Lack of association between E148Q MEFV variant and Kawasaki disease

山口, 賢一郎

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Brief Communications

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Running Title: E148Q *MEFV* variant and Kawasaki disease

Authors: Kenichiro Yamaguchi, MD, Kazuyuki Ikeda, MD, PhD, Kenji Ihara, MD, PhD, Hidetoshi Takada, MD, PhD, Koichi Kusuhara, MD, PhD, Toshiro Hara, MD, PhD.

Affiliation: Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

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Correspondence: Kenichiro Yamaguchi

E-mail: yamaken@pediatr.med.kyushu-u.ac.jp

TEL: +81-92-642-5421 FAX: +81-92-642-5435

ABSTRACT

We investigated a possible association between Kawasaki disease (KD), a systemic vasculitis of unknown etiology, or its coronary artery lesions (CAL) and *MEFV* gene variants including E148Q, a commonest and mildest mutation in *MEFV* gene for Familial Mediterranean Fever (FMF) or vasculitis-related disorders. The study population comprised a total of 138 Japanese patients with KD including 45 patients with CAL and 93 patients without CAL, and 170 normal controls. Sequence variations for *MEFV* gene were detected by direct sequencing followed by TaqMan SNP genotyping assay. Then, the genotype and allele frequencies of *MEFV* gene variants (E148Q, L110P, R202Q, P369S, R408Q) were compared between KD patients with and without CAL, or between KD patients with CAL and controls. E148Q heterozygotes and homozygotes were observed in 37.1% and 5.5% of healthy controls, 33.3% and 5.1% of KD patients, and 37.8% and 4.4% of KD patients with CAL, respectively. No significant differences were observed in the genotype and allele frequencies of other *MEFV* gene variants (L110P, R202Q, P369S, R408Q) between KD patients with and without CAL, or between KD patients with CAL and controls. No associations were detected between the *MEFV* gene variants and the

development of KD or CAL formation in KD.

Keywords

Kawasaki disease

Coronary artery lesion

MEFV gene

Polymorphism

Japanese

Introduction

Kawasaki disease (KD) is an acute self-limited systemic vasculitis that occurs predominantly in infants and young children. Coronary artery aneurysm or ectasia develops in ~15% to 25% of untreated children with the disease [1, 2]. Its etiology remains unknown, however, clinical and epidemiological features strongly suggest that it is caused by one or several widely distributed infectious agents [2]. It is likely that KD results from an abnormal immunologic response to certain microbial agents in genetically susceptible individuals. Actually, the higher rate of KD in the siblings of KD patients and the racial difference in its incidence support this consideration [2]. Recently, several host genetic factors have been identified in the development of KD and coronary artery lesions (CAL) [3-6].

Familial Mediterranean fever (FMF) is an inherited inflammatory disease that is common in Arabs, Non-Ashkenazi Jews, Armenians, and Turks, whereas uncommon in East Asia including Japan. It is characterized by self-limited periodic fever and various symptoms such as peritonitis, arthritis, rash, pleurisy, and pericarditis. The *MEFV* gene is responsible for FMF [7, 8]. Among the *MEFV* mutations, the role of E148Q (c.442 G>C) is still controversial. Although

some reports suggested that E148Q was only one of the gene polymorphisms, other reports suggested that E148Q was associated with the mildest disease with a low penetrance, or it usually required another additional *MEFV* mutation to cause the classical manifestation of FMF [9, 10]. Although FMF is an uncommon disorder in Japan, the frequency of E148Q is higher in Japanese than in European or Arab [11-13]. *MEFV* was predominantly expressed in granulocytes and monocytes [7], both of which play major roles in the pathophysiology of KD at the acute phase [2]. Several reports revealed that *MEFV* mutations were associated with vasculitis-related disorders such as Behçet's disease, Henoch-Schönlein purpura, and polyarteritis nodosa [14-16], suggesting that *MEFV* gene mutations contribute to the development of a broader spectrum of vasculitis. Furthermore, it was reported that *MEFV* mutations might increase baseline of inflammation, induce the development of rheumatic diseases and affect the clinical course of inflammatory disorders [17].

To clarify the role of *MEFV* gene in the development of KD as one of the host genetic factors, we investigated the associations between KD and *MEFV* gene variants, particularly E148Q that is common in Japanese.

