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ARTICLE

Investigation of novel variations of *ORAI1* gene and their association with Kawasaki disease

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Abstract

ORAI1 encodes a calcium channel essential in the store-operated calcium entry mechanism. A previous genetic association study identified a rare in-frame insertion variant of *ORAI1* conferring Kawasaki disease (KD). To deepen our understanding of the involvement of rare variants of *ORAI1* in KD pathogenesis, we investigated 3812 patients with KD and 2644 healthy individuals for variations in the protein-coding region of *ORAI1*. By re-sequencing the study participants' DNA, 27 variants with minor allele frequencies (MAFs) < 0.01 that had not been examined in the previous study were identified. Although no significant association with KD was observed either in single-variant analyses or in a collapsing method analysis of the 27 variants, stratification by MAFs, variant types, and predicted deleteriousness revealed that six rare, deleterious, missense variants (MAF < 0.001, CADD C-score ≥ 20) were exclusively present in KD patients, including three refractory cases (OR = ∞, $P = 0.046$). The six missense variants include p.Gly98Asp, which has been demonstrated to result in gain of function leading to constitutive Ca^{2+} entry. Conversely, five types of frameshift variants, all identified near the N terminus and assumed to disrupt ORAI1 function, showed an opposite trend of association (OR = 0.35, $P = 0.24$). These findings support our hypothesis that genetic variations causing the upregulation of the Ca^{2+}/NFAT pathway confer susceptibility to KD. Our findings also provide insights into the usefulness of stratifying the variants based on their MAFs and on the direction of the effects on protein function when conducting association studies using the gene-based collapsing method.

Introduction

Kawasaki disease (KD, MIM#611775) is an acute systemic vasculitis syndrome which mainly affects children younger than 5 years of age. Due to the cardiac complication represented by coronary artery aneurysms, KD has become the leading cause of acquired heart diseases during childhood in developed countries [1, 2]. Genome-wide studies have identified several common variants associated with KD susceptibility [3–5]. However, the involvement of rare genetic variants, which has been recognized as one of the explanations for the "missing heritability" problem in common diseases, in KD pathogenesis has been largely uninvestigated. In 2016, we reported associations of a common single-nucleotide variant (SNV) and a rare in-frame insertion variant of the *ORAI1* gene with susceptibility to KD [6]. *ORAI1* is a gene that encodes a plasma membrane calcium channel essential to the store-operated calcium entry (SOCE) mechanism in immune cells [7–9] and skeletal muscle cells [10, 11], and is the first gene in which both common

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and rare variants have been associated with KD. Owing to the design of our previous study, where we identified variants by re-sequencing 94 patients with KD and evaluating their association in a larger case and control panel, it was assumed that there remained other rare variants of *ORAI1* that were not examined. In this study, to deepen the understanding of the involvement of rare genetic variants in KD susceptibility and to obtain insight into the role of ORAI1 in KD pathogenesis, a large-scale genetic association study was conducted.

Materials and methods

The flow of this study is demonstrated in Fig. 1.

Samples

First, 2434 KD samples were collected at several cooperating medical institutes in Japan, and 1378 KD samples were obtained from the Japan Kawasaki Disease Genome Consortium, which comprises 49 participating medical institutes/hospitals.

The control samples were all from adult individuals without a history of KD and were collected at Keio University ($n = 374$) or obtained from the Midosuji Rotary Club ($n = 940$) and Health Science Research Resources Bank, Osaka ($n = 1330$).

Ethics

The ethical committees or institutional review boards at RIKEN, Chiba University, and all medical institutes that contributed to the patient samples approved the study. Written informed consent was given by all participants. Since KD is a childhood disease and the patients were infants or children at the time of enrollment, in most cases, written informed consent was obtained from the patients' parents. When the patients were 16 to 20 years old, we received written informed consent from both the patients themselves and their parents.

