and an *in vitro* lymphocyte apoptotic defect (Su & Anderson, 2009). ALPS is classified into several groups, according to the genetic defects. ALPS type 0 is caused by homozygous mutations of *FAS* (Rieux-Laucat *et al.*, 1995; Kasahara *et al.*, 1998; van der Burg *et al.*, 2000), type Ia by heterozygous mutations of *FAS* (Jackson *et al.*, 1999; Rieux-Laucat *et al.*, 1999; Vaishnaw *et al.*, 1999) and type Ib by heterozygous mutations in the Fas ligand (*FasL*) gene (Wu *et al.*, 1996). Heterozygous *CASP10* mutants are classified as ALPS type IIa, and homozygous *CASP8* mutations cause ALPS type IIb. In ALPS type III, the genetic defect is unknown.

Recently, genomic structural variations such as copy number variations (CNVs) are recognized as important causes for many human diseases including autoimmune diseases (Stankiewicz & Lupski, 2010). In this study, we performed genome-wide SNP array analysis to detect CNVs for the first time in s-JIA patients. In this process, we found an intragenic deletion of *CASP10* in one patient, a causative gene for ALPS type IIa, raising a question of the pathogenic significance of *CASP10* mutation in s-JIA.

Materials and method

Subjects

A total of 50 patients with s-JIA who had disease refractory to conventional treatment and were given tocilizumab were enrolled with informed consent in IRB-approved protocols at Yokohama City University Hospital. There were no family histories in each patient. Genomic DNA of peripheral blood leucocytes from all patients were isolated using DNA isolation systems (Quick Gene-800; Fujifilm, Tokyo, Japan). DNA of nail tissues and buccal cells from the patient with the *CASP10* deletion was isolated using ISOHAIR (Wako, Tokyo, Japan) and Puregene Kit C (Quiagen, MD, USA), respectively, according to each manufacturer's protocol.

SNP array

To detect CNVs, two different commercially available SNP array platforms, the Genechip Human Mapping 250K array (Affymetrix Inc., Santa Clara, CA, USA) (23 patients) and the Genome-wide Human SNP array 6.0 (Affymetrix Inc.) (27 patients) were used following the manufacture's protocols. In brief, for the Genome-wide Human SNP array 6.0, 500-ng DNA was digested with *Nsp* I and *Sty* I (only *Nsp* I was used for 250K array). The adaptors were ligated to the digested DNA, and the ligation-mediated PCR with singleprimer was performed. PCR products were purified by magnetic beads (Ampure; Beckman Coulter Company, Beverly, MA, USA). Microcon YM-100 (Millipore Corporation, Bedford, MA, USA) was used for purification for the 250K array. The product was fragmented, end labelled and hybridized to an array. CNAG3.0 (Nannya *et al.*, 2005), Genotyping Console (Copy Number Analyser for GeneChip, Affymetix Inc.) and Partek Genomic Suite (Partek Inc., St. Louis, MO, USA) were used to validate copy number alterations. The qualities of the results were high in every sample [250K array: SNP call rate >95%, MDR >99%, (MDR–MCR) <5%, SNP array 6.0: Contrast QC >2, QC call rate >93%, MAPD <0.4].

Quantitative real-time PCR

The deletion breakpoints were analysed using genomic DNAs by quantitative real-time polymerase chain reaction (qPCR) with Quantifast SYBR Green PCR kit on Rotor-Gene™ 6200 HRM (Corbett Life Science, Sydney, Australia). The delta–delta Ct relative quantitative method was employed according to the manufacturer's protocol. Averages of duplicates were calculated by ROTOR-GENE 6000 SERIES software (Corbett Life Science).

Direct sequencing of a deletion junction

Fragments containing the deletion break point were amplified by PCR for direct sequencing. Long PCR primers adjacent to presumed deleted regions by qPCR were generated. PCR was cycled once at 94°C for 2 min, 35 times at 98°C for 10 s, and at 68°C for 3 min in 20-μL mixture using KODFX (Toyobo, Osaka, Japan). PCR products were purified with ExoSAP™ (USB Co., Cleveland, OH, USA) and sequenced using BigDye™ terminator (Applied Biosystems, Foster City, CA, USA) on the ABI 3100 automatic DNA sequencer (Applied Biosystems).

RT-PCR analysis

Total RNA was extracted from lymphoblastoid cell line (LCL) of all patients using TRIzol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed with 3 μg of total RNA using PrimeScript™ first-strand cDNA Synthesis kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's protocol. PCR was cycled once at 94°C for 2 min, 35 times at 94°C for 30 s, at 64°C for 30 s, and at 68°C for 2 min in 20-μL mixture using KODFX. Pre treatment of cells with cycloheximide (protein synthesis inhibitor, 150 μg/1.0 × 10^6 cells) for 4 h was done to examine the influence of nonsense-mediated mRNA decay (NMD). Primers are listed below: *CASP10*-forward, 5′-CCTGTAGACAAGGAAGCCGAGTCGT-3′ and *CASP10*-reverse, 5′-TTCGACTCACATCATCGTTGACAGC-3′.

Mutation search for *CASP10* and *CASP8*

Mutation of *CASP10* and *CASP8* was screened by high-resolution melt analysis. As *CASP10* and *CASP8*

are both causative for ALPS, showing similarity at the nucleotide level, we also looked for *CASP8* mutations. PCR and HRM were performed on Rotor-Gene™ 6200 HRM. PCR was cycled 35–40 times with denaturation for 10 s at 95°C, annealing for 20 s at 60°C, and extension for 30 s at 72°C in 12-μL mixture using ExTaq (Takara Bio Inc.) and SYTO™ 9 green fluorescent (Invitrogen). The annealing temperature varied according to the amplicon. Variants were selected for sequencing when the melting profile deviated from control samples. PCR products showing variant melting profiles were sequenced using BigDye terminators by standard methods with the same primers used in HRM-PCR.

As *CASP8* mutations in ALPS were reported to be homozygous mutations (Chun *et al.*, 2002), we performed HRM with samples which were spiked with 10% control DNA to detect homozygous mutations.

T-cell apoptosis assay

Peripheral blood mononuclear cells from the patient were activated with phytohemagglutinin and IL-2 for 10 days, and Fas-mediated apoptosis in these activated T cells was evaluated by a flow cytometric method after their incubation with anti-Fas monoclonal antibody (CH-11; MBL, Nagoya, Japan) for 24 h as previously described (Kasahara *et al.*, 1998).

Results

CASP10 intragenic deletion

A 13.4-kb intragenic deletion was detected in a patient with s-JIA who is unlikely to be affected with ALPS using Genome-wide Human SNP array 6.0 (Fig. 1a). We also confirmed the deletion by qPCR (Table 1, Fig. 1b). Sequencing a deletion junction successfully amplified by long PCR revealed that the deleted region contained exons 6–9 of *CASP10* (Figs 1a & 2). Proximal and distal breakpoints were located in two directly oriented *Alu*Y and *Alu*Sx elements. Identity between these *Alu* elements was 97% and the possible crossing-over region was 36 bp in length (Fig. 2). *Alu*-mediated nonallelic homologous recombination was the likely mechanism of this microdeletion. The father and sister, who had no symptoms of ALPS or s-JIA, also had the same deletion (Fig. 1d,e). This deletion was seen in the DNA extracted from blood, buccal cells and nails of the proband (Fig. 1e), suggesting that it was indeed a germline change (not somatic). RT-PCR of the mRNA extracted from the patient's LCL revealed that *CASP10* mRNA was truncated (Fig. 3). This was seen in both samples pretreated with or without cycloheximide (data not shown), indicating that the truncated *CASP10* mRNA does not suffer from NMD. We further sequenced the mRNA, and found that this deletion resulted in a frame shift with an early stop codon (the termination codon appeared at the second amino acid in exon 10). No deletions containing *CASP10* were observed in 54 patients (108 alleles) with other diseases (29 patients with autism, 21 patients with mental retardation and multiple congenital disorders, and four patients with premature ovarian failure) by Genome-wide Human SNP array 6.0 or Nimblegen 385K array.

Mutation search for *CASP10* and *CASP8*

We could not detect any mutations in *CASP10* as well as *CASP8* in the other 49 s-JIA patients. Furthermore, to search for abnormal *CASP10* transcripts, we performed RT-PCR using mRNA extracted from LCLs of s-JIA patients, but no truncated mRNAs were found.

Clinical features of the proband and her family members with *CASP10* deletion

The proband is a 9-year-old girl who developed s-JIA with high fever, liver damage and enlargement of lymph nodes at 4 years of age. Although she recovered after symptomatic treatment, she suffered a recurrence of spiking fever, erythematous skin rash, pain and swelling of the knee and foot and was diagnosed as s-JIA at 6 years of age. The patient recovered after being given methylprednisolone pulse therapy twice. However, when the oral administration of prednisolone 15 mg day^{-1} was reduced to 13 mg day^{-1}, the swelling of her knee worsened. Therefore, she was admitted to Yokohama City University Hospital. As she was resistant to conventional therapies, she received tocilizumab (anti-IL-6 receptor antibody) therapy. Her condition got stable since tocilizumab was medicated. The level of IgG was normal, and rheumatoid factor and antinuclear antibodies were not detected (Table 2). The levels of IL-10, IL-5, IL-4 and TNF-α were normal (Table 2), showing no shift to a Th2 cytokine production pattern. TCR $\alpha\beta^+$ CD4/CD8 double-negative T cells in the peripheral blood were not high in this patient (Fig. 4a), and lymphocyte apoptosis induced by anti-Fas antibody was normal (Fig. 4b). The patient's father and her sister, both having the same partial *CASP10* deletion, are totally healthy.

Discussion

In our study, we detected an intragenic deletion of *CASP10*, a causative gene for ALPS (OMIM #601859) type IIa. Although the exonic deletion may produce a truncated protein (if translated) in this patient lacking the entire CASc domain where all the reported missense mutations harboured (Wang *et al.*, 1999; Zhu *et al.*, 2006) (Fig. 1c), she had no symptoms for ALPS. TCR $\alpha\beta^+$ CD4/CD8 double-negative T cells in her peripheral blood were not high, and lymphocyte apoptosis induced by anti-Fas antibody was normal (Fig. 4), denying ALPS.