Patients and Methods

One hundred and thirty-eight KD patients who were treated with oral aspirin plus intravenous immunoglobulin (IVIG:1-2g/kg/total in CAL- patients and 3-4g/kg/total in CAL+ patients) in Kyushu University Hospital or its affiliated hospitals, from 1991 through 2003 were enrolled. Informed consent was obtained from their parents, and the Ethical Committees of Kyushu University approved the study. All patients were Japanese, and met the appropriate diagnostic criteria for KD [18]. The study population consisted of 92 boys and 46 girls; the median age at diagnosis was 19 months (range: 1 to 151 months). Forty-five patients developed CAL, and 93 patients did not. According to the criteria of Japanese Ministry of Health, Labour and Welfare, the coronary artery was considered abnormal if the diameter of the initial lumen was >3mm in a child younger than 5 years or >4mm in a child at or over 5 years, or if the initial diameter of a segment was at least 1.5 times larger than that of an adjacent segment [19]. Clinical and laboratory data are shown in Table 1.

Peripheral blood was collected from KD patients and 170 randomly selected healthy Japanese volunteers. The donors and their families had no episodes of periodic fever similar to FMF. Genomic DNA was extracted from whole-blood

leukocytes with a QIAamp blood kit (Qiagen GmbH, Hilden, Germany).

We screened coding regions of *MEFV* gene for polymorphisms from genomic DNA of KD patients by direct sequencing with an ABI PRISM 3100 Genetic Analyzer (PerkinElmer, Foster City, CA, USA), as described previously [7, 20]. The forward and reverse oligonucleotide primers used to amplify the each exon were as follows (all oligonucleotide sequences are given 5' to 3'): exon 1 forward, AACCTGCCTTTTCTTGCTCA; exon 1 reverse, CACTCAGCACTGGATGAGGA; exon 2a forward, ATCATTTTGCATCTGGTTGTCCTTCC; exon 2a reverse, TCCCCTGTAGAAATGGTGACCTCAAG; exon 2b forward, GGCCGGGAGGGGGCTGTCGAGGAAGC; exon 2b reverse, TCGTGCCCGGCCAGCCATTCTTTCTC; exon 3 forward, GAACTCGCACATCTCAGGC; exon 3 reverse, AAGGCCCAGTGTGTCCAAGTGC; exon 4 forward, TTGGCACCAGCTAAAGATGGC; exon 4 reverse, TCTCCCTCTACAGGGATGAGC; exon 5 forward, TATCGCCTCCTGCTCTGGAATC; exon 5 reverse, CACTGTGGGTCACCAAGACCAAG; exon 6 forward, TCCAGGAGCCCAGAAGTAGAG; exon 6 reverse,

TTCTCCCTATCAAATCCAGAG; exon 7 forward, AGAATGTAGTTCATTTCCAGC; exon 7 reverse, CATTTCTGAACGCAGGGTTT; exon 8 forward, GCATGCTCACTTCCTCCCTA; exon 8 reverse CTTTGCTCCAGGTGTTTGGT; exon 9 forward, TTAGACCACAGTCCCCAACA; exon 9 reverse, CAGGAAACAGGGACAGGGTA; exon 10a forward, CCAGAAGAACTACCCTGTCCC; exon 10a reverse, AGAGCAGCTGGCGAATGTAT; exon 10b forward, GAGGTGGAGGTTGGAGACAA; exon 10b reverse, TCCTCCTCTGAAATCCATGG. Exon 2 and 10 were amplified in two overlapping PCR fragments. The PCR condition were as follows; initial denaturation at 94°C for 1 minute followed by 35 cycles of denaturation at 94°C for 30 second annealing at 60°C for exon 1, 3, 4, 5 and each part of exon 10, 58℃ for exon 6, 7, 8 and 9, 55°C for each part of exon 2 for 30 second and extension at 72°C for 30 second, followed by a final extension step at 72°C for 5 minute.

Genotyping of each variant was carried out by TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA); *MEFV* L110P (rs11466018)

Applied Biosystems code c_11186727_10, *MEFV* R202Q (rs224222) c_2394721_10, *MEFV* P369S (rs11466023) c_2394737_10 and *MEFV* R408Q (rs11466023) c_45171223_10. Detecting probes and primers for *MEFV* E148Q (rs3743930) was made by Custom TaqMan® SNP Genotyping Assays, and each oligonucleotide sequence was as follows (given 5' to 3'); forward primer, CCAGCCTGCGGTGCA; reverse, GCCTTCTCTCTGCGTTTGCT; reporter 1 (VIC), CAGCCCGAGGCCG; reporter 2 (FAM), CAGCCCCAGGCCG. Genotyping by the TaqMan method was performed with an ABI PRISM 7700 Sequence Detection System.

