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Association between adult-onset Still's disease and interleukin-18 gene polymorphisms

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Recently, we reported that serum concentration of IL-18 is strikingly high in patients with adult-onset Still's disease (AOSD). The aim of the present study was to screen for genetic polymorphisms in the human IL-18 (hIL-18) gene and to determine the association of polymorphisms with susceptibility to AOSD. We investigated the 6.7 kb region upstream of exon 2 of hIL-18 gene, in which a promoter activity had been reported. Sixteen AOSD patients, 144 rheumatoid arthritis (RA) patients and 92 healthy control individuals were studied. We found seven single nucleotide polymorphisms and a single 9 bp insertion which were frequently present in the AOSD patients. Three haplotypes including a unique combination of these polymorphisms were also determined. Of them, haplotype S01 contained all eight of these polymorphisms. The frequency of individuals carrying a diplotype configuration, ie a combination of two haplotypes, of S01/S01 was significantly higher in the AOSD patients than in the healthy controls (P = 0.00059, Fischer's exact probability test, odds ratio [OR] = 7.81, 95% confidence interval [95% CI] = 2.48–24.65) and the RA patients (P = 0.015, Fischer's exact probability test, OR = 4.0, 95% CI = 1.39–11.54). We therefore conclude that possession of the diplotype configuration of S01/S01 is a major genetic risk factor for susceptibility to AOSD.
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Keywords: *adult-onset Still's disease; IL-18; polymorphisms; rheumatoid arthritis*

Introduction

Adult-onset Still's disease (AOSD) is an acute inflammatory disorder characterized by high spiking fever, polyarthralgia, evanescent salmon-colored rash, liver dysfunction, and splenomegaly.[1] This entity was first described by Bywaters[2] as a syndrome seen in adults similar to the one in children with acute, febrile onset of juvenile rheumatoid arthritis (JRA). Serological studies for autoantibodies including anti-nuclear antibody and rheumatoid factor are negative. Leukocytosis and high levels of acute-phase proteins are usually present. Especially, serum ferritin levels can be enormously elevated, well beyond those expected when compared with other acute-phase reactants in the same individuals.[3] Although its pathogenesis is still unclear, high serum levels of several cytokines such as IL-1β, IL-6, tumor necrosis factor (TNF)-α and interferon (IFN)-γ have been suggested to play roles in the disease.[4,5] However, elevated levels of these cytokines are not specific for AOSD since they are often elevated in other systemic inflammatory diseases such as sepsis, rheumatoid arthritis (RA) and JRA. Recently, we reported that serum IL-18 levels were significantly elevated (more than 1000-fold) in AOSD patients compared with those in patients with other inflammatory disorders such as RA and JRA as well as healthy individuals.[6] Serum levels of IL-18 in AOSD patients correlated well with disease severity. Several investigators also demonstrated that serum IL-18 was increased in AOSD patients compared to other systemic inflammatory diseases and that it was well correlated to serum ferritin or C-reactive protein levels.[5,7] These findings suggest that elevated serum concentrations of IL-18 would be closely specific for AOSD among systemic inflammatory diseases.

IL-18 was first described as an (IFN-γ)-inducing factor,[8] and has multiple functions including induction of the synthesis of IFN-γ by T cells and natural killer (NK) cells,[8,9] promotion of Th1-type immune responses, augmentation of proliferative response and cytokine production of activated T cells.[10] Recently, it was reported that bioactive IL-18 could cause acute liver injury,[11] inflammatory arthritis,[12] allergic inflammation,[13,14] and the other autoimmune diseases,[15,16] These biologic effects of IL-18 might be implicated with the most important characteristics of AOSD, suggesting that aberrant expression of IL-18 can be related to the pathogenesis of this disease.

The human IL-18 (hIL-18) gene is located on chromosome 11q22.2-q22.3,[17] and is composed of six exons and five introns. The translation-starting site is present in exon 2.[18] Two promoter regions of the hIL-18 have been reported, one of which is located within the 6.7 kb region upstream of exon 2[19] and the other in the 5′-flanking region.[18] This 6.7 kb region includes recognition sites such as *PU.1* (purine-rich sequence), *NF-κB* (nuclear factor), *AP1* (activator protein) and *Sp-1* (specificity

