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

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Abstract

Aneurysms of the vascular wall represent a final common pathway for a number of inflammatory processes including atherosclerosis and idiopathic vasculitis syndromes. Kawasaki disease is an acute, self-limited vasculitis in children and the leading cause of acquired coronary artery

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aneurysms. We sought to identify shared molecular mechanisms of aneurysm formation by genotyping 8 polymorphisms in *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 Kawasaki disease patients (aneurysm+: n=111, aneurysm−: n=371) and tested our findings in an independent cohort of 200 Japanese Kawasaki disease 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= 2-4 $\times$ 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 Kawasaki disease. The region encompassing these risk haplotypes is a prime candidate for re-sequencing to look for rare genetic variation that may influence aneurysm formation.

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 (ECM) and elastic fibers. In children with Kawasaki disease (KD), coronary artery aneurysms (CAA) form in 25% of untreated patients and 5% of patients treated with intravenous immunoglobulin (IVIG) 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 [1]. For this reason, enzymes that cleave elastin have been implicated in the pathogenesis of KD [2, 3]. Proteases capable of degrading elastin include neutrophil elastase and the matrix metalloproteinases (MMP)-2, 3, 7, 9 and 12 [4]. MMPs are zinc-dependent endopeptidases produced by a wide variety of cell types. In addition to the degradation of (ECM), MMPs also cleave cytokines and chemokines [5] and influence recruitment of inflammatory cells. Therefore MMPs play 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[6]. 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 [7-11]. 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 UK and Japan to collect DNA from KD patients and determined the contribution of 5 MMP genes (*MMP-1, 3, 7, 12* and *13*) to CAA formation as these MMPs have been implicated in atherosclerotic coronary artery and abdominal aneurysms or are known to have elastase activity [4, 12, 13]. We genotyped 8 single nucleotide polymorphisms (SNPs) in these 5 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.

Methods

Subjects

Details of the US-UK subjects and their clinical presentation and case definition have been previously described 9. 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 9 (Table 1). The distribution of different ethnic groups was similar between the CAA+ and CAA− cohorts. For the US subjects, coronary artery 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 (LAD) coronary arteries (CA) 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 4.0 in the first year after KD onset in association with an internal diameter 1.5 times the adjacent segment 14. For the UK and Japanese subjects, Z scores were not available and coronary artery lesions (aneurysm or ectasia) were defined according to the Japanese Ministry of Health criteria (internal lumen diameter 3 mm for children < 5 yrs and 4 mm for children 5 yrs or the internal diameter of one or more segments 1.5 times the diameter of the adjacent segment15.

Genotyping

Genomic DNA from whole blood or mouth wash samples was extracted as previously described 9. We chose 8 SNPs from 5 MMP genes (Figure 1) that had positive disease associations in the published literature 16-23 (Figure 2). Genotyping was performed using a multiplex PCR-based, sequence-specific oligonucleotide hybridization research assay (Roche Molecular Systems, Pleasanton, CA) as previously described 9. To genotype *MMP13* rs2252070 (A/G) we used a TaqMan allele discrimination assay (Applied Biosystem, 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 discontinued by the manufacturer before the study was completed (Supplemental Table 1 and 2). Data quality control was performed as previously described 9. SNPs with allele frequencies less than 1%, genotype call rate less than 93%, or deviation from HWE were excluded from further analysis.

Statistical analyses

Case-control association studies between MMP SNPs and CA status were analyzed via 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 α represents the intercept, X_i is the ith subject's genotype score for a given marker, and ϵ_i is normally distributed with mean 0 and variance σ^2. Under the dominant model, $X_i=1$ if the ith subject has at least one minor allele or $X_i=0$ otherwise. Under the additive model, X_i indicates the ith 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 the tables. Analysis was performed using the R software (version 2.6.2, http://www.r-project.org/) and the R package SNPassoc.

Haplotype associations between MMP SNPs and CA status were analyzed using a moving window approach. The EM algorithm 24 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 25. 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 coronary arteries) was performed and genotype data met quality control criteria on 8 SNPs in 5 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, OR=0.72, 95%CI 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, OR=0.76, 95% CI 0.56-1.05; genotype nominal *p*=0.042) (Supplemental 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 US-UK subjects

We analyzed SNPs in the 5 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 2 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 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 3 to 8 SNPs that were associated with CAA all included the del allele of *MMP-3* rs3025058 (–/T). Haplotypes containing the T allele were not significantly associated. Two haplotypes were significant after correction for multiple sample testing (Table 2, nominal p= $2\text{-}4 \times 10^{-5}$). 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 were again showed association with CAA (nominal *p*=0.0034 and *p*=0.0039) in the all-Caucasian subset. Four 5-7 SNP haplotypes were associated with protection against aneurysm formation while 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, OR=4.92, 95%CI 1.54-15.73) (Supplemental 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 coronary artery 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 re-sequencing 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 ECM proteins, MMP-12 may promote macrophage recruitment to the vessel wall by activating TNF-α or by modulating levels of proinflammatory cytokines such as monocyte chemotactic protein-1 (MCP-1) 26. 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 17. *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 *MMP3*. *MMP-3* also has elastolytic capabilities and plays an important role in other diseases involving vascular wall inflammation including AAA, atherosclerosis, and Takaysu's arteritis 27. *MMP-3* rs3025058 (−/T) is a well-established functional variant. The T allele in this promoter region preferentially binds to the transcriptional repressor, NFκB p50 homodimer, and is associated with reduced transcript abundance 19. *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) 28. 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, different alleles were implicated suggesting a difference in LD structure in this region. As for *MMP-12*, this region should be considered for re-sequencing 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)29. MMP-13 rs2252070 (A/G) was only genotyped in the US-UK cohort in our study and 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. 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, while the G allele was associated with increased risk. One possible interpretation is that the LD 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 based

