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Association of *COL11A2* polymorphisms with susceptibility to Kawasaki disease and the development of coronary artery lesions

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Summary

Kawasaki disease is a pediatric systemic vasculitis of unknown etiology for which a genetic influence is suspected. Gene clusters within the MHC region at chromosome 6p21.3 have been linked to many autoimmune diseases, including Kawasaki disease. In this study, we tested the hypothesis that single-nucleotide polymorphisms (SNPs) of a collagen gene, *COL 11A 2*, located in this MHC region may exert influence on susceptibility to Kawasaki disease and its arterial sequelae. Two SNP sites, rs2294478 and rs2076311, were genome-typed on 93 KD patients and 680 healthy subjects. Genotypic and allelic frequencies of rs2076311 have been found significant different between normal controls and patients. Clinical association study found that patients with C allele at rs2076311 have lower probability to develop coronary artery lesions. Although the SNP site of rs2294478 did not show association with KD, the A allele could be used as the determinant genetic indicator to differentiate KD patients with or without CALs. C-A haplotype of *COL 11A 2* gene associates with KD development and determines CAL formations in patients. Our findings suggest the involvement of genetic variations of *COL 11A 2* in Kawasaki disease and CAL formation.

Introduction

Kawasaki disease (KD) is an acute febrile vasculitis primarily affecting infants and children under the age of 5 years. Clinical symptoms include skin rashes, inflammation of mucous membranes in the mouth, nose or throat, and swollen lymph nodes with persistent high fever. There is no specific test for KD as its etiology remains unknown, although viral infection is suspected as one risk factor (Rowley, 2008; Rowley & Shulman, 2007). Most clinical diagnoses entail evaluating symptoms and ruling out other conditions. Though children with KD can fully recover within days when detected early, about 25% of untreated cases develop serious complications that can cause myocardial infarction. As such, this disease is the leading cause of acquired heart disease in children (Burns & Glode, 2004; Chang *et al*., 2004).

Epidemiologic studies indicate that KD affects individuals in all ethnic groups, yet it is more prevalent among children of Asian and Pacific Island descent. This observation suggests that there is a genetic influence on disease susceptibility (Hata & Onouchi, 2009). KD has been hypothesized to be an autoimmune disorder, where aneurysm/cardiac complications arise from vascular inflammation. Importantly, genetic variations involved in regulating immune functions and inflammation have been found to be related to KD susceptibility (Chen *et al*., 2009; Lin *et al*.,2009; Hseueh *et al*.,2008;Onouchi *et al*.,2008; Cheung *et al*.,2008). Some genes that play roles in cardiovascular pathogenesis, such as coronary artery lesion (CAL) formation, likewise are important for the development of KD (Hsueh *et al*.,2008; Burgner *et al*.,2009). These prior studies provide molecular evidence that KD is a genetic disease, and therefore justify further study of potentially related genetic variations.

COL11A2 encodes the alpha-2 chain of human fibrillar collagen type XI, belonging to the fibril-forming class of collagens. Collagen is a strong autoantigen that can induce chronic inflammation in patients with vasculitis. Its accumulation correlates with the development of CALs (Direskeneli *et al*.,1994; Wisnieski & Jones,1992; Siegert *et al*.,1990), such that it is reasonable to presume that collagen genes could play a role in the development of KD. In fact, abnormally upregulated collagen proteins and auto-antibodies against collagens have been detected in the serum of patients with KD (Kobayashi *et al*.,1992; Lin *et al*.,2008). In addition, genetic mapping of *COL11A2* located it at the centromeric border of the MHC region on chromosome 6, 6p21.3, an area linked to immune-mediated vascular diseases (Porto *et al*.,2005; Mizuki *et al*.,1997; Papiha *et al*.,1992). While MHC genes within the 6p21.3 region have been scrutinized for possible association with KD development, it is still unknown whether genetic variations in *COL11A2* determine susceptibility.

The incidence of KD in Taiwan is the third highest in the world, after Japan and Korea, with an annual diagnosis rate of 66 out of 100,000 children (Chang *et al*.,2004; Park *et al*.,2005). Our study investigated the association between genetic polymorphisms of *COL11A2* and KD, including the development of CALs, in 93 KD children and 680 unrelated individuals from Taiwan. Two unique SNP sites, rs2294478 and rs2076311, as well as their relationship to clinical features in the patients, were also analyzed in this study.

