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2  
3 25. Japanese Kawasaki disease Research Committee. Diagnostic Guidelines of  
4  
5  
6 Kawasaki Disease, 5<sup>th</sup> ed. Tokyo: Japanese Kawasaki disease Research  
7  
8  
9 Committee, 2002.  
10  
11
- 12 26. Japan Circulation Society Joint Working Groups. : Guideline for diagnosis and  
13  
14  
15 management of cardiovascular sequelae in Kawasaki disease (JCS2008).  
16  
17
- 18 27. Igarashi T.: Hyperkalemia. In: Igarashi T, ed. Clinical Pediatric Nephrology.  
19  
20  
21 3<sup>rd</sup> ed. Tokyo: SHINDAN TO CHIRYO Sha Inc.; 2008:39-40. (in Japnasese)  
22  
23
- 24 28. Ishikura K, Ikeda M, Hattori S, Yoshikawa N, Sasaki S, Iijima K, et al.: Effective  
25  
26  
27 and safe treatment with cyclosporine in nephrotic children: a prospective,  
28  
29  
30  
31 randomized multicenter trial. Kidney Int. 2008; 73(10):1167-1173.  
32  
33
- 34 29. Hamasaki Y, Yoshikawa N, Hattori S, Sasaki S, Iijima K, Nakanishi K, et al.:  
35  
36  
37 Cyclosporine and steroid therapy in children with steroid-resistant nephritic  
38  
39  
40  
41 syndrome. Pediatr Nephrol. 2009; 24(11):2177-2185.  
42  
43
- 44 30. Schwartz D, Conelly NR, Manikantan P, Nichols JH.: Hyperkalemia and pyloric  
45  
46  
47 stenosis. Anesth Analg 2003;97(2):355-357.  
48  
49
- 50 31. Shimizu T, Yamashiro Y and Yabuta K.: Pseudohyperkalemia in Kawasaki  
51  
52  
53 disease. Eur J Pediatr 1992;151:497-498  
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Figure and Table legends

Figure 1: Study protocol:

KD: Kawasaki disease, IVIG: intravenous immunoglobulin, CyA: Cyclosporin A,

IC: informed consent.

\*: Responders to each treatment. \*\*: Patients resistant to initial IVIG or CyA.

\*\*\*: Patients resistant to additional IVIG. IC: informed consent.

All patients with KD received initial IVIG infusion (2 g/kg) and aspirin (30-50 mg/kg/day). Patients resistant to initial IVIG received additional IVIG (2 g/kg). In addition, patients who were resistant to additional IVIG and were 4 months old or more, were treated with CyA. Patients who were resistant to additional IVIG and aged less than 4 months were treated with a 3<sup>rd</sup> course of IVIG.

Figure 2: Protocol outcomes.

KD: Kawasaki disease, IVIG: intravenous immunoglobulin, CyA: Cyclosporin A,

CAL: coronary arterial lesions.

Of 329 patients with KD, 245 (74.5%) became afebrile after initial IVIG. Eighty-four patients resistant to initial IVIG received additional IVIG, of whom 30 failed to become afebrile within the treatment completion time. Among these 30 patients,

28 who were 4 months old or more, were treated with CyA, and the other two patients, who were less than four months of age, received a third course of IVIG (2 g/kg).

\*: CAL developed in these two patients (no. 11 and no. 19 in Table 2) before CyA treatment (during additional IVIG).

Table 1: Characteristics of 8 patients treated with CyA between January and December 2008.

CyA: Cyclosporin A, IVIG: intravenous immunoglobulin, An: Aneurysm, ID: illness day, CAL: coronary arterial lesions.

These 8 patients ranged in age from 5 to 39 months. The male to female ratio was 6:2. CyA treatment was started in days 7-12 of illness, and the dose of CyA was 4-8 mg/kg/day. We increased and decreased the dose of CyA according to both clinical responses such as fever and the trough levels themselves. One patient (no.3) received a 3<sup>rd</sup> IVIG infusion. Two patients (nos. 3 and 8) developed CAL.

Table 2. Characteristics of 20 patients treated with CyA between January 2009 and June 2010.

CyA: Cyclosporin A, IVIG: intravenous immunoglobulin, An: Aneurysm, AnG:

giant Aneurysm, ID: illness day, CAL: coronary arterial lesions

These 20 patients ranged in age from 4 to 93 months. The male to female ratio was 14:6. CyA treatment was started in days 7-11 of illness, and the dose of CyA was 4-8 mg/kg/day. 4 patients (nos. 10, 19, 26, and 28) failed to become afebrile within 5 days after the start of CyA and/or high fever returned after becoming afebrile within 5 days. Three patients (nos. 10, 26 and 28) received a third course of IVIG infusion. Two patients (nos. 11 and 19) developed CAL before CyA treatment (during additional IVIG).

Table 3. Summary of clinical parameters in 28 KD patients treated with CyA.

CyA: Cyclosporin A, IVIG: intravenous immunoglobulin

Values are ranges and (medians). P values\* were calculated by Wilcoxon's signed rank test.



Table 1. Characteristics of 8 patients treated with CyA between January and December 2008.

Case No.	age	Sex	Initial IVIG (illness day of IVIG)	additional IVIG (illness day of IVIG)	(illness day of CyA)	CyA dose (mg/kg/day)	duration (days)	duration (days) until afebrile after CyA	CAL
1)	3y 3 m	M	4	6	9	4	21	1	(-)
2)	2y 0 m	M	5	8	12	4	21	1	(-)
3)	1y 11m	F	6	9	11	4 - 8	50	13	An (5mm)
4)	1y 10m	M	4	6	9	4	21	3	(-)
5)	3y 0 m	M	5	7	9	4	21	2	(-)
6)	1y 3 m	M	4	7	8	4 - 6	13	5	(-)
7)	5 m	F	6	8	9	4	21	3	(-)
8)	1y 2m	M	3	5	7	4 - 8	21	13	An (4mm)

Table 2. Characteristics of 20 patients treated with CyA between January 2009 and June 2010.

Case No.	age	Sex	Initial IVIG		CyA		duration (days) until afebrile after CyA	CAL
			(illness day of IVIG)	(illness day of CyA)	dose (mg/kg/day)	duration (days)		
9)	1y 5m	F	5	10	4 - 6	14	1	(-)
10)	1y 4m	M	4	6	4 - 7	14	4	(-)
11)	5y 3 m	F	5	8	4	14	1	AnG(10mm)
12)	6 m	M	4	6	4	14	2	(-)
13)	4y10m	M	4	7	4	14	1	(-)
14)	1y 3 m	M	4	6	4 - 6	13	5	(-)
15)	2y11m	M	5	7	4 - 6	14	4	(-)
16)	4y 3 m	M	4	7	4	10	1	(-)
17)	2y 6m	F	5	7	4 - 6	11	1	(-)
18)	1y 4 m	M	3	6	4 - 5	13	5	(-)
19)	5y 4 m	M	5	7	4 - 5	14	6	An (6mm)
20)	2y 7 m	M	5	7	4	14	3	(-)
21)	1y11m	F	4	7	4	5	1	(-)
22)	4 m	M	4	6	4	14	1	(-)
23)	7y9m	M	4	7	4	14	1	(-)
24)	2y6m	M	4	6	4	14	3	(-)
25)	5m	F	5	8	4	14	1	(-)
26)	1y1m	F	4	6	4 - 8	14	7	(-)
27)	1y8m	M	5	7	4	8	1	(-)
28)	1y1m	M	5	7	4 - 8	14	7	(-)
							3 <sup>rd</sup> -IVIG(22th ID)	
							3 <sup>rd</sup> -IVIG(26th ID)	

Table 3. Summary of clinical parameters in 28 KD patients treated with CyA.

1) age (months)	4-93 (23)	
2) male:female	20 : 8	
3) illness day of initial IVIG	3 - 6 (4.5)	
4) illness day of additional IVIG	5 -10 (7)	
5) illness day of the start of CyA treatment	7 -12 (8)	
6) duration (days) of CyA treatment	5 -50 (14)	
7) duration (days) until afebrile after CyA treatment	1 -13 (2)	
8) axillary temperature (centigrade)		
on the day of the start of CyA treatment	38.0-40.3 (38.9)	} p<0.01*
2 days after the start of CyA treatment	36.1-40.4 (37.6)	
9) CRP (mg/dL)		
on the day of the start of CyA treatment	1.2-16.8 (9.3)	} p<0.01*
2 days after the start of CyA treatment	0.4-16.1 (5.2)	
10) serum potassium levels		
on the day of the start of CyA treatment	2.9-5.1 (4.1)	} p<0.01*
maximum potassium levels	4.9-6.1 (5.4)	
duration (days) until maximum potassium levels	3 -13 (6.5)	
11) serum creatinine levels		
on the day of the start of CyA treatment	0.1-0.33 (0.23)	} p=0.156*
on the day of maximum potassium levels	0.12-0.33 (0.24)	
12) estimated GFR:		
on the day of the start of CyA treatment	85.5-152.3 (109.9)	} p=0.123*
on the day of maximum potassium levels	83.4-135 (103.9)	

ranges (median)

\*: Wilcoxon's signed rank test

Figure 1

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graph TD
    A[Diagnosis of KD] -- IC(+) --> B[Initial IVIG]
    B -- ** --> C[Additional IVIG]
    B -- * --> D[No additional therapy]
    C -- *** --> E["CyA 4 mg/kg/day  
(oral administration)  
(4 months old or more)"]
    C -- ** --> F["Other therapy: 3rd IVIG  
(less than 4 months)"]
    C -- * --> G[No additional therapy]
    E -- ** --> H["Other therapy  
(3rd IVIG)"]
    E -- * --> I[No additional therapy]
  
