Serum amyloid A1 (SAA1) gene polymorphisms in Japanese patients with adult-onset Still’s disease

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Abstract
Adult-onset Still’s disease (AOSD) is a rare systemic inflammatory disorder in which inflammasome activation plays a pathophysiological role. In view of the inflammatory nature of AOSD, we investigated whether serum amyloid A (SAA) gene polymorphisms affect the susceptibility of patients with AOSD.

Eighty-seven Japanese patients with AOSD and 200 healthy Japanese subjects were recruited in this study. The genotypes of the -13C/T SNP in the 5′-flanking region of the SAA1 gene (rs12218) and two SNPs within exon 3 of SAA1 (2995C/T and 3010C/T polymorphisms) were determined using polymerase chain reaction fragment length polymorphism (PCR-RFLP) assay in all subjects. In AOSD patients, exons 1, 2, 3, and 10 of the MEFV gene were also genotyped by direct sequencing.

The frequency of the SAA1.3 allele was increased in AOSD patients compared with that in healthy subjects (43.1% versus 37.5%), but the difference was not significant. The –13T allele was more frequently observed in AOSD patients than in healthy subjects (50.6% versus 41.0%, P = .0336). AOSD patients with the –13T allele had been treated with immunosuppressants more frequently than those without this allele. MEFV mutations were detected in 49 patients with AOSD (49/87, 57.3%), AOSD patients with MEFV variants frequently exhibit macrophage activation syndrome, but the difference was not significant (34.7% versus 18.4%, P = .081). Also, there was no significant difference in SAA1 -13C/T allele frequency between AOSD patients with and without MEFV mutations.

Our data shows a significant association between T allele of rs12218 and AOSD in Japanese population.

Abbreviations: AOSD = adult-onset Still’s disease, PCR-RFLP = PCR restriction fragment length polymorphism, SAA = serum amyloid A, SNPs = single-nucleotide polymorphisms.

Keywords: adult-onset still’s disease, inflammasome, MEFV, polymorphisms, serum amyloid A

1. Introduction
Adult-onset Still’s disease (AOSD) is a systemic inflammatory disease characterized by spiking fever, skin rash, and arthritis.[1] Although its pathogenesis is largely unknown,[2] a growing number of studies support the hypothesis that dysregulation of inflammasome activation and the related overproduction of cytokines play a pivotal role in it, similar to other autoimmune inflammatory diseases.[3] Serum amyloid A (SAA) is an acute-phase reactant that is synthesized in the liver by inflammatory cytokines, the level of which can increase 1000-fold during inflammatory conditions.[4] Within the SAA gene cluster, only the SAA1 and SAA2 genes encode acute-phase SAA.[5] The presence of two single-nucleotide polymorphisms (SNPs) within exon 3 of the SAA1 gene, 2995C/T and 3010C/T, define 3 haplotypes that correspond to SAA1.1, SAA1.3, and SAA1.5.[6] Several studies have shown that the development of AA amyloidosis is positively related to the frequency of SAA1.3 alleles in the Japanese population.[7,8] Subsequent investigations indicated that another SNP (rs12218) in the SAA1 gene, at position –13 in the 5′ regulatory region of this gene, is in linkage disequilibrium with the SAA1.3 allele and is closely associated with the occurrence of AA amyloidosis in Japanese and Caucasian rheumatoid arthritis patients.[9,10] Moreover, a functional study suggested that the T variant at this SNP is associated with greater transcriptional activity of the SAA1 gene.[11] G-protein-coupled formyl peptide receptor 2 (FPR2) is one of the receptors for SAA.[12] In addition to FPR2, several receptors...
for SAA have been identified.\cite{13} Recent studies have suggested that SAA may play a role in the modulation of inflammation and immunity through these SAA receptors.\cite{14} In this study, we investigated the relationship between SAA1 gene polymorphism and AOSD.