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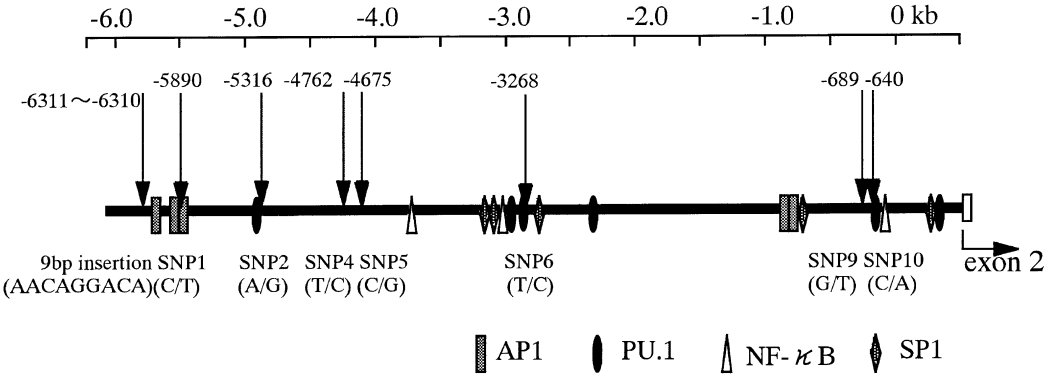

Figure 1 A schematic drawing of the 6.7 kb region upstream of exon 2 of the hIL-18 gene as well as major transcriptional factor recognition sequences. Arrows indicate eight different sites of polymorphism including seven SNPs and a single 9 bp insertion which were frequently present in the AOSD patients. Nucleotides at the seven SNP sites and the presence or absence of the 9 bp insertion for the wild-type allele (S02) were as follows: C, A, T, C, T, G and C without the 9 bp insertion.

protein). However, the exact position of the promoter activity within this wide 6.7 kb region and critical transcriptional factor-binding sequences remain to be determined. The other promoter activity within the 5′-flanking region has been shown to act constitutively.[18]

In the present study, we performed a systematic search for polymorphisms of the promoter regions of the hIL-18 gene, to assess whether the genetic components of IL-18 could contribute to the pathogenesis of AOSD.

Results

Polymorphisms at intron 1 of the hIL-18 gene

We investigated the entire 6.7 kb region upstream of exon 2 of the hIL-18 gene that had been submitted to the DDBJ nucleotide sequence databases with accession number AB015961. The genomic sequences of this region were numbered from the first nucleotide of exon 2 as position +1. By screening 40 Japanese genomic DNAs obtained from PBMCs, we determined the common nucleotide sequences of the 6.7 kb region upstream of exon 2 of the hIL-18 gene. They contained several differences from deposited sequences, which were determined using a sample from human placenta. The newly determined sequences included an additional *PU.1* binding site at −3411.

In the 6.7 kb region upstream of exon 2 of the hIL-18 gene, we initially found 11 polymorphisms, which included 10 single nucleotide polymorphisms (SNPs) and a single 9 bp insertion as follows: C to T substitution at nt position −5890 (SNP1), A to G at nt position −5316 (SNP2), T to C at nt position −5207 (SNP3), T to C at nt position −4762 (SNP4), C to G at nt position –4675 (SNP5), T to C at nt position −3268 (SNP6), C to T at nt position −2835 (SNP7), T to C at nt position −2565 (SNP8), G to T at nt position −689 (SNP9), C to A at nt position −640 (SNP10), and a 9 bp (AACAGGACA) insertion between nt positions −6311 and −6310. Among these polymorphisms, SNP1, 2, 4, 5, 6, 9 and 10 and the 9 bp insertion were more frequently present in the AOSD patients than in the healthy controls. The frequencies of homozygosity for SNP1, 6, 9 and 10 and the 9 bp insertion in the AOSD patients were 62.5%, whereas those for the healthy controls were 30.4% ($P = 0.021$, by Fisher's exact probability test). For SNP2, 4 and 5, the frequencies in the AOSD patients were 56.3%, while those of the healthy controls were only 14.1% ($P = 0.00059$, by Fisher's exact probability test). The positions of these polymorphisms as well as the major transcriptional factor recognition sequences are schematically shown in Figure 1. These single nucleotide substitutions neither alter known transcriptional factor recognition sequences nor result in new recognition sequences.