Direct sequencing

Protein-coding regions of the *ORAI1* gene distributed in exons 1 (303 bp) and 2 (600 bp) were re-sequenced by a PCR direct sequencing method. For exon 1, PCR primers were also used for direct sequencing, while for exon 2, primers for sequencing were additionally designed. The sequencing reactions were performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific), and sequencing was carried out by an ABI 3770xL or ABI 3130xL sequencer (Applied Biosystems). Variants were identified on the SEQUENCHER V4 software (HITACHI). Primer sequences for PCR and direct sequencing were demonstrated, and PCR conditions are summarized in Supplementary Figure 1.

Association analyses

Lack of statistical power is a major obstacle in performing association studies of rare variants when the number of subjects is not sufficiently large. Combining information of multiple rare variants to evaluate the effect as a single variant is one method to overcome this problem [12]. We applied the collapsing method to variants for which ≥0.75 of statistical power was not expected to detect association with $P < 0.05$ under the assumption of the odds ratio of 3.0. Association of the individual, as well as collapsed multiple variants with KD, were assessed by Fisher's exact test using the R 3.4.0 statistical computing environment. P values < 0.05 were considered significant.

In silico prediction of deleteriousness of the identified variants

Predictions of the deleteriousness of the *ORAI1* variants identified in this study were carried out using the Combined Annotation Dependent Depletion (CADD) tool [13]. The prediction score (C-score) of 20 corresponded to the top 1% of harmfulness when evaluating all known single-base substitutions, and was set as the threshold for creating a stratified variants subgroup for collapsing.

Results

Chromosomal locations, nucleotide and protein changes, the allele frequencies in cases of KD and controls, and the protein domains together with the CADD C-scores of the identified 34 variants, including 11 novel ones, are summarized in Table 1. These include all of the known five common variants (rs3741595, rs3741596, rs3741597, rs3825174, and rs3825175) as well as two rare variants (rs141919534 and c.59G>C) that have already been identified in our previous study [6]. The 27 variants that have never been evaluated for their association with KD include 12 missense (44%), 9 synonymous (33%), 5 frameshift (19%), and 1 in-frame insertion (4%) variant, and all of them had minor allele frequencies (MAFs) < 0.01 in the subjects in this study (Table 1). The position of these rare variants on the ORAI1 protein domains and the number of subjects presenting each variant are shown in Fig. 2. All of the alternative alleles of insertion or deletion variants were determined by sequencing of the PCR amplicons cloned into plasmids (data not shown).