2. Patients and methods

2.1. Design, setting, patients, and measurements

We retrospectively evaluated 87 patients, who were diagnosed as AOSD and be treated at the Rheumatology Units of participating hospital group, between 2010 and 2017. The patients had all been diagnosed with AOSD according to the Yamaguchi criteria,\cite{15} after exclusion of infectious, hematologic, and inflammatory diseases. All patients had undergone laboratory tests, including a complete blood count, biochemistry, urinalysis, erythrocyte sedimentation rate (ESR) and ferritin. The demographic data of the enrolled patients, including clinical manifestations, treatments, prognosis, and complications, were collected using a standardized form. Each patient was assessed for the systemic score proposed by Pouchot et al\cite{26} for AOSD. This score assigns 1 point to each of 12 manifestations: fever, typical rash, pleuritis, pneumonia, pericarditis, hepatomegaly or abnormal liver function tests, splenomegaly, lymphadenopathy, leukocytosis > 15,000/mm\(^3\), sore throat, myalgia, and abdominal pain (maximum score: 12 points). This study was approved by the institutional review board of the Sasebo City Hospital institutional review board (No. 2012-A22) and participating hospitals. Two hundreds of healthy Japanese individuals without pre-existing medical diseases (90 men and 110 women 14 to 64 years, with a mean age of 38.6 ± 13.9 years) from East Japan (n = 86) and West Japan (n = 114) were enrolled in the study after obtaining informed consent.

2.2. Genotyping of SAA1 gene

The SAA1.1, 1.3, and 1.5 alleles, corresponding to the T-C, C-T, and C-C haplotypes of the C2995T (rs1136743) and G3010T (rs1136747) polymorphisms were analyzed by the PCR-RFLP methods.\cite{8} The primers used for the PCR reaction were 5'-GCC AATTACATCGGCCTCAG-3' (sense) and 5'-TGGCCA AAGAATCTCCTGG AT-3' (antisense). The 518-bp PCR products were digested with restriction enzyme BclI (Promega, San Luis Obispo, CA, USA) and BanII (Promega) and electrophoresed on a agarose gel. The genotypes of the SAA1 -13C/T in the 5'-flanking region of the SAA1 gene, (rs12218) were also determined by the PCR-RFLP method.\cite{8} The primers used for PCR were 5'-ACATCT TGTTTCCCTC AGGTTG-3' (sense) and 5'-GCTGTA AGCTGCCC GG-3' (antisense). The 229-bp PCR products were subjected to the restriction enzyme AciI (BioLabs, Beverly, MA, USA) and electrophoresed on a 12.5% polyacrylamide gel.

2.3. MEFV gene analysis

Peripheral blood samples (collected in vacutainers containing EDTA as anticoagulant) were used for genomic DNA isolation. Genomic DNA was isolated by the Promega Wizard Genomic DNA Purification Kit (Madison, WI, USA). Polymerase chain reaction (PCR) was performed using forward and reverse primers for each exon of the MEFV gene, as described previously.\cite{16} The polymerase chain reaction (PCR) products were purified and directly sequenced using ABI 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). The MEFV genetic analysis was approved by the Ethics Committee of Fukushima Medical University School of Medicine (2016, No. 2920).

2.4. Statistical analysis

Statistical analyses were out using SPSS version 17.0 (SPSS; Chicago, IL, USA). Hardy-Weinberg equilibrium was assessed by the chi-square test. Differences in the distribution of alleles between AOSD patients and control subjects were analyzed using the chi-square test. Differences among AOSD characteristics were analyzed by performing a Mann-Whitney’s U test or Chi-squared test using 2 × 2 contingency tables.

3. Results

3.1. Demographic findings of enrolled AOSD patients

A total of 87 Japanese patients (18 men and 69 women) who fulfilled the Yamaguchi criteria for AOSD\cite{13} were recruited into this study. All patients were ethnic Japanese. Routine blood tests including tests of C-reactive protein, serum ferritin, and liver enzymes were performed. The clinical manifestations of the enrolled patients are shown in Table 1. Among the 87 enrolled patients with AOSD, 69 (79.3%) were women and the mean age at diagnosis was 49.3 ± 19.9 years old. Among these 87 patients, 51 patients (58.6%) were treated with immunosuppressants (Cyclosporin A 38, Tacrolimus 4, Methotrexate 9).

3.2. SAA1 gene haplotypes in Japanese patients with AOSD

A segment of the SAA1 gene with polymorphic sites was subjected to PCR restriction fragment length polymorphism (PCR-RFLP) analysis. Table 2 shows the frequencies of individuals with various genotypes and alleles at the SAA1 locus in either AOSD patients (n = 87) or healthy Japanese subjects (n = 200). The frequency of the SAA1.3 allele was increased in AOSD patients compared with that in healthy subjects (43.1% versus 37.5%), but the difference was not significant.