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Aspirin (30-50mg/kg/day, p.o.)		<After afebrile, 5mg/kg/day, p.o.>	
Initial therapy, for 48 hours	Additional therapy, for 24 hours	CyA therapy, for 2-3 weeks	observation
Initial IVIG 2 g/kg (24 hours) + observation (24 hours)	Additional IVIG 2 g/kg (24 hours)	4 - 8 mg/kg/day trough level (60-200 ng/ml)	

Total periods: 4 weeks after initial IVIG therapy

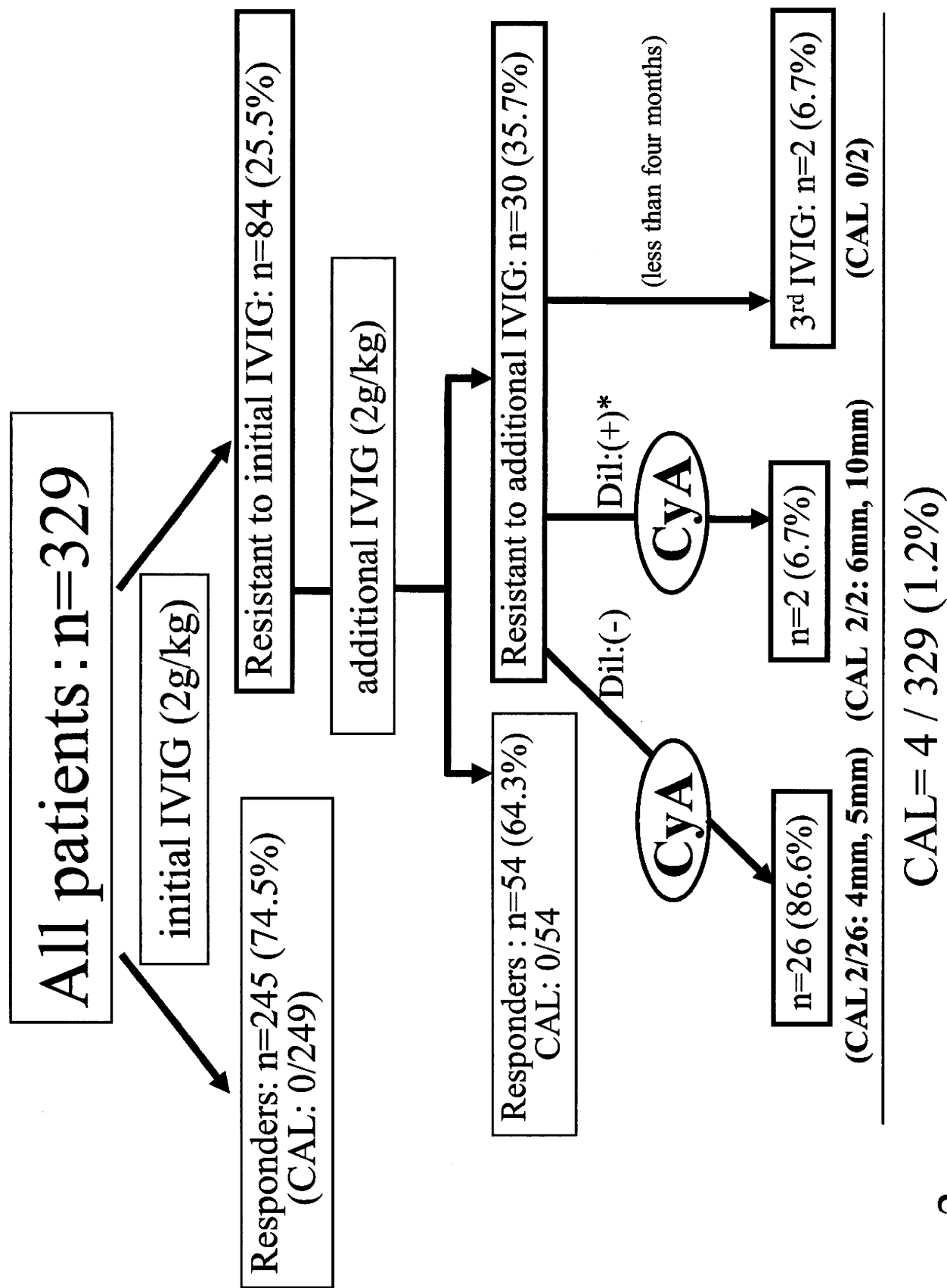


Figure 2

ORIGINAL ARTICLE

# Matrix metalloproteinase haplotypes associated with coronary artery aneurysm formation in patients with Kawasaki disease

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Aneurysms of the vascular wall represent a final common pathway for a number of inflammatory processes, including atherosclerosis and idiopathic vasculitis syndromes. Kawasaki disease (KD) is an acute, self-limited vasculitis in children and the leading cause of acquired coronary artery aneurysms. We sought to identify shared molecular mechanisms of aneurysm formation by genotyping eight polymorphisms in *matrix metalloproteinase (MMP)*-1, 3, 7, 12 and 13 in the gene cluster on Chr.11q22, whose gene products have been implicated in aneurysm formation or are known to have elastase activity. We genotyped 482 US–UK KD patients (aneurysm+:  $n=111$ , aneurysm–:  $n=371$ ) and tested our findings in an independent cohort of 200 Japanese KD patients (aneurysm+:  $n=58$ , aneurysm–:  $n=142$ ). Analysis of the five MMP genes identified modest trends in allele and genotype frequencies for *MMP-3* rs3025058 (–/T) and haplotypes containing *MMP-3* rs3025058 (–/T) and *MMP-12* rs2276109 (A/G) (nominal  $P=2$  to  $4 \times 10^{-5}$ ) that conferred increased risk of aneurysm formation in US–UK subjects. This finding was validated in Japanese subjects and suggests the importance of this locus in aneurysm formation in children with KD. The region encompassing these risk haplotypes is a prime candidate for resequencing to look for rare genetic variation that may influence aneurysm formation.

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**Keywords:** coronary artery aneurysm; haplotype; Kawasaki disease; matrix metalloproteinase

## INTRODUCTION

Aneurysms of the vascular wall complicate many different diseases that involve vessel wall inflammation and destruction of extracellular matrix and elastic fibers. In children with Kawasaki disease (KD), coronary artery aneurysms (CAA) form in 25% of untreated patients and in 5% of patients treated with intravenous immunoglobulin within the first 10 days after fever onset. A hallmark of CAA is focal destruction of the internal elastic lamina with early neutrophil infiltration followed by macrophages and cytotoxic T lymphocytes.<sup>1</sup> For this reason, enzymes that cleave elastin have been implicated in the pathogenesis of KD.<sup>2,3</sup> Proteases capable of degrading elastin include neutrophil elastase and the matrix metalloproteinases (MMPs)-2, 3, 7, 9 and 12.<sup>4</sup> MMPs are zinc-dependent endopeptidases produced by a wide variety of cell types. In addition to the degradation of

extracellular matrix, MMPs also cleave cytokines and chemokines<sup>5</sup> and influence recruitment of inflammatory cells. Therefore, MMPs have important roles in both inflammation and tissue remodeling.

According to a current paradigm, KD is triggered by an infectious agent that elicits an inflammatory response directed at cardiovascular tissues in genetically susceptible hosts.<sup>6</sup> A genetic influence on disease susceptibility in KD has been explored in candidate gene association studies, a genome-wide linkage analysis of siblings concordant for KD followed by linkage disequilibrium mapping, and a genome-wide association study.<sup>7–11</sup> However, fewer studies have explored the impact of genetic variation on aneurysm formation because of the difficulty in collecting a sufficient sample size of patients with this phenotype for genotyping. To bridge this gap in knowledge, we collaborated with groups in the United Kingdom and Japan to collect DNA from KD

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patients and determined the contribution of five MMP genes (*MMP-1*, 3, 7, 12 and 13) to CAA formation, as these MMPs have been implicated in atherosclerotic coronary artery (CA) and abdominal aneurysms or are known to have elastase activity.<sup>4,12,13</sup> We genotyped eight single-nucleotide polymorphisms (SNPs) in these five MMP genes clustered on chromosome 11 and analyzed haplotypes in the US-UK cohort. These results were tested in an independent cohort of Japanese KD subjects to examine the association of this region with CAA across different ethnic populations.

MATERIALS AND METHODS

Subjects

Details of the US-UK subjects and their clinical presentation and case definition have been previously described.<sup>9</sup> Briefly, KD patients (*n*=482) were recruited at Rady Children's Hospital San Diego, CA; Boston Children's Hospital, Boston, MA; and Imperial College School of Medicine, London, UK. KD patients from Japan (*n*=200) were recruited by investigators at Yamaguchi University and Oita Children's Hospital Japan. Parental consent and subject assent when appropriate were obtained for all subjects. The Institutional Review Boards of the participating centers reviewed and approved this study.