A Chi-squared test was used to compare the genotype and allele frequency distributions of each variant between the KD patients and controls, between the KD patients with and without CAL, and between KD patients with CAL and controls. We also used Mann-Whitney *U* test to compare the clinical and laboratory data between patients with and without minor allele of E148Q or other *MEFV* variants.

Results

We first analyzed the allelic frequency of E148Q (c.442 G>C), which is a common SNP in Japanese [21], compared with other populations, in 138 KD patients and 170 controls by Taqman genotyping assay. E148Q heterozygotes and homozygotes were observed in 37.1% and 5.5% of healthy controls, 33.3% and 5.1% of KD patients, and 37.8% and 4.4% of KD patients with CAL, respectively (Table 2). There were no significant differences in the genotype and allele frequencies of E148Q variant between KD patients and healthy controls, KD patients with and without CAL, or KD patients with CAL and controls.

As it might be still possible that other *MEFV* gene variants were associated with the development of KD in combination with E148Q, we investigated other *MEFV* gene variants by direct sequencing of PCR-amplified genomic DNA from 53 KD patients who had at least one Q148 allele. We found four types of non-synonymous variants, L110P (c.329T>C), R202Q (c.606G>A), P369S (c.1105C>T), and R408Q (c.1223G>A), in 23, 1, 9, and 6 among these 53 patients, respectively. Then, we performed genotyping of these four gene variants for the 138 KD patients and 170 controls by TaqMan SNP genotyping

assay. As a result, the genotype and allele frequencies of each variant showed no significant differences between all KD patients and healthy controls, or between KD patients with and without CAL (Table 2). We performed haplotype analysis of these five *MEFV* variants, but the assembly of these alleles showed no significant differences between KD and healthy controls (data not shown). Clinical and laboratory data were analyzed by the comparison of subgroups with E148/E148, Q148/Q148, E148/Q148 or Q148/Q148, (E148/Q148 or Q148/Q148) and (L110/P110 or P110/P110), (E148/Q148 or Q148/Q148) and (P369/S369) (Table 3). The median serum CRP concentration (18.2 mg/dl) tended to be higher in Q148 homozugous or heterozygous group (E/Q or Q/Q) than that (9.3 mg/dl) in wild type group (E/E) among KD patients with CAL (P=0.059), but there were no significant differences between the other groups in other variables.

There are several limitations that need to be acknowledged and addressed regarding the present study. The sample sizes in this study did not have a sufficient power of statistical analysis especially for the L110P, R202Q, P369S, R408Q because their minor allele frequencies in the controls were as low as 0.10 or less. Based on the minor allele frequencies of E148Q (0.24), L110P

(0.10), R202Q (0.02), P369S (0.05), R408Q (0.04) or the cohort size, the estimated smallest detectable risks of their variants were calculated to 1.45, 1.67, 2.54, 1.89, 2.07 for KD development (Control: 2N=340 vs. KD: 2N=276); 1.68, 2.01, 3.40, 2.34, 2.45 for CAL formations (Control: 2N=340 vs. KD CAL(+): 2N=90), respectively. Alternatively, 81, 432, >1000, >1000 and >1000 KD patients were calculated to be required to reach the statistical significances ($P<0.05$), respectively, if their relative risks for KD development were set to 1.50.

Discussion

Ozen et al. reported that among 70 individuals with *MEFV* gene mutations, 28 (40.0%) had some form of rheumatic complaints and 15 (21.4%) developed rheumatic diseases or vasculitis including Behçet's disease. They also reported that 30.5% of the children with rheumatic diseases and 25.4% of patients with juvenile idiopathic arthritis (JIA) had mutations of *MEFV* gene [17]. FMF and Behçet's disease are not very rare disorders in the Eastern Mediterranean and it was suggested that *MEFV* mutation played a role in the pathogenesis of Behçet's disease [16]. Furthermore, Rosenbaum et al. reported that the prognosis of 3 JIA patients who had M694V mutation of *MEFV* gene was extremely poor [22]. In addition, Tunca et al. showed an increased acute phase response in carriers for the *MEFV* gene [23]. These reports suggested that *MEFV* gene mutation was also related to enhanced inflammatory responses and severity in these disorders. On the other hand, we found that *MEFV* gene variants in Japanese were not related to the development of KD or CAL formation in KD, or played no major role in the inflammatory responses in KD.