3.3. Association of SAA1 –13T allele with AOSD in Japanese

The -13C/T polymorphism, in the 5'-flanking region of the SAA1 gene, which is in linkage disequilibrium with the SAA1.3 allele. The -13C allele was increased in AOSD patients compared with that in healthy subjects (43.1% versus 37.5%), but the difference was not significant.

Table 1

| Number | 87 |
|---|---|
| Female, n (%) | 69 (79.3) |
| Age at onset (years) | 49.3 ± 19.9 |
| Ferritin (ng/mL) | 1125.1 ± 1699.7 |
| CRP (mg/dL) | 12.4 ± 8.2 |
| ESR (mm/h) | 71.1 ± 50.1 |
| Liver dysfunction, n (%) | 68 (78.1) |
| MAS, n (%) | 23 (26.4) |
| Initial dose of PSL (mg/day) | 41.8 ± 12.2 |
| Steroid pulse, n (%) | 55 (63.2) |
| Immunosuppressant, n (%) | 51 (58.6) |
| Biologics, n (%) | 26 (29.8) |

Mean ± standard deviation or number (%) is shown. CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, MAS = macrophage activation syndrome, PSL = prednisolone. AOSD = adult onset Still’s disease.
allele, has been shown to be associated with susceptibility to AA amyloidosis in Japanese RA patients. We thus analyzed the -13C/T polymorphisms in AOSD patients and healthy Japanese subjects. The observed genotype frequencies did not differ from the expected frequencies of the genotypes under the assumption of Hardy–Weinberg equilibrium (data not shown). Allele frequencies of -13C/T differed between the 2 groups (Table 3), with the -13T allele being significantly more common in AOSD patients than in healthy subjects (50.6% versus 41.0%, P = 0.0336). This suggests that the -13T allele is associated with susceptibility to AOSD in the Japanese population. To evaluate the potential correlations of -13C/T alleles with the clinical manifestations of AOSD, we stratified the AOSD patients according to the presence of the -13T allele (Table 4). AOSD patients with this allele were found to have been treated more frequently (64.7% versus 36.8%, P = 0.029) with immunosuppressants (Cyclosporin A 33, Tacrolimus 2, Methotrexate 9), but there was no significant difference in the other clinical manifestations. We also compared the systemic score according to the genotypes of SAA -13C/T among AOSD patients. As demonstrated in Figure 1, we could not find a significant difference in systemic score according to the genotype SAA -13C/T allele in Japanese patients with AOSD.

### Table 2

| Genotypes | AOSD patients (n=87) (%) | Control (n=200) (%) | P value |
|-----------|-------------------------|--------------------|---------|
| 1.1/1.1   | 8 (9.2)                 | 24 (12.0)          |         |
| 1.1/1.3   | 25 (28.7)               | 49 (24.5)          |         |
| 1.1/1.5   | 12 (13.8)               | 39 (19.5)          |         |
| 1.3/1.3   | 14 (16.1)               | 27 (13.5)          |         |
| 1.3/1.5   | 22 (25.3)               | 47 (23.5)          |         |
| 1.5/1.5   | 6 (6.9)                 | 14 (7.0)           |         |

### Table 3

| Genotype and allele frequencies of –13 C/T SAA1 in AOSD patients and the healthy subjects. | AOSD patients (n=87) (%) | Healthy subjects (n=200) (%) | χ² | P value |
|-----------------------------------------------|-------------------------|-----------------------------|---|--------|
| Genotypes at -13C/T SAA1                       |                         |                             |   |        |
| C/C                                           | 19 (21.8)               | 67 (33.5)                   |   |        |
| C/T                                           | 48 (55.1)               | 102 (51.0)                  |   |        |
| T/T                                           | 20 (22.9)               | 31 (15.3)                   |   |        |
| Alleles at -13C/T SAA1                        |                         |                             |   |        |
| C                                             | 86 (94.9)               | 236 (59.0)                  |   |        |
| T                                             | 88 (60.6)               | 164 (41.0)                  | 4.513 | .0336 |

