Next-generation sequencing of the whole MEFV gene in Japanese patients with familial Mediterranean fever: a case-control association study

T. Koga1,2, S. Sato3, H. Mishima4, K. Migita5, Y. Endo1, M. Umeda1, R. Sumiyoshi1, F. Nonaka1,6, S. Fukui1, S. Kawashiri1, N. Iwamoto1, K. Ichinose1, M. Tama1, H. Nakamura1, T. Origuchi1, Y. Ueki7, J. Masumoto8, K. Agematsu9, A. Yachie10, K. Yoshiura4, K. Eguchi7, A. Kawakami1

Affiliations: page S40.

ABSTRACT

Objectives. We aimed to identify the whole nucleotide sequence of the Mediterranean Fever (MEFV) gene in familial Mediterranean fever (FMF) and reveal novel single nucleotide variants (SNVs) associated with the susceptibility of FMF.

Methods. SeqCap capturing technique followed by Illumina next-generation sequencing have been used to assess two hundred SNVs in the whole region of MEFV in 266 Japanese patients with FMF and 288 ethnically matched controls. We performed an association analysis using these SNVs to identify genetic variants that predispose to FMF.

Results. We identified the two most significant SNVs [rs28940578; M694I in exon 10, odds ratio (OR) = 153, p=2.47x10−21 and rs3743930; E148Q in exon 2, OR = 1.65, p<0.0005]. Stratified analysis identified rs28940578 as a risk allele in typical FMF. Haplotype AG, defined by rs401298 and rs28940578, was the most significant and prevalent among patients with typical FMF compared with controls (22.4% vs. 0%, respectively; OR = 137, p=1.44x10−11). Haplotype GTC, defined by rs11466018, rs224231, and rs401877, was the most significant among patients with typical FMF without the rs28940578 mutation compared with controls (15.9% vs. 6%, respectively; OR = 12.4, p=0.004).

Conclusions. rs28940578 is associated with the highest risk in typical FMF cases. This is consistent with results from previous studies in Japan. We found a novel MEFV gene haplotype that confers susceptibility of FMF among typical FMF without the rs28940578 mutation. There were no relevant SNVs identified in MEFV among the atypical FMF group.

Introduction

Familial Mediterranean fever (FMF) is a typical hereditary autoinflammatory disease characterised by recurrent manifestations of fever with arthritis, abdominal pain, skin rash, and/or serositis (1, 2). The Mediterranean Fever (MEFV) gene, coding pyrin that acts as a major regulatory component of the inflammasome, is responsible for FMF (3, 4). Numerous variants have been identified in the region of MEFV in the INFEVERS database (https://infevers.umai-montpellier.fr/web/), and mutations in exon 10 reportedly correlate with disease severity and prognosis (5). Accordingly, genetic diagnostic testing is important in the diagnosis and treatment of FMF.

The identification of single nucleotide variants (SNVs) has permitted the characterisation of various disease-related genes, greatly contributing to the fields of clinical medicine and preventive medicine (6). Risk variants located within exons may be synonymous or non-synonymous, and only the latter one usually associates with altered activity of the gene product. Thus far, the analysis of MEFV in FMF was mainly focused on the coding region through short-range sequencing using capillary sequencers. A whole genomic analysis of MEFV, including the promoter and intron regions, has not yet been performed. Long-range sequencing using next-generation massive parallel sequencing (NGS) may provide the method to identify DNA sequences in the whole of the gene, including the enhancer-promoter region and the whole gene covering all exon and intron regions. In the present study, we analysed the
whole region of MEFV using the NGS after target capturing and attempted to identify new disease-related variants in MEFV among Japanese patients with FMF.