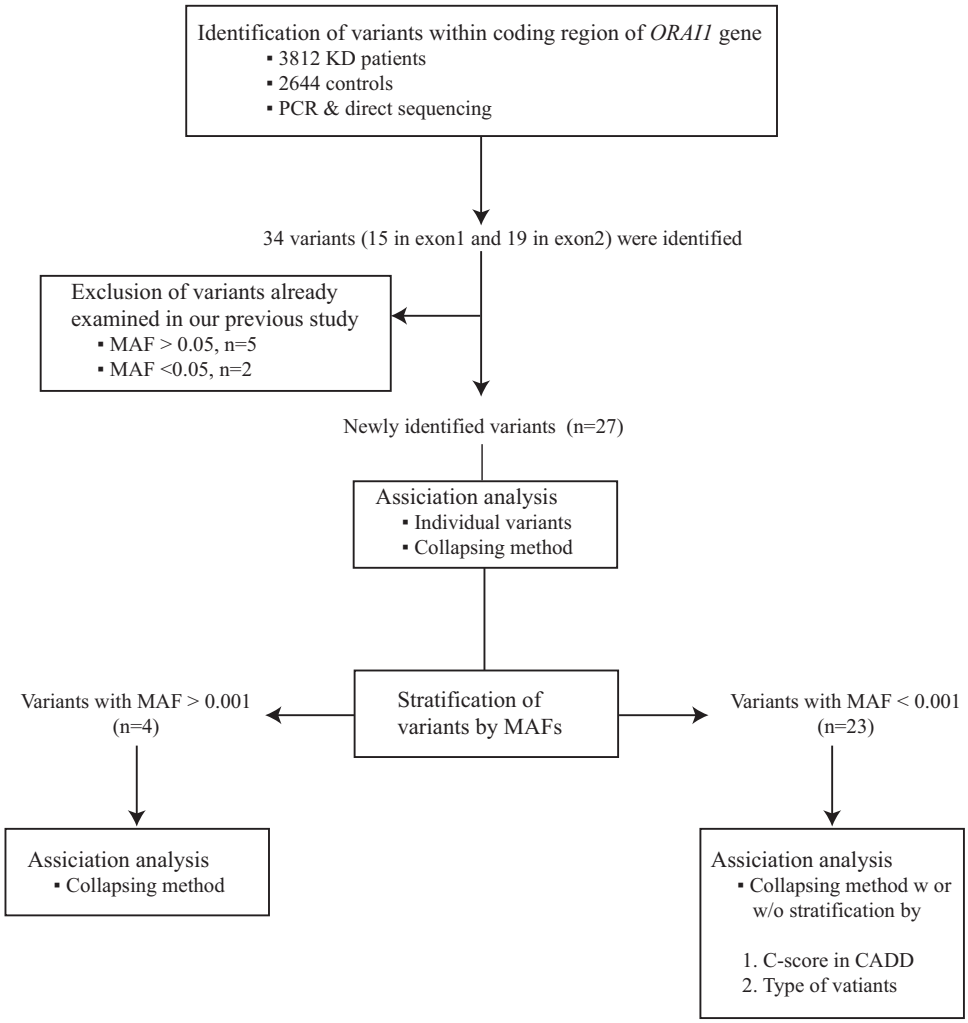

Fig. 1 Flowchart of the study

Of the 27 variants, 4 (c.12G>T, c.264C>G, c.397G>A, and c.889G>A) had relatively high allele frequencies (MAF > 0.001) in the controls, and the others were so rare that only up to three minor alleles were observed in the entire sample sets. None of the four relatively common variants showed significant association with KD (data not shown). When all 27 variants and the variants stratified based on their MAFs (>0.001 or <0.001) were evaluated as a single variant by the collapsing method, none of them had a significant association (Table 2).

Next, the variants were further stratified based on their deleteriousness using the CADD prediction score (C-score). Of the 23 variants with MAFs < 0.001, 11 had higher C-scores ≥ 20 and included 6 missense and 5 frameshift variants. The six deleterious missense variants were exclusively identified in patients with KD (odds ratio (OR) = ∞, $P = 0.046$). The clinical features of the six KD patients is summarized in Table 3. Three of the six were refractory cases who did not respond to at least two rounds of the standard intravenous immunoglobulin (IVIG) treatment (2 g/kg) and required additional therapies. The three included two patients who had medium-sized to giant coronary artery aneurysms as cardiac complications. Although not significant, the five frameshift variants showed a negative trend of association (OR = 0.35, $P = 0.24$).

Discussion

ORAI1, as part of the SOCE mechanism, plays an essential role in T-cell activity. Homozygous loss of functional *ORAI1* alleles causes an autosomal recessive immune deficiency syndrome [9]. *ORAI1* is also expressed in hematopoietic cells including B cells [14], dendritic cells [15], neutrophils [16], natural killer cells [17], platelets [18], and mast cells [19]. In our recent study, one common non-synonymous variant (rs3741596) and a rare 6-bp insertion/deletion variant (rs141919534) were associated with KD [6]. Based on previous studies on Ca^{2+}/nuclear factor of activated T-cells (NFAT) pathway upregulation by susceptibility variants of *ITPKC* and *CASP3*, in addition to successful clinical studies on cyclosporine A, which is an