Most of Japanese FMF patients have compound heterozygous *MEFV* mutations [21, 24, 25]. Among them, E148Q is the most common, which is

reported to have a weak effect only to enhance the development and severity of FMF that was primarily induced by another mutation [25]. We found that Q148 was not associated with the development of KD that occurs in a higher frequency in Japanese. It is possible that the functional effect of Q148 might be too small to accelerate the inflammation for the development of KD or to exacerbate vasculitis in KD. Since Q148 was not significantly associated with enhanced inflammatory responses in KD patients, it is less likely that these Q148 homozygotes will develop FMF, rheumatic disease or vasculitis in future. Rather, the high frequency of Q148 variant and the low incidence of FMF, and the presence of many asymptomatic Q148 homozygotes in Japanese strongly suggest that E148Q is one of the genetic polymorphisms of *MEFV* gene with little functional significance. Further long-term evaluation of Q148 homozygotes would be necessary to clarify a role of Q148 in the pathogenesis of rheumatic or vasculitis disorders.

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Table 1. Clinical and laboratory data of KD patients

Variables	With CAL (n=45) Median (range)	Without CAL (n=93) Median (range)	*P* value*
Age (mo)	19(1-151)	19(2-105)	0.614
Admission (day of illness)	4(1-13)	4(1-9)	0.552
Start of IVIG (day of illness)	5(2-15)	5(1-9)	0.993
Duration of fever (days)	10(4-27)	7(3-15)	***7.60 ×10^{-7}***†
Peak of white blood cell (×10^3/μL)	16.7(7.3-35.9)	15.6(7.9-31.0)	0.357
Peak of C-reactive protein (mg/dl)	15.5(3.3-32.4)	9.6(2.0-33.3)	***0.00534***†

*Mann-Whitney *U* test N.S：no significant difference †Significant difference

Table 2. Polymorphisms of the *MEFV*genes in KD patients with and without CAL, and control subjects

Gene	Genotype /Allele	Ctrl (n=170)	KD total (n=138)	KD CAL (+) (n=45)	KD CAL (-) (n=93)	Ctrl vs KD total			KD CAL (+) vs Ctrl			KD CAL (+) vs CAL (-)		
						p Value	OR	95% C.I.	*p* Value	OR	95% C.I.	*p* Value	OR	95% C.I.
E148Q	GG	98 (0.58)	85 (0.62)	26 (0.58)	59 (0.63)									
	GC	63 (0.37)	46 (0.33)	17 (0.38)	29 (0.31)	0.496			0.824			0.512		
	CC	9 (0.05)	7 (0.05)	2 (0.04)	5 (0.05)									
	Allele G	259 (0.76)	216 (0.78)	69 (0.77)	147 (0.79)									
	Allele C	81 (0.24)	60 (0.22)	21 (0.23)	39 (0.21)	0.540	0.89	0.608-1.30	0.923	0.97	0.562-1.68	0.655	1.15	0.628-2.10
L110P	TT	139 (0.82)	115 (0.83)	36 (0.80)	79 (0.85)									
	TC	29 (0.17)	22 (0.16)	9 (0.20)	13 (0.14)	0.598			0.445			0.321		
	CC	2 (0.01)	1 (0.01)	0	1 (0.01)									
	Allele T	307 (0.90)	252 (0.91)	81 (0.90)	171 (0.92)									
	Allele C	33 (0.10)	24 (0.09)	9 (0.10)	15 (0.08)	0.667	0.89	0.510-1.54	0.933	1.03	0.475-2.25	0.593	1.27	0.532-3.02
R202Q	GG	163 (0.96)	132 (0.96)	43 (0.96)	89 (0.96)									
	GA	7 (0.04)	6 (0.04)	2 (0.04)	4 (0.04)									
	AA	0	0	0	0									
	Allele G	333 (0.98)	270 (0.98)	88 (0.98)	182 (0.98)									
	Allele A	7 (0.02)	6 (0.02)	2 (0.02)	4 (0.02)	0.855	1.05	0.351-3.18	0.751	1.08	0.221-5.30	0.688	1.03	0.186-5.75
P369S	CC	153 (0.90)	120 (0.87)	38 (0.84)	82 (0.88)									
	CT	17 (0.10)	18 (0.13)	7 (0.16)	11 (0.12)									
	TT	0	0	0	0									
	Allele C	323 (0.95)	258 (0.93)	83 (0.84)	175 (0.94)									
	Allele T	17 (0.05)	18 (0.07)	7 (0.16)	11 (0.06)	0.417	1.33	0.670-2.62	0.307	1.60	0.643-3.99	0.557	1.34	0.502-3.59
R408Q	GG	157 (0.92)	123 (0.89)	40 (0.89)	83 (0.89)									
	GA	13 (0.08)	15 (0.11)	5 (0.11)	10 (0.11)									
	AA	0	0	0	0									
	Allele G	327 (0.96)	261 (0.95)	85 (0.94)	176 (0.95)									
	Allele A	13 (0.04)	15 (0.05)	5 (0.06)	10 (0.05)	0.340	1.45	0.676-3.09	0.466	1.48	0.513-4.27	0.825	1.04	0.343-3.12

CI = confidence interval; Ctrl = Control subjects; OR = odds ratio.