Typing of haplotype and diplotype configurations

We typed haplotypes of the gene including these eight polymorphisms using the LDSUPPORT program. We identified three haplotypes (S01, S02 and S03), each of which contained a unique combination of nt changes at the sites (SNPs 1, 2, 4, 5, 6, 9 and 10) and did or did not have the 9 bp insertion. Nucleotides at the 7 SNP sites and the presence or absence of the 9 bp insertion for each haplotype were as follows: haplotype S01, T, G, C, G, C, T and A with the 9 bp insertion; haplotype S02, C, A, T, C, T, G and C without the 9 bp insertion; haplotype S03, T, A, T, C, C, T and A with the 9 bp insertion (Figure 2). With the LDSUPPORT program, the 9 bp insertion, SNP1, SNP6, SNP9 and SNP10 were found to be in complete linkage disequilibrium, while SNP2, SNP4 and SNP5 were also in complete linkage disequilibrium. The allelic frequencies of these three haplotypes in the AOSD patients, the RA patients and the healthy controls are summarized in Table 1. The frequencies of haplotype S01 were significantly higher in the AOSD patients than in the healthy controls ($P = 0.0072$, Fisher's exact probability test, odds ratio [OR] = 2.90, 95% confidence interval 95% CI = 1.32–6.38). The RA patients also tended to have higher frequencies of haplotype S01, though not to a statistically significant extent ($P = 0.088$, Fisher's exact probability test, OR = 1.39, 95% CI = 0.96–2.03). A diplotype configuration, ie a combination of two haplotypes, was also determined for each subject using the LDSUPPORT program. The frequency of the diplotype configuration of S01/S01 was significantly higher in the AOSD patients than that of the healthy controls ($P = 0.00059$, Fischer's exact probability test, OR = 7.81, 95% CI = 2.48–24.65) or the RA patients ($P = 0.015$, Fischer's exact

probability test, OR = 4.0, 95% CI = 1.39–11.54), (Table 2). The RA patients tended to have a higher frequency of the diplotype configuration of S01/S01, though not to a statistically significant extent ($P = 0.069$, Fisher's exact probability test OR = 1.95, 95% CI = 0.97–3.93). The populations of the normal controls and the RA patients were found to be in Hardy–Weinberg's equilibrium, while that of the AOSD patients was not in Hardy–Weinberg's equilibrium.

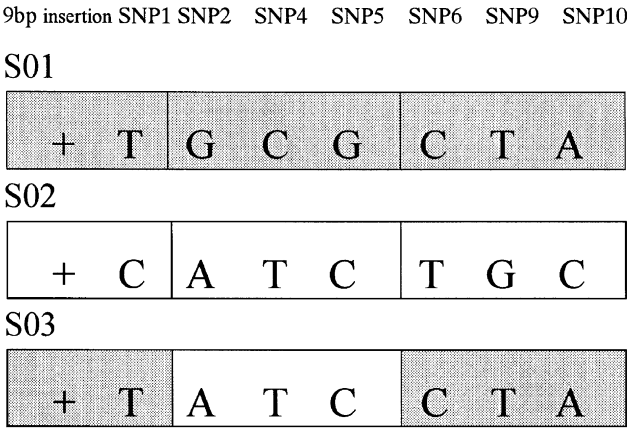

Figure 2 A unique combination of eight novel polymorphisms constructed three different haplotypes, S01, S02 and S03. The 9 bp insertion, SNP1, 6, 9 and 10, and SNP2, 4 and 5 were in linkage disequilibrium. Gray box, mutant-type allele; open box, wild-type allele; +, 9 bp (AACAGGACA) is inserted;−, 9 bp is not inserted.

Table 1 Numbers and frequencies of three haplotypes at the IL-18 geneintron 1 in the AOSD, RA patients and normal controls

	Haplotype	*AOSD total* (%)	*RA total* (%)	*Control total* (%)
S01	+TGCGCTA	21 (65.6)	138 (47.9)	73 (39.7)
S02	+CATCTGC	8 (25)	101 (35.1)	82 (44.6)
S03	+TATCCTA	3 (9.4)	49 (17.0)	29 (15.8)

+, There is 9 bp insertion between nt position –6311 and –6310; −, There is no 9 bp insertion between nt position –6311 and –6310. Hapotypes are expressed by seven bases at seven SNP sites (SNP1, 2, 4, 5, 6, 9 and 10) and having or not having 9 bp insertion.