Materials and Methods

Patients and sample collection

The study subjects included a total of 93 KD patients and 680 healthy participants, and were recruited from China Medical University Hospital in Taiwan. The disease patients all met the criteria for KD as defined in a previous study (Lin et al., 2009; Wu et al., 2004). The healthy control group was selected from a pool of healthy persons based on examinations conducted at the same hospital. All individual blood samples were collected by venipuncture for genomic DNA isolation. Informed consent was obtained from all subjects participating in this study, which was approved by the Institutional Review Board (IRB) at China Medical University.

Genomic DNA extraction and genotyping

Genomic DNA was extracted from peripheral blood leukocytes according to standard protocols (Genomic DNA kit; Qiagen, Valencia, CA, USA). DNA fragments containing rs2294478 and rs2076311 SNP sites were amplified by PCR using the *Taqman* SNP genotyping assay system from Applied Biosystems, Inc (Carlsbad, CA, USA). The probe IDs for rs2294478 and rs2076311 are C16187114-10 and CMU-Sheu-001, respectively. PCR amplification conditions consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 56°C for 10 s, and 72°C for 20 s, with one additional cycle of 72°C for 5 min. Genetic variations were detected by reading the fluorescence signals of the PCR products. A positive signal indicates a perfect match between the probe and the tested DNA, thus identifying wild type alleles.

Clinical symptoms and association study

The clinical information of KD patients studied in this study was collected from clinical note, including blood tests, liver function and fever duration. All patients in this study were treated with intravenous immunoglobulin (IVIG; 2 g/kg infused over 8–12 hr) and oral aspirin (80–100mg/kg/day). Echocardiographs were obtained by the pediatric cardiologist before or within 2 weeks of IVIG administration. CALs were diagnosed according to the criteria proposed by the Japanese Kawasaki Disease Research Committee (Research Committee on KD). Coronary arteries were classified as abnormal if the internal lumen diameter was > 3mm in children younger than 5 years or > 4mm in children older than 5 years, if the internal diameter of a segment measured $\geq$ 1.5 times that of an adjacent segment, or if the coronary lumen was clearly irregular.

Statistic analysis

The allelic and genotypic frequency distributions for the two polymorphisms in KD patients and controls were performed by χ^2 analysis using SPSS software (version 10.0, SPSS Inc. Chicago, Illinois, US). A *p* value of less than 0.05 was considered statistically significant. Allele and genotype frequencies are expressed as percentages of the total number of alleles and genotypes. Odds Ratios (OR) were calculated from allelic and genotypic frequencies with 95% confident interval (95% CI). Haplotypes were determined using the Bayesian statistical method available in the program Phase 2.1 (Stephens & Scheet, 2005). Adherence to the Hardy-Weinberg equilibrium constant was tested using a χ^2 test with one degree of freedom.

Results

Allelic and genotypic frequencies of COL11A2 in KD patients

The gene locus for *COL11A2* was mapped to the MHC region on chromosome 6p, an area linked to a variety of autoimmune diseases. Within this region, 201 reliable, polymorphic, and evenly spaced SNPs have been previously genotyped (Walsh *et al*., 2003). Among these defined SNPs, rs2294478 and rs2076311 are unique to *COL11A2*. Genotyping PCR was performed to analyze the genetic variations of these two SNPs in the study subjects. No significant differences in allelic and genotypic frequencies for rs2294478 were observed between patients and controls. In contrast, there was a significant difference between patients and controls in the frequency of C/C (OR = 0.38, 95% CI = 0.18-0.79) and A/C (OR = 0.42, 95% CI = 0.19-0.91) at rs2076311 (p = 0.029). In addition, KD patients had a lower frequency of the C allele at rs2076311 as compared to the controls (p = 0.044, OR = 0.70, 95% CI = 0.50-0.99) (Table 1).