**Patients and methods**

**Study population**

From May 2010 to October 2015 we prospectively enrolled 272 consecutive Japanese patients with FMF in Nagasaki University, Shinshu University, Kanazawa University, and Nagasaki Medical Centre and 288 ethnically matched controls in Nagasaki University. All patients with FMF were diagnosed according to the Tel Hashomer criteria (7). We excluded 6 patients with homozygous (5 patients: homozygous M694I mutation) or double heterozygous pathogenic variants (1 patient: heterozygous M680I/V726A mutations) in exon 10 of the MEFV gene because they were genetically confirmed FMF according to the current guidelines (8). We divided the study patients (n=266) into two groups, namely, typical FMF and atypical FMF, as previously described (9, 10). Patients with typical FMF exhibited typical episodes of peritonitis, pleuritis, arthritis, or fever, as specified in the Tel Hashomer criteria. Patients with atypical FMF exhibited “incomplete” episodes with clinical manifestations such as temperature <38°C, episode duration longer or shorter than specified periods (12 hours to 3 days) but not shorter than 6 hours or longer than a week, and absence of signs of peritonitis during an abdominal episode.

All patients provided written informed consent prior to their enrollment in the study. The study protocol was approved by the Institutional Review Board of Nagasaki University (approval no. 14092956-3) and other participating centres.

**Targeted region enrichment for sequencing**

The SeqCap (Roche Diagnostics, Basel, Switzerland) solution hybridisation system was used for the enrichment of the whole MEFV genomic region. RNA baits were designed to correspond to chr16: 3,280,000-3,318,000 in the GRCh37 human genomic reference sequence. The whole region, except the Alu-repetitive sequence in 3'‐UTR of MEFV (chr16: 3292081-3292690), was sequenced. Library construction and hybridisation capturing were performed according to the protocol provided by the manufacturer of SeqCap.

**Next-generation sequencing**

The HiSeq2500 (Illumina, San Diego, CA, USA) system was used for the acquisition of DNA base information. HiSeq2500 (101 + 101 base-paired ends) raw data files were converted to FASTQ files using the Bcl2Fastq software package (v. 1.8.4, Illumina). The GRCh37 human genome reference sequence of canonical chromosomes (mitochondrial genome and chromosomes 1–22, X, and Y) were downloaded from the UCSC Genome Browser (11). Reads in FASTQ files were subjected to mapping and base quality score recalibration using SNV sites not registered in dbSNP version 136 mapped by the NovoAlign software (version 3, Novocraft Technologies, Petaling Jaya, Selangor, Malaysia). Unsorted BAM files were subjected to position-wise sorting and marking polymerase chain reaction and optical deduplication using the NovoSort software (v. 1.3, Novocraft). Sorted and deduped BAM files were processed using a workflow of the Genome Analysis Tool Kit (GATK) software package version 3.4-46 (12). Variant calling was performed using the HaplotypCaller with default settings to generate single sample genomic VCF (g.VCF) files. Whole g.VCF files were combined and genotyped in the SeqCap target regions using the GenotypeGVCFs to generate a VCF file. Subsequently, detected SNVs were extracted using SelectVariants. Genotypes and characteristic files were converted to ped and fam files using R. These files were analysed using the PLINK software package version 1.9. The PLINK parameters were as follows: minor allele frequency (--maf) ≥0.05 and Hardy-Weinberg equilibrium test p-value (--hwe) ≤0.001.

**Statistical analysis**

Statistical analysis was performed using the R and PLINK 1.9 (13). For the association analyses, we used Fisher’s exact test for quality control (QC)-passed SNVs. Fisher’s exact test was also used to analyse differences in the distribution of genotypes and alleles between cases and controls. We excluded SNVs with significant deviation from the Hardy-Weinberg equilibrium (p<0.001) and did not consider SNVs with minor allele frequency (MAF) <0.05 in both cases and controls. The pairwise linkage disequilibrium (LD) was calculated using the Haplovew software version 4.2 (Broad Institute, Cambridge, MA, USA) (14). The results are expressed as p-values, odds ratios (OR), and 95% confidence intervals (CI). To account for multiple testing, we used the Bonferroni correction and considered significant QC-passed SNVs (n=65) those with p<0.000769. Alleles not used in this association study were evaluated using individual mutation analysis, which did not identify new pathogenic mutations. Accordingly, the following results are from the association analysis, not the individual mutation testing.