Table 1 Variants of *ORAI1* identified in this study

dbSNP rsID	Position[a]	Alleles		Allele counts[b]				Minor allele frequency		Substitution[c]		C-score[d]
		Reference	Alternative	Reference		Alternative		KD	Control	Nucleotide	Protein	
				KD	Control	KD	Control					
rs868908978	122064659	G	T	7369	5154	53	32	0.0071	0.0062	c.12G>T	p.Glu4Asp	22.5
–	122064700	C	CT	7422	5185	0	1	0	0.00019	c.53_54insT	p.Ser19Lysfs*69	22.3
–[e]	122064706	G	C	7421	5185	1	1	0.00013	0.00019	c.59G>C	p.Gly20Ala	17.7
–	122064722	C	T	7421	5186	1	0	0.00013	0	c.75C>T	p.Ser25Ser	18.2
–[e]	122064723	G	A	7421	5186	1	0	0.00013	0	c.76G>C	p.Gly26Ser	22.4
–	122064742	G	GGAGCCGCCG	7419	5186	3	0	0.00040	0	c.95_96insGAGCCGCCG	p.Arg32_Arg33insSerArgArg	17.6
–	122064772	C	CGCCGCCGCCGCAG CGGGGACGGGGA GCCCCCGGGGGC	7422	5185	0	1	0	0.00019	c.125_126insGCCGCCGCCGCAGCG GGGACGGGGAGCCCCCGGGGGC	p.Pro46Glnfs*54	22.7
rs141919534	122064782	GCCACCG[f]	G	23	4	7399	5182	0.0031	0.00077	c.135_136insCCACCG[f]	p.Pro45_Pro46insProPro	16.2
–	122064796	CCGCCGTC	C	7421	5186	1	0	0.00013	0	c.144_150delCGCCGTC	p.Ala49Pro*13	28.1
–	122064803	C	CCGCCGTC	7421	5185	1	1	0.00013	0.00019	c.150-151insCGCCGTC	p.Thr51Argfs*39	25.6
rs782675422	122064812	G	C	7420	5186	2	0	0.00027	0	c.159G>C	p.Pro53Pro	15.4
–	122064827	GAGTTA	TTTT	7422	5185	0	1	0	0.00019	c.174_179delinsTTTT	p.Gln58Hisfs*29	29.7
–	122064837	G	A	7421	5186	1	0	0.00013	0	c.184G>A	p.Glu62Lys	21.9
rs543433737	122064917	C	G	7413	5178	9	8	0.0012	0.0015	c.264C>G	p.Ala88Ala	16.0
–	122064946	G	A	7421	5186	1	0	0.00013	0	c.293G>A	p.Gly98Asp	28.5
–[e]	122079007	G	A	7421	5186	1	0	0.00013	0	c.364G>A	p.Ala122Thr	25.2
–	122079030	G	A	7420	5186	2	0	0.00027	0	c387G>A	p.Val129Val	11.2
–[e]	122079040	G	A	7419	5180	3	6	0.00040	0.0012	c.397G>A	p.Val133Met	26.5
rs781980977	122079102	C	T	7421	5186	1	0	0.00013	0	c.459C>T	p.Asn153Asn	11.3
rs3741595	122079189	C	T	5805	3996	1617	1190	0.22	0.23	c.546C>T	p.Ile182Ile	7.8
rs782238081	122079291	C	T	7422	5185	0	1	0	0.00019	c.648C>T	p.Pro216Pro	0.050
rs3741596	122079295	A	G	5893	4242	1529	944	0.21	0.18	c.652A>G	p.Ser218Gly	0.18
rs782308800	122079300	C	T	7422	5185	0	1	0	0.00019	c.657C>T	p.Gly219Gly	0.0040
rs782722476	122079313	G	T	7421	5186	1	0	0.00013	0	c.670G>T	p.Val224Phe	11.1
rs377456337	122079325	G	A	7421	5186	1	0	0.00013	0	c.682G>A	p.Gly228Ser	18.1
rs200214435	122079336	G	A	7421	5186	1	0	0.00013	0	c.693G>A	p.Pro231Pro	0.27
rs3741597	122079348	T	C	5896	4242	1526	944	0.21	0.18	c.705T>C	p.Ala235Ala	0.14
rs781789915	122079419	G	A	7421	5186	1	0	0.00013	0	c.776G>A	p.Arg259His	25.6
rs3825174	122079429	T	C	5890	4240	1532	946	0.21	0.18	c.786T>C	p.Val262Val	11.3
rs3825175	122079441	T	C	2870	1993	4552	3193	0.39	0.38	c.798T>C	p.Thr266Thr	3.9
–[e]	122079510	A	G	7421	5185	1	1	0.00013	0.00019	c.867A>G	p.Arg289Arg	12.4
–	122079523	C	G	7422	5185	0	1	0	0.00019	c.880C>G	p.Leu294Val	18.4
rs555170508	122079527	C	T	7421	5186	1	0	0.00013	0	c.884C>T	p.Thr295Met	23.5
rs375464035	122079532	G	A	7413	5172	9	14	0.0012	0.0027	c.889G>A	p.Gly297Ser	19.8