Polymorphisms of COL11A2 and coronary artery lesion (CAL) formation

Formation of coronary artery lesions (CALs) is a leading cause of heart attacks in KD patients and carries a significant risk of death or disability. Therefore, we next studied the impact of *COL11A2* polymorphisms on this clinical symptom. As shown in Table 2, we analyzed both the genotypic and allelic frequency at rs2294478 and rs2076311 with respect to CAL symptoms in KD patients. Our data did not indicate any relationship between rs2294478 or rs2076311 with CAL formation in KD patients, neither in genotypic frequency (rs2294478: p = 0.186; rs2076311: p = 0.485) nor in allelic frequency (rs2294478: p = 0.069; rs2076311: p = 0.438). Surprisingly,

individuals with the A/A or C/A genotype at rs2294478 were more likely to develop CAL-free KD (p = 0.007; A/A: OR = 0.33, 95% CI = 0.15-0.72; A/C: OR = 0.30, 95% CI = 0.13-0.70), even though the allele analysis did not indicate a significant difference. Our data also suggest that polymorphisms at rs2294478 can differentiate between KD patients with and without CALs (p = 0.047) (Table 2). In particular, KD patients with the A allele at rs2294478 have a higher probability of developing CALs (p = 0.010; OR = 2.91, 95% CI = 1.26-6.71).

In contrast to rs2294478, we observed an association between polymorphisms at rs2076311 with CAL-free KD. Table 2 shows that patients who carry the C allele have a lower probability of developing CAL-free KD (p = 0.048; OR = 0.67, 95% CI = 0.45-1.00), whereas both C/C and C/A genotypes at rs2076311 were observed less frequently in CAL-free KD patients at a significant level (p = 0.037; C/C: OR = 0.34, 95% CI = 0.15-0.80; C/A: OR = 0.40, 95% CI = 0.16-0.96). Our data suggest that polymorphisms at rs2076311 can determine the genetic susceptibility of KD development, and polymorphisms at rs2294478 could be used as a genetic indicator to predict CAL formation in KD patients.

The C-A haplotype of COL11A2 is associated with KD development and predicts CAL formation

Because genetic variations at both rs2294478 and rs2076311 were found to control CAL formation in KD patients, we next studied the impact of different genetic combinations on KD development and CAL formation. Haplotype analyses of these two SNPs in the *COL11A2* gene revealed that the C-A haplotype was significantly different between normal controls and KD

patients (Table 3). In addition, this haplotype was significantly associated with the absence of CALs in KD patients. Therefore, the C-A haplotype could be used as a genetic marker to differentiate KD patients with CALs from those lacking such lesions.

Discussion

This study defined the relationship between SNPs in *COL11A2* and KD development, and analyzed the impact of genetic variations on CAL formation. Previously, we identified critical MHC gene clusters linked to KD within the MHC region of chromosome 6p21.3, where *COL11A2* is located (Hanson *et al*., 1989). The data presented here further support the potential functional SNPs of *COL11A2*, a non-MHC gene, in KD. Genotypic and allelic frequencies at rs2076311 were observed to be significantly different between normal controls and patients with KD, and individuals carrying the C allele at this SNP site had a lower probability of developing KD. Although the rs2294478 SNP site was not associated with KD, the A allele could be used as a determinant genetic indicator to differentiate between KD patients with CAL formation from those without such lesions. Finally, a clinical association study found that KD patients with the C allele at rs2076311 had a lower probability of developing CALs, and inflammation responses in these patients were also reduced.

CAL-induced heart attack is the most serious complication in KD patients. Although the molecular mechanism underlying such events is unclear, weakness of coronary arteries due to inflammation of heart vessels has been observed to be a leading cause for CAL formation (Tulloh *et al*., 2004). As collagen is important for maintaining the primary structure of the heart, especially for blood vessels and mitral valves, alterations in the composition of the extracellular matrix by enhanced collagen synthesis plays a crucial role in CAL formation (Lin et al., 2008). Since COL11A2 is one component of fibrillar collagen in the myocardial matrix, our data provide the first evidence to support the potential roles of genetic variations in collagen genes during KD

development, particularly with respect to CAL formation. Importantly, previous studies also indicate that SNPs in *COL3A1* are associated with mitral valve prolapse (Chou *et al*., 2004). Relationships between KD and genetic variations in other collagen genes are under investigation.