Numbers in parenthesis indicate percentages of the genotype or allele frequencies. After Bonferroni's correction of multiple comparison, $P < 0.0166$ was considered to be statistically significant.

All the evaluated SNPs in controls were under the Hardy Weinberg's disequilibrium.

Table3. Clinical and laboratory data of all KD patients or KD patients with CAL, sub-grouped by combined genotypes of MEFV gene

Population		**All KD patients**								
Sub-group		(1)	(2)	(3)	(4)	(5)	Comparison	(***P*-values)**		
Genotype	**E148Q**	E/E	Q/Q	E/Q or Q/Q	E/Q or Q/Q	E/Q or Q/Q				
	L110P	-	-	-	L/P or P/P	-				
	P369S	-	-	-	-	P/S				
Comparison							(1) vs (2)	(1) vs (3)	(1) vs (4)	(1) vs (5)
Number		85	7	53	23	9				
Variables	Age (months)	18 (2-151)	22 (7-66)	22 (1-96)	20 (2-88)	40 (2-96)	0.473	0.38	0.669	0.338
	Admission (day of illness)	4 (1-13)	3 (2-8)	4 (1-10)	4 (2-10)	4 (1-6)	0.348	0.382	0.243	0.870
	Start of IVIG (day of illness)	5 (2-10)	4 (3-8)	5 (1-15)	4 (1-15)	4 (3-6)	0.552	0.518	0.485	0.218
	Duration of fever (days)	7 (3-27)	6 (5-12)	7 (4-20)	7 (4-17)	7 (5-20)	0.781	0.574	0.451	0.55
	Peak of white blood cell ($\times 10^3/\mu L$)	16.2 (7.3-35.9)	12.5 (10.8-21.3)	15.7 (8.8-31.0)	15.4 (8.8-22.6)	15.6 (12.5-19.1)	0.381	0.992	0.328	0.931
	Peak of C-reacted protein (mg/dl)	9.3 (2.5-33.3)	12.2 (3.5-23.9)	13.4 (2.0-32.4)	5.4 (2.0-22.6)	10.7 (9.2-13.3)	0.664	0.157	0.518	0.495

Population		**KD patients with CAL**								
Sub-group		(1)	(2)	(3)	(4)	(5)	Comparison	(***P*-values)**		
Genotype	**E148Q**	E/E	Q/Q	E/Q or Q/Q	E/Q or Q/Q	E/Q or Q/Q				
	L110P	-	-	-	L/P or P/P	-				
	P369S	-	-	-	-	P/S				
Comparison							(1) vs (2)	(1) vs (3)	(1) vs (4)	(1) vs (5)
Number		26	2	19	9	2				
Variables	Age (months)	17 (2-151)	(13, 66)	38 (1-96)	17 (5-77)	(40, 96)	0.459	0.186	0.752	0.152
	Admission (day of illness)	4 (2-13)	(3, 3)	4 (1-10)	4 (3-10)	(3, 4)	0.345	0.600	0.779	0.629
	Start of IVIG (day of illness)	5 (2-10)	(3, 6)	5 (3-15)	5 (3-15)	(3, 5)	0.694	0.757	0.779	0.459
	Duration of fever (days)	10 (4-27)	(6, 12)	11 (5-20)	12 (5-17)	(11, 20)	0.629	0.990	0.690	0.247
	Peak of white blood cell ($\times 10^3/\mu L$)	16.2 (7.3-35.9)	(10.8, 21.3)	16.8 (10.8-22.6)	16.9 (9.9-22.6)	(16.7, 19.1)	0.636	0.843	0.607	0.776
	Peak of C-reacted protein (mg/dl)	9.3 (3.9-29.3)	(11.8, 12.7)	18.2 (3.3-32.4)	10.7 (9.2-13.3)	(12.5, 13.7)	0.784	0.059	0.836	0.717

Patients who had Q148 + Q202 and Q148 + Q408 were so rare (n=1 and 6) that we did not analyze these group.