dbSNP single-nucleotide polymorphism database, *KD* Kawasaki disease

[a]Chromosomal positions were based on GRCh37 Patch Release 13 (GRCh37.p13)

[b]Subjects with missing genotype data at variant positions in this table were excluded from the calculation of the allele counts

[c]Substitutions were named according to the Human Genome Variation Society guidelines [39]

[d]C-scores were calculated as a scale of deleteriousness of single-nucleotide variants (SNVs) and insertion/deletion variants in the human genome by Combined Annotation Dependent Depletion (CADD)

[e]The variants have been registered in the Japanese Multi Omics Reference Panel (jMORP) database (https://jmorp.megabank.tohoku.ac.jp/201901/)

[f]The reference sequences of human genome (NC.000012.11; with 6 bp insertion) and transcript (NM_032790.3; without 6 bp insertion) differ for this variant

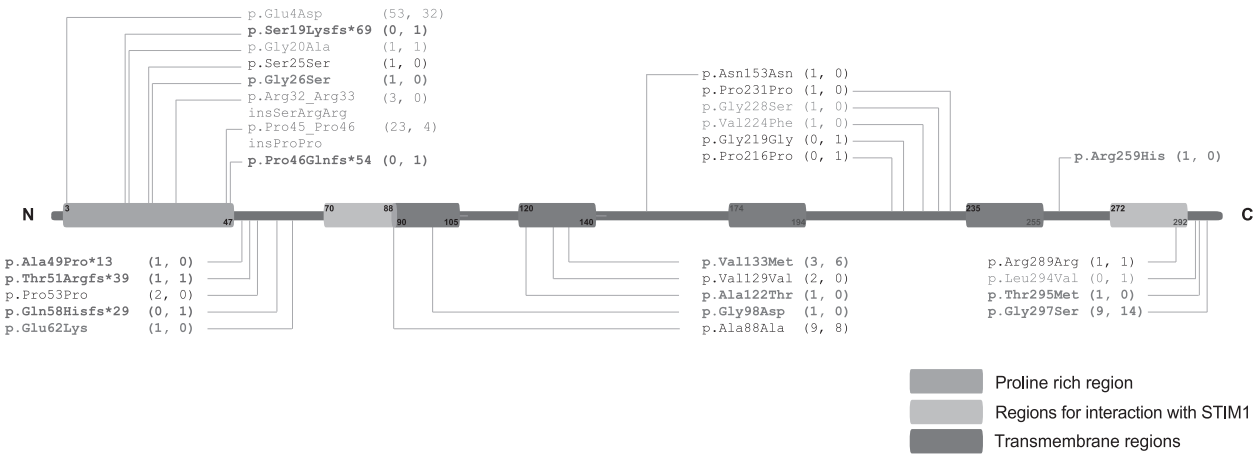

Fig. 2 ORAI1 protein domains and variants identified in this study. Positions and amino acid substitutions of the 27 *ORAI1* variants identified in this study on the protein domains are shown. Two previously identified rare variants (p.Pro45_Pro46insProPro4 and p.Gly20Ala) are also shown. For each modification, the number of individuals with the variant in the 3812 cases of Kawasaki disease and 2644 controls is provided between parentheses. The black, red, and purple letters indicate synonymous, missense, and deletion/insertion variants, respectively. Variants with a Combined Annotation Dependent Depletion (CADD) C-score ≥ 20 (5 frameshift and 8 missense variants) are indicated in boldface (color figure online)