Altered or abnormal production of collagen is associated with autoimmune diseases (Griffiths & Remmers, 2001; Corthay *et al*., 2001). Among the collagen family members, mutations in *COL11A2* are linked to Stickler syndrome (Brunner *et al*., 1994), which is characterized by spinal abnormalities due to arthritis development. Therefore, it is possible that variations in COL11A2 sequences could also play some role in the induction of auto-immunity. Future studies will focus on the detection of autoantibodies specific for COL11A2 to evaluate the possibility of developing a diagnosis marker for KD.

Although our data support the idea that KD is a disease caused by genetic variations, we cannot exclude the possible involvement of one or more infectious agents. Protein mimicry between viral antigens and host proteins has been frequently observed as one method to circumvent immune tolerance in the host during viral infection. As there is increasing evidence in support of an infectious etiology for KD, verification of the contribution of these defined SNP sites to the structural or linear epitopes shared by viral antigens to trigger immune responses is warranted.

Taken together, our data suggest the possibility that genetic variations in *COL11A2* present genomic elements involved in susceptibility to KD, and provides the first evidence that polymorphisms in collagen genes may contribute to KD development.

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Table 1. Polymorphisms of *COL11A2* in Kawasaki disease (KD) and control

SNP	genotype /allele	Control	KD	KD *vs.* Control		
				p value	OR[1]	95% CI[2]
rs2294478		(n=670)	(n=93)			
	AA	394 (58.8%)	56 (60.2%)	0.113	0.54	0.25-1.14
	CA	238 (35.5%)	27 (29.0%)		0.43	0.19-0.96
	CC	38 (5.7%)	10 (10.8%)		1.00	
	allele A	1026 (76.6%)	139 (74.7%)	0.581	0.91	0.64-1.29
	allele C	314 (23.4%)	47 (25.3%)		1.00	
rs2076311		(n=659)	(n=93)			
	CC	397 (60.2%)	50 (53.8%)	***0.029****	0.38	0.18-0.79
	AC	229 (34.7%)	32 (34.4%)		0.42	0.19-0.91
	AA	33 (5.0%)	11 (11.8%)		1.00	
	allele C	1023 (77.6%)	132 (71.0%)	***0.044****	0.70	0.50-0.99
	allele A	295 (22.4%)	54 (29.0%)		1.00	

Numbers in parentheses indicate percentage of the genotypic or allelic frequency.

*Numbers in bold italics indicate significant differences.

[1]OR, odds ratio.

[2]CI, confidence interval.

Table 2. Polymorphisms of *COL11A2* in Kawasaki disease (KD) patients and associations with coronary artery lesion (CAL) formation

genotype /allele	Control	KD CAL(+)	KD CAL (–)	KD-CAL (+) *vs.* Control			KD-CAL (–) *vs.* Control			KD-CAL (+) *vs.* KD-CAL (–)		
SNP				P^1	OR[2]	95% CI[3]	P^1	OR[2]	95% CI[3]	P^1	OR[2]	95% CI[3]
rs2294478	(n=670)	(n=30)	(n=63)									
AA	394 (58.8%)	22 (73.3%)	34 (54.0%)	0.186	--	--	***0.007****	0.33	0.15-0.72	***0.047****	--	--
CA	238 (35.5%)	8 (26.7%)	19 (30.2%)		--	--		0.30	0.13-0.70		--	--
CC	38 (5.7%)	0 (0.0%)	10 (15.8%)		1.00			1.00			1.00	
allele A	1026 (76.6%)	52 (86.7%)	87 (69.0%)	0.069	1.99	0.93-4.23	0.059	0.68	0.46-1.02	***0.010****	2.91	1.26-6.71
allele C	314 (23.4%)	8 (13.3%)	39 (31.0%)		1.00						1.00	
rs2076311	(n=659)	(n=30)	(n=63)									
CC	397 (60.2%)	17 (56.7%)	33 (52.4%)	0.485	0.47	0.13-1.69	***0.037****	0.34	0.15-0.80	0.901	1.37	0.32-5.86
AC	229 (34.7%)	10 (33.3%)	22 (34.9%)		0.48	0.13-1.84		0.40	0.16-0.96		1.21	0.26-5.56
AA	33 (5.0%)	3 (10.0%)	8 (12.7%)		1.00		***0.048****	1.00			1.00	
allele C	1023 (77.6%)	44 (73.3%)	88 (69.8%)	0.438	0.79	0.44-1.43		0.67	0.45-1.00	0.624	1.19	0.6-2.36
allele A	295 (22.4%)	16 (26.7)	38 (30.2%)		1.00			1.00			1.00	

Numbers in parentheses indicate percentage of the genotypic or allelic frequency.