Demographic and clinical data were collected on all subjects as previously described<sup>9</sup> (Table 1). The distribution of different ethnic groups was similar between the CAA+ and CAA- cohorts. For the US subjects, CA status was assessed by echocardiography during the acute, subacute and convalescent phase of the illness. Measurements of the internal diameters of the proximal

right (R) and left anterior descending CAs were normalized for body surface area and expressed as standard deviation units from the mean (Z-scores). For US subjects, CAA was defined as a Z-score  $\geq 4.0$  in the first year after KD onset in association with an internal diameter  $\geq 1.5$  times the adjacent segment.<sup>14</sup> For the UK and Japanese subjects, Z-scores were not available and CA lesions (aneurysm or ectasia) were defined according to the Japanese Ministry of Health criteria (internal lumen diameter  $\geq 3$  mm for children < 5 years and  $\geq 4$  mm for children  $\geq 5$  years or the internal diameter of one or more segments  $\geq 1.5$  times the diameter of the adjacent segment.<sup>15</sup>

Genotyping

Genomic DNA from whole blood or mouth wash samples was extracted as previously described.<sup>9</sup> We chose eight SNPs from five MMP genes (Figure 1) that had positive disease associations in the published literature<sup>16-23</sup> (Figure 2). Genotyping was performed using a multiplex PCR-based, sequence-specific oligonucleotide hybridization research assay (Roche Molecular Systems, Pleasanton, CA, USA) as previously described.<sup>9</sup> To genotype *MMP13* rs2252070 (A/G), we used a TaqMan allele discrimination assay (Applied Biosystem, Foster City, CA, USA, Assay ID: C\_25474083\_10) according to the manufacturer's instructions. Different numbers of subjects were genotyped for different loci because of limited availability of some of the genotyping reagents that were

Table 1 Characteristics of subjects with KD

	US-UK KD (n=482)		Japanese KD (n=200)	
	CAA- (n=371)	CAA+ (n=111)	CAA- (n=142)	CAA+ (n=58)
Male	235 (63%)	77 (69%)	75 (53%)	40 (69%)
Self-reported ethnicity				
Caucasian	215 (58%)	75 (68%)		
Caucasian-Hispanic	56 (15%)	12 (11%)		
Asian, unspecified	42 (11%)	13 (12%)		
Mixed	43 (12%)	8 (7%)		
Others	14 (4%)	3 (2%)		
Japanese	1 (0.2%)	0 (0%)	142 (100%)	58 (100%)

Abbreviations: CAA, coronary artery aneurysms; KD, Kawasaki disease.

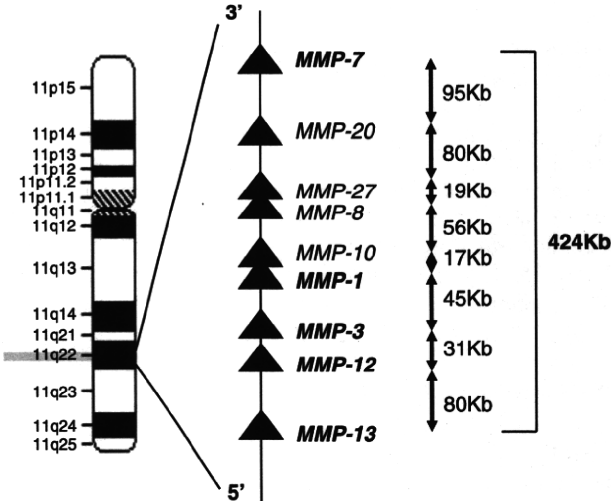


Figure 1 Matrix metalloproteinase (MMP) gene cluster on 11q22. Arrowheads show the direction of genes. Arrows on the right indicate intergenic distance in the 424 Kb region. Genes in bold were genotyped in this study.

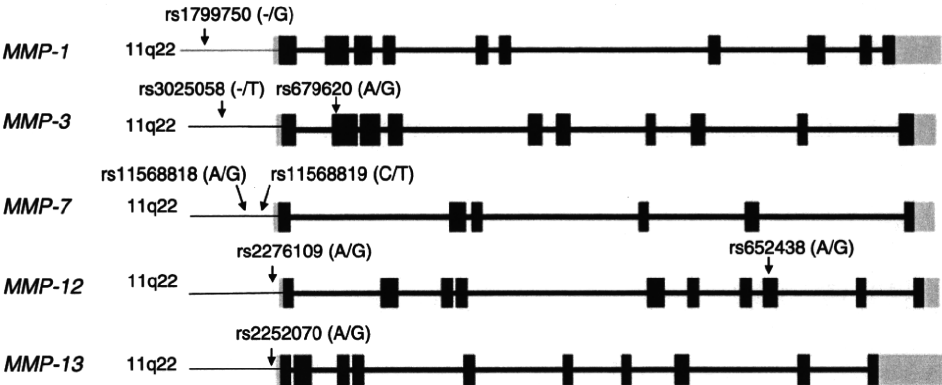


Figure 2 Eight single-nucleotide polymorphisms (SNPs) from five matrix metalloproteinase (MMP) genes genotyped in Kawasaki disease patients. Gene structure and the location of SNPs: black boxes, exons; gray boxes, 3' and 5' untranslated regions.

discontinued by the manufacturer before the study was completed (Supplementary Tables 1 and 2). Data quality control was performed as previously described.<sup>9</sup> SNPs with allele frequencies <1%, genotype call rate <93% or deviation from Hardy–Weinberg equilibrium were excluded from further analysis.

### Statistical analyses

Case–control association studies between MMP SNPs and CA status were analyzed by the general linear model:  $Y_i = \alpha + \beta X_i + \epsilon_i$ . The genetic models that were considered were the codominant model (genotype) and the additive model (allele). Under the codominant model, a particular SNP is considered a categorical variable with one level for each genotype for a total of three levels. To assess the association between phenotype Y and each SNP, the above general linear model is used, where  $\alpha$  represents the intercept,  $X_i$  is the  $i$ th subject's genotype score for a given marker and  $\epsilon_i$  is normally distributed with mean 0 and variance  $\sigma^2$ . Under the dominant model,  $X_i = 1$  if the  $i$ th subject has at least one minor allele or  $X_i = 0$  otherwise. Under the additive model,  $X_i$  indicates the  $i$ th subject's number of minor alleles. This is equivalent to the test based on the allele frequency. Multiple testing corrections were not applied and nominal P-values are shown in tables. Analysis was performed using the R software (version 2.6.2, <http://www.r-project.org/>) and the R package SNPpassoc.

Haplotype associations between MMP SNPs and CA status were analyzed using a moving window approach. The expectation–maximization algorithm<sup>24</sup> was used to estimate haplotype frequencies and to account for missing genotypes. Score statistics were computed to test associations between the haplotypes and various traits. Analyses were performed using the R package haplo.stats.<sup>25</sup> Correction for multiple testing was performed for single locus and haplotype analyses according to the following calculation: single locus: 0.05/number of SNPs tested; haplotypes: 0.05/number of observed haplotypes/number of haplotypes with nominal  $P < 0.05$ .

## RESULTS

### Single-locus analysis in the US–UK cohort

Case–control analysis of the multiethnic US–UK cohort (111 KD subjects with aneurysms vs 371 KD subjects with normal or transiently

dilated CAs) was performed and genotype data met quality control criteria on eight SNPs in five MMP genes. Modest trends in allele and genotype frequencies were noted between the two groups for MMP-3 rs3025058 (–/T) (allele nominal  $P = 0.031$ , odds ratio = 0.72, 95% confidence interval 0.54–0.97; genotype nominal  $P = 0.015$ ), suggesting that the T allele in the MMP-3 promoter contributes to protection against aneurysm formation. Another SNP in MMP-3, rs679620 (A/G) was associated with a trend towards protection (allele nominal  $P = 0.089$ , odds ratio = 0.76, 95% confidence interval 0.56–1.05; genotype nominal  $P = 0.042$ ) (Supplementary Table 1). None of these associations remained significant after correction for multiple testing. To explore the possible effect of population stratification on our analysis, we performed single-locus analyses using only self-reported Caucasian subjects (CAA+  $n = 75$ , CAA–  $n = 215$ ). No significant differences between the CAA+ and CAA– groups were detected with this smaller sample (data not shown).

### Haplotype analysis in the US–UK subjects

We analyzed SNPs in the five MMP genes located in a cluster on chromosome 11q22 (MMP-1, 3, 7, 12 and 13) (Figure 1). Several haplotypes were associated with CAA formation in the US–UK subjects (Table 2). Analysis using a window of two SNPs identified two haplotypes both with the del allele (–allele) of MMP-3 rs3025058 (–/T) that were associated with increased risk of aneurysm formation (nominal  $P = 0.03$  and 0.04, respectively). The association of the del allele of MMP-3 rs3025058 (–/T) with the A allele of rs679620 (A/G) and the A allele of rs2276109 (A/G) in MMP-12 remained constant, suggesting a strong linkage disequilibrium in this region ( $D'$  between MMP-3 rs3025058 (–/T) and rs679620 (A/G): 0.92, MMP-3 rs3025058 (–/T) and MMP-12 rs2276109 (A/G): 0.85). Haplotypes containing three to eight SNPs that were associated with CAA all included the del allele of MMP-3 rs3025058 (–/T). Haplotypes

**Table 2 Haplotypes on Chr. 11q22 associated with coronary artery aneurysms in the multiethnic US–UK cohort**

Haplotype								Total number of observed haplotypes <sup>a</sup>	Frequency		P-value
MMP-7		MMP-1	MMP-3		MMP-12		MMP-13		CAA–	CAA+	
rs11568819C/T	rs11568818A/G	rs1799750–G	rs679620A/G	rs3025058–T	rs652438A/G	rs2276109A/G	rs2252070A/G				
			A	—				4	0.4	0.49	0.03
				—	A			4	0.4	0.48	0.04
			A	—	A			7	0.4	0.48	0.04
				—	A	A		7	0.4	0.47	0.05
				—	A	A	G	11	0.05	0.12	0.002
			A	—	A	A	G	16	0.05	0.12	0.004
		—	A	—	A	A	G	27	0.05	0.08	0.03
	A	—	A	—	A	A	G	37	0.01	0.08	0.001
C	A	—	A	—	A	A	G	46	0.01	0.08	0.001
C	A	G	A	—				18	0.03	0.07	0.03
C	A	G	A	—	A			24	0.03	0.07	0.02
C	A	G	A	—	A	A		30	0.03	0.06	0.02
	A	G	A	—	A	A		25	0.03	0.06	0.04
	A	G	A	—	A			19	0.03	0.06	0.05
		G	A	—	A	A	G	27	0.01	0.04	0.001
	A	G	A	—	A	A	G	37	0.005	0.04	0.00002 <sup>b</sup>
C	A	G	A	—	A	A	G	46	0.004	0.05	0.00004 <sup>c</sup>

Abbreviations: CAA, coronary artery aneurysms; KD, Kawasaki disease; MMP, matrix metalloproteinase.