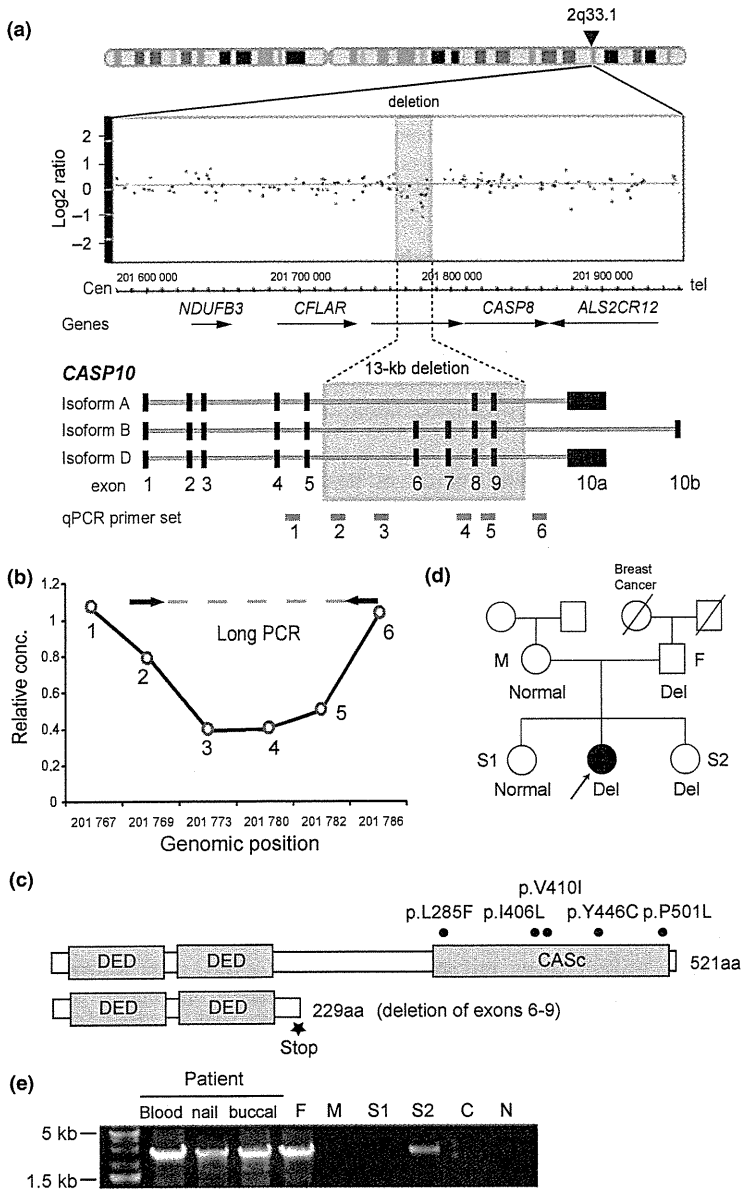

Figure 1. Characterization of the 2q33.1 microdeletion. (a) Result of Genome-wide human SNP array 6.0. The position (*x*-axis matching the genomic location of chromosome 2) and log2 ratio (*y*-axis) of each SNP probes are indicated. The 13-kb deletion is within *CASP10* encompassing exons 6–9. (b) Breakpoint analysis of the s-JIA patient. Result of quantitative RT-PCR is shown. Heterozygous deletion of positions 3, 4 and 5 was implied. Arrows show the primer positions for long PCR. (c) Protein structure of caspase 10. All the reported mutations clustered at the CASc domain. The deletion of exons 6–9 results in protein truncation lacking the entire CASc domain. (d). Family pedigree of the patient. Patient is indicated by arrow. Normal: no deletion, del: caspase 10 intragenic deletion. F: father, M: mother, S1: older sister, S2: younger sister. (e) Result of long PCR using DNAs of the patient's blood leucocytes, nails and buccal cells. Long PCR could successfully amplify 3.3-kb fragments from the patient and the patient's father (F) and sister (S2) respectively. DNA from father, mother and control was extracted from blood leucocytes, and DNA from the two sisters was extracted from their nails. F: father, M: mother, S1: older sister, S2: younger sister, C: control, N: negative control.

Table 1. Primers for quantitative real-time PCR and long PCR

Position (kb)		Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
Common primer			
1	2017671–2017674	AGTCAAACCTGGCTGCCTTA	TGCTCCTCAACTCATTCTGTG
2	2017694–2017695	GCAAGGGTTTCTGGTTTCTG	CCAAGTCTGCTGGAAGAACC
3	2017734–2017737	ACGCCCACCTGAAGACTATG	AGGCGGAGGTGTTACCATTT
4	2017809–2017811	GATCCATTGGAGTGGTTGGT	TCAGGGAGGTAAAGCTGTGG
5	2017822–2017824	AGTGCCCTAGACTGGCTGAA	GTGGCCAGACCAAGTAGGAA
6	2017859–2017861	GAAAGTGCATGCGACAGCTA	ATGCCTCCATGCCTAACAAC
Long PCR primer	2017695–2017855	GGGATTTGTGGTTCTTCCAGCAGAC	GACATGGCCAAGCAGATGCTAACAC

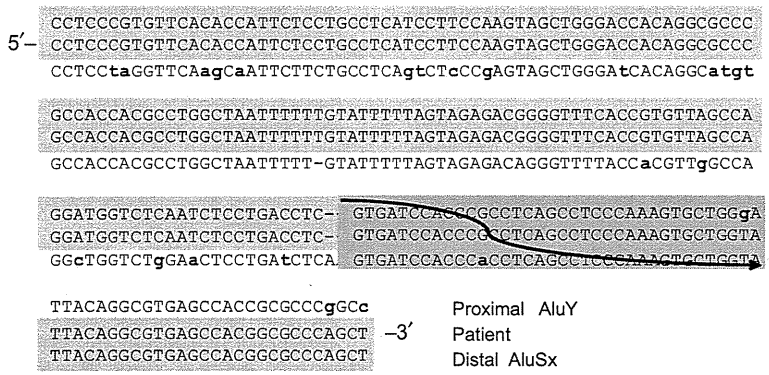

Figure 2. Result of deletion breakpoint sequence. The top, middle and bottom nucleotide strands show the proximal, recombined and distal sequences respectively. Matched sequences are shown as uppercase letters and unmatched ones as lowercase letters. Pale grey boxes show the same sequences and darker grey ones indicate a possible crossing-over region. Curved arrow shows recombination.

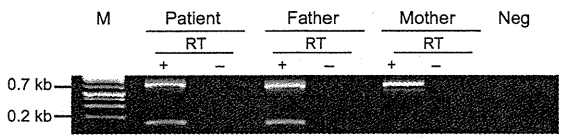

Figure 3. Result of *CASP10* RT-PCR. The lymphoblastoid cell lines which were not treated with cycloheximide were used. Forward primer was designed at exon 5 and reverse primer was designed at exon 10. The predicted size of the PCR product was 742 bp for normal cDNA and 145 bp for the deletion product. M: marker, Neg: negative control.

Table 2. Laboratory findings for the patient

Subject	Data	Normal range
IL-6	24.3 pg mL^{-1}	<2.0 pg mL^{-1}
sIL-6-R	37.5 pg mL^{-1}	14–46 pg mL^{-1}
IFNr	<0.1 IU mL^{-1}	<0.1 IU mL^{-1}
IL-5	<7.8 pg mL^{-1}	<10 pg mL^{-1}
IL-4	7.3 pg mL^{-1}	<6.0 pg mL^{-1}
IL-10	<2 pg mL^{-1}	<5 pg mL^{-1}
TNF-α	0.8 pg mL^{-1}	0.6–2.8 pg mL^{-1}
sTNF-RI	828 pg mL^{-1}	749–1966 pg mL^{-1}
sTNF-R2	1720 pg mL^{-1}	1003–3170 pg mL^{-1}
IgG	1492 mg dL^{-1}	870–1700 pg mL^{-1}
RF	–	–
Antinuclear antibody	–	–

IL, interleukin.

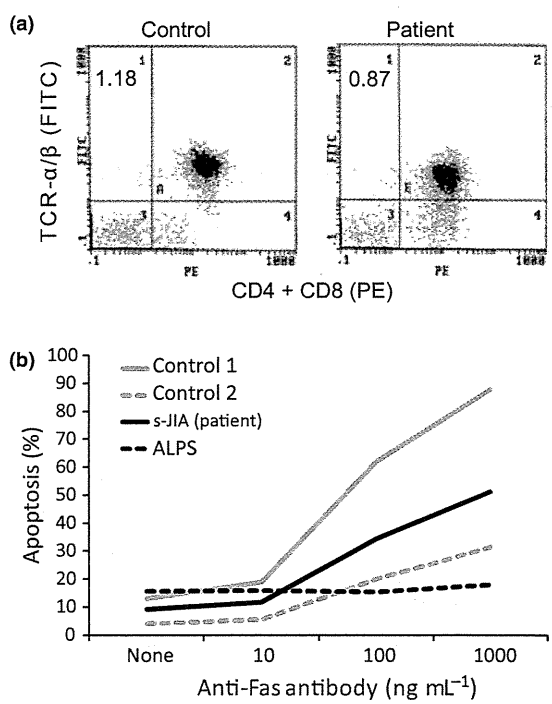

Figure 4. (a) Fluorescence-activated cell sorting (FACS) analysis of TCR αβ$^+$ CD4/CD8 double-negative T cells. TCR αβ$^+$ CD4/CD8 double-negative T cells in the peripheral blood were not high in this patient. (b) Fas-induced T-cell apoptosis assay. Apoptosis of activated T cells were induced by anti-Fas monoclonal antibody for 24 h and percentage of apoptotic cells was analysed as previously described (Kasahara *et al.*, 1998).

The *CASP10* mutations are characterized by resistance to Fas-mediated apoptosis despite the presence of normal FasL and Fas. The reported mutations for *CASP10* are missense mutations within the CASc domain (Wang *et al.*, 1999; Zhu *et al.*, 2006). Only two previous studies show ALPS patients having *CASP10* mutation so far, and both of them are reported to be inherited from nonaffected parents (Wang *et al.*, 1999; Zhu *et al.*, 2006). Although both mutations decreased caspase 10 activity and exerted a dominant negative effect on the wild-type protein, neither report was sufficient enough to prove that the mutations consistently induced the overt disease, as several mutated familial members were healthy, and some showed multiple autoantibodies and defective lymphocyte apoptosis. Moreover, in one previous report, two ALPS patients carried double heterozygous mutations in the *CASP10* and *FAS* genes, showing that mild *CASP10* mutations alone were not enough to exert a dominant

negative effect to the wild protein, and the concurrent effect of mutations hitting different genes involved in Fas function causes ALPS (Cerutti *et al.*, 2007). In our study, we detected a truncation mutation of *CASP10* in one s-JIA patient, which was inherited from the healthy father, and also was seen in the healthy sister. Although the *CASP10* mRNA extracted from the patient's LCL results in an early stop codon, the patient had no evidence of ALPS. As both previous studies and ours show mutations sharing with nonaffected parents and siblings, we need further evidence for supporting the pathogenic significance of *CASP10* mutations.

Approximately 24% of ALPS patients are classified as ALPS type III, in which no gene defects are found (Puck & Straus, 2004). In ALPS type III patients, somatic mutations of *Fas* in isolated double-negative T cells have been reported (Holzelova *et al.*, 2004). These mutations were found in a fraction of $CD4^+$ and $CD8^+$ T cells, monocytes, and $CD34^+$ hematopoietic precursors, but not in hair or mucosal epithelial cells (Holzelova *et al.*, 2004). Therefore, in our study, we investigated whether the *CASP10* deletion is somatic by examining not only blood leucocyte DNA but also nail and buccal cell DNAs, but no evidence of somatic changes was obtained.

As the phenotype and laboratory data of the patient with *CASP10* intragenic deletion were different from those of ALPS, we hypothesized that *CASP10* could be responsible for s-JIA. However, *CASP10* was not mutated at the level of genomic DNA and transcripts in other s-JIA patients. Furthermore, we searched for *CASP8* mutations, but no mutations were found. In conclusion, a 13.4-kb intragenic deletion of *CASP10* was detected in the s-JIA patient using genome-wide human SNP array. Our report provides a new insight into the pathogenic significance of caspase 10 in relation to apoptosis and human diseases. Further investigation is absolutely necessary.

Acknowledgements

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Conflict of Interest

Authors declare no conflict of interest in this study.

References

van der Burg, M., de Groot, R., Comans-Bitter, W.M., den Hollander, J.C., Hooijkaas, H., Neijens, H.J. *et al.* (2000) Autoimmune lymphoproliferative syndrome (ALPS) in a child from consanguineous parents: a dominant or recessive disease? *Pediatric Research*, **47**, 336.

Cerutti, E., Campagnoli, M.F., Ferretti, M., Garelli, E., Crescenzio, N., Rosolen, A. *et al.* (2007) Co-inherited mutations of Fas and caspase-10 in development of the autoimmune lymphoproliferative syndrome. *BMC Immunology*, 8, 28.