immunosuppressant targeting this pathway for KD [4, 5, 20–22], the two above-mentioned variants were hypothesized to upregulate ORAI1 channel function. In this study, by selecting variants for collapsing according to their MAFs, types, and predicted deleteriousness, we observed a significant association in six rare deleterious missense SNVs with KD. Although conclusions should be carefully drawn because of a lack of exact information on the directions and sizes of the effects due to individual variants, the results in this study further support our hypothesis that genetic variants leading to Ca^{2+}/NFAT pathway upregulation may confer susceptibility to KD. The idea was based on the following two studies. First, we obtained the information showing that one of the six rare and deleterious missense variants has a significant gain-of-function effect [23]. The p.Gly98Asp mutation was located within the first transmembrane region of the protein, where amino acids were well conserved throughout the species (Supplementary Figure 2). The Gly98 of ORAI1 is a key amino acid that acts as a gating hinge for channel opening and closing, and the change from glycine (neutral and hydrophobic) to aspartate (acidic and hydrophilic) is known to result in constitutive Ca^{2+} permeability independently of STIM1 [23, 24]. Second, the five frameshift variants, which tended to be negatively associated with KD, were collectively identified in the N-terminal cytoplasmic region (Supplementary Figure 3). None of the four transmembrane regions of ORAI1, all located downstream of the frameshift-initiating points on the variant proteins, would be properly translated because of the incorrect reading frames. Thus, the truncated ORAI1 proteins, which had the correct amino acid sequence of ORAI1 only in the N-terminal cytoplasmic region and lacked the membrane protein properties, would not be expressed on the plasma membrane (Supplementary Figure 4), and could not engage in ion conduction. Therefore, the two types of variants, which were supposed to have at least partly different functional effects on ORAI1 protein function, had the opposite directions of association with KD, and thereby provided an insight into the role of *ORAI1* variants in KD pathogenesis. Of the six KD patients with the rare deleterious missense variants, three were refractory cases requiring repeated IVIG administration and other treatment options. In particular, an early-onset case, who was affected at 2 months of age, suffered from extremely severe KD. The patient developed giant coronary aneurysms in three coronary artery branches and had an acute myocardial infarction by occlusion of the right coronary artery on day 34 of illness (Table 3). Considering the low frequencies of the deleterious alleles, the usefulness of sequencing the *ORAI1* gene at the bedside of acute KD patients seems to be limited. However, the accumulation of knowledge about the functional significance of the rare variants, their association with KD, as well as the severer manifestations and the degrees of genetic penetrance will pave the way for genetic counseling or testing of the relatives of the variant carriers.

In contrast to the six rare and deleterious missense variants exclusively observed in patients with KD, frameshift variants were observed in patients with KD as well as in controls. Loss-of-function mutations of *ORAI1* have been known to cause an autosomal recessive congenital immune deficiency syndrome (IMD9; MIM #61782) [9]. In the familial case of IMD9, both parents of the patients, who were heterozygous for the causal mutation and in whose T cells SOCE was partially impaired, were immunologically normal. Furthermore, it is also known that some gain-of-

Table 2 Association of the newly identified rare variants of *ORAI1* gene with KD

	Number of variants	Number of alternative alleles in the subjects		OR[a]	*P*[a]
		KD[b] $n = 3711$	Control[b] $n = 2593$		
All variants[c]	27	95	68	0.98	0.87
Variants with MAF > 0.001	4	74	60	0.86	0.43
C-score < 20	2	18	22	0.57	0.08
C-score ≥ 20	2	56	38	1.03	0.92
Variants with MAF < 0.001	23	21	8	1.84	0.19
C-score < 20	12	13	4	2.27	0.22
In-frame variants	1	3	0	∞	0.27
Synonymous variants	8	8	3	1.86	0.54
Missense variants	3	2	1	1.40	1
C-score ≥ 20	11	8	4	1.40	0.77
Frameshift variants	5	2	4	0.35	0.24
Missense variants	6	6	0	∞	0.046