**Results**

**Association of MEFV with FMF**

Forty-seven cases in our cohort had a family history. Among them, there were three families with autosomal dominant inheritance pattern. Other familial cases were not confirmed the mode of inheritance because parental DNAs were not available. We analysed 554 samples (266 cases and 288 controls). After filtering all SNVs with MAF <0.05 in all individuals or SNVs with significant deviation from the Hardy-Weinberg equilibrium (i.e., p<0.001 in the control group), we analysed the distribution of allele frequencies of 65 SNVs within the MEFV gene in the FMF cases and controls. Table I shows dbSNP rs numbers, protein name defined by Human Genome Variation Society nomenclature, genomic locations defined by the Genome Reference Consortium human genome (GRCh37.p12, hg19), and MAF in missense SNVs and supplementary Table S1 shows dbSNP rs numbers, genomic locations, and MAF in all 65 SNVs. The two most significant SNVs identified in the coding region of MEFV genes were rs28940578
Table I. The distribution of single nucleotide missense variants in the MEFV gene among cases and controls.

<table>
<thead>
<tr>
<th>SNV name</th>
<th>Gene: Consequence</th>
<th>HGVS protein name</th>
<th>exon Location (GRCh37.p12)</th>
<th>Minor Allele</th>
<th>Allele Count (2n)</th>
<th>MAF (%)</th>
<th>p-value</th>
<th>Odds ratio 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs28940578</td>
<td>MEFV: Missense Variant</td>
<td>p.Met694Ile</td>
<td>3293405</td>
<td>T 0 62 0 0 62 0</td>
<td>0.117 0 2.47×10⁻⁴ 153.16 NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs3743930</td>
<td>MEFV: Missense Variant</td>
<td>p.Glu148Gln</td>
<td>3304626</td>
<td>G 22 138 16 106 182 138</td>
<td>0.342 0.24 0.00002* 1.65 1.27 - 2.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs11466018</td>
<td>MEFV: Missense Variant</td>
<td>p.Leu110Pro</td>
<td>3304739</td>
<td>G 1 53 2 32 55 36</td>
<td>0.103 0.06 0.015 1.73 1.12 - 2.68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs11466024</td>
<td>MEFV: Missense Variant</td>
<td>p.Arg408Gln</td>
<td>3294968</td>
<td>T 0 41 0 27 41 27</td>
<td>0.077 0.047 0.046 1.7 1.03 - 2.80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs11466026</td>
<td>MEFV: Missense Variant</td>
<td>p.Pro369Ser</td>
<td>3295856</td>
<td>A 0 44 2 28 44 32</td>
<td>0.083 0.056 0.076 1.53 0.96 - 2.46</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1231123</td>
<td>MEFV: Missense Variant</td>
<td>p.Asp24Glu</td>
<td>3293922</td>
<td>T 35 114 30 137 197 184</td>
<td>0.37 0.319 0.077 1.25 0.98 - 1.61</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CI: confidence interval; FMF: familial Mediterranean fever; GRC: Genome Reference Consortium; HGVS: Human Genome Variation Society; MAF: minor allele frequency; SNV: single nucleotide variant.

*SNVs reached the Bonferroni-corrected significance level.

Table II. Single nucleotide variants significantly associated with typical FMF (case-control analysis).

<table>
<thead>
<tr>
<th>SNV name</th>
<th>Gene: Consequence</th>
<th>HGVS protein name</th>
<th>exon Location (GRCh37.p12)</th>
<th>Minor Allele</th>
<th>MAF (%)</th>
<th>p-value</th>
<th>Odds ratio 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs28940578</td>
<td>MEFV: Missense Variant</td>
<td>p.Met694Ile</td>
<td>3293405</td>
<td>T 43 0</td>
<td>0.224 0 2.63×10⁻⁵ 332.79 NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs224230</td>
<td>MEFV: 2kB Upstream Variant</td>
<td>p.Glu148Gln</td>
<td>3308357</td>
<td>A 41 260</td>
<td>0.214 0.451 2.74×10⁻⁵ 0.33 0.23 - 0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs401298</td>
<td>ZNF200: Intron Variant</td>
<td>p.Leu110Pro</td>
<td>3280974</td>
<td>G 59 94</td>
<td>0.307 0.163 4.01×10⁻⁵ 2.28 1.56 - 3.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs224231</td>
<td>None</td>
<td>3309979</td>
<td>T 81 153</td>
<td>0.422 0.266 6.53×10⁻³ 2.02 1.44 - 2.84</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs3743930</td>
<td>MEFV: Missense Variant</td>
<td>p.Glu148Gln</td>
<td>3304626</td>
<td>G 75 138</td>
<td>0.391 0.24 8.55×10⁻³ 2.04 1.44 - 2.88</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CI: confidence interval; FMF: familial Mediterranean fever; MAF: minor allele frequency; SNV: single nucleotide variant.