Discussion

The present study is the first report describing novel genetic polymorphisms of the hIL-18 gene which frequently exist in AOSD patients in the 6.7 kb region upstream of exon 2. Three haplotypes (S01, S02 and S03) which consist of unique combinations of these polymorphisms were also determined. The frequency of haplotype S01 as well as that of the diplotype configuration of S01/S01 was significantly higher in the AOSD patients than in the healthy controls and the RA patients. Considering from the statistical viewpoints, homozygosity for S01 is more important for susceptibility to AOSD than only carrying S01 haplotype, since the P value for the former was smaller than that of the latter. Among the eight polymorphisms which construct haplotype S01, SNPs 2, 4 and 5 are the key polymorphisms responsible for susceptibility to AOSD, since these three SNPs were specific to haplotype S01, as shown in Figure 2.

These findings of the present study raise the question as to how these genetic polymorphisms contribute to the pathogenesis of AOSD. First, the possibility that the SNPs induce higher transcriptional activity in the promoter region of hIL-18 gene is considered. These polymorphisms are within intron 1 of the hIL-18 gene, in which promoter activity is reported to exist. Although the nucleotide substitutions do not create new transcription recognition sequences, they could alter binding capacities of transcriptional factors to their recognition sites, leading to inappropriate IL-18 mRNA expression and protein production, which would in turn contribute to the pathogenesis of AOSD. Obviously, alternation of transcriptional activity by genetic polymorphisms is reported in the human TNF-α gene. The *TNFA* promoter with SNP at –308 (TNF2) has much stronger transcriptional activity than the common allele (TNF1).[20] TNF2 is a component of an extended MHC haplotype HLA-A1-B8-DR3-DQ2, which is associated with high TNF-α production.[20] The second possibility is that undefined genetic polymorphisms in linkage disequilibrium with haplotype S01 exist in other regions of the hIL-18 gene including coding exons, and contribute to high bioactivity of IL-18 seen in AOSD.

Whether possession of the diplotype configuration of S01/S01 is associated with high IL-18 production *in vivo* must also be considered. We compared serum levels of IL-18 among three types of diplotype configurations, ie those with two S01 haplotypes, one S01 or no S01, in 10 of 16 AOSD patients for whom serum IL-18 data were available, but detected no significant differences among them. This might have been explained by the small study populations which was examined in the present study.

Table 2 Numbers and frequencies of diplotype configurations in the AOSD, RA patients and normal controls

Diplotype configurations	*AOSD total* (%) (*n=16*)	*RA total* (%) (*n=144*)	*Control total* (%) (*n=92*)
S01/S01	**9 (56.3)**	**35 (24.3)**	**13 (14.1)**
S01/S02	3 (18.6)	50 (34.7)	35 (38.0)
S01/S03	0 (0)	18 (12.5)	12 (13.0)
S02/S02	2 (12.5) **7 (43.7)**	18 (12.5) **109 (75.7)**	18 (19.6) **79 (85.9)**
S02/S03	1 (6.3)	15 (10.4)	11 (12.0)
S03/S03	1 (6.3)	8 (5.6)	3 (3.3)

Table 3 Primers for direct sequencing

Primers for PCR		*Primers for sequencing*	
Primer name	Sequence 5′ to 3′	Primer name	Sequence 5′ to 3′
−6574F	ACTTGCCTTAAAAGCTTTGC	−6554F	ATAGGTAGACAACATTAGAT
−5361R	GTCCTACAACATAGGAGGA	−6167F	GGAAATTTACAGGCCCAAG
		−6149R	CTTGGGCCTGTAAATTTCC
		−5749F	AGCGTCTGGTATCTTGAACC
−5846F	GGCACAGACTCACTTCTG	−5423F	TGGAGGCCTATACTTAGTG
−4473R	GAGGTGGAGTCTCGCTCTG	−4895R	GGCCATATCATGGAATTTC
		−4983F	GGGTAATCTTTCAAAAC
		−4513R	GGCACAATCTCGGCTCACTGC
−4535F	TTGCAGTGAGCCGAGATTG	−4313F	TCACTCGAGTCTGAGGTTAT
−3562R	CAGAGGGGACAGTGCTTAGTA	−4156F	GCCCTGTCCATATTCAAGTT
		−4137R	AACTTGAATATGGACAGGGC
−3674F	ATCCAGGCCTGGTGGACGGGCT	−3624F	ACTGCCCTGGCTCTGCCA
−2631R	GTGAGCCATGTGTGTTATGC	−3106R	CCGGGAAAAAGCTGCCTTTCCG
		−3203F	GCACGAGTCACGTGACAGCT
		−2698R	CCCGGGGTAAGAATTTTC
−2786F	GATCCAGAGCCTCAGTTACT	−2766F	GCCCCCTCTTCCTCTTTGGT
−1894R	CACAGTGGACAACTCTACAT	−2367F	GTCTTGGAATTATTCTGTGTG
		−2224R	GGCTAGTATTATGTGGCCTA
−1985F	GCCAGGTGAGATAAGTTTTATG	−1943F	CTTACATTTTTAGTGTCCTT
−1004R	GTTTTGCCAGTACAGAGTTG	−1784R	ACAGTGTGAAGGCTGTGAGA
		−1630F	GCTAAATGGGTAGGAATAAG
		−1338F	GCTTTCATGTTAATTGGCCC
−1058F	GTTCCCTGACTCTAGGAACCCCT	−990F	GCCACCTTGCTAATTCCC
+66R	AGGGCAAAATGCACTGGGAGAC	−473R	CCTCATTCAGGACTTCCC
		−538F	TGGCCTGTATCAACTATCC
		+40R	CCTTGCTGACTGTCCAGGCAG