KD Kawasaki disease, *MAF* minor allele frequency, *OR* odds ratio

[a]Association in allelic model was evaluated using Fisher's exact test

[b]Subjects with missing genotype data at variant positions identified in this study were excluded from this analysis

[c]Variants with minor allele frequencies < 0.01 except for c.59C>G and rs141919534 were included in this analysis

function mutations of *ORAI1* cause an autosomal dominant tubular aggregate myopathy (TAM2; MIM #615883) [25]. Three independent patients with TAM2 due to a p.Gly98Ser of ORAI1 have been reported [26, 27]. In in vitro experiments using exogenously expressed ORAI1 mutant proteins, both p.Gly98Ser and p.Gly98Asp similarly lead to a constitutive activation of the channel [23, 27]. Given that five other rare deleterious missense variants also have a gain-of-function effect on ORAI1 function, and some dominant effects, it was reasonable for the control population to lack these types of variants. The different distribution patterns of these stratified variants in the study populations might reflect the difference in impact of the variants on the cellular activity as well as on the phenotype that individuals with the variants would have. It has been reported that ORAI1 has two isoforms, ORAI1α with 303 amino acids translated from the first methionine, and ORAI1β, a shorter isoform that uses the second (64th position) or the third (71st position) methionine residue as a translation initiation codon [28]. The impact of the five frameshift variants in this study might be milder than expected, because the shorter isoform that also supports SOCE can be translated from all alternative alleles of these variants.

Collapsing the rare variants has been recognized to be effective in assessing the association of rare genetic variants [29]. Usually, common variants are not included in the collapsing analysis because their high allele frequencies in cases or controls, whether they are associated with the trait of interest or not, may interfere with the detection of the association of other rare variants. In this study, we found it useful to further stratify the rare variants by their MAFs. The MAFs of the rare variants in the controls in this study vary from 0.00617 for c.12G>T, with 53 and 32 alternative alleles seen in the cases of KD and controls, respectively, to 0.0 for multiple variants observed only in a single or few patients with KD. There is a well-recognized model of the relationship between the risk allele frequencies and the strength of the genetic effect, in which the smaller allele frequencies of the risk-associated variants result in a larger effect size [30, 31]. In this context, it seemed reasonable to evaluate the association of rare variants by the collapsing method after stratifying them by their MAFs. In contrast to the lack of difference in the alternative allele frequencies when the 27 rare variants were collapsed without stratification (OR = 0.98, $P = 0.87$), 23 variants with MAFs < 0.001 showed a positive trend of association (OR = 1.84, $P = 0.19$).

The opposite trends of association in both missense and frameshift variants were indicative of the potential importance of stratifying rare variants in association studies, according to their magnitude and the direction of their effects on the protein function. Currently, there are a number of computer tools to predict the deleteriousness of genetic variants. The results of stratifying the nine rare (MAF < 0.001) missense variants were inconsistent among the commonly used prediction tools, including Polyphen-2, SIFT, and Mutation Taster [32] (Supplementary Table 1). In this study, we used CADD, recognizing an advantage of the tool in performing the prediction by integrating multiple prediction results and scores, including those from SIFT and PolyPhen-2. The option to evaluate synonymous SNVs and deletion/insertion variants was another reason for selecting CADD. However, the items utilized to calculate the C-scores during deleteriousness evaluations of known causal variants of TAM2 (gain of function) [26, 27, 33, 34] and IMD9 (loss of function) [35–38] with CADD did not seem to be suited to discriminating the two variant categories. This resulted in difficulty in precisely inferring the direction of the variant effects by in silico tools (Supplementary Table 2). At present, determining the true functional effect of each variant by functional assays with Ca^{2+} imaging or patch-clamp techniques using the variant carriers' lymphocytes, or cultured cell lines transduced with the variant channels, is the most suitable and reliable method of stratifying the variants for efficient association studies.