*SNVs reached the Bonferroni-corrected significance level.

Table III. Single nucleotide variants significantly associated with typical FMF (case-case analysis).

<table>
<thead>
<tr>
<th>SNV name</th>
<th>Gene: Consequence</th>
<th>HGVS protein name</th>
<th>exon Location (GRCh37.p12)</th>
<th>Minor Allele</th>
<th>Allele Count (2n)</th>
<th>MAF (%)</th>
<th>p-value</th>
<th>Odds ratio 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs28940578</td>
<td>MEFV: Missense Variant</td>
<td>p.Met694Ile</td>
<td>3293405</td>
<td>T 43 1</td>
<td>0.224 0 2.42×10⁻⁵ 66.6 9.08-489</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs224230</td>
<td>MEFV: 2kB Upstream Variant</td>
<td>p.Glu148Gln</td>
<td>3308357</td>
<td>A 41 96</td>
<td>0.214 0.413 1.11×10⁻⁵ 0.39 0.24 - 0.59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs224227</td>
<td>MEFV: 2kB Upstream Variant</td>
<td>p.Glu148Gln</td>
<td>3307566</td>
<td>G 38 87</td>
<td>0.198 0.375 7.15×10⁻⁵ 0.41 0.26 - 0.64</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CI: confidence interval; FMF: familial Mediterranean fever; MAF: minor allele frequency; SNV: single nucleotide variant.

*SNVs reached the Bonferroni-corrected significance level.

(M694I in exon 10, OR = 153, p=2.47×10⁻³⁴) and rs3743930 (E148Q in exon 2, OR = 1.65, p=0.0005). We also identified three significant SNVs rs224230 (2kB upstream variant of MEFV, OR = 0.55, p=1.39×10⁻⁵), rs224231 (OR = 1.63, p=0.0002224), and rs72774487 (intron variant of LINC00921, OR = 1.68, p=0.000181) located outside of the coding region of the MEFV gene. None of the other SNVs in our analysis reached the Bonferroni-corrected significance level. Of the 266 patients with FMF, 62 patients (23%) had one M694I allele and none of them were homozygous. Overall, 138 patients (52%) with FMF had one E148Q allele and 22 patients (8%) had two alleles. In familial cases (n=47), the prevalence of M694I heterozygous mutations was significantly higher than in non-familial cases (32% vs. 15%, p=0.011). The LD pattern of the 65 SNVs within the region of the MEFV revealed six main haplotype blocks in all patients and three haplotype blocks in the controls; of these blocks, the block 1 which contains from the MEFV promoter region to intron 2 region, was consistent with a hotspot previously identified (15). Block 3 included from MEFV intron 2 region to the 3'UTR region of MEFV, which was also consistent with a hotspot noted previously (Supplementary Fig. S1) (15).

Identification of factors associated with the susceptibility of typical Japanese FMF cases

Although it is suggested that patterns of MEFV mutation differ between typical cases and atypical cases in Japanese patients, there are no studies investigating the whole/complete genomic sequence of MEFV using NGS. To address this, we subsequently examined the factors associated with the susceptibility of typical FMF. We excluded 54 cases in which we could not distinguish between