Threshold for significance after correction for multiple testing:  $b = 8 \times 10^{-5}$ ;  $c = 6 \times 10^{-5}$ . KD subjects with normal or dilated coronary arteries (CAA–,  $n = 371$ ) were compared with KD subjects with aneurysms (CAA+,  $n = 111$ ).

<sup>a</sup>Not all theoretical haplotypes were observed among the study subjects in our cohort.



**Table 3 Haplotypes on Chr. 11q22 associated with coronary artery aneurysms in the Caucasian-only US-UK cohort**

Haplotype								Total number of observed haplotypes <sup>a</sup>	Frequency		
MMP-7		MMP-1	MMP-3		MMP-12		MMP-13		CAA−	CAA+	P-value
rs11568819 C/T	rs11568818 A/G	rs1799750 -/G	rs679620 A/G	rs3025058 -/T	rs652438 A/G	rs2276109 A/G	rs2252070 A/G				
C	A	G	A	—	A	A	G	30	0.01	0.10	0.0034 <sup>b</sup>
	A	G	A	—	A	A	G	36	0.02	0.09	0.0039 <sup>b</sup>
C	A	—	G	T	A			17	0.12	0.05	0.041
	A	—	G	T	A			18	0.11	0.05	0.047
C	A	—	G	T	A	A		22	0.12	0.05	0.039
	A	—	G	T	A	A		23	0.11	0.05	0.041

Abbreviations: CAA, coronary artery aneurysms; KD, Kawasaki disease; MMP, matrix metalloproteinase.

KD subjects with normal or dilated coronary arteries (CAA-, *n*=215) were compared with KD subjects with aneurysms (CAA+, *n*=75).

<sup>a</sup>Not all theoretical haplotypes were observed among the study subjects in our cohort.

<sup>b</sup>Haplotype also associated with CAA formation in the multiethnic cohort (Table 2).

**Table 4 Haplotypes on Chr. 11q22 associated with CAA in Japanese subjects**

Haplotypes				Total number of haplotypes <sup>a</sup>	Frequency		P-value
MMP-3		MMP-12			CAA—	CAA+	
rs679620 A/G	rs3025058 -/T	rs652438 A/G	rs2276109 A/G				
		A	G	3	0.03	0.11	0.003 <sup>b</sup>
	T	A	G	6	0.01	0.10	0.0009 <sup>c</sup>
G	T	A	G	8	0.01	0.10	0.001 <sup>d</sup>

Abbreviations: CAA, coronary artery aneurysms; KD, Kawasaki disease; MMP, matrix metalloproteinase.

KD subjects with CAA (*n*=58) was compared with KD subjects without CAA (*n*=142). Threshold for significance after correction for multiple testing: *b*=0.006, *c*=0.003, *d*=0.002.

<sup>a</sup>Not all theoretical haplotypes were observed among the study subjects in our cohort.

containing the T allele were not significantly associated. Two haplotypes were significant after correction for multiple sample testing (Table 2, nominal *P*=2 to  $4 \times 10^{-5}$ ). The risk haplotypes consistently included the G allele of *MMP-13* rs2252070 (A/G), the A and C alleles of *MMP7* rs11568818 (A/G) and rs11568819 (C/T), respectively. To explore the possible effects of population stratification on our analysis, we performed haplotype analyses using only self-reported Caucasian subjects from the larger, multiethnic cohort (CAA+ *n*=75, CAA- *n*=215) (Table 3). Two haplotypes identified in the multiethnic cohort again showed association with CAA (nominal *P*=0.0034 and 0.0039) in the all-Caucasian subset. Four 5–7 SNP haplotypes were associated with protection against aneurysm formation, whereas these same haplotypes with the opposite alleles at *MMP-1* rs1799750 (-/G), *MMP-3* rs679620 (A/G) and rs3025058 (-/T) were associated with aneurysm formation in the larger, multiethnic cohort.

#### Single-locus analysis in Japanese cohort

To test the influence of genetic variation in *MMP-3* and *MMP-12* in a different ethnic cohort, single-locus analysis for CAA in the Japanese cohort was performed for SNPs in *MMP-3* and *MMP-12*. *MMP-12* rs2276109 (A/G) was associated with CAA formation with the G allele conferring increased risk (*P*=0.006, odds ratio=4.92, 95% confidence interval 1.54–15.73) (Supplementary Table 2).

#### Haplotype analysis in Japanese subjects

We analyzed the haplotypes of *MMP-3* and *MMP-12* in a Japanese cohort and found that haplotypes containing the *MMP-12* rs2276109 (A/G) G allele and *MMP-3* rs3025058 (-/T) T allele were associated

with CAA after correction for multiple testing (nominal *P*=0.003–0.0009); however, these haplotypes were rare in the study cohort (frequencies: CAA-: 0.01, CAA+: 0.10) (Table 4).

#### DISCUSSION

We tested the hypothesis that genetic variation in MMP genes influences CA damage in patients with KD. Haplotypes in *MMP-3* and *MMP-12*, both with elastolytic activity, were associated with aneurysm formation in our mixed ethnic cohort from the US-UK and an all-Caucasian subset of the cohort. Analysis of an independent cohort of Japanese KD subjects validated the influence of haplotypes in *MMP-3* and *MMP-12* on aneurysm formation, although different alleles were associated with increased risk in the Japanese cohort. This may be due to a difference in haplotype structure with the consequence that the functional genetic variant is linked differently to these alleles in the two populations. Fine mapping or resequencing of this region in these two populations may uncover the specific genetic variation associated with CAA formation.

We found a relationship between haplotypes, including SNPs in *MMP-12*, and aneurysm formation in KD. *MMP-12*, which encodes for a macrophage elastase, has multiple functions that could be important in aneurysm pathogenesis. In addition to degrading extracellular matrix proteins, *MMP-12* may promote macrophage recruitment to the vessel wall by activating tumor necrosis factor- $\alpha$  or by modulating levels of proinflammatory cytokines such as monocyte chemoattractant protein-1.<sup>26</sup> The A allele of *MMP-12* rs2276109 (A/G) shows a higher affinity for the transcription factor activator protein-1 (AP-1) and higher expression levels in reporter gene assays.<sup>17</sup> *MMP-12*

rs652438 (A/G) in exon 8 changes the neutral amino acid, asparagine, to the neutral amino acid, serine, in the hemopexin domain, which is thought to bind to TIMP-1. Functional studies have not been performed to determine the consequence of this amino-acid substitution.

The haplotypes associated with aneurysm formation also included MMP-3. MMP-3 also has elastolytic capabilities and has an important role in other diseases involving vascular wall inflammation, including AAA, atherosclerosis, and Takayasu's arteritis.<sup>27</sup> MMP-3 rs3025058 (–/T) is a well-established functional variant. The T allele in this promoter region preferentially binds to the transcriptional repressor, nuclear factor-κB p50 homodimer, and is associated with reduced transcript abundance.<sup>19</sup> MMP-3 rs679620 (A/G) is located close to a cleavage site, and the substitution of positively charged lysine for negatively charged glutamic acid may alter protein function. In our US–UK subjects, haplotype analysis identified the del-A as the risk haplotype. Our findings are in agreement with a study of MMP-3 rs3025058 (–/T) in a small Korean cohort of KD subjects (CAA+, *n*=34; CAA–, *n*=49).<sup>28</sup> Homozygotes for the MMP-3 T allele had increased risk of CAA. Although this locus was associated with CAA in both the Japanese and US–UK subjects, different alleles were implicated suggesting a difference in linkage disequilibrium structure in this region. As for MMP-12, this region should be considered for resequencing efforts to further understand the role of genetic variation on CAA.

Recently, the A allele of MMP-13 rs2252070 (A/G) was reported to be associated with CAA formation in Japanese cohorts (CAA+, *n*=44; CAA–, *n*=92).<sup>29</sup> MMP-13 rs2252070 (A/G) was only genotyped in the US–UK cohort in our study and was not significantly associated in the single-locus analysis (CAA+, *n*=62; CAA–, *n*=202). The risk haplotype for CAA formation included the G allele of MMP-13 rs2252070 (A/G) in our US–UK cohort, which differs from the published results in Japanese cohorts. Variation in population structure or our larger sample size may account for these differences. The effect of genetic variation in MMP-13 on CAA formation in Japanese subjects should be explored in future studies.