on 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 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 MMP1329, 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 re-sequencing of *MMP-3* and *MMP-12* should be considered in future studies. Finally, the coronary artery 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. In this case, it is better to describe false discovery rate of the results. *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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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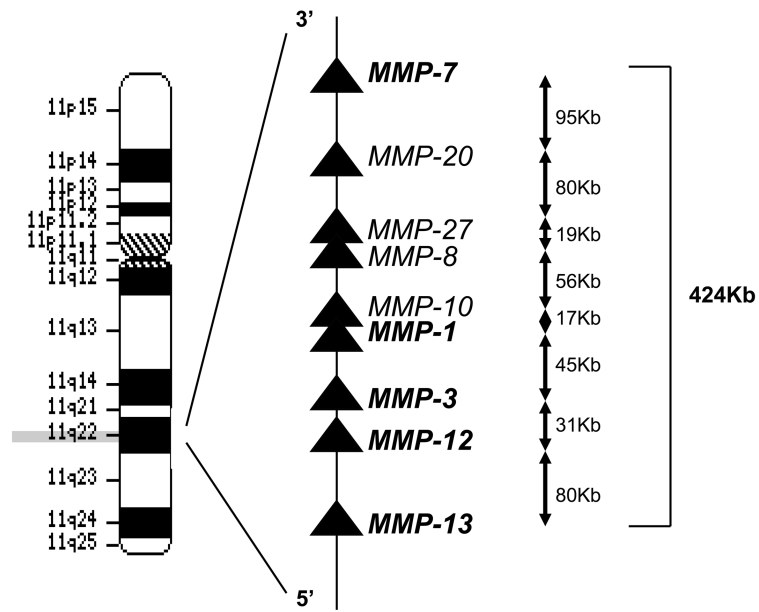
Figure 1. MMP Gene cluster on 11q22

Arrowheads show the direction of genes. Arrows on right indicate intergenic distance in the 424Kb region. Genes in bold were genotyped in this study.
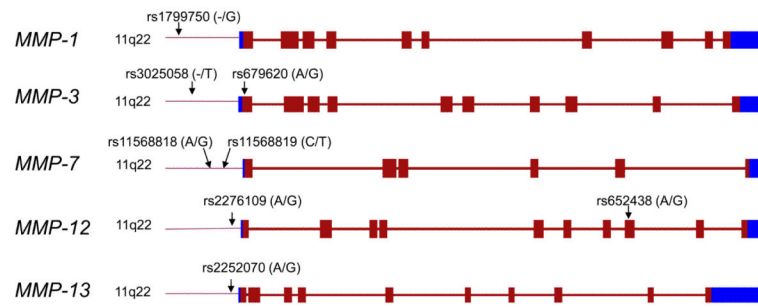
Figure 2. 8 SNPs from 5 MMP genes genotyped in KD patients
Gene structure and the location of SNPs are color-coded: red boxes= exons; blue boxes= 3′ and 5′ untranslated regions.

Table 1

Characteristics of subjects with Kawasaki disease.

	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%)

Table 2

Haplotypes on Chr. 11q22 associated with coronary artery aneurysms in the multiethnic US-UK cohort

KD subjects with normal or dilated coronary arteries (CAA− n=371) were compared with KD subjects with aneurysms (CAA+ n=111).

| Haplotype | | | | | | | | Total number of observed haplotypes[a] | Frequency | | p |
| MMP-7 | MMP-7 | MMP-1 | MMP-3 | MMP-3 | MMP-12 | MMP-12 | MMP-13 | | | | |
rs11568819 C/T	rs11568818 A/G	rs1799750 −/G	rs679620 A/G	rs3025058 −/T	rs652438 A/G	rs2276109 A/G	rs2252070 A/G		CAA−	CAA+	
			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	G	37	0.01	0.08	0.001
			A	-	A	A	G	46	0.01	0.08	0.001
	A	-	A	-	A			18	0.03	0.07	0.03
C	A	G	A	-	A	A		24	0.03	0.07	0.02
C	A	G	A	-	A	A		30	0.03	0.06	0.02
C	A	G	A	-	A	A		25	0.03	0.06	0.04
C	A	G	A	-	A			19	0.03	0.06	0.05
	A	G	A	-	A	A	G	27	0.01	0.04	0.001
C	A	G	A	-	A	A	G	37	0.005	0.04	0.00002[b]
C	A	G	A	-	A	A	G	46	0.004	0.05	0.00004[c]

[a] Not all theoretical haplotypes were observed among the study subjects in our cohort

Threshold for significance after correction for multiple testing:

[b] $= 8 \times 10^{-5}$

[c] $= 6 \times 10^{-5}$

Table 3

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

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

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

[a]Not all theoretical haplotypes were observed among the study subjects in our cohort

[b]Haplotype also associated with CAA formation in the multiethnic cohort (Table 2)

Table 4

Haplotypes on Chr. 11q22 associated with CA aneurysm in Japanese subjects

KD subjects with CAA (n=58) was compared with KD subjects without CAA (n=142).

Haplotypes						Frequency		
MMP-3		*MMP-12*						
rs679620 A/G	rs3025058 –/T	rs652438 A/G	rs2276109 A/G	Total number of haplotypes[a]		CAA–	CAA+	*P*
		A	G	3		0.03	0.11	0.003[b]
	T	A	G	6		0.01	0.10	0.0009[c]
G	T	A	G	8		0.01	0.10	0.001[d]

[a]Not all theoretical haplotypes were observed among the study subjects in our cohort

Threshold for significance after correction for multiple testing:

[b]=0.006

[c]=0.003

[d]=0.002.