Clinical and Experimental Rheumatology 2020

S-37
typical and atypical cases. Among the 212 cases, 96 patients with FMF were classified as clinically typical cases (median age at onset: 23 years; females: 42.1%), whereas 116 patients with FMF were classified as clinically atypical cases (median age at onset: 34 years; females: 41.4%). In the typical FMF group, five SNVs had allele frequencies that differed significantly between patients and controls (Table II and Suppl. Table S2). We also performed case (typical cases)-case (atypical cases) analysis and found three SNVs that differed significantly between typical cases and atypical cases (Table III). Moreover, we examined differences in the frequency distribution of all common haplotypes between the typical FMF patient group and the controls (Fig. 1A). Within block 1, haplotype AG defined by rs401298 and rs28940578 was the most significant and was more prevalent in the typical FMF patient group than in the control group (22.4% vs. 0%, respectively; OR=137, p=1.44 × 10⁻³¹). These two SNVs were only modestly correlated (r²=0.23). Collectively, these observations indicate that the rs28940578 mutation is the most significant factor associated with the susceptibility of typical FMF in Japanese patients.

Identification of factors associated with the susceptibility of typical FMF in Japanese patients without the rs28940578 mutation

Japanese patients with FMF reportedly have a higher prevalence of the rs28940578 mutation in exon 10 (10). Accordingly, we further investigated potential associations of other variants with the susceptibility of typical FMF. Among the 96 patients with typical FMF, 53 did not have the rs28940578 mutation (median age at onset: 31 years; females: 30.8%). In this group, four SNVs had allele frequencies that differed significantly between the patients and controls. The risk allele frequencies of rs11466018, rs401877, rs3743930, and rs224231 were significantly higher in patients with typical FMF than in the controls (Table IV and Suppl. Table S3). We examined differences in the frequency distribution of all common haplotypes between the typical FMF patient group without the rs28940578 mutation and the controls (Fig. 1B). Within block 1, haplotype GTC defined by rs11466018, rs224231, and rs401877 was the most significant and was more prevalent among patients with typical FMF without the rs28940578 mutation than the controls (15.9% vs. 6%, respectively; OR=12.4, p=0.004). The rs11466018 variant was in weak pairwise LD with other SNVs (rs224231, and rs401877) within the LD block (r²=0.17 and 0.14, respectively). In contrast, the rs224231 was in moderate pairwise LD with rs401877 (r²=0.75). In all FMF patients in this study, 14.9% had the GTC haplotype. The effect on this GTC haplotype on the susceptibility of typical FMF was also observed in the group with rs28940578 (OR=5.2, p=0.022), but this GTC haplotype strongly affected the susceptibility in those without the rs28940578 mutation. Collectively, rs11466018, rs401877, rs3743930, and rs224231 may be markers conferring susceptibility to typical FMF in Japanese patients without the rs28940578 mutation.

Identification of factors associated with the susceptibility of atypical FMF in Japanese patients

Finally, we investigated the single-site variant and the haplotype associated with the susceptibility of atypical FMF. In the atypical FMF group, none of the
SNVs had allele frequencies that differed significantly between patients and controls (Suppl. Table S4). We examined the difference in the frequency distribution of all common haplotypes between the typical FMF patient group and the controls. The results did not identify haplotypes significantly associated with the susceptibility of atypical FMF.

**Discussion**

Although *MEFV* has been extensively studied in Japanese patients with FMF (10, 16-18), NGS of the whole region of *MEFV* sequence in a large number of patients with FMF has not been performed previously. In the stratified analysis of our study, rs28940578 was identified as a risk allele in typical FMF cases. This finding is consistent with the results of previous studies conducted in Japan using Sanger sequencing (10). Most importantly, we identified novel disease-related haplotype GTC defined by rs11466018, rs224231, and rs401877 conferring susceptibility of FMF among Japanese patients with typical FMF without the rs28940578 mutation. This haplotype may characterise FMF in Japan and measuring this haplotype may be a useful diagnostic tool in the future. However, the GTC haplotype and the common missense variants in exon 2 and 3 are not decisive factors in the diagnosis of FMF, and the importance of clinical diagnosis should be emphasised. Accordingly, further investigation using more cases is required to determine the significance of this haplotype for the development of typical FMF.