To determine whether the genotypes are related to phenotypes with high IL-18 production, we are attempting to compare *in vitro* IL-18 production of monocytes obtained from subjects with different genotypes in conditions optimized to induce maximum IL-18 production. Since IL-18 production is regulated at multiple levels including transcription, translation and posttranslational modification by caspase 1,[11,21] it might be difficult to directly correlate IL-18 production at the protein level and the diplotype configuration.

The genotypes of the hIL-18 gene in the RA patients were also determined as a control for systemic inflammatory disease. The frequencies of the haplotypes and the diplotype configurations did not differ between the RA patients and the healthy individuals. We found no significant increase in the frequency of the diplotype configuration of S01/S01 in patients with RA who carried a DR antigen referred to as 'shared epitope' (data not shown). A high concentration of IL-18 has been found in synovial fluid obtained from patients with RA.[12,22,23] Although some investigators have reported that RA patients have high concentrations of serum IL-18,[22] it is commonly accepted that IL-18 concentrations are higher in synovial fluid than in serum.[12] IL-18 would exert pathological effects only within affected synovium in RA, while systemic and abundant production of IL-18 would be specific for AOSD. These phenomena are consistent with the present data showing that a certain diplotype configuration is frequently observed only in AOSD.

In summary, we identified skewing of the diplotype configuration of the hIL-18 gene in AOSD, which might explain the extraordinary high serum IL-18 levels in this disease.

Patients and methods

Patients and control subjects

The present study has been approved by the institutional Genome-Ethics Committee of Tokyo Women's Medical University. We examined 16 patients with AOSD, who were followed up at the Institute of Rheumatology, Tokyo Women's Medical University, with informed consent. Eleven patients (69%) were female, and all patients met both the criteria of Cush *et al*[1] and those of Yamaguchi *et al*[3] for AOSD. We also examined 92 healthy individuals (female, 67%). As a control for inflammatory disease, 144 patients with RA (female, 81%) randomly selected from among those visiting our outpatient department were also examined. All RA patients met the 1987 classification criteria for RA of the American College of Rheumatology.[24] All subjects were Japanese, and there was no significant differences in sex ratio among the patients with AOSD and RA and healthy individuals.

DNA isolation and genotyping

Genomic DNA was isolated from peripheral blood using a commercial kit (DnaQuick, Dainippon, Japan). Genomic sequences for the region 6.7 kb region upstream of exon 2 of the hIL-18 gene were obtained from the GenBank DNA database (http:www.ncbi.nlm.nih.gov/; accession:AB015961). For direct sequencing, polymerase chain reaction (PCR) was performed to amplify a segment of the 6.7 kb region upstream of exon 2 using genomic DNA as a template. Primers used for direct sequencing are listed in Table 3. The sequences were analyzed using PCR products by the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, CA,

USA). On preliminary examination by direct sequencing, we found 10 SNPs and a single 9 bp insertion. To determine the genotype for an individual, we used either direct sequencing, restriction enzyme digestion or allelic discrimination chemistries using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) as described below.

