8-4.2)	2.8 (1.6-4.5)	NS
Absolute Mono, $\times 10^3/mm^3$	0.8 (0.5-1.1)	0.6 (04-1.1)	NS
Platelet count, $\times 10^3/mm^3$	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

*: p-values were calculated by Mann–Whitney U test for continuous variables and chi 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 follows: East Asians: 40.3%, European descent: 6.6%, and Hispanic: 10.4%. §: Ethnicities of five patients were two Asians, two Hispanics and one mixture of Asian and Caucasian. ||: Fisher's exact T-test. aneurysm+Dilated comparing to Normal. Relative risk:1.9, 95% CI: 1.2-2.9. IVIG: Intra venous immunoglobulin G therapy CRP: C-reactive protein, ESR: Erythrocyte sedimentation rate, WBC: White blood cell count, NS: not significant, NA: not applicable.

Figure legends

Figure 1. Workflow of study

A. Pathway analysis and gene stability selection

B. Influence of *SLC8A1* on KD pathogenesis

CAA: coronary arterial aneurysms, SMC: smooth muscle cells, ECG: electrocardiogram, GO: Gene Ontology, *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, *SLC8A1*: solute Carrier Family 8, Member 1, *KCNMA1:* potassium channel, calcium activated large conductance subfamily M alpha, member 1, *RYR2*: ryanodine receptor 2, *PPP2R2A:* protein phosphatase 2 regulatory subunit B, alpha*, PRKG1:* protein kinase, cGMP-dependent, type I. Font in red: calcium signaling pathway genes

Figure 2. Association with KD susceptibility was validated for three SNPs in *SLC8A1* in the same LD block

A. Association results using imputed 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 three validated SNPs and their LD block in European decent (EUR) and Japanese (JPT).

Figure 3. Immunohistochemical staining of aneurysmal wall from KD autopsy

Aneurysmal wall of coronary arteries (A-D) and myocardium (G-J) from KD autopsies of a 3y7m-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 NCX1 antibody (A, C, E, G, I, and K) and rabbit IgG for control (B, D, F, H, J, and L). Squares 1-4 in A, C and E are magnified (x400) 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 (E1-3). Cardiomyocytes from KD autopsies showed positive staining of NCX1 but not from infant who died from pneumonia (G, I and K). lu: lumen, IEL: internal elastic lamina, m: media, a: adventitia, i: intima, Black bars: 100μm for A-F, 10μm for magnified photos and 50μm for G-L.

Figure 4. *SCL8A1* genotype is associated with differences in intracellular Ca^{2+} levels at rest and with stimulation.

Mean fluorescence Intensity (MFI) of Fluo-4AM (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.

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 boxies go from the first quartiles to the third quartiles, the horizontal lines in the boxies 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. *UST2, SLC8A1: see text.*