Chun, H.J., Zheng, L., Ahmad, M., Wang, J., Speirs, C.K., Siegel, R.M. *et al.* (2002) Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency. *Nature*, **419**, 395.

De Benedetti, F., Meazza, C., Vivarelli, M., Rossi, F., Pistorio, A., Lamb, R. *et al.* (2003) Functional and prognostic relevance of the -173 polymorphism of the macrophage migration inhibitory factor gene in systemic-onset juvenile idiopathic arthritis. *Arthritis and Rheumatism*, **48**, 1398.

Donn, R.P., Shelley, E., Ollier, W.E. & Thomson, W. (2001) A novel 5′-flanking region polymorphism of macrophage migration inhibitory factor is associated with systemic-onset juvenile idiopathic arthritis. *Arthritis and Rheumatism*, **44**, 1782.

Donn, R., Alourfi, Z., De Benedetti, F., Meazza, C., Zeggini, E., Lunt, M. *et al.* (2002) Mutation screening of the macrophage migration inhibitory factor gene: positive association of a functional polymorphism of macrophage migration inhibitory factor with juvenile idiopathic arthritis. *Arthritis and Rheumatism*, **46**, 2402.

Fishman, D., Faulds, G., Jeffery, R., Mohamed-Ali, V., Yudkin, J.S., Humphries, S. *et al.* (1998) The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. *Journal of Clinical Investigation*, **102**, 1369.

Glass, D.N. & Giannini, E.H. (1999) Juvenile rheumatoid arthritis as a complex genetic trait. *Arthritis and Rheumatism*, **42**, 2261.

Hinks, A., Barton, A., Shephard, N., Eyre, S., Bowes, J., Cargill, M. *et al.* (2009) Identification of a novel susceptibility locus for juvenile idiopathic arthritis by genome-wide association analysis. *Arthritis and Rheumatism*, **60**, 258.

Holzelova, E., Vonarbourg, C., Stolzenberg, M.C., Arkwright, P.D., Selz, F., Prieur, A.M. *et al.* (2004) Autoimmune lymphoproliferative syndrome with somatic Fas mutations. *New England Journal of Medicine*, **351**, 1409.

Jackson, C.E., Fischer, R.E., Hsu, A.P., Anderson, S.M., Choi, Y., Wang, J. *et al.* (1999) Autoimmune lymphoproliferative syndrome with defective Fas: genotype influences penetrance. *American Journal of Human Genetics*, **64**, 1002.

Kasahara, Y., Wada, T., Niida, Y., Yachie, A., Seki, H., Ishida, Y. *et al.* (1998) Novel Fas (CD95/Apo-1) mutations in infants with a lymphoproliferative disorder. *International Immunology*, **10**, 195.

Nannya, Y., Sanada, M., Nakazaki, K., Hosoya, N., Wang, L., Hangaishi, A. *et al.* (2005) A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. *Cancer Research*, **65**, 6071.

Puck, J.M. & Straus, S.E. (2004) Somatic mutations – not just for cancer anymore. *New England Journal of Medicine*, **351**, 1388.

Rieux-Laucat, F., Le Deist, F., Hivroz, C., Roberts, I.A., Debatin, K.M., Fischer, A. *et al.* (1995) Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science*, **268**, 1347.

Rieux-Laucat, F., Blachere, S., Danielan, S., De Villartay, J.P., Oleastro, M., Solary, E. *et al.* (1999) Lymphoproliferative syndrome with autoimmunity: a possible genetic basis for dominant expression of the clinical manifestations. *Blood*, **94**, 2575.

Stankiewicz, P. & Lupski, J.R. (2010) Structural variation in the human genome and its role in disease. *Annual Review of Medicine*, **61**, 437.

Su, M.A. & Anderson, M.S. (2009) Monogenic autoimmune diseases: insights into self-tolerance. *Pediatric Research*, **65**, 20R.

Symmons, D.P., Jones, M., Osborne, J., Sills, J., Southwood, T.R. & Woo, P. (1996) Pediatric rheumatology in the United Kingdom: data from the British Pediatric Rheumatology Group National Diagnostic Register. *Journal of Rheumatology*, **23**, 1975.

Thomson, W. & Donn, R. (2002) Juvenile idiopathic arthritis genetics – what's new? What's next? *Arthritis Research*, **4**, 302.

Vaishnaw, A.K., Orlinick, J.R., Chu, J.L., Krammer, P.H., Chao, M.V. & Elkon, K.B. (1999) The molecular basis for apoptotic defects in patients with CD95 (Fas/Apo-1) mutations. *Journal of Clinical Investigation*, **103**, 355.

Vastert, S.J., Kuis, W. & Grom, A.A. (2009) Systemic jia: new developments in the understanding of the pathophysiology and therapy. *Best Practice & Research. Clinical Rheumatology*, **23**, 655.

Vastert, S.J., van Wijk, R., D'Urbano, L.E., de Vooght, K.M., de Jager, W., Ravelli, A. *et al.* (2010) Mutations in the perforin gene can be linked to macrophage activation syndrome in patients with systemic onset juvenile idiopathic arthritis. *Rheumatology (Oxford)*, **49**, 441.

Wang, J., Zheng, L., Lobito, A., Chan, F.K., Dale, J., Sneller, M. *et al.* (1999) Inherited human caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. *Cell*, **98**, 47.

Wu, J., Wilson, J., He, J., Xiang, L., Schur, P.H. & Mountz, J.D. (1996) Fas ligand mutation in a patient with systemic lupus erythematosus and lymphoproliferative disease. *Journal of Clinical Investigation*, **98**, 1107.

Zhang, K., Biroschak, J., Glass, D.N., Thompson, S.D., Finkel, T., Passo, M.H. *et al.* (2008) Macrophage activation syndrome in patients with systemic juvenile idiopathic arthritis is associated with MUNC13-4 polymorphisms. *Arthritis and Rheumatism*, **58**, 2892.

Zhu, S., Hsu, A.P., Vacek, M.M., Zheng, L., Schaffer, A.A., Dale, J.K. *et al.* (2006) Genetic alterations in caspase-10 may be causative or protective in autoimmune lymphoproliferative syndrome. *Human Genetics*, **119**, 284.

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ORIGINAL ARTICLE

De novo 19q13.42 duplications involving *NLRP* gene cluster in a patient with systemic-onset juvenile idiopathic arthritis

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Systemic-onset juvenile idiopathic arthritis (s-JIA) is a rare inflammatory disease classified as a subtype of chronic childhood arthritis, manifested by spiking fever, erythematous skin rash, pericarditis and hepatosplenomegaly. The genetic background underlying s-JIA remains poorly understood. To detect disease-related copy number variations (CNVs), we performed single-nucleotide polymorphism array analysis in 50 patients with s-JIA. We detected many CNVs, but most of them were inherited from either of normal-phenotype parents. However, in one patient, we could identify two *de novo* microduplications at 19q13.42 with the size of 77 and 622 kb, separated by a 109-kb segment of normal copy number. The duplications encompass *NLRP* family (*NLRP2*, *NLRP9* and *NLRP11*) as well as *IL11* and *HSPBP1*, all of which have an important role in inflammatory pathways. These genes may significantly contribute to the pathogenesis of s-JIA.
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Keywords: arthritis; *de novo*; duplication; systemic-onset JIA

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is a chronic rheumatic disease of childhood. Approximately 11% of JIA patients show systemic-onset JIA (s-JIA),[1] which is clinically manifested by spiking fever, erythematous skin rash, pericarditis and hepatosplenomegaly in addition to arthritis. Abnormal innate immunity involving cytokines such as interleukin (IL)-1 and IL-6, neutrophils and monocytes/macrophages may play a major role in the pathogenesis of s-JIA. One of the major features of s-JIA is its progression to macrophage activation syndrome. On the basis of all these evidences, it is now generally accepted that s-JIA should be classified as an autoinflammatory syndrome rather than a classical autoimmune disease.

Two genetic factors, *HLA* and *PTPN22*, have been found as JIA susceptibility genes in multiple populations.[2] For example, HLA-B27, HLA-DR1 and HLA-DR4 have been reported to increase risk for polyarticular JIA.[3] However, such associations are seen mostly in polyarticular JIA, but not in s-JIA. Other genes including *MIF*, *IL6*, *IL10* and *TNF* are reported to be associated with s-JIA in different populations and subtypes,[4–8] although these genes account for only a small part of the total genetic contribution to JIA. Therefore, the genetic background underlying the s-JIA remains mostly undetermined.

We performed genome-wide copy number variations (CNVs) analysis in s-JIA patients. Two *de novo* microduplications at 19q13.42 encompassing 77 and 622 kb were identified in one patient by single-nucleotide polymorphism (SNP) array 6.0 and confirmed by other methods. The duplications encompass *NLRP* (Nucleotide-binding oligomeriztion domain, Leucine rich Repeat and Pyrin domain) family (*NLRP2*, *NLRP9* and *NLRP11*), which have important roles in inflammatory processes as well as *IL11*, which was reported to correlate with arthritis severity in s-JIA patients. This is the first report of *de novo* CNVs in relation to s-JIA.

MATERIALS AND METHODS

Subjects

A total of 50 patients with s-JIA, which was refractory to conventional treatment and was treated with tocilizumab, were enrolled with informed consent based on the IRB-approved protocols at Yokohama City University Hospital. There were no family histories in each patient. Genomic DNA of peripheral blood leukocytes from all patients were isolated using DNA isolation systems (Quick Gene-800; Fujifilm, Tokyo, Japan).

Whole-genome SNP array and custom array analyses

To detect CNVs, two different SNP array platforms, the Genechip Human Mapping 250K array (Affymetrix, Santa Clara, CA, USA) (for 23 patients) and

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the Genome-wide Human SNP array 6.0 (Affymetrix) (for the other 27 patients), were used following the manufacturer's protocols. In brief, for the Genome-wide Human SNP array 6.0, 500-ng DNA was digested with *Nsp*I and *Sty*I (only *Nsp*I was used for 250K array). The adaptors were ligated to the digested DNA, and the ligation-mediated polymerase chain reaction (PCR) with single primer was performed. PCR products were purified by magnetic beads (Ampure; Beckman Coulter Company, Beverly, MA, USA). Microcon-YM100 (Millipore Corporation, Bedford, MA, USA) was used for purification for the 250K array. The product was fragmented, end-labeled and hybridized to an array. CNAG3.0 (ref. 9), Genotyping Console (Copy Number Analyzer for GeneChip) (Affymetrix) and Partek Genomic Suite (Partek, St Louis, MO, USA) were used to validate copy number alterations. The qualities of the results were high in every sample (250K array: SNP call rate >95%, multifactor dimensionality reduction >99%, (multifactor dimensionality reduction−minimum candidate region) <5%; SNP array 6.0: contrast quality control >2, quality control call rate >93%, median absolute pairwise difference (MAPD) <0.4).

We also performed custom high-density oligonucleotide array-comparative genomic hybridization (CGH) analysis using custom-made 4×72K array (Roche NimbleGen, Madison, WI, USA) based on the manufacturer's protocol. This slide covered the genomic region of chromosome 19 between 59 510 019 and 61 490 039 bp with 71 891 probes. The average probes spacing are 20 bp, and the probes encompassing two duplication regions (60 144 400–60 429 000 and 60 860 000–61 051 000 bp) are 10 bp. DNA (500 ng) was sonicated with the condition of 10 s, power level 1, pulse 1 s and duty 50% using SONIFIRE-250D (Branson, Danbury, CT, USA). Patient's DNA was labeled with Cy3-random nonamers and patient's parent (father or mother) was labeled Cy5-random nonamers. Dyes were swapped in the combination of father/patient and mother/patient (patient's DNA with Cy5 and parent DNA with Cy3). Labeled DNA was hybridized at 42 °C for 16–20 h, and washed. The data analysis was carried out by NimbleScan (Roche NimbleGen) and visualized by SignalMap (Roche NimbleGen).