### Table 4

| Demographic and clinical features of AOSD patients with or without -13T allele. | C/C (n=19) (%) | C/T, T/T (n=68) (%) | P value |
|------------------------------------------------------------------------------|---------------|---------------------|--------|
| Age, years (SD)                                                             | 51.12 (21.9)  | 53.50 (28.9)        | .513   |
| Onset age, years (SD)                                                       | 47.47 (22.3)  | 49.88 (19.3)        | .662   |
| Gender (Female/Male)                                                        | 14/5          | 5/13                | .493   |
| CRP mg/dl (SD)                                                              | 12.34 (8.3)   | 11.78 (8.3)         | .442   |
| Ferritin ng/ml (SD)                                                         | 7292.2 (12535.2) | 11199.2 (7787.8) | .969   |
| Liver dysfunction, (%)                                                      | 13 (68.4%)    | 55 (80.9%)          | .246   |
| MAS, (%)                                                                    | 6 (31.5%)     | 18 (26.5%)          | .659   |
| Initial dose of PSL mg/dl (SD)                                              | 31.8 (14.8)   | 34.8 (11.8)         | .679   |
| Steroid pulse therapy, (%)                                                  | 6 (31.5%)     | 26 (38.2%)          | .594   |
| Immunosuppressants, (%)                                                      | 7 (36.8%)     | 44 (64.7%)          | .029   |

Association was tested between AOSD patients with or without –13 T allele by χ²-squared test using 2 × 2 contingency tables or Mann-Whitney’s U test. CRP=C-reactive protein, MAS=microphage activation syndrome, PSL=prednisolone, SD=standard deviation. AOSD=adult onset Still’s disease.

3.4. MEFV variant analysis

All AOSD patients were successfully genotyped for the MEFV gene. MEFV variants were identified in 46 AOSD patients (57.6%), the distribution of which is shown in Table 5. We stratified the AOSD patients according to the presence or absence of MEFV variants, and then compared the clinical features between these groups (Table 6). Although there was no statistically significant difference, AOSD patients with MEFV variants were more frequently associated with macrophage activation syndrome (34.7% versus 18.4%, P = .092) and the initial doses of prednisolone were higher compared with those without MEFV variants (35.9 ± 12.8 mg/day versus 31.8 ± 11.2 mg/day, P = .081). We also compared the frequencies of -13C/T polymorphisms between the AOSD patients stratified according to the presence of MEFV variants, but no significant differences were found (Table 7).

4. Discussion

A major acute-phase reactant, SAA, is produced when the immune system senses potential danger signals including trauma, infection, and severe stress. Prior studies accumulated substantial evidence that SAA is involved in inflammatory disorders including autoinflammatory diseases. SAA has proinflammatory activities, including those involving cell migration and phagocytosis. It is also considered to be a danger signal that activates inflammasomes. Specifically, SAA is capable of activating NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome and subsequent IL-1β production, which play important roles in the pathogenesis of AOSD. Therefore, it is possible that SAA is important in the pathogenesis of autoinflammatory diseases including AOSD. Evidence has also been presented that the risk of AOSD is influenced by polymorphisms in proinflammatory genes including cytokines and components of the innate immune system.

In this study, we found that polymorphisms of the SAA1 gene were associated with the susceptibility to AOSD in the Japanese population. We demonstrated that the frequency of the T allele at position –13 in the SAA1 gene (rs122118) was significantly increased in AOSD patients compared with that in healthy subjects. Previous studies also demonstrated the strong linkage disequilibrium between the SAA1.3 allele and the SAA1 –13T.
allele in the Japanese population.[9] Our data also indicate that the allele frequency of SAA1.3 was increased in AOSD patients compared with that in healthy controls, although this did not reach statistical significance.

The overproduction of IL-1β is responsible for a variety of autoinflammatory disorders, including AOSD.[23] Recent studies have shown that SAA activates the NLRP3 inflammasome and subsequent IL-1β production in innate immune cells,[24] suggesting a link between SAA and autoinflammatory diseases.

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**Table 5**

**MEFV genotypes of AOSD patients.**

| Mutations                      | Number of patients (%) (n=87) |
|-------------------------------|------------------------------|
| M694I/normal                  | 2 (2.3)                      |
| G632S/E408Q                   | 1 (1.1)                      |
| P369S/R408Q                   | 4 (4.6)                      |
| E148Q/P369S                   | 1 (1.1)                      |
| E148Q/E148Q/P369S/R408Q       | 1 (1.1)                      |
| L110P/E148Q/E148Q/P369S/R408Q | 1 (1.1)                      |
| E148Q/R202Q                   | 2 (2.3)                      |
| E148Q/R202Q                   | 1 (1.1)                      |
| R202Q/normal                  | 3 (3.4)                      |
| E148Q/normal                  | 19 (21.8)                    |
| E148Q/E148Q                   | 2 (2.3)                      |
| L110P/E148Q                   | 6 (6.9)                      |
| L110P/E148Q/E148Q             | 2 (2.3)                      |
| L110P/E148Q/R202Q             | 1 (1.1)                      |
| L110P/L110P/E148Q/E148Q       | 1 (1.1)                      |
| E84K/normal                   | 2 (2.3)                      |
| E84K/L110P/E148Q              | 1 (1.1)                      |
| Normal                        | 38 (43.7)                    |

AOSD = adult onset Still’s disease.