*Numbers in bold italics indicate significant differences.

[1]*p*, *p* value.

[2]OR, odds ratio.

[3]CI, confidence interval.

Table 3. Haplotype analysis of *COL11A2* in KD patients and healthy control subjects

Haplotype rs2294478-rs2076311	Estimated haplotype frequency				*p* value			
	Control (n=680)	KD total (n-93)	KD-CAL(+) (n=30)	KD-CAL(−) (n=63)	KD total *vs.* Control	KD-CAL(+) vs. Control	KD-CAL(−) vs. Control	KD-CAL(+) vs. KD-CAL(−)
A-A-	121 (17.8%)	19 (20.4%)	7 (23.3%)	11 (17.5%)	0.317	0.085	0.888	0.196
A-C-	400 (58.8%)	50 (53.8%)	18 (60.0%)	32 (50.8%)	0.204	0.842	0.079	0.231
C-A-	31 (4.6%)	8 (8.6%)	1 (3.3%)	8 (12.7%)	***0.033****	0.098	***< 0.001****	***0.006****
C-C-	128 (18.8%)	16 (17.2%)	4 (13.3%)	12 (19.0%)	0.564	0.276	0.938	0.321

Numbers in parentheses indicate percentage of haplotype frequency.

*Numbers in bold italics indicate significant differences.

Table 4. Association between rs2076311 alleles and clinical parameters in children with KD CAL(+) and KD CAL(–)

Clinical parameters[1]	KD CAL(+)		KD CAL(–)		*P* value (KD CAL(+) *vs.* KD CAL (–)	
	AA (*n*=3)	AC+CC (*n*=27)	AA (n=8)	AC+CC (n=55)	AA	AC+CC
Age, year	1.18 ± 0.9	2.08 ± 1.89	1.47 ± 1.01	1.79 ± 1.45	0.674	0.479
WBC, $x10^3/mm^3$	21.48 ± 4.78	16.43 ± 6.03	12.01 ± 2.59	13.67 ± 5.08	***0.006****	0.053
Hemoglobin, g/dL	10.80 ± 2.44	11.02 ± 1.31	11.92 ± 0.95	11.25 ± 0.99	0.245	0.520
Platelet, $x10^3/mm^3$	577.00 ± 287.07	508.99 ± 233.12	371.4 ± 78.07	393.63 ± 128.75	0.216	***0.034****
ESR, mm/h	70 ± 56.57	93.83 ± 29.23	82.5 ± 30.16	76.33 ± 33.98	0.685	0.062
CRP, mg/dL	17.5 ± 1.1	14.26 ± 6.99	8.98 ± 4.55	6.74 ± 4.99	***0.040****	***< 0.001****
GOT, IU/L	21.5 ± 9.19	86.53 ± 106.62	132.67 ± 187.38	67.89 ± 90.16	0.456	0.492
GPT, IU/L	24.5 ± 4.95	71.22 ± 78.84	83.5 ± 88.28	71.08 ± 102.56	0.405	0.996
Fever duration (before IVIG)	7.21 ± 1.53	6.71 ± 2.31	6.14 ± 1.21	5.67 ± 1.37	***0.025****	***0.027****
Fever duration (after IVIG)	1.87 ± 2.78	1.86 ± 2.48	2.14 ± 2.73	1.15 ± 1.53	0.709	0.196
Total fever duration	9.08 ± 2.32	8.57 ± 3.17	8.29 ± 2.06	6.78 ± 1.98	0.264	***0.008****

[1]Data for each group are expressed as mean ± SD.

Abbreviations: WBC, white blood cell; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; GOT, glutamate oxaloacetate transaminase; GPT, Glutamic Pyruvic Transaminase; IVIG, intravenous immunoglobulin.

*Numbers in bold italics indicate significant differences.