In the present study, we demonstrated that rs28940578 and rs3743930 are disease-associated variants significantly associated with the susceptibility of FMF. A previous study involving 311 Japanese patients with FMF showed that of 126 Japanese FMF patients with the rs28940578 (M694I) variant in exon 10 of the *MEFV* gene, 50 patients (40%: 50/126) simultaneously carried the rs3743930 (E148Q) variant in exon 2 (10). A similar study involving 216 Japanese patients with FMF also found that 29 patients (13%) had the two mutant alleles M694I and E148Q (16). These results suggest an association between M694I and E148Q. However, our study indicated very low LD between the rs28940578 and rs3743930 (r²=0.02, D’=0.44).

The present NGS analysis detected numerous intron regions and downstream variants. However, these are polymorphisms common observed (30–40%) in healthy individuals, and this, the diagnostic significance of these polymorphisms may be poor. Regarding non-synonymous mutations, our data revealed that p.Leu110Pro in exon 2, p.Pro369Ser and p.Asp408Gln in exon 3, and p.Asp424Glu in exon 4 are frequently found in patients with FMF. Although genomic analysis of the promoter region of *MEFV* has been reported (19), this study is the first entire genome analysis of the *MEFV* region including upstream regions, downstream regions, and all intronic regions. Importantly, the authors did not find significant variants in these regions that could explain the FMF phenotype in some patients, suggesting that it is not currently necessary to search for deep-intronic variants.

The mutation in exon 10 is pathologically significant and useful for the diagnosis of FMF. Moreover, it is useful as a risk factor of amyloidosis (5). In contrast, the role of other variants involved in the pathology of FMF remains unknown. Accordingly, the functional impact of these variants on the activation of the inflammasome needs to be biologically examined. Most recently, a variety of in silico tools such as Rare Exome Variant Ensembler Learner (REVEL) have been developed to predict genetic variant pathogenicity (20) and a recent report has indicated that the possibility in which the diagnostic accuracy of FMF heightens by classifying the missense mutation of the *MEFV* gene using REVEL (21). In addition, a study to classify the clinical significance of gene mutations based on expert consensus have also been reported (22) and are available as the INFEVERS database (https://fmf.igh.cnrs.fr/ISSAID/infevers/). Therefore, these predictor tools and the database may be useful in understanding clinical consequences of *MEFV* gene variants. In this study, we performed independent analyses for typical cases, typical cases without M694I, and atypical cases. Among the 96 typical FMF cases in Japan, 43 mutations of M694I were observed. This suggests that 45% of the typical cases can be explained by a single mutation of rs28940578 (M694I). Notably, we identified four relevant single-site variants (rs11466018, rs401877, rs3743930, and rs224231) and the haplotype GTC defined by rs11466018, rs224231, and rs401877 in the typical FMF cases without an M694I mutation. However, the GTC

---

Table IV. The distribution of single nucleotide variants in the *MEFV* gene among typical FMF without the rs28940578 mutation and controls (case-control analysis).

<table>
<thead>
<tr>
<th>SNV name</th>
<th>Gene:</th>
<th>Location (GRCh37.p12)</th>
<th>Minor Allele</th>
<th>Consequence</th>
<th>Allele count (2n)</th>
<th>MAF (%)</th>
<th>p-value</th>
<th>Odds ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cases: (typical FMF without M694I)</td>
<td>Controls: (typical FMF without M694I)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs3743930</td>
<td>MEFV: Missense Variant</td>
<td>3304626</td>
<td>G</td>
<td>43</td>
<td>138</td>
<td>0.406</td>
<td>0.24</td>
<td>0.00072*</td>
<td>2.17</td>
</tr>
<tr>
<td>rs11466018</td>
<td>MEFV: Missense Variant</td>
<td>3304739</td>
<td>G</td>
<td>18</td>
<td>36</td>
<td>0.17</td>
<td>0.063</td>
<td>0.00061*</td>
<td>3.07</td>
</tr>
<tr>
<td>rs224231</td>
<td>None</td>
<td>3309979</td>
<td>T</td>
<td>51</td>
<td>153</td>
<td>0.491</td>
<td>0.266</td>
<td>8.99 × 10⁻⁶</td>
<td>2.66</td>
</tr>
<tr>
<td>rs401877</td>
<td>LINCO00921: 2KB Upstream Variant</td>
<td>3312480</td>
<td>C</td>
<td>57</td>
<td>185</td>
<td>0.538</td>
<td>0.321</td>
<td>3.63 × 10⁻⁶</td>
<td>2.46</td>
</tr>
</tbody>
</table>

CI: confidence interval; FMF: familial Mediterranean fever; MAF: minor allele frequency; SNV: single nucleotide variant.