Discrepant results were obtained for the effect of MMP-1 rs1799750 on CAA in different cohorts. In the multiethnic US–UK cohort, both alleles of MMP-1 rs1799750 (–/G) were in haplotypes associated with CAA (Table 2). However, in the Caucasian-only analysis (Table 3), the deletion allele MMP-1 rs1799750 (–/G) was associated with protection against CAA, whereas the G allele was associated with increased risk. One possible interpretation is that the linkage disequilibrium structure in Caucasians is different, thus driving the difference in results.

We recognize several limitations to our study. First, we performed case–control analyses using a multiethnic US–UK cohort to increase the statistical power. We justified this on the basis of similar allele frequencies in HapMap for the MMP SNPs in Caucasian–Hispanics and Caucasians, which comprised 85% of our cohort. Analyses performed on an all-Caucasian subset with reduced sample size supported the results from the analysis of the multiethnic cohort. Although this is one of the largest studies on genetic determinants influencing CAA formation, the number of subjects with CAA was still limited and results will need to be validated in additional, independent cohorts. Our study did not replicate the association of SNPs in MMP13,<sup>29</sup> but a smaller cohort was genotyped for this region in our study, thus reducing our power to detect an association. This should be addressed in future studies with a larger sample size. As another limitation, our genotyping approach did not include all haplotype-tagging SNPs across the MMP gene cluster and not all genes/SNPs were genotyped in both cohorts. Detailed fine mapping or

resequencing of MMP-3 and MMP-12 should be considered in future studies. Finally, the CA phenotyping varied slightly between the western countries and Japan. Some subjects in the Japanese aneurysm group might have been classified as transiently dilated according to the US criteria and therefore included with the normal group. However, the number of subjects who might have been differently assigned in the US and Japanese cohorts would have been small.

In summary, haplotype analyses suggested an influence of genetic variation in genes for the elastolytic MMPs in the gene cluster on Chr.11 on formation of CAA in KD patients, which was validated in an independent cohort. MMP-3 and MMP-12 haplotypes have been associated with susceptibility to aneurysms in other conditions, which suggests a shared molecular mechanism that unifies these inflammation-associated aneurysm syndromes.

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- 1 Takahashi, K., Oharaseki, T., Naoe, S., Wakayama, M. & Yokouchi, Y. Neutrophilic involvement in the damage to coronary arteries in acute stage of Kawasaki disease. *Pediatr Int.* **47**, 305–310 (2005).
- 2 Inamo, Y., Harada, K., Okuni, M., Kimoto, K., Takeuchi, S. & Sakurabayashi, I. Immunoreactive polymorphonuclear leukocyte elastase in complex with alpha 1-antitrypsin in Kawasaki disease. *Acta Paediatr Jpn.* **29**, 202–205 (1987).
- 3 Biezeveld, M. H., van Mierlo, G., Lutter, R., Kuipers, I. M., Dekker, T., Hack, C. E. et al. Sustained activation of neutrophils in the course of Kawasaki disease: an association with matrix metalloproteinases. *Clin Exp Immunol.* **141**, 183–188 (2005).
- 4 Shapiro, S. D. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. *Curr Opin Cell Biol.* **10**, 602–608 (1998).
- 5 Manicone, A. M. & McGuire, J. K. Matrix metalloproteinases as modulators of inflammation. *Semin Cell Dev Biol.* **19**, 34–41 (2008).
- 6 Burns, J. C. & Glode, M. P. Kawasaki syndrome. *Lancet.* **364**, 533–544 (2004).
- 7 Burgner, D., Davila, S., Breunis, W. B., Ng, S. B., Li, Y., Bonnard, C. et al. A genome-wide association study identifies novel and functionally related susceptibility loci for Kawasaki disease. *PLoS genetics.* **5**, e1000319 (2009).
- 8 Burns, J. C., Shimizu, C., Gonzalez, E., Kulkarni, H., Patel, S., Shike, H. et al. Genetic variations in the receptor-ligand pair CCR5 and CCL3L1 are important determinants of susceptibility to Kawasaki disease. *J Infect Dis.* **192**, 344–349 (2005).
- 9 Burns, J. C., Shimizu, C., Shike, H., Newburger, J. W., Sundel, R. P., Baker, A. L. et al. Family-based association analysis implicates IL-4 in susceptibility to Kawasaki disease. *Genes Immun.* **6**, 438–444 (2005).
- 10 Onouchi, Y., Gunji, T., Burns, J. C., Shimizu, C., Newburger, J. W., Yashiro, M. et al. ITPKC functional polymorphism associated with Kawasaki disease susceptibility and formation of coronary artery aneurysms. *Nat Genet.* **40**, 35–42 (2008).
- 11 Onouchi, Y., Tamari, M., Takahashi, A., Tsunoda, T., Yashiro, M., Nakamura, Y. et al. A genome-wide linkage analysis of Kawasaki disease: evidence for linkage to chromosome 12. *J Hum Genet.* **52**, 179–190 (2007).
- 12 Aziz, F. & Kuivaniemi, H. Role of matrix metalloproteinase inhibitors in preventing abdominal aortic aneurysm. *Ann Vasc Surg.* **21**, 392–401 (2007).
- 13 Thompson, R. W. & Parks, W. C. Role of matrix metalloproteinases in abdominal aortic aneurysms. *Ann NY Acad Sci.* **800**, 157–174 (1996).
- 14 Newburger, J. W., Takahashi, M., Gerber, M. A., Gewitz, M. H., Tani, L. Y., Burns, J. C. et al. Diagnosis, treatment, and long-term management of Kawasaki disease: a statement for health professionals from the Committee on Rheumatic Fever, Endocarditis and Kawasaki Disease, Council on Cardiovascular Disease in the Young, American Heart Association. *Circulation.* **110**, 2747–2771 (2004).
- 15 Disease, R.C.o.K. Report of subcommittee on standardization of diagnostic criteria and reporting of coronary artery lesions in Kawasaki disease (Ministry of Health and Welfare, Tokyo, Japan, 1984).
- 16 Jormsjo, S., Whattling, C., Walter, D. H., Zeiher, A. M., Hamsten, A. & Eriksson, P. Allele-specific regulation of matrix metalloproteinase-7 promoter activity is associated with coronary artery luminal dimensions among hypercholesterolemic patients. *Arterioscler Thromb Vasc Biol.* **21**, 1834–1839 (2001).
- 17 Jormsjo, S., Ye, S., Moritz, J., Walter, D. H., Dimmeler, S., Zeiher, A. M. et al. Allele-specific regulation of matrix metalloproteinase-12 gene activity is associated with

- coronary artery luminal dimensions in diabetic patients with manifest coronary artery disease. *Circ Res.* **86**, 998–1003 (2000).
- 18 Rutter, J. L., Mitchell, T. I., Buttice, G., Meyers, J., Gusella, J. F., Ozelius, L. J. *et al.* A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter creates an Ets binding site and augments transcription. *Cancer Res.* **58**, 5321–5325 (1998).
  - 19 Ye, S., Eriksson, P., Hamsten, A., Kurkinen, M., Humphries, S. E. & Henney, A. M. Progression of coronary atherosclerosis is associated with a common genetic variant of the human stromelysin-1 promoter which results in reduced gene expression. *J Biol Chem.* **271**, 13055–13060 (1996).
  - 20 Yoon, S., Kuivaniemi, H., Gatalica, Z., Olson, J. M., Buttice, G., Ye, S. *et al.* MMP13 promoter polymorphism is associated with atherosclerosis in the abdominal aorta of young black males. *Matrix Biol.* **21**, 487–498 (2002).
  - 21 Haq, I., Chappell, S., Johnson, S. R., Lotya, J., Daly, L., Morgan, K. *et al.* Association of MMP-2 polymorphisms with severe and very severe COPD: a case control study of MMPs-1, 9 and 12 in a European population. *BMC Medical Genetics.* **11**, 7.
  - 22 Joos, L., He, J. Q., Shepherdson, M. B., Connett, J. E., Anthonisen, N. R., Pare, P. D. *et al.* The role of matrix metalloproteinase polymorphisms in the rate of decline in lung function. *Hum. Mol Genetics.* **11**, 569–576 (2002).
  - 23 Ricketts, C., Zeegers, M. P., Lubinski, J. & Maher, E. R. Analysis of germline variants in CDH1, IGFBP3, MMP1, MMP3, STK15 and VEGF in familial and sporadic renal cell carcinoma. *PLoS One.* **4**, e6037 (2009).
  - 24 Dempster, A., Laird, N. & Rubin, D. Likelihood from incomplete data via the EM algorithm. *J. Royal Stat. Soc. Series B.* **39**, 1–38 (1977).
  - 25 Sinnwell, J. P. & Schaid, D. J. haplo.stats: statistical analysis of haplotypes with traits and covariates when linkage phase is ambiguous R package version 1.2.2., (2005).
  - 26 Hautamaki, R. D., Kobayashi, D. K., Senior, R. M. & Shapiro, S. D. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science.* **277**, 2002–2004 (1997).
  - 27 Matsuyama, A., Sakai, N., Ishigami, M., Hiraoka, H., Kashine, S., Hirata, A. *et al.* Matrix metalloproteinases as novel disease markers in Takayasu arteritis. *Circulation.* **108**, 1469–1473 (2003).
  - 28 Park, J. A., Shin, K. S. & Kim, Y. W. Polymorphism of matrix metalloproteinase-3 promoter gene as a risk factor for coronary artery lesions in Kawasaki disease. *J Korean Med Sci.* **20**, 607–611 (2005).
  - 29 Ikeda, K., Ihara, K., Yamaguchi, K., Muneuchi, J., Ohno, T., Mizuno, Y. *et al.* Genetic analysis of MMP gene polymorphisms in patients with Kawasaki disease. *Pediatr Res.* **63**, 182–185 (2008).