Fluorescence *in situ* hybridization analysis

Fluorescence *in situ* hybridization (FISH) analysis was performed on metaphase chromosomes and interphase nuclei of the patient's and parental peripheral blood leukocytes. The bacterial artificial chromosome clone, RP11-384F2, mapped to the duplicated segment was used as a probe (60 374 564–60 616 811 bp at 19q13.42, UCSC Genome Browser on Human, March 2006: hg18). Bacterial artificial chromosomes were labeled with Cy3-dUTP (Invitrogen, Carlsbad, CA, USA) by Nick translation kit (Vysis, Des Plaines, IL, USA). Probe-hybridization mixtures (16 µl) were mounted on chromosomes/nuclei, incubated at 37 °C for 16–72 h and washed. Chromosomes and nuclei were counterstained with 4′,6-diamidino-2-phenylindol containing antifade solution. Fluorescence photomicroscopy was performed under an AxioCam MR CCD fitted to Axioplan2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Quantitative real-time PCR to confirm copy number changes

The deletion breakpoints were analyzed using genomic DNAs by quantitative real-time qPCR with Quantifast SYBR Green PCR kit on Rotor-Gene™ 6200 HRM (Corbett Life Science, Sydney, Australia). The delta-delta Ct relative quantitative method was employed according to the manufacturer's protocol. Averages of duplicates were calculated by ROTOR-GENE 6000 SERIES software (Corbett Life Science).

Real-time reverse transcription-PCR

Lymphoblastoid cell lines established from the patient and her parents, and three normal controls were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1×antibiotic–antimycotic (Invitrogen) and 8 µg ml^{-1} tylosin (Sigma, St Louis, MO, USA) at 37 °C in a 5% CO_2 incubator. Total RNA was independently extracted twice using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). Total RNA (2 µg) was subjected to reverse transcription (RT) using PrimeScript first-strand synthesis kit with random hexamers (Takara, Ohtsu, Japan). Quantitative real-time RT-PCR was performed using TaqMan Gene expression assays (Applied Biosystems) (FAM label: Hs00174148_m1 for *IL11*, Hs00215284_m1 for *NLRP2*, Hs00603423_m1 for *NLRP9*, Hs00935472_m1 for *NLRP11*; VIC-label: Human ACTB (β-actin) Endogenous Control). Multiplex quantitative real-time RT-PCR was carried out by the two standard curve methods on RoterGene-6200 HRM (Corbett Life Science). Relative gene expression was calculated in comparison with that of the reference (used in standard curve) cDNA. The data from duplicated experiments using two distinctive RNA samples were averaged and the standard deviation was calculated.

RESULTS

Clinical features of the proband with *de novo* duplications

The proband is a 15-year-old female subject who developed s-JIA at 2 years old, with swelling of her left knee and limping. She was resistant to antibiotics, anti-inflammatory therapy, methylprednisolone pulse therapy, cyclosporin A, mizoribine, methotrexate and azathioprine. As she was resistant to all the conventional therapies, she was admitted to Yokohama City University Hospital at 9 years old and received tocilizumab (anti-IL-6 receptor antibody) therapy. After starting tocilizumab, her condition got recovered and stable since then.

Whole-genome oligonucleotide SNP array

We performed analysis of 50 patients of s-JIA using whole-genome oligonucleotide SNP array (250K *Nsp*I or SNP 6.0; Affymetrix). The total copy number abnormalities were 9 deletions and 12 duplications (Table 1).

Confirmation of CNVs by FISH and qPCR

Copy number changes were confirmed by other methods like FISH or qPCR and their origin was also checked using parental DNA if available. Most of the copy number abnormalities were of either paternal or maternal origin (Table 1), but two duplications at 19q13.42 on the patient (ID1395) occurred *de novo*, being confirmed by FISH analysis (Figure 1) and qPCR (data not shown).

Custom array-CGH

In addition, we performed custom oligonucleotide 72K array-CGH (Roche NimbleGen), to check the precise size of the duplications. CGH was performed using the following combination of test DNA/reference DNA: patient/father and patient/mother. Dye-swap analysis was also performed (Figure 2). As a result, two *de novo* microduplications at 19q13.42 with the size of 77 kb (Genome browser version hg18, chromosome 19 coordinates: 60 190 370–60 267 627 bp) and 622 kb (60 377 092–60 991 185 bp), separated by a 109-kb segment of normal copy number (60 267 627–60 377 092 bp), were identified. Two sets of test DNA/reference DNA combinations consistently showed the duplications, indicating that the duplications occurred *de novo*. Parentage of the family was confirmed using microsatellite markers (data not shown). Similar duplication was never deposited to the DECIPHER database (http://decipher.sanger.ac.uk/syndrome), although only one overlapping deletion was found in a patient with autism.

Expression analysis of duplicated genes

IL11, *NLRP2*, *NLRP9* and *NLRP11* were chosen to see their expression as they were mapped to the duplication. Low expression of *IL11* and *NLRP9* in lymphoblasts hindered the proper evaluation. *NLRP2* and *NLRP11* did show variable expression patterns depending on lymphoblastoid cells; thus, it was indeed difficult to see the effect of duplication of these genes (data not shown).

DISCUSSION

We identified two *de novo* microduplications in one out of 50 s-JIA patients. The duplications contain several interesting genes, including

Table 1 CNVs found in s-JIA patients

Patient ID	*CNV overlap (%)*	*Size*	*Deletion*	*Duplication*	*Confirmation*	*Inheritance*	*Array type*
1239	Partial CNV	267 kb		dup(3)(p12.2)	qPCR	Not available	250K
1247	0	275 b	del(11)(q21)		FISH	Inherit (father)	250K
1285	0	235 kb		dup(18)(q23)	qPCR	Inherit (father)	250K
1287	Partial CNV	1.58 mb	del(2)(q13)		FISH	Inherit (mother)	250K
	0	259 kb		dup(3)(p26.1)	qPCR	Inherit (mother)	
1317	0	13 kb	del(16)(q24.1)		qPCR	Not available	SNP 6.0
1333	0	93 kb		dup(5)(q34)	qPCR	CNV	SNP 6.0
1344	36	205 kb		dup(15)(q13q15.1)	qPCR	Inherit (father)	SNP 6.0
1350	0	50 kb	del(4)(q34.2)		qPCR	Not available	SNP 6.0
1361	0	128 kb	del(2)(q13)		qPCR, FISH	Inherit (mother)	SNP 6.0
1383	0	362 kb		dup(2)(q11.2)	qPCR, FISH	Inherit (mother)	SNP 6.0
	0	558 kb		dup(2)(q11.2)	qPCR, FISH	Inherit (mother)	
1395	**27**	**622 kb**		**dup(19)(q13.42)**	**qPCR, FISH**	***De novo***	**SNP 6.0**
	58	**77 kb**		**dup(19)(q13.42)**	**qPCR, FISH**	***De novo***	
1406	0	169 kb	del(1)(q25.3)		FISH	CNV	SNP 6.0
1407	0	144 kb		dup(13)(q12.11)	qPCR	Inherit (mother)	SNP 6.0
1433	0	13 kb	del(2)(q33.1)		qPCR	Inherit (father)	SNP 6.0
1434	42	5.5 mb	del(10)(q11.21q11.23)		FISH	Inherit (mother)	SNP 6.0
1439	62	906 kb		dup(1)(q43)	qPCR	Not available	SNP 6.0
1620	0	695 kb		dup(3)(q26.31)	qPCR	Not available	SNP 6.0
1669	0	85 kb	del(16)(q24.1)		qPCR	Not available	SNP 6.0

Abbreviations: CNV, copy number variation; FISH, fluorescence *in situ* hybridization; qPCR, quantitative real-time polymerase chain reaction; s-JIA, systemic-onset juvenile idiopathic arthritis; SNP, single-nucleotide polymorphism.
CNV, overlap shows overlapping ratio of reported CNVs. Inheritance indicates parental origin of CNVs. Not available: parental samples were unavailable.
De novo change is shown in bold.

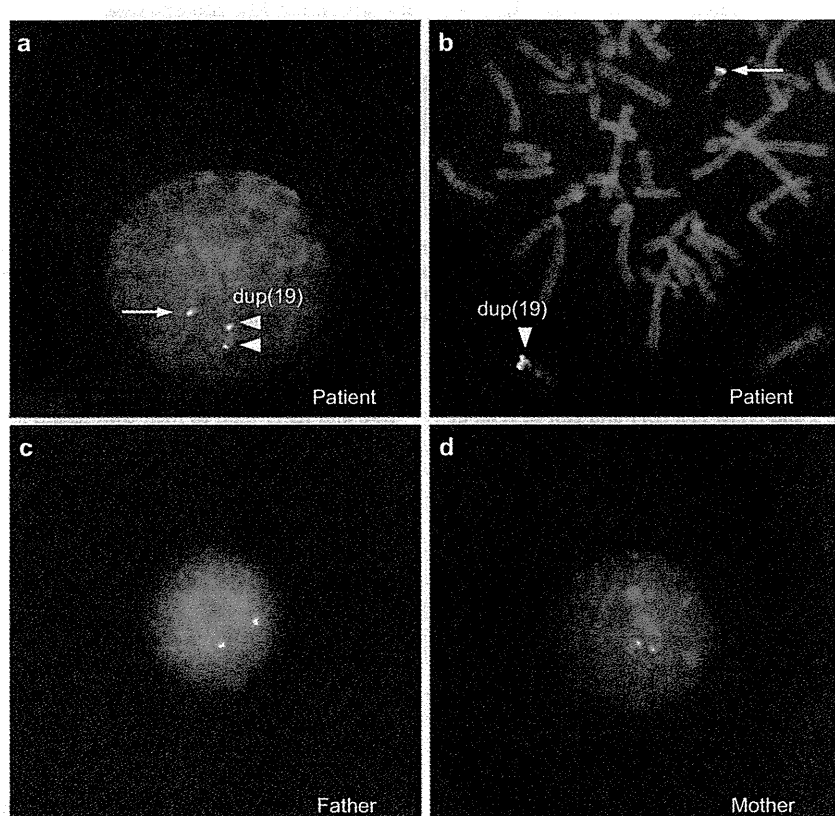

Figure 1 Fluorescence *in situ* hybridization (FISH) analysis using a bacterial artificial chromosome (BAC) clone, RP11-384F2, on cells of the family. (**a**) Interphase nucleus of the patient, (**b**) metaphase chromosomes of the patient and (**c**, **d**) father's and mother's interphase nuclei, respectively. Arrowhead indicates double signals showing duplication at 19q13.42 and arrow indicates a single signal of normal chromosome.