In this study, six insertion/deletion variants were newly identified. Because all of them were located within the N-terminal cytoplasmic region, this domain might be a hot spot for these types of variants (Supplementary Figure 3). One of these variants was a novel insertion of 37 bases

Table 3 Characteristics of the KD patients with rare deleterious missense variants of *ORAI1*

Patients	Variants	Age of onset	Gender	IVIG regimen	Additional therapies	CAL	Cardiovascular events
#1	p.Gly26Ser	1y 2m	F	400 mg/kg × 5	–	–	–
#2	p.Glu62Lys	5m	M	2 g/kg × 2	mPSL pulse, IFX, UTI	Medium-sized aneurysms (5–6 mm) on RCA and LAD	–
#3	p.Gly98Asp	1y 8m	F	2 g/kg × 1		–	–
#4	p.Ala122Thr	2m	M	2 g/kg × 4	–	Giant aneurysms (>8 mm) on 3 coronary artery branches	AMI (day 34)
#5	p.Arg259His	1y 11 m	F	2 g/kg × 1	–	–	–
#6	p.Thr295Met	1y 11 m	F	2 g/kg × 2	CsA	–	–

KD Kawasaki disease, *IVIG*intravenous immunoglobulin, *CAL* coronary artery lesion, *y* year, *m* month, *F* female, *M* male, *mPSL* methylprednisolone, *IFX* infliximab, *UTI* urinary trypsin inhibitor, *CsA* cyclosporine A, *RCA* right coronary artery, *LAD* left anterior descending coronary artery, *AMI* acute myocardial infarction

(c.125_126ins), which was identical to the upstream sequence from the inserted position. In ClinVar, two other insertion variants of 37 bases in similar positions of *ORAI1* (c.126_127insCCGCCGCCGCAGCGGGGACGGGGAGC CCCCGGGGGCC; Variation ID: 594718, and c.141_142insCCGCCGCCGCAGCGGGGACGGGGAGC CCCCGGGGGCC; Variation ID: 470188) had been registered. Although these three variants were not identical, the high similarity of the inserted sequences (Supplementary Figure 5) suggests some common mechanism in the generation of the variants.

Previously, we reported a positive association of a six-base insertion variant of *ORAI1* (rs141919534; p.Pro45_Pro46insProPro) with KD. Interestingly, another novel in-frame insertion variant (p.Arg32_Arg33insSerArgArg), identified within the same cytoplasmic region, was observed in three patients with KD but not in controls. Within the cytoplasmic region, there is a domain for interactions with STIM1 (Supplementary Figure 3). The similar association trend observed for these two in-frame insertion variants within the N-terminal cytoplasmic region may reflect the modulated interaction between ORAI1 and STIM1 due to the elongation of this domain and the subsequent increase in Ca^{2+} influx or the duration of the channel opening.

There are several limitations to this study. First, the exact impacts of the rare and deleterious missense variants were not evaluated by biological assays and the prediction of deleteriousness was carried out with a single tool. Second, the frequencies of the deleterious missense variants identified in this study were so low that the findings cannot be immediately applied in clinical diagnosis, even when the gain-of-function effect was proven for all six variants. Finally, a correction of multiple testing was not conducted.

In conclusion, we observed that *ORAI1* deleterious missense variants with MAFs < 0.001, including one known gain-of-function mutation in the first transmembrane region, had a positive association with KD in a collapsing method analysis. Although it was not significant, an in-frame insertion variant in the N-terminal cytoplasmic region showed the same association trend as the other in-frame insertion variant within the same protein domain that had been identified in our previous study [6]. Conversely, five frameshift variants causing N-terminal protein truncation showed the opposite trend of association. These observations support our hypothesis that genetic variants leading to upregulation of the Ca^{2+}/NFAT pathway confer susceptibility to KD. The usefulness of the collapsing method in evaluating the association of mutations and rare variations with diseases may be enhanced when MAFs and variant types are taken into account. Currently, there is no in silico tool that can predict the direction of the variants' effect on the protein function. Future advances in the precise prediction of the functional impact of the identified variants

(i.e., neutral, gain of function, or loss of function) may facilitate the assessment of their association with diseases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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