Restriction enzyme genotyping

The polymorphism at nt position −4762 (SNP4) constituted a T to C substitution, while that at −4675 (SNP5) was a C to G substitution. The genotype of each polymorphism was determined by restriction fragment length polymorphism using restriction enzymes. Primers were established to amplify the segment from −4916 to −4473, which contained both SNP4 and SNP5: forward 5′-GTTGAAATTCCATGATATGGCCT-3′; reverse 5′-GAGGTGGAGTCTCGCTCTG-3′. A 10 μl portion of each PCR product of 444 bp was digested with 1 μl of *MboII* (Toyobo, Tokyo, Japan) for SNP4, or 1 μl of *TaqI* (Toyobo) for SNP5. SNP4 generated a *MboII* restriction site, and digestion resulted in 290 bp and 154 bp fragments, while PCR products without SNP4 were resistant to digestion. SNP5 destroyed a *TaqI* restriction site, which prevented the PCR products from being digested into 203 and 241 bp fragments. The digested products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide.

Allelic discrimination chemistries

For allelic discrimination chemistries, a set of forward and reverse primers and fluorescent-labeled probes which hybridize either a wild-type (wt) or a variant-type (vt) sequence were prepared. The polymorphism at nt position –5316 constituted a A to G substitution (SNP2), while that at –3268 was a T to C substitution (SNP6). Primers for SNP2 and SNP6 were as follows: SNP2 forward 5′-TCCTAGCTCTGGGCATACGAAT-3′; SNP2 reverse 5′-AGCTGTATGCCTATGGAGCCTTA-3′; SNP6 forward 5′-TCCCTACTGTTGTTTCCGCC-3′; SNP6 reverse 5′-CCCGAAGTCCGAGCACC-3′. Probes for SNP2 and SNP6 were as follows: SNP2 wt 5′-VIC-CCACTTATCTATAGAGCTT-MGB-3′; SNP2 vt 5′-FAM-CACTTATCTGTAGAGCTT-MGB-3′; SNP6 wt 5′-VIC-TGAAGACCCTGGGC-MGB-3′; SNP6 vt 5′-FAM-TGAA-GACCCCGGGC-MGB-3′. A 10 μl portion of PCR mixture contained 9 pM each of the forward and reverse primers, 2 pM each of probes, 10 ng of genomic DNA as a template and TaqMan PCR Universal Master Mix containing AmpliTaq Gold DNA polymerase (Applied Biosystems). During PCR, each probe annealed specifically to complementary sequences between the forward and reverse primer sites. AmpliTaq Gold DNA polymerase cleaved probes that hybridize to the target, and the cleavage separated the reporter dye from the probe. By comparing the fluorescent signals generated during PCR amplification, it was possible to determine the sequences that were present in the sample. When the fluorescent signal was VIC only, the sample was homozygous for the wt allele. Similarly, with FAM fluorescence only, the sample was homozygous for the vt allele. When both fluorescent signals were increased, the sample was heterozygous.

Detection of 9 bp insertion

For detection of the 9 bp insertion between nt position –6311 and –6310, PCR primers were established for amplification between –6347 and –6201: forward 5′-AGAGAGGACAGCTGTGGACTATC-3′; reverse 5′-GCTTTCTAGCATGTTTGCCTT -3′. Genomic DNA with and without the 9 bp insertion generated 156 and 147 bp fragments, respectively. If a subject was heterozygous, PCR products of both sizes would be generated. PCR products were separated by 1.5% Nusieve GTG agarose (BioWhittaker Molecular Application, Rockland, ME, USA) gel and visualized by ethidium bromide.

Typing of haplotype and diplotype configurations

We demonstrated eight novel polymorphisms including seven SNPs and a single 9 bp insertion which frequently exist in the AOSD patients. We applied the genotypic data to the LDSUPPORT program[25] to estimate the haplotype frequencies in the population and to calculate the posterior probability of diplotype distribution for each subject. This program was designed for typing of haplotype using a maximum likelihood estimation method which is based on the expectation maximization (EM) algorithm assuming Hardy–Weinberg's equilibrium for the population. This method estimates the frequencies of haplotypes in the population from which the sample was obtained. Based on the estimated haplotype frequencies, the posterior distribution of the diplotype configuration, ie a combination of two haplotypes, can be calculated for each subject. The haplotype frequencies estimated by LDSUPPORT were the same as those obtained by a previous EM-based program EH[26] as shown using both simulation and observed data.

Statistical analysis

To compare the frequencies of the haplotype or the diplotype configurations, Fisher's exact probability test was used. Differences were considered significant at $P < 0.05$. OR were determined for disease susceptibility in carriers of a specific diplotype configuration. The 95% CIs for OR were also calculated.

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