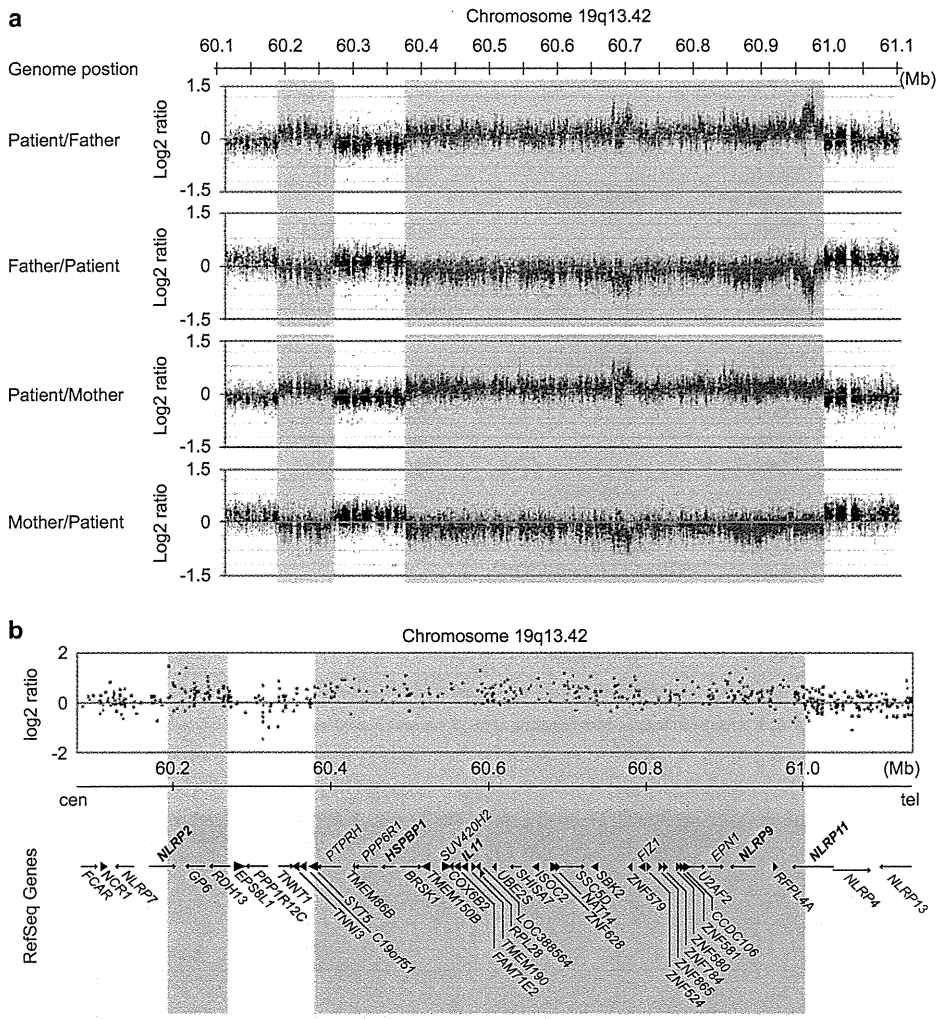

Figure 2 (**a**) Custom array-comparative genomic hybridization (CGH) results showing two *de novo* duplications at 19q13.42. Combinations of test DNA/reference DNA from top to bottom are patient/father, father/patient (dye-swapped), patient/mother and mother/patient (dye-swapped). (**b**) Characterization of 19q13.42 duplications. Upper panel shows the result of GeneChip Human SNP array 6.0 of chromosome 19 clearly showing duplications. Lower panel shows the RefSeq gene list at 19q13.42 corresponding to duplications. Duplications include *NLRP* families, *IL11* and *HSPBP1*.

NRLP family (*NLRP2*, *NLRP9* and *NLRP11*), *IL11* and *HSPBP1*, all of which are correlated with inflammatory pathways.

The *NLRP* family is composed of 14 members, including *NLRP2*, *NLRP9* and *NLRP11* (ref. 10). Most *NLRPs* are encoded by two gene clusters on chromosome 11p15 (*NLRP6*, *NLRP10* and *NLRP14*) and 19q13.4 (*NLRP2*, *NLRP4*, *NLRP5*, *NLRP7*, *NLRP8*, *NLRP9*, *NLRP11*, *NLRP12* and *NLRP13*) (ref. 10). *NLRPs* are evolutionally conserved through *Caenorhabditis elegans*, *Drosophila melanogaster*, rat, mouse and human. *NLRP1* and *NLRP3* are known as components of the inflammasome implicated in early detection of extracellular pathogens and intracellular noxious compounds and driving inflammatory and immune responses.[11] Germline mutations in *NLRP3* and *NLRP12* are associated with hereditary periodic fever syndromes.[12,13] Although the function of *NLRP9* and *NLRP11* is not well understood, *NLRP2* is suggested to function as a modulator of macrophage nuclear factor-κB activation and caspase-1 activation,[14] which may cause inflammation. All the evidences support that duplications of the *NLRP* gene cluster may significantly contribute to s-JIA pathogenesis in the patient.

IL-6 and IL-11 bind to their own ligand-specific receptor (IL-6R or IL-11R) and recruit a homodimer of gp130, which is responsible for intracellular signaling.[15] The gp130 signaling cytokines contribute to inflammation and bone homeostasis. IL-6 and IL-11 play an important role in osteoclast development, such as promoting osteoclastogenesis and bone resorption in bone marrow cultures.[16,17] IL-6 and IL-11 are capable of inducing osteoclast formation from peripheral blood mononuclear cells by a receptor activator of nuclear factor-κB ligand-independent mechanism.[18] IL-11 is produced by fibroblasts, mesenchyme-derived stromal cells of bone marrow, osteoblasts and chondrocytes.[16,19] The expression levels of IL-11 are upregulated in patients with s-JIA compared with healthy children and positively correlated with the number of joints with active arthritis.[20] Duplication of IL-11 possibly associated with its higher expression in inflammatory cells is likely to be related to the arthritis in the patient, although the roles of IL-11 are not well clarified in rheumatoid arthritis pathogenesis and alteration of IL-11 expression could not be confirmed in the patient's lymphoblastoid cells owing to the low

expression level. It is possible that the anti-IL-6 receptor antibody therapy effectively suppressed the upregulated gb130 signaling in part by IL-11 duplication in this patient.

Heat-shock proteins (Hsps) are essential to prokaryotic and eukaryotic cellular organisms during intracellular (un)folding, assembly and translocation of proteins.[21] Their synthesis is greatly enhanced in response to a variety of stressful stimuli such as temperature, hypoxia, infection and inflammation.[22] Microbial Hsps are a potential inducer of crossreactive immune responses to host self-molecules that may lead to autoimmunity.[23] In several experimental models, T cells responding to Hsps play an important role in the regulation of peripheral tolerance and suppressing pathogenic immune response.[24] HspBP1 (Hsp70 binding protein 1) has an inhibitory effect in Hsp70-assisted refolding reactions in the cytosol,[25,26] suggesting that HspBP1 plays an important role in regulating immune responses. HspBP1 duplication may alter immune responses in the s-JIA patient.

Genomic duplications can be pathogenic through both increased dosage (whole gene duplication, duplication of regulatory elements) and disruption of coding regions (intragenic duplications). Intronic duplications potentially disrupting the splicing machinery have also been reported to be pathogenic.[27] In our study, we found *de novo* duplications in one s-JIA patient, which involve many important genes for the regulation of the immune system. Other patients without any pathological CNVs in this study may have point mutations of a gene(s) mapped to the duplications, which may lead to upregulated gene expression causative for s-JIA as previously described in a different disease.[28] Further analysis is absolutely necessary.

In conclusion, this is the first report describing a possible relationship between CNVs and s-JIA, and we believe such abnormal genotypes are important to solve the pathogenesis of s-JIA.

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1 Symmons, D. P., Jones, M., Osborne, J., Sills, J., Southwood, T. R. & Woo, P. Pediatric rheumatology in the United Kingdom: data from the British Pediatric Rheumatology Group National Diagnostic Register. *J. Rheumatol.* **23,** 1975–1980 (1996).
2 Hinks, A., Barton, A., Shephard, N., Eyre, S., Bowes, J., Cargill, M. *et al.* Identification of a novel susceptibility locus for juvenile idiopathic arthritis by genome-wide association analysis. *Arthritis Rheum.* **60,** 258–263 (2009).
3 Glass, D. N. & Giannini, E. H. Juvenile rheumatoid arthritis as a complex genetic trait. *Arthritis Rheum.* **42,** 2261–2268 (1999).
4 Thomson, W. & Donn, R. Juvenile idiopathic arthritis genetics—What's new? What's next? *Arthritis Res.* **4,** 302–306 (2002).
5 Fishman, D., Faulds, G., Jeffery, R., Mohamed-Ali, V., Yudkin, J. S., Humphries, S. *et al.* The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. *J. Clin. Invest.* **102,** 1369–1376 (1998).
6 Donn, R. P., Shelley, E., Ollier, W. E. & Thomson, W. A novel 5′-flanking region polymorphism of macrophage migration inhibitory factor is associated with systemic-onset juvenile idiopathic arthritis. *Arthritis Rheum.* **44,** 1782–1785 (2001).
7 Donn, R., Alourfi, Z., De Benedetti, F., Meazza, C., Zeggini, E., Lunt, M. *et al.* Mutation screening of the macrophage migration inhibitory factor gene: positive association of a functional polymorphism of macrophage migration inhibitory factor with juvenile idiopathic arthritis. *Arthritis Rheum.* **46,** 2402–2409 (2002).
8 De Benedetti, F., Meazza, C., Vivarelli, M., Rossi, F., Pistorio, A., Lamb, R. *et al.* Functional and prognostic relevance of the −173 polymorphism of the macrophage migration inhibitory factor gene in systemic-onset juvenile idiopathic arthritis. *Arthritis Rheum.* **48,** 1398–1407 (2003).
9 Nannya, Y., Sanada, M., Nakazaki, K., Hosoya, N., Wang, L., Hangaishi, A. *et al.* A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. *Cancer Res.* **65,** 6071–6079 (2005).
10 Tschopp, J., Martinon, F. & Burns, K. NALPs: a novel protein family involved in inflammation. *Nat. Rev. Mol. Cell. Biol.* **4,** 95–104 (2003).
11 Drenth, J. P. & van der Meer, J. W. The inflammasome—a linebacker of innate defense. *N. Engl. J. Med.* **355,** 730–732 (2006).
12 Jeru, I., Duquesnoy, P., Fernandes-Alnemri, T., Cochet, E., Yu, J. W., , Lackmy-Port-Lis, M. *et al.* Mutations in NALP12 cause hereditary periodic fever syndromes. *Proc. Natl Acad. Sci. USA* **105,** 1614–1619 (2008).
13 Hoffman, H. M., Mueller, J. L., Broide, D. H., Wanderer, A. A. & Kolodner, R. D. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle–Wells syndrome. *Nat. Genet.* **29,** 301–305 (2001).
14 Bruey, J. M., Bruey-Sedano, N., Newman, R., Chandler, S., Stehlik, C. & Reed, J. C. PAN1/NALP2/PYPAF2, an inducible inflammatory mediator that regulates NF-kappaB and caspase-1 activation in macrophages. *J. Biol. Chem.* **279,** 51897–51907 (2004).
15 Sims, N. A. & Walsh, N. C. GP130 cytokines and bone remodelling in health and disease. *BMB Rep.* **43,** 513–523.
16 Girasole, G., Passeri, G., Jilka, R. L. & Manolagas, S. C. Interleukin-11: a new cytokine critical for osteoclast development. *J. Clin. Invest.* **93,** 1516–1524 (1994).
17 Heymann, D. & Rousselle, A. V. gp130 cytokine family and bone cells. *Cytokine* **12,** 1455–1468 (2000).
18 Kudo, O., Sabokbar, A., Pocock, A., Itonaga, I., Fujikawa, Y. & Athanasou, N. A. Interleukin-6 and interleukin-11 support human osteoclast formation by a RANKL-independent mechanism. *Bone* **32,** 1–7 (2003).
19 Hill, P. A., Tumber, A., Papaioannou, S. & Meikle, M. C. The cellular actions of interleukin-11 on bone resorption *in vitro*. *Endocrinology* **139,** 1564–1572 (1998).
20 Mima, T., Ishikawa, S., Aoki, C., Yoshio-Hoshino, N., Adachi, Y., Imagawa, T. *et al.* Interleukin 11 and paired immunoglobulin-like type 2 receptor alpha expression correlates with the number of joints with active arthritis in systemic juvenile idiopathic arthritis. *Ann. Rheum. Dis.* **68,** 286–287 (2009).
21 Gething, M. J. & Sambrook, J. Protein folding in the cell. *Nature* **355,** 33–45 (1992).
22 Welch, W. J., Kang, H. S., Beckmann, R. P. & Mizzen, L. A. Response of mammalian cells to metabolic stress; changes in cell physiology and structure/function of stress proteins. *Curr. Top. Microbiol. Immunol.* **167,** 31–55 (1991).
23 Albani, S. & Carson, D. A. A multistep molecular mimicry hypothesis for the pathogenesis of rheumatoid arthritis. *Immunol. Today* **17,** 466–470 (1996).
24 van Eden, W., van der Zee, R., Paul, A. G., Prakken, B. J., Wendling, U., Anderton, S. M. *et al.* Do heat shock proteins control the balance of T-cell regulation in inflammatory diseases? *Immunol. Today* **19,** 303–307 (1998).
25 Shomura, Y., Dragovic, Z., Chang, H. C., Tzvetkov, N., Young, J. C., Brodsky, J. L. *et al.* Regulation of Hsp70 function by HspBP1: structural analysis reveals an alternate mechanism for Hsp70 nucleotide exchange. *Mol. Cell* **17,** 367–379 (2005).
26 Raynes, D. A. & Guerriero, V. Jr Inhibition of Hsp70 ATPase activity and protein renaturation by a novel Hsp70-binding protein. *J. Biol. Chem.* **273,** 32883–32888 (1998).
27 Bovolenta, M., Neri, M., Fini, S., Fabris, M., Trabanelli, C., Venturoli, A. *et al.* A novel custom high density-comparative genomic hybridization array detects common rearrangements as well as deep intronic mutations in dystrophinopathies. *BMC Genom.* **9,** 572 (2008).
28 Gimelli, S., Caridi, G., Beri, S., McCracken, K., Bocciardi, R., Zordan, P. *et al.* Mutations in SOX17 are associated with congenital anomalies of the kidney and the urinary tract. *Hum. Mutat.* **31,** 1352–1359.