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# Association of *CCR2-CCR5* Haplotypes and *CCL3L1* Copy Number with Kawasaki Disease, Coronary Artery Lesions, and IVIG Responses in Japanese Children

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## Abstract

**Background:** The etiology of Kawasaki Disease (KD) is enigmatic, although an infectious cause is suspected. Polymorphisms in CC chemokine receptor 5 (*CCR5*) and/or its potent ligand *CCL3L1* influence KD susceptibility in US, European and Korean populations. However, the influence of these variations on KD susceptibility, coronary artery lesions (CAL) and response to intravenous immunoglobulin (IVIG) in Japanese children, who have the highest incidence of KD, is unknown.

**Methodology/Principal Findings:** We used unconditional logistic regression analyses to determine the associations of the copy number of the *CCL3L1* gene-containing duplication and *CCR2-CCR5* haplotypes in 133 Japanese KD cases [33 with CAL and 25 with resistance to IVIG] and 312 Japanese controls without a history of KD. We observed that the deviation from the population average of four *CCL3L1* copies (i.e., < or > four copies) was associated with an increased risk of KD and IVIG resistance (adjusted odds ratio (OR) = 2.25,  $p=0.004$  and OR=6.26,  $p=0.089$ , respectively). Heterozygosity for the *CCR5* HHF\*2 haplotype was associated with a reduced risk of both IVIG resistance (OR=0.21,  $p=0.026$ ) and CAL development (OR=0.44,  $p=0.071$ ).

**Conclusions/Significance:** The *CCL3L1-CCR5* axis may play an important role in KD pathogenesis. In addition to clinical and laboratory parameters, genetic markers may also predict risk of CAL and resistance to IVIG.

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## Introduction

Kawasaki disease (KD) is an acute, self-limiting systemic vasculitis of infants and children [1,2]. The most serious complication of KD is the development of coronary artery lesions (CAL) that range from transient dilatation to destruction of the vessel wall architecture resulting in aneurysms [3]. Indeed, the primary goal of KD treatment is to prevent this complication [1,2]. There is significant inter-individual variation in KD susceptibility as well as CAL development. Moreover, although administration of a combination of a high dose intravenous immunoglobulin (IVIG) and aspirin is the standard therapy for acute KD, 15–30% of KD patients have persistent or recurrent fever after IVIG treatment [4,5,6,7,8,9,10]. Also, such patients are at increased risk of developing CAL [11]. Thus, identification of

host factors that influence KD susceptibility, CAL development and resistance to IVIG treatment may provide new insights into KD pathogenesis, novel means for prognostication of clinical outcome, and therapeutic targets.

According to a current paradigm, KD is thought to be triggered by an infectious agent that elicits an inflammatory response directed at cardiovascular tissues in genetically susceptible hosts [1,12,13]. Polymorphisms in various genes have been shown to influence KD susceptibility in different populations [14,15,16,17,18,19,20,21,22]. Similarly, variations in the genes encoding CD14 [23], matrix metalloproteinase (MMP)-3 [24], vascular endothelial growth factor (VEGF) and its receptor kinase insert domain receptor (KDR) [21] have been implicated in CAL development in KD. With respect to response to IVIG, several studies have reported laboratory and demographic predictors associated with

IVIG failure [6,7,8]. However, the generalization of scoring systems based on such predictors to multiethnic U.S. populations has not been successful [10]. The genetic basis of IVIG resistance in the setting of KD or other inflammatory, autoimmune and infectious diseases in which IVIG has been empirically used (e.g. Idiopathic thrombocytopenic purpura), including pediatric HIV and post-infectious complications [25], has not been fully elucidated.

There is evidence to suggest that recruitment of inflammatory cells in KD may be mediated through CC chemokine receptor 5 (CCR5) [15,19,26]. Chemotactic gradients for homing of CCR5+ cells are provided by a variety of chemokines, the most potent of which is its ligand - CC ligand 3 like 1 (CCL3L1) [27]. The genes encoding CCR5 and CCL3L1 demonstrate two distinct types of polymorphisms: single nucleotide polymorphisms in CCR5 [28] and copy number variation (CNV) in the CCL3L1 gene containing segmental duplication [29]. There is a growing interest in understanding the contribution of CNV in disease pathogenesis since it is recognized that 12% of the human genome may have undergone segmental duplications [30,31]. We previously found that variations in CCR5 and CCL3L1 affect susceptibility to KD in parent-child trios from the United States [15].

However, there is significant variation in the prevalence of KD as well as the frequency of CCR5 genotypes and CCL3L1 copy number in different populations [15,27,32]. Consequently, whether the observations made in US trios can be generalized to Japanese children is unknown. To address this, we conducted a case-control study in subjects from Japan, a geographic region where the prevalence of KD is at least 10 times higher than the Western world [1,2]. We tested the hypothesis that CCR5 haplotypes and CCL3L1 copy number influence KD susceptibility and two disease-related outcomes: development of CAL and IVIG resistance.

## Materials and Methods

### Ethics Statement

This study was approved by the institutional review boards of Yamaguchi and Kurume University Hospitals in Japan and the University of California San Diego and the University of Texas Health Science Center in San Antonio in the U.S. and written informed consent was given by the parents of all KD subjects and controls.

### Study subjects

We conducted an unmatched case-control study of 133 cases of KD and 312 controls collected between January 2002 and April 2005. The KD patients were recruited from three sites: the Department of Pediatrics, Yamaguchi University Hospital; Oita Children's Hospital; and Kurume University Hospitals, Japan. All patients met the Japanese criteria for the diagnosis of KD [33]. CAL was defined as a luminal diameter >3 mm for patients <4 years or >4 mm for patients >5 years of age, or an internal diameter of one or more segments at least 1.5 times larger than the adjacent segment [34]. IVIG-resistant subjects were defined as KD patients who had persistent fever ( $\geq 38.0^{\circ}\text{C}$ ) for at least 36 hours after completion of the IVIG infusion and who received secondary treatment after the initial treatment with IVIG. KD patients who did not receive secondary treatment were considered to have responded to the initial IVIG treatment. The initial IVIG was administered as a single infusion of 2 g/kg/day. All KD patients also received oral aspirin (30 mg/kg/d). Controls were Japanese adults without a history of KD recruited from three centers: San Diego, CA, and Yamaguchi University and RIKEN in Tokyo,

Japan. Most of the controls of Japanese origin (28% from Yamaguchi University, 60% from Riken, and 12% from San Diego) were healthy adults and some had common diseases of adulthood unrelated to KD.

### Genotyping

The methods for genotyping CCR5 polymorphisms are described elsewhere [15,27,32]. The variations in CCR5 were categorized into haplotypes as described previously and were designated as CCR5 human haplogroups A (HHA), HHB, HHC, HHD, HHE, HHF\*1, HHF\*2, HHG\*1, and HHG\*2 [35]. The CCR5 haplotypes that bear the CCR5-Δ32 or CCR2-64I polymorphisms are designated as the CCR5 HHG\*2 and HHF\*2 haplotypes, respectively [32,35]. Copy number of the CCL3L1 gene-containing segmental duplication was estimated as described previously [27]. The assay used to genotype CCL3L1 copy number captures three separate CCL3L genes (CCL3L1, CCL3L2 and CCL3L3) as described previously [27].

### Statistical analysis

Allele frequency and Hardy-Weinberg equilibrium for all the CCR5 haplotypes was estimated using the PowerMarker software [36]. We used unconditional multiple logistic regression analysis to evaluate the association of CCR5 haplotypes and CCL3L1 copy number with KD-related outcomes. The median number of CCL3L1 copies in the study population was 4 and for this reason the study subjects were trichotomized into those possessing <4, 4 and >4 CCL3L1 copies. In these regression analyses, we included CCR5 haplotypes (HHA, HHC, HHE, HHF\*1, HHF\*2 and HHG1) and CCL3L1 copy number (less than 4 and greater than 4) in the same regression model. To determine whether CCL3L1 gene copy number modified the KD-influencing effects of CCR5 haplotypes, we used the Mantel-Haenszel test of homogeneity. We used Stata 10.0 (Stata Corp, College Station, Texas) for the statistical analysis.