*SNVs reached the Bonferroni-corrected significance level.
haplotype was also found in 12.9% of the cases of FMF with M694I mutation. Therefore, the relationship between the GTC haplotype and M694I mutation is not mutually exclusive. In addition, because the minor allele frequencies are high in the general population, it is unlikely that these related alleles are responsible for the susceptibility of FMF. Of note, E148Q (rs5743930) and L110P (rs11466018) are frequent in the general population, particularly in East Asian population (MAF: respectively 29.1% and 8.4% according to GnomAD database (http://gnomad. broadinstitute.org) and considered as susceptibility factors of inflammation (22). The role of two other variants, rs401877 and rs224231, still unknown. Therefore, revealing the biological significance of these variants in the future is important. The DNA analysis of the whole MEFV revealed that typical cases without M694I and atypical cases were not caused by an abnormality on MEFV alone. Other factors including genetic factors may be involved in the susceptibility of FMF. The limitations of our study must be acknowledged. First, this research exclusively included Japanese patients. The genetic background of Japanese and Mediterranean populations is different (16). Therefore, the results of this study may not be extrapolated to non-Japanese populations. Second, longitudinal analyses assessing the long-term prognosis and therapeutic response of patients were not conducted in this study. Investigation of the impact of MEFV profile on the rate of FMF50, which is a score for assessing outcome in FMF (23), disease activity evaluated using the ISSF score (24), and the risk of amyloidosis is warranted. In conclusion, analysis of the whole MEFV using NGS revealed that M694I is definitely related to the susceptibility of typical FMF in Japanese patients and that the haplotype GTC conferred susceptibility of typical FMF patients without the M694I mutation. However, their effect on the susceptibility of FMF remains unknown. There are certain cases of FMF which may not be explained only by abnormalities in MEFV. These cases need to be examined using other methods such as whole exome and whole genome analyses.

Acknowledgements
We thank Kaori Furukawa (Research Assistant, Department of Immunology and Rheumatology, Division of Advanced Preventive Medical Sciences, Nagasaki University Graduate School of Biomedical Sciences; Nagasaki University Graduate School of Biomedical Sciences; Nagasaki University Hospital, Clinical Research Centre; Department of Human Genetics, Nagasaki University Atomic Bomb Disease Institute; Department of Rheumatology, Fukushima Medical University School of Medicine, Fukushima; Department of Internal Medicine, Sasebo City General Hospital, Sasebo; Centre for Rheumatic Disease, Sasebo Chuo Hospital, Sasebo; Department of Pathology, Ehime University Graduate School of Medicine and Proteo-Science Centre, Toon, Ehime; Department of Infectious Immunology, Shinshu University, Graduate School of Medicine, Matsumoto; Department of Paediatrics, School of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Japan) for her technical assistance.

Affiliations
1Department of Immunology and Rheumatology, Division of Advanced Preventive Medical Sciences, Nagasaki University Graduate School of Biomedical Sciences;
2Centre for Bioinformatics and Molecular Medicine, Nagasaki University Graduate School of Biomedical Sciences;
Nagasaki University Graduate School of Biomedical Sciences;
3Nagasaki University Hospital, Clinical Research Centre;
4Department of Human Genetics, Nagasaki University Atomic Bomb Disease Institute;
5Department of Rheumatology, Fukushima Medical University School of Medicine, Fukushima;
6Department of Internal Medicine, Sasebo City General Hospital, Sasebo;
7Centre for Rheumatic Disease, Sasebo Chuo Hospital, Sasebo;
8Department of Pathology, Ehime University Graduate School of Medicine and Proteo-Science Centre, Toon, Ehime;
9Department of Infectious Immunology, Shinshu University, Graduate School of Medicine, Matsumoto;
10Department of Paediatrics, School of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Japan.