Association of *IRF5* Polymorphisms with Susceptibility to Macrophage Activation Syndrome in Patients with Juvenile Idiopathic Arthritis

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ABSTRACT. ***Objective.*** Systemic-onset juvenile idiopathic arthritis (systemic JIA) and macrophage activation syndrome (MAS), the most devastating complication of systemic JIA, are characterized by abnormal levels of proinflammatory cytokines. Interferon regulatory factor 5 (IRF5) is a member of the IRF family of transcription factors, and acts as a master transcription factor in the activation of genes encoding proinflammatory cytokines. Polymorphisms in the *IRF5* gene have been associated with susceptibility to autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis. Our aim was to assess associations of *IRF5* gene polymorphisms with susceptibility to systemic JIA and MAS.

Methods. Three *IRF5* single-nucleotide polymorphisms (rs729302, rs2004640, and rs2280714) were genotyped using TaqMan assays in 81 patients with systemic JIA (33 with MAS, 48 without) and 190 controls.

Results. There were no associations of the *IRF5* gene polymorphisms or haplotypes under study with susceptibility to systemic JIA. There was a significant association of the rs2004640 T allele with MAS susceptibility (OR 4.11; 95% CI 1.84, 9.16; p = 0.001). The *IRF5* haplotype (rs729302 A, rs2004640 T, and rs2280714 T), which was reported as conferring an increased risk of SLE, was significantly associated with MAS susceptibility in patients with systemic JIA (OR 4.61; 95% CI 1.73, 12.3; p < 0.001).

Conclusion. *IRF5* gene polymorphism is a genetic factor influencing susceptibility to MAS in patients with systemic JIA, and IRF5 contributes to the pathogenesis of MAS in these patients. (First Release Jan 15 2011; J Rheumatol 2011;38:769–74; doi:10.3899/jrheum.100655)

Key Indexing Terms:
INTERFERON REGULATORY FACTOR 5 — POLYMORPHISMS
MACROPHAGE ACTIVATION SYNDROME — JUVENILE IDIOPATHIC ARTHRITIS

Systemic-onset juvenile idiopathic arthritis (systemic JIA) is one of the most perplexing diseases in childhood, manifesting as spiking fever, rash, arthritis, pericarditis, and hepatosplenomegaly[1].

The systemic symptoms frequently recur in conjunction with exacerbation of the arthritis symptoms. Some studies have observed that abnormal expression of the proinflammatory cytokines such as interleukin 6 (IL-6) and IL-1ß was characteristic of systemic JIA[2,3].

The most devastating complication of JIA is macrophage activation syndrome (MAS), which is strongly associated with systemic JIA, but rarely with polyarthritis[4]. MAS is accompanied by serious morbidity and sometimes death. The increased levels of several proinflammatory cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and others correlate with the rapid development of clinical symptoms and the progression of abnormal laboratory measurements[4,5]. MAS closely resembles a reactive or an acquired form of familial hemophagocytic lymphohistiocytosis, considered to be caused by diminished natural killer (NK) cell function, and mutations of perforin (*PRF1*), *UNC13D*, and *STX11* genes[6]. Because patients with systemic JIA have decreased levels of perforin in NK cells and diminished NK cell function, it was recently suggested that *PRF1* mutations also play a role in the development of MAS in patients with systemic JIA[7,8,9]. Munc13-4 polymorphism was also associated with MAS in patients with JIA[10]. There

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is a clinical impression, however, that there are at least 2 subsets of patients with systemic JIA, one never experiencing MAS and the other with recurring MAS.

Interferon regulatory factor 5 (IRF5) is a member of the IRF family of transcription factors, and is known to have a crucial role in the Toll-like receptor (TLR) signaling pathway[11]. The activation of TLR is central to innate and adaptive immunity. IRF5 acts as a master transcription factor in the activation of proinflammatory cytokine genes. In *IRF5*-knockout mice, a severely impaired induction of IL-6, IL-12, and TNF-α was observed[11]. Recent investigations revealed associations of single-nucleotide polymorphism (SNP) in the *IRF5* gene with susceptibility to systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA)[12,13]. Thus, IRF5 has a regulatory potential for proinflammatory cytokines in certain inflammatory diseases that manifest with abnormal expression of proinflammatory cytokines.

We hypothesized that polymorphisms in the *IRF5* gene may constitute the genetic differences between the 2 tentative subsets of systemic JIA. We found a close relationship between *IRF5* gene polymorphism/haplotype and susceptibility to MAS in patients with systemic JIA.

MATERIALS AND METHODS

Study population. Patients were eligible if they met the International League of Associations for Rheumatology classification criteria for systemic JIA[14]. A total of 81 children, 40 boys and 41 girls, enrolled in this study were followed at the Yokohama City University Hospital between December 2007 and December 2009. The mean age of the patients was 4.7 years at onset of systemic JIA. The observation period of patients without MAS was at least 25 months, with a mean observation period of 102.2 months (range 25–284 mo).

Patients were diagnosed as having MAS based on the clinical symptoms and laboratory abnormalities as suggested in the preliminary diagnostic guidelines for MAS complicating systemic JIA[15], as follows: (1) clinical criteria including central nerve dysfunctions, hemorrhages, and hepatomegaly; and (2) laboratory criteria including decreased platelet counts (< 26.2 × 10^9/l), elevated levels of aspartate aminotransferase (> 59 U/l), decreased white blood cell counts (< 4.0 × 10^9/l), and hypofibrinogenemia (< 2.5 g/l). The diagnosis of MAS requires the presence of 2 or more criteria. Evidence of hemophagocytosis in bone marrow aspirates was sought only for confirmation of doubtful cases.

We conducted our study in accordance with the Declaration of Helsinki and with the approval of the Ethics Committee of the Yokohama City University School of Medicine. Written informed consent was obtained from each patient and/or their guardians.

Genotyping. Three SNP (rs729302, rs2004640, and rs2280714) in the *IRF5* gene were selected based on previous research associating them with SLE and RA[12,13]. The patients with systemic JIA (n = 81) and 190 healthy controls were genotyped. Genomic DNA was isolated from peripheral blood using the QIAamp DNA Mini kit (Qiagen K.K., Tokyo, Japan). Genotyping was performed using the TaqMan SNP Genotyping Assays (AB assay ID: C_2691216_10 for rs729302, C_9491614_10 for rs2004640, and C_2691243_1 for rs2280714). These SNP were analyzed by real-time polymerase chain reaction (PCR) using the AB7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA) under the conditions recommended by the manufacturer. The TaqMan SNP Genotyping Assay for rs2004640 was performed by TaqMan gene expression master mix instead of by TaqMan genotyping master mix. Results of genotyping at rs2004640 by TaqMan gene expression master mix were consistent with results from direct sequencing, while results by TaqMan genotyping master mix were not consistent with results from direct sequencing. The rs41298401 SNP, located 6 base pairs downstream of rs2004640, influenced these conflicting results, presumably because rs41298401 is in the base sequence annealing with TaqMan probe and causes the annealing to be insecure.

Allele discrimination was done using SDS software version 1.4 (Applied Biosystems). Confirmation of which bases were present for 5 cases of each genotype at each of these SNP sites of the genomic DNA sample was carried out using direct sequencing in the Applied Biosystems 3730xl and Sequence Scanner version 1.0 under the conditions recommended by the manufacturer.

Statistical analysis. The SNPassoc package using the R-language version 2.8 (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org) was used to evaluate associations between systemic JIA/MAS and these SNP, by logistic regression analysis[16]. Haplotype phases and haplotype frequencies were estimated using the Expectation-Maximization algorithm as implemented in the haplostat package (minimum haplotype frequency: > 0.05; www.docstoc.com)[17]. Haplotype blocks were assessed using Haploview (The Broad Institute, Cambridge, MA, USA; www.broadinstitute.org). Logistic regression analysis was also performed to evaluate the association between systemic JIA/MAS and the *IRF5* haplotypes. Association between MAS and *IRF5* gene polymorphism was analyzed by Kaplan-Meier curves with log-rank test.

RESULTS

Of the 81 patients with systemic JIA, 33 (13 boys and 20 girls) developed MAS during the followup period, according to the preliminary diagnosis guideline (Table 1)[15]. The mean lengths of followup were 97.8 months in patients with MAS and 102.2 months in patients without MAS (Table 2). MAS was recognized at a mean of 24.8 months (range 0–166 mo) after the onset of systemic JIA. However, the remaining 48 patients did not develop MAS during the followup. Age at onset of systemic JIA (p = 0.92, Welch's t test) and sex (p = 0.54, Fisher's exact test) were not associated with susceptibility to MAS in our study population (Table 2).

The genotype frequencies for the 3 SNP of the patients with systemic JIA and the healthy controls were both in Hardy-Weinberg equilibrium (p > 0.05). These results were consistent with the findings of a recent Japanese population

Table 1. The frequency of clinical, laboratory, and histopathological features of macrophage activation syndrome (MAS) in the preliminary diagnostic guideline[15]. Total number of patients was 81.

Features	No. Patients (%)
Laboratory criteria	
Decreased platelet count (< 26.2 × 10^9/l)	27 (81.8)
Elevated levels of aspartate aminotransferase (> 59 U/l)	25 (75.8)
Decreased white blood cell count (< 4.0 × 10^9/l)	11 (33.3)
Hypofibrinogenemia (< 2.5 g/l)	9 (27.3)
Clinical criteria	
Central nervous system dysfunction (seizure)	1 (3.0)
Hemorrhages (purpura, mucosal bleeding)	1 (3.0)
Hepatomegaly (> 3 cm below the costal arch)	3 (9.1)
Histopathological criterion	
Evidence of macrophage hemophagocytosis in the bone marrow aspirate	3 (9.1)

Table 2. Clinical characteristics of patients with systemic JIA with or without macrophage activation syndrome (MAS).