**Table 6**

**Demographic and clinical features of AOSD patients with or without MEFV variants.**

| Demographic/clinical features | MEFV variants (+) n=49 | MEFV variants (−) n=38 | P value |
|-------------------------------|------------------------|------------------------|---------|
| Age years, (SD)               | 53.3 (10.6)            | 53.1 (19.9)            | .687    |
| Onset age years, (SD)         | 48.2 (19.2)            | 50.9 (20.9)            | .598    |
| Gender (Female/Male)          | 38/11                  | 31/7                   | .645    |
| CRP mg/dl, (SD)               | 11.4 (7.9)             | 12.6 (8.7)             | .459    |
| Ferritin ng/ml, (SD)          | 11908.7 (20164.5)      | 9211.6 (14374.5)       | .528    |
| Liver dysfunction, (%)        | 38 (77.6%)             | 30 (78.9%)             | .875    |
| MAS, (%) activation syndrome  | 17 (34.7%)             | 7 (18.4%)              | .092    |
| Initial dose of PSL mg/dl, (SD)| 35.9 (12.8)            | 31.8 (11.2)            | .081    |
| Steroid pulse therapy, (%)    | 32 (65.3%)             | 23 (60.5%)             | .646    |
| Immunosuppressant, (%)        | 30 (61.2%)             | 21 (53.1%)             | .575    |
| Biologics, (%)                | 14 (28.6%)             | 12 (31.6%)             | .761    |

Association was tested between AOSD patients with or without MEFV variants by Chi-squared test using 2 × 2 contingency tables or Mann-Whitney’s U test. CRP = C-reactive protein, MAS = macrophage activation syndrome, PSL = prednisolone, SD = standard deviation. AOSD = adult onset Still’s disease.
SNAI1 polymorphisms have been shown to be associated with a predisposition to AA amyloidosis.\(^5\) The mechanisms by which SNAI1 gene polymorphism (-13C/T, rs12218) may be associated with AOSD are unknown. Moriguchi et al demonstrated that a C/T switch at -13 of SNAI1 gene (-13C/T) is associated with increased transcription activity of this gene correlating susceptibility to AA amyloidosis in Japanese patients with rheumatoid arthritis. They found 4 major haplotypes of SNAI1 promoter based on 3 SNPs at -61, -13, and -2 of the SNAI1 promoter region and designed them -14C1 (C-C-G), -13C2 (G-C-G), -13C3 (G-C-A), and -13T (C-T-G). They demonstrated that -13T promoter exhibited greater transcriptional activity compared to other cis-regulating elements promoter haplotypes -13C1, -13C2, and -13C3 in luciferase reporter gene assays under IL-1\(\beta\) or IL-6 stimulation. The serum concentration of A-SAA to C-reactive protein (CRP) were demonstrated to be significantly higher in subjects carrying SAA1.5 allele than in those carrying SAA1.3 allele. The data from luciferase reporter assay seem inconsistent with these laboratory data. However, an increased transcriptional ability of A-SAA does not necessarily seem inconsistent with these laboratory data. However, an increased transcriptional activity of A-SAA correlated susceptibility to AA amyloidosis in Asian patients.

5. Conclusions

In conclusion, we suggest that the SNPs in the promoter region of SNAI1 gene (-13C/T) may correlate with AOSD in the Japanese population. Further studies are necessary to determine, which are the predominant SNPs with the susceptibility to AOSD in various ethnic population.

| Alleles at –13C/T | AOSD with MEFV variants | AOSD without MEFV variants |
|------------------|-------------------------|--------------------------|
|                  | \((n=49)\)               | \((n=38)\)               |
| C                | 51 (52.0%)               | 35 (46.1%)               |
| T                | 47 (53.1%)               | 41 (53.9%)               |

Allele carrier frequencies are shown in parenthesis (%). Association was tested by \(\chi^2\) test. AOSD = adult onset Still’s disease.
Writing – review, and editing: Kiyoshi Migita and Hiroshi Furukawa.

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