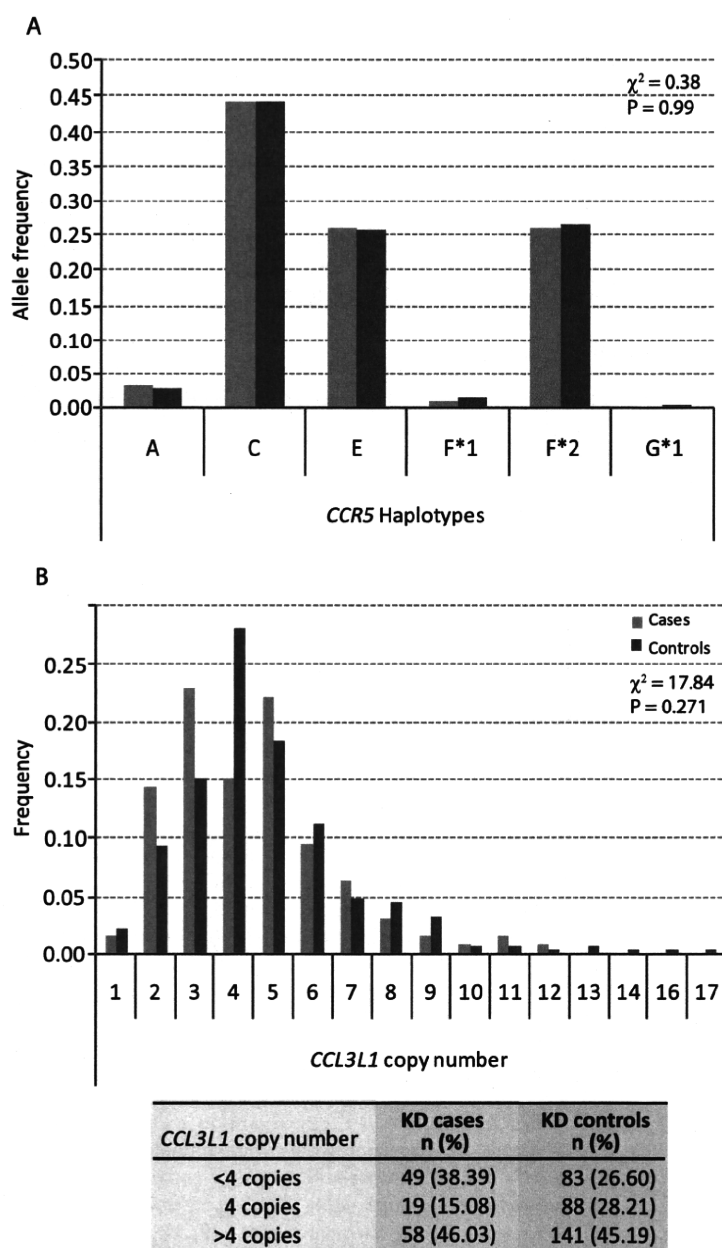
## Results

Among the cases there were 55 (41.35%) females and 78 (58.65%) males whereas in the control group there were 190 (60.90%) females and 122 (39.10%) males. KD patients with available echocardiographic data were categorized into 2 groups according to the presence of CAL. There were 33 (27.5%) and 87 (72.5%) patients with and without CAL, respectively. Mean age of disease onset was 43.5 months (range 2–270 months). Of the 95 cases who were treated with IVIG within the first 10 days of onset of fever, 25 (26.32%) were resistant to treatment.

The most common CCR5 haplotype was CCR5 HHC, followed by HHF\*2 and HHE (Fig. 1A). In the Japanese population the HHG\*2 haplotype bearing the CCR5-Δ32 mutation is very rare. The CCR5 locus was in Hardy-Weinberg equilibrium (Exact  $P = 0.9808$  in controls and  $0.5624$  in cases). The median CCL3L1 copy number in both cases and controls was four (Fig. 1B).

To determine whether CCR5 haplotypes or copy number of the CCL3L1 gene-containing segmental duplication was associated with an altered risk of developing KD, we first performed stepwise unconditional logistic regression analyses. We found that both possession of <4 (OR = 2.73, 95% CI = 1.49–5.03,  $p = 0.001$ ) and >4 CCL3L1 copies (OR = 1.91, 95% CI = 1.06–3.41,  $p = 0.03$ ) was associated with an increased risk of developing KD (Table 1, Final model). Since gender distribution in the case and control groups was different, we adjusted for this covariate, and the adjusted odds ratios indicated that possession of <4 (OR<sub>adjusted</sub> = 2.64, 95% CI = 1.42–4.88,  $p = 0.002$ ) and >4 CCL3L1 copies (OR<sub>adjusted</sub> = 2.00, 95%





**Figure 1. Distribution of *CCR5* haplotypes and *CCL3L1* copy number in cases (blue bars) and controls (purple bars).** (A) Distribution of *CCR5* haplotypes and (B) Distribution of *CCL3L1* copy number. The overall difference of distribution between cases and controls was tested for significance using the  $\chi^2$  test. The table at the bottom of Panel B shows frequencies of *CCL3L1* copy number categories in cases and controls. The categories were derived since 4 was the median copy number in the study population.  
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CI = 1.11–3.61,  $p = 0.022$ ) remained associated with a significantly higher risk of developing KD. Thus, departure from the population average of 4 *CCL3L1* copies (i.e., either < or >4 copies) was associated with a significantly increased risk of KD before (OR = 2.21, 95% CI = 1.28–3.82,  $p = 0.004$ ) and after adjustment for gender (OR<sub>adjusted</sub> = 2.25, 95% CI = 1.29–3.91,  $p = 0.004$ ).

The results in Table 1 indicated that none of the *CCR5* haplotypes had a significant association with the risk of KD. In previous studies, we found that the copy number of *CCL3L1* modified the SLE-, Kawasaki disease-, and HIV-1-disease-influencing effects of *CCR5* haplotypes ([15,37] and data not

shown). Thus, one possibility was that the association of *CCR5* haplotypes with KD susceptibility is present only when it is present in the context of a specific *CCL3L1* copy number. To assess this possibility, we conducted the analysis shown in Table 2. We found that *CCR5* haplotypes did not influence KD susceptibility in subjects possessing <4, >4 or 4 copies of *CCL3L1* (Table 2).

In our cohort, of the 25 subjects who were resistant to IVIG, 18 (72%) developed CAL. By contrast, of the 68 who responded to IVIG treatment, only 5 (7.3%) developed CAL. This association between IVIG resistance and CAL development was highly significant (Fisher's exact  $p = 1.4 \times 10^{-9}$ ). Evaluation of the

**Table 1.** Association of *CCR5* haplotypes and *CCL3L1* copy number with Kawasaki disease susceptibility.

<i>CCR5</i> haplotype/ <i>CCL3L1</i> copy number	OR	95% CI	P value
<b>Full Model</b>			
<i>CCR5</i> HHA	1.13	0.44–2.87	0.802
<i>CCR5</i> HHC	0.70	0.39–1.26	0.236
<i>CCR5</i> HHE	0.80	0.47–1.36	0.407
<i>CCR5</i> HHF*1	0.58	0.12–2.91	0.512
<i>CCR5</i> HHF*2	0.75	0.44–1.27	0.283
<i>CCL3L1</i> <4copies	2.71	1.47–4.99	0.001
<i>CCL3L1</i> >4 copies	1.90	1.06–3.42	0.031
<b>Final Model (Probability Criterion of 0.1)</b>			
<i>CCL3L1</i> <4copies	2.73	1.49–5.03	0.001
<i>CCL3L1</i> >4 copies	1.91	1.06–3.41	0.030

Full model shows results from a logistic regression model including all the indicated predictors while final model indicates the results from the stepwise regression using a retention criterion of 0.1; OR, Odds Ratio; CI, Confidence Interval.

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association for the outcome of CAL revealed that possession of the *CCR2*-64I-bearing *CCR5* HHF\*2 haplotype was associated with a reduced risk of developing CAL which trended towards statistical significance (OR = 0.44, 95% CI = 0.18–1.07,  $p = 0.071$ ). However, we did not observe a significant association between *CCL3L1* copy number and the risk of developing CAL.

We next determined whether *CCR5* haplotypes and *CCL3L1* copy number associated with IVIG responses. In the full model (Table 3), possession of *CCR5* HHF\*2 haplotype was associated with beneficial IVIG responses (OR = 0.21, 95% CI = 0.54–0.83,  $p = 0.026$ ). We also found that possession of <4 *CCL3L1* copies was significantly associated with an increased risk of IVIG resistance (OR = 10.93, 95% CI = 1.17–101.99,  $p = 0.036$ ). Although possession of >4 *CCL3L1* copies was also associated with an increased risk of IVIG resistance (OR = 5.12, 95% CI = 0.57–46.34,  $p = 0.146$ ) (Table 3, Full Model), this did not achieve statistical significance. In the final model (Table 3), possession of <4 *CCL3L1* copies remained associated with an increased risk of IVIG resistance (OR = 2.56, 95% CI = 0.96–6.87,  $p = 0.061$ ) while possession of *CCR5* HHF\*2 haplotype was associated with a salutary IVIG response (OR = 0.34, 95% CI = 0.12–

0.95,  $p = 0.040$ ). Departure from the population average of 4 copies (i. e., < or >4 copies) was associated with a higher risk of IVIG resistance (OR = 6.26, 95% CI 0.76–51.9,  $p = 0.089$ ).

Because we observed that the *CCR5* HHF\*2 haplotype was associated with a reduced risk of IVIG resistance as well as development of CAL, we next examined whether these associations were due to homozygosity and/or heterozygosity of the HHF\*2 haplotype. We observed that heterozygosity but not homozygosity for HHF\*2 was associated with a reduced risk of both CAL (OR = 0.37, 95% CI 0.14–0.97,  $p = 0.042$ ) and IVIG resistance (OR = 0.39, 95% CI 0.14–1.11,  $p = 0.078$ ).