Characteristics	Systemic JIA with MAS (n = 33)	Systemic JIA without MAS (n = 48)	p
Male, n (%)	16 (48.5)	24 (50.0)	0.54
Age at systemic JIA onset, yrs, mean	4.8	4.7	0.92
Observation period, mo, mean	97.8	102.2	0.43
Time interval between JIA onset and MAS development, mo, mean	24.8	—	—

JIA: juvenile idiopathic arthritis.

study[18]. None of the gene polymorphisms under study was associated with susceptibility to systemic JIA (Table 3).

However, the rs2004640 SNP was found to be associated with MAS susceptibility (Table 4). Patients with the rs2004640 T allele had a high risk of developing MAS compared to those without this allele even after the Bonferroni correction (p_c = 0.003, OR 4.12, 95% CI 1.84, 9.16). Moreover, all the patients with the TT genotype at rs2004640 finally developed MAS (Table 4, Figure 1). Patients carrying the TT genotype at rs2004640 had an early onset of MAS (a mean of 12.1 mo after onset of JIA). Additionally, the ATT haplotype of the *IRF5* gene (rs729302-rs2004640-rs2280714) showed a statistically significant association with susceptibility to MAS ($p < 0.001$, OR 4.61, 95% CI 1.73, 12.3; Table 5). A haplotype block showed the correlation between the SNP genotyped (Figure 2).

DISCUSSION

In the clinical setting, MAS apparently develops under the influence of systemic inflammatory responses of systemic JIA together with environmental factor(s), supposedly viral infection[5]. Susceptibility to these environmental factors may be subject to genetic influences. The combined pres-

Table 3. Association of polymorphisms in the *IRF5* gene with susceptibility to systemic juvenile idiopathic arthritis (JIA).

SNP, Subject Subset	No. (%) with Genotype			Allelic Association			
				Total	OR	(95% CI)	p
rs729302	AA	AC	CC				
Systemic JIA	42 (51.9)	33 (40.7)	6 (7.4)	81	0.84	0.56–1.24	0.37
Control	116 (61.1)	57 (30.0)	17 (8.9)	190	1.0	—	—
rs2004640	GG	GT	TT				
Systemic JIA	36 (44.4)	36 (44.4)	9 (11.1)	81	1.05	0.71–1.55	0.80
Control	82 (43.2)	85 (44.7)	23 (12.1)	190	1.0	—	—
rs2280714	CC	CT	TT				
Systemic JIA	28 (34.6)	44 (54.3)	9 (11.1)	81	1.19	0.81–1.73	0.38
Control	58 (30.5)	94 (49.5)	38 (20.0)	190	1.0	—	—

IRF5: interferon regulatory factor 5; SNP: single-nucleotide polymorphism.

Table 4. Association of polymorphisms in the *IRF5* gene with susceptibility to macrophage activation syndrome (MAS) in patients with systemic juvenile idiopathic arthritis (JIA).

SNP, Subject Subset	No. (%) with Genotype			Allelic Association				
				Total	OR	(95% CI)	p	p_c
rs729302	AA	AC	CC					
MAS	22 (66.7)	10 (30.3)	1 (3.0)	33	2.45	1.11–5.42	0.03	0.08
Non-MAS	20 (41.7)	23 (47.9)	5 (10.4)	48	1.0	—	—	—
rs2004640	GG	GT	TT					
MAS	9 (27.3)	15 (45.5)	9 (27.3)	33	0.24	0.11–0.54	0.001	0.003
Non-MAS	27 (56.3)	21 (43.8)	0 (0.0)	48	1.0	—	—	—
rs2280714	CC	CT	TT					
MAS	14 (42.4)	17 (51.5)	2 (6.1)	33	2.12	1.08–4.40	0.045	0.13
Non-MAS	14 (29.2)	27 (56.3)	7 (14.6)	48	1.0	—	—	—

p_c: corrected combined p value using the Bonferroni method. *IRF5*: interferon regulatory factor 5; SNP: single-nucleotide polymorphism.

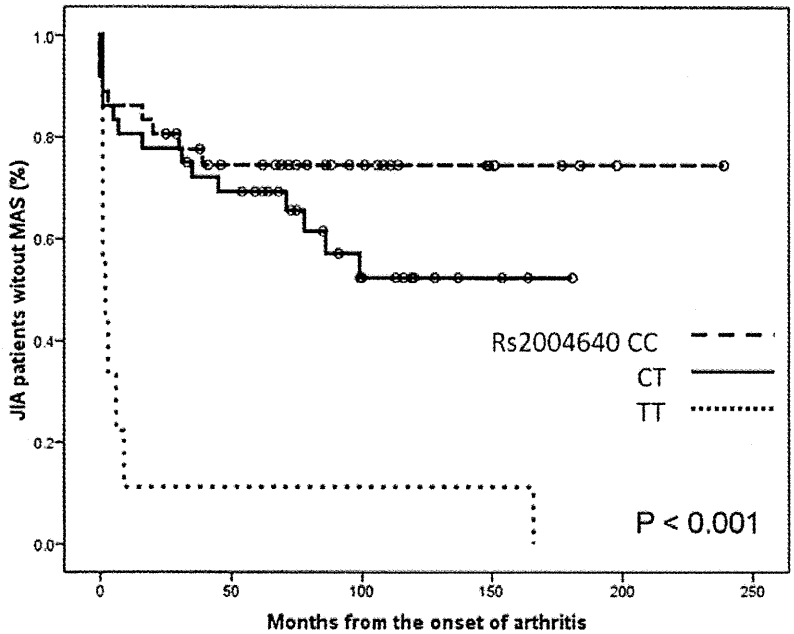

Figure 1. Kaplan-Meier analysis of survival without macrophage activation syndrome and rs2004640 genotypes. JIA: juvenile idiopathic arthritis.

Table 5. Comparison of IRF5 haplotypes in patients with systemic juvenile idiopathic arthritis (JIA) with or without MAS. The order of SNP (single-nucleotide polymorphisms) in the haplotype is rs729302-rs2004640-rs2280714.

Haplotype	Haplotype Frequencies in Patients without MAS	Haplotype Frequencies in Patients with MAS	p	OR	95% CI
A-G-C	0.438	0.303	0.06	1.0	—
C-G-T	0.330	0.182	0.04	0.92	0.35–2.39
A-T-C	0.017	0.015	0.93	1.19	0.08–17.6
A-T-T	0.201	0.485	< 0.001	4.61	1.73–12.3

IRF5: interferon regulatory factor 5; MAS: macrophage activation syndrome.

ence of fairly frequent polymorphisms in multiple genes involved in the regulation of innate and adaptive immunity may be one of the major determinants in the initiation of rheumatic diseases[19]. To develop innate and adaptive immune responses, the activation of a TLR signaling pathway is essential. The transcription factor IRF5 is generally involved downstream of the TLR signaling pathway for induction genes for proinflammatory cytokines such as IL-6, IL-12, and TNF-α[11,20]. As mentioned, proinflammatory cytokines such as IFN-γ and TNF-α are responsible for the clinical and laboratory abnormalities seen in MAS[4,5]. Thus, we examined the association of polymorphisms in the *IRF5* gene with susceptibility to MAS in patients with systemic JIA.

Our investigation revealed that the rs2004640 T allele and the ATT haplotype in the *IRF5* gene were associated with MAS developing in patients with systemic JIA. All the patients with the TT genotype at rs2004640 had MAS, and they had an early onset of MAS compared to those with non-TT genotypes (Figure 1). While the ATT haplotype of the *IRF5* gene was associated with susceptibility to MAS in patients with systemic JIA in our study, it was also reported that this is the common haplotype conferring increased risk of SLE[12]. The T alleles of both rs2004640 and rs2280714 were associated with higher levels of IRF5 messenger RNA expression[12]. Further, the other IRF5 haplotype was associated with high serum IFN-α activity in patients with SLE[21]. Although we did not address the association between proinflammatory cytokine activity and genotype/haplotype in the *IRF5* gene, there may be the potential role of IRF5-associated immune response in the pathogenesis of MAS. Further research is needed to determine the influence of gene polymorphisms in the *IRF5* gene on proinflammatory cytokine activities.

Although several drugs, such as tolmetin and tocilizumab, and viral infections were considered the triggering caus-

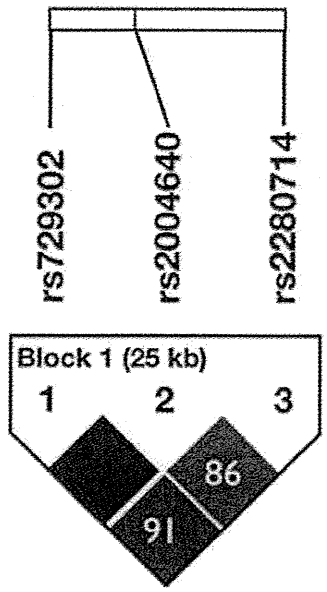

Figure 2. Haplotype blocks of interferon regulatory factor 5 gene polymorphisms.

es of MAS in our cohort, the triggers were not determined in most cases. All patients with MAS recovered from their severe complication. We could not find any association between IRF5 genotypes/haplotypes and the characteristics of clinical symptoms and severity of MAS in this study, presumably because of a small study population. We need a larger cohort to determine this association.

There are some limitations to our study. The incidence of MAS (40.7%) was significantly higher than generally seen (about 10%)[4,5]. Our hospital is one of the pediatric rheumatology centers in Japan and we have treated many patients with severe systemic JIA. The incidence of MAS is so high partly because of the characteristics of our hospital. In addition, there are ethnic differences in the incidence by the subtypes of JIA. Specifically, systemic JIA accounts for about 20% of JIA in Japan but for only about 10% in Europe and the United States[22]. Therefore there may also be ethnic differences in susceptibility to MAS.

A second issue is that we could not carry out a validation study. Although the genetic association study should be validated, the incidence of MAS complicating systemic JIA is too low to validate this association in a single institution. Therefore it is important for the association between the IRF5 genotype/haplotype and MAS susceptibility to be confirmed by other groups.

We found a strong association between polymorphisms in the *IRF5* gene and susceptibility to MAS in patients with systemic JIA. This finding suggests a potentially important role of the IRF5-associated immune response in the pathogenesis of MAS.