## Discussion

Our results suggest that in Japanese children, copy number variation of the segmental duplication bearing *CCL3L1* associates with susceptibility to KD and IVIG response whereas the *CCR2*-64I-containing *CCR5*-HHF\*2 haplotype is associated with a reduced risk of both CAL development and IVIG resistance. Our finding that deviation from the average *CCL3L1* copy number (i.e., < or >4 copies) found in the Japanese population is associated with increased risk of KD is noteworthy because we have previously found that deviation from median copy number of *CCL3L1* is also associated with an increased risk of systemic lupus erythematosus (SLE) [37] – a disease with broad immunological underpinnings – in three separate cohorts (TX, USA; Ohio, USA; and Medellin, Colombia). The notion that haploinsufficiency and higher gene dosages of immune response genes may influence susceptibility to immune-mediated diseases is also highlighted by our recent observation that both low and high copy numbers of the gene encoding *FCGR3B* was associated with increased susceptibility to SLE and primary Sjogren's syndrome [38]. Together these observations underscore the concept that departure of the gene copy number from a homeostatic copy number, i.e., higher or lower than the average found in the population, may be an important determinant of susceptibility to diseases with a strong immunologic component.

The precise mechanistic basis by which deviation from the average copy number of the *CCL3L1*-containing segmental duplication in our study population was associated with increased KD susceptibility as well as an increased risk of IVIG failure is unknown. As noted, *CCL3L1* is the most potent *CCR5* ligand and *CCR5* ligands are associated with pro-inflammatory effects [39]. Additionally, a copy number of the *CCL3L1*-containing segmental duplication that is higher than the population average is associated with increased leukocyte chemoattraction [29], circulating levels of *CCL3* [27] and *CCL3L1* transcript (data not shown). In this light,

**Table 2.** Lack of a modifying influence of the *CCL3L1* gene copy number on the association of five common *CCR5* haplotypes found in the study population with the risk of KD.

<i>CCR5</i> haplotype	<4 <i>CCL3L1</i> copies		4 <i>CCL3L1</i> copies		>4 <i>CCL3L1</i> copies		M-H Test	
	OR	95% CI	OR	95% CI	OR	95% CI	$\chi^2$	P
HHA	0.41	0.01–4.34	5.06	0.34–72.7	1.38	0.35–4.87	2.75	0.2531
HHC	1.22	0.54–2.81	0.93	0.30–3.10	0.65	0.32–1.33	1.54	0.4627
HHE	0.86	0.39–1.86	1.24	0.40–3.78	0.98	0.50–1.89	0.35	0.8398
HHF*1	—	—	0.00	0.00–6.08	0.48	0.01–4.42	3.08	0.2139
HHF*2	0.92	0.42–1.98	0.66	0.21–2.02	0.98	0.50–1.91	0.43	0.8064

The last column shows the results of Mantel-Haenszel test for homogeneity of results across *CCL3L1* copy number.

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**Table 3.** Association of *CCR5* haplotypes and *CCL3L1* copy number with IVIG response.

<i>CCR5</i> haplotype/ <i>CCL3L1</i> copy number	OR	95% CI	P value
<b>Full Model</b>			
<i>CCR5</i> HHA	0.83	0.12–5.79	0.851
<i>CCR5</i> HHC	0.62	0.15–2.52	0.499
<i>CCR5</i> HHE	0.45	0.13–1.51	0.194
<i>CCR5</i> HHF*2	0.21	0.54–0.83	0.026
<i>CCL3L1</i> <4copies	10.93	1.17–101.99	0.036
<i>CCL3L1</i> >4 copies	5.12	0.57–46.34	0.146
<b>Final Model (Probability Criterion of 0.1)</b>			
<i>CCL3L1</i> <4copies	2.56	0.96–6.87	0.061
<i>CCR5</i> HHF*2	0.34	0.12–0.95	0.040

Full model shows results from a logistic regression model including all the indicated predictors while final model indicates the results from the stepwise regression using a retention criterion of 0.1; OR – Odds Ratio; CI – Confidence Interval.

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it is conceivable that subjects bearing higher *CCL3L1*-containing segmental duplications may express higher levels of chemokines following antigenic stimulus that in turn may increase the risk of developing KD and possibly, IVIG resistance. In addition to causing an immunologic blockade of Fc receptor and inducing further antibody production, IVIG therapy is also known to play an important role in down-regulation of the cytokine/chemokine levels [40]. Conceptually then, in persons possessing high *CCL3L1* gene copy numbers the currently used dose of IVIG may be insufficient to induce the desired degree of down-regulation of chemokines leading to IVIG resistance. The latter along with increased *CCL3L1* associated inflammation may provide a partial explanation as to why we observed a trend for possession of a high *CCL3L1* copy number and reduced clinical responsiveness to IVIG.

On the other hand, a low *CCL3L1* copy number is associated with reduced *CCL3*-*CCL3L1* chemokine expression levels [29] resulting in reduced inflammatory responses. It has been shown that there is a surge in levels of several cytokines/chemokines during the acute phase of KD [40,41,42,43,44] and we have observed that the *CCL3* surge is a key feature of the acute phase of KD (data not shown). Thus, it is possible that an impaired *CCL3*-*CCL3L1*-dependent inflammatory response may partly explain increased risk of KD and reduced clearance of antigen. Consequently, the increased and decreased inflammation associated with a high and low *CCL3L1*-containing segmental duplication, respectively, may explain why all subjects do not respond to a single dose of IVIG and require additional treatments. This hypothesis is supported by the fact that greater than half of IVIG-resistant patients who receive an additional dose of IVIG become afebrile [10]. While appealing, laboratory or clinical data that directly evaluates these hypotheses regarding mechanisms by which a high or low *CCL3L1* gene copy associates with KD and IVIG non-responsiveness are currently lacking and require validation.

The role of *CCR5* polymorphisms in KD susceptibility has been investigated previously. Significant attention has focused on the widely recognized 32-bp deletion ( $\Delta 32$ ) mutation present in the coding region of *CCR5* that is found mainly in populations of

European ancestry. We reported previously that there was an inverse relationship between the global distribution of  $\Delta 32$  allele and the incidence of KD [15]. Also, in our large family-based study in US-trios we had observed an asymmetric transmission of the *CCR5*- $\Delta 32$  allele across generations [15]. Further, we had found that the KD-influencing effects of the *CCR5*- $\Delta 32$ -bearing HHG\*2 haplotype were modified by *CCL3L1* copy number [15]. Breunis et al [26] replicated our observations in a Northern European population and observed that the frequency of the *CCR5*- $\Delta 32$  allele was lower in cases (6.5%) compared to controls (10.7%).

The *CCR5*- $\Delta 32$ -bearing HHG\*2 haplotype is rarely found in Asian populations. The results of two prior studies in subjects with KD of European ancestry [15,26] and a separate study of KD patients from Korea [19] suggested that other polymorphisms at the *CCR5* locus also associate with susceptibility to KD. However, in the present study of Japanese subjects, we did not find an association between *CCR5* haplotypes and KD susceptibility. By contrast, we did find an association of *CCR5* haplotypes with KD outcomes and IVIG-resistance.

Early coronary lesions demonstrate marked infiltration of neutrophils [45] whereas at later time points show infiltration predominantly of T-cells and monocytes/macrophages [43]. Members of the chemokine system, including *CCR5* and *CCL3L1* play an important role in leukocyte trafficking and activation as well as the pathogenesis of coronary artery diseases such as arteriosclerosis, hypertension and myocardial infarction [46]. In our previous study of European-descent KD patients, we found that the  $\Delta 32$ -bearing *CCR5*-HHG\*2 haplotype was associated with not only reduced KD susceptibility, but also a lower risk of CAL [15]. In the present study, we observed that the *CCR5* HHF\*2 haplotype which bears the *CCR2*-64I polymorphism is associated with a reduced the risk of IVIG-resistance and CAL formation. Whether this association suggests an involvement of *CCR2*, a receptor critically involved in monocyte trafficking and activation, in KD pathogenesis and therapy responses is unclear because the *CCR2*-64I polymorphism is in linkage disequilibrium with promoter polymorphisms in *CCR5* [32]. Notwithstanding this quandary, it is conceivable that the beneficial associations observed for the *CCR2*-64I-bearing *CCR5* HHF\*2 haplotype with KD-related outcomes may relate either directly or indirectly to inflammation.

Many demographic and laboratory factors such as patient age, white blood cell count, and plasma levels of aspartate amino transferase and C-reactive protein have been identified as risk factors for IVIG resistance [3,6,8,47,48,49]. Onouchi et al [22] observed that a functional polymorphism in the *ITPKC* gene was associated with response to IVIG in US KD children. The results of the present study extend the notion that host genetic factors may influence IVIG resistance. IVIG has been shown to be effective across a range of autoimmune, inflammatory and infectious conditions, as well as for post-infectious complications [25]. This suggests that IVIG may have a broad immunomodulatory mechanism of action, beyond merely inhibiting antibody-triggered inflammation. Park-Min et al showed recently that IVIG blocks cellular activation by interferon- $\gamma$  (IFN $\gamma$ ) [50], a proinflammatory cytokine that plays a key role in cellular immune responses and Th1-type-driven inflammatory/infectious diseases [51,52]. In this respect it is notable that *CCR5* is expressed on Th1 cells [53], and thus it is conceivable that polymorphisms in this gene and its ligands by influencing Th1 pathways may influence IVIG responses. Because IVIG is far from an optimized therapeutic, and responses to IVIG vary considerably among patients, future studies are warranted to identify the broader range of host genetic