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REFERENCES

1. Cassidy JT, Petty RE. Juvenile idiopathic arthritis. In: Cassidy JT, Petty RE, editors. Textbook of pediatric rheumatology. Philadelphia: WB Saunders; 2005:291-303.
2. De Benedetti F. Targeting interleukin-6 in pediatric rheumatic diseases. Curr Opin Rheumatol 2009;21:533-7.
3. Pascual V, Allantaz F, Arce E, Punaro M, Banchereau J. Role of interleukin-1 (IL-1) in the pathogenesis of systemic onset juvenile idiopathic arthritis and clinical response to IL-1 blockade. J Exp Med 2005;201:1479-86.
4. Kelly A, Ramanan AV. Recognition and management of macrophage activation syndrome in juvenile arthritis. Curr Opin Rheumatol 2007;19:477-81.
5. Ravelli A, Martini A. Macrophage activation syndrome. In: Lehman TJ, Cimaz R, editors. Pediatric rheumatology. Amsterdam: Elsevier; 2008:55-63.
6. Henter JI, Horne A, Arico M, Egeler RM, Filipovich AH, Imashuku S, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer 2007;48:124-31.
7. Grom AA, Villanueva J, Lee S, Goldmuntz EA, Passo MH, Filipovich A. Natural killer cell dysfunction in patients with systemic-onset juvenile rheumatoid arthritis and macrophage activation syndrome. J Pediatr 2003;142:292-6.
8. Vastert SJ, van Wijk R, D'Urbano LE, de Vooght KM, de Jager W, Ravelli A, et al. Mutations in the perforin gene can be linked to macrophage activation syndrome in patients with systemic onset juvenile idiopathic arthritis. Rheumatology 2010;49:441-9.
9. Grom AA, Mellins ED. Macrophage activation syndrome: advances towards understanding pathogenesis. Curr Opin Rheumatol 2010;22:561-6.
10. Zhang K, Biroschak J, Glass DN, Thompson SD, Finkel T, Passo MH, et al. Macrophage activation syndrome in patients with systemic juvenile idiopathic arthritis is associated with MUNC13-4 polymorphisms. Arthritis Rheum 2008;58:2892-6.
11. Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, et al. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature 2005;434:243-9.
12. Graham RR, Kozyrev SV, Baechler EC, Reddy MV, Plenge RM, Bauer JW, et al. A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. Nat Genet 2006;38:550-5.
13. Han SW, Lee WK, Kwon KT, Lee BK, Nam EJ, Kim GW. Association of polymorphisms in interferon regulatory factor 5 gene with rheumatoid arthritis: a metaanalysis. J Rheumatol 2009;36:693-7.
14. Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J, et al. International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. J Rheumatol 2004;31:390-2.
15. Ravelli A, Magni-Manzoni S, Pistorio A, Besana C, Foti T, Ruperto N, et al. Preliminary diagnostic guidelines for macrophage activation syndrome complicating systemic juvenile idiopathic arthritis. J Pediatr 2005;146:598-604.
16. Gonzalez JR, Armengol L, Sole X, Guino E, Mercader JM, Estivill X, et al. SNPassoc: an R package to perform whole genome association studies. Bioinformatics 2007;23:644-5.
17. Lake SL, Lyon H, Tantisira K, Silverman EK, Weiss ST, Laird NM, et al. Estimation and tests of haplotype-environment interaction when linkage phase is ambiguous. Hum Hered 2003;55:56-65.

18. Shimane K, Kochi Y, Yamada R, Okada Y, Suzuki A, Miyatake A, et al. A single nucleotide polymorphism in the IRF5 promoter region is associated with susceptibility to rheumatoid arthritis in the Japanese population. Ann Rheum Dis 2009;68:377-83.
19. Gutierrez-Roelens I, Lauwerys BR. Genetic susceptibility to autoimmune disorders: clues from gene association and gene expression studies. Curr Mol Med 2008;8:551-61.
20. Yanai H, Chen HM, Inuzuka T, Kondo S, Mak TW, Takaoka A, et al. Role of IFN regulatory factor 5 transcription factor in antiviral immunity and tumor suppression. Proc Natl Acad Sci USA 2007;104:3402-7.
21. Niewold TB, Kelly JA, Flesch MH, Espinoza LR, Harley JB, Crow MK. Association of the IRF5 risk haplotype with high serum interferon-alpha activity in systemic lupus erythematosus patients. Arthritis Rheum 2008;58:2481-7.
22. Yokota S, Mori M, Imagawa T, Takei S, Murata T, Tomiita M, et al. Proposal for juvenile idiopathic arthritis guidance on diagnosis and treatment for primary care pediatricians and nonpediatric rheumatologists. Mod Rheumatol 2007;17:353-63.

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ORIGINAL ARTICLE

Association of HLA-A*02:06 and HLA-DRB1*04:05 with clinical subtypes of juvenile idiopathic arthritis

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Juvenile idiopathic arthritis (JIA) is one of the most common forms of pediatric chronic arthritis. JIA is a clinically heterogeneous disease. Therefore, the genetic background of JIA may also be heterogeneous. The aim of this study was to investigate associations between human leukocyte antigen (HLA) and susceptibility to JIA and/or uveitis, which is one of the most devastating complications of JIA. A total of 106 Japanese articular JIA patients (67 with polyarthritis and 39 with oligoarthritis) and 678 healthy controls were genotyped for HLA-A, -B and -DRB1 by PCR-sequence-specific oligonucleotide probe methodology. HLA-A*02:06 was the risk factor for JIA accompanied by uveitis after adjustment for clinical factors (corrected *P*-value <0.001, odds ratio (OR) 11.7, 95% confidence interval (CI) 3.2–43.0). On the other hand, HLA-DRB1*04:05 was associated with polyarticular JIA (corrected *P*-value <0.001, OR 2.9, 95% CI 1.7–4.8). We found an association of HLA-A*02:06 with susceptibility to JIA accompanied by uveitis, which might be considered a separate clinical JIA entity. We also found an association between HLA-DRB1*04:05 and polyarticular JIA. Thus, clinical subtypes of JIA can be classified by the presence of the specific HLA alleles, HLA-A*02:06 and DRB1*04:05.
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Keywords: HLA-A*02:06; HLA-DRB1*04:05; juvenile idiopathic arthritis; uveitis

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is one of the most common forms of pediatric chronic arthritis, with an incidence of approximately about 10 per 100 000 children less than 16 years old.[1] JIA is clinically classified into subtypes, such as systemic arthritis, oligoarthritis and polyarthritis, according to the International League of Associations for Rheumatology (ILAR) classification criteria for JIA.[2]

JIA is the leading cause of chronic anterior uveitis in pediatric cases, accounting for 30–40% of incidence.[3,4] The prevalence of JIA-associated uveitis varies among different ethnic groups. Children of European ancestry have a higher relative risk for developing JIA-associated uveitis than those of non-European ancestry.[5] Risk factors for developing JIA-associated uveitis include antinuclear antibody positivity, early age at onset of JIA and female gender.[3,5] However, the contribution of each risk factor may also vary among ethnic groups, presumably because of variations in genetic background.[6]

Human leukocyte antigen (HLA) typing is considered useful for assisting in the diagnosis of autoimmune disease-associated uveitis such as HLA-B27-associated uveitis.[7] It has also been reported that HLA-DRB1*13 is associated with susceptibility to JIA-associated uveitis in a Caucasoid population.[8] Because the distribution of HLA alleles vary in different ethnic groups, the HLA-linked genetic background for JIA is also likely to vary among different populations. Currently, there is no information in the literature on the association between JIA-associated uveitis and HLA in Japanese. The aim of this study was therefore to investigate the association between HLA genes and clinical subtypes of JIA, especially JIA accompanied by uveitis in Japanese children.

This is the first report on the association between HLA alleles and JIA accompanied by uveitis in the Asian population.

MATERIALS AND METHODS

Studied population

Patients were eligible, if they met the ILAR classification criteria for JIA oligoarthritis or polyarthritis (articular JIA).[2] All patients enrolled in this study were followed-up at the Yokohama City University Hospital between December 2006 and November 2009. A total of 106 Japanese articular JIA patients (86 female and 20 male; 67 with polyarthritis and 39 with oligoarthritis) were included in this study. Clinical data, including age at onset of arthritis and uveitis, gender, antinuclear antibody, rheumatoid factor and anti-cyclic citrullinated peptide status, were reviewed. Presence of uveitis was diagnosed by ophthalmologists at routine visits every 6 months. Written informed consent

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was obtained from each patient and/or their guardians. Japanese healthy control subjects (n=678) were randomly selected, and an informed consent was obtained from each subject before blood sampling.

The study was performed in accordance with the Declaration of Helsinki, and the study protocol was approved by the Yokohama City University School of Medicine Ethics Committee and the Ethics Committee of Medical Research Institute, Tokyo Medical and Dental University.

HLA genotyping

JIA patients were genotyped for HLA-A, -B and -DRB1 by the PCR-sequence-specific oligonucleotide probe (SSOP)–Luminex method using Genosearch HLA-A, -B and -DRB1 Ver. 2 (Medical & Biological Laboratories, Nagoya, Japan), as described previously.[9] HLA genotyping data from 678 healthy Japanese controls selected at random were obtained for HLA-A and -DRB1 by the PCR-SSOP-based typing method as described previously[10,11] and for HLA-B using PCR-reverse SSOP-based RELI SSOP HLA-B typing kits (Invitrogen, Carlsbad, CA, USA).

Statistical analysis

Frequencies of HLA allele carriers were calculated for each locus in patients and controls. The statistical significance of the differences in the frequencies between patients and controls was evaluated by Fisher's exact test. A corrected P-value (P_c) was calculated by multiplying the P-value by the number of alleles tested at each locus. A two-locus analysis was carried out to investigate interactions of the disease-associated HLA alleles for increasing disease susceptibility, according to the method of Svejgaard and Ryder.[12]

Associations between JIA-associated uveitis and clinical variables were analyzed by Student's t-test and Fisher's exact test. Multiple logistic regression analysis was performed to investigate associations with JIA-associated uveitis. All statistical analyses were carried out using SAS system version 9 (SAS Institute, Cary, NC, USA).

RESULTS

Association between HLA and JIA susceptibility

A total of 106 articular JIA patients and 678 healthy controls were genotyped for HLA-A, -B and -DRB1 to investigate associations of HLA with JIA susceptibility. It was found that frequencies of HLA-DRB1*04:05 and HLA-A*02:06 tended to increase in the patients ($P<0.05$, P_c=not significant) (Table 1). Because the marginal association between HLA and JIA overall might reflect a stronger association between HLA and a specific clinical subtype of JIA, patients were stratified into polyarticular and oligoarticular types. This approach revealed that HLA-DRB1*04:05 was indeed highly significantly associated with polyarticular JIA ($P_c<0.001$, odds ratio (OR) 2.9, 95% confidence interval (CI) 1.7–4.8), but tended to decrease in oligoarticular JIA (Table 1). In the latter, HLA-A*02:06 and HLA-DRB1*09:01 showed a tendency toward increased frequencies, but this did not reach significance after correction for the number of alleles tested ($P<0.05$, P_c=not significant) (Table 1). No other HLA-A, -B or -DRB1 alleles showed any significant associations with either form of articular JIA.

Clinical and genetic characteristics of JIA accompanied by uveitis

Because different HLA alleles showed associations with different subtypes of articular JIA, it is likely that the association of HLA with JIA would reflect the association with other specific clinical manifestations. Therefore, we investigated the clinical and genetic characteristics of JIA accompanied by uveitis.

Of 106 patients with articular JIA, 13 (12.2%) developed uveitis. All the patients with uveitis were female, but the gender difference was not statistically significant (P=0.055). Mean age at onset of JIA in the patients with uveitis was 3.2 years (range 0–14) (Table 2). The mean age for developing uveitis was 5.2 years (range 1–14), and the mean time interval between the onset of JIA and development of uveitis was 21.9 months (range −4 to 48), with one case developing uveitis 4 months before the onset of JIA. Oligoarticular onset, rheumatoid factor negativity ($<14\,IU\,ml^{-1}$) and low anti-cyclic citrullinated peptide ($<4.5\,U\,ml^{-1}$), as well as younger age at onset of JIA were all significantly associated with JIA accompanied by uveitis (Table 2). Nine patients with JIA accompanied by uveitis responded well to topical corticosteroids and could discontinue medications for uveitis, but the remaining four additionally required systemic glucocorticoids or anti-tumor necrosis factor-α therapy for uveitis in the follow-up period.[13]

Table 1 Association of HLA with susceptibility to JIA

	Articular JIA (N=106)				*Polyarticular JIA (N=67)*				*Oligoarticular JIA (N=39)*				*Control (N=678)*
	N *(%)*	*OR (95% CI)*	P-*value*	P_c	N *(%)*	*OR (95% CI)*	P-*value*	P_c	N *(%)*	*OR (95% CI)*	P-*value*	P_c	N *(%)*
HLA-A*02:06	24 (22.6)	1.9 (1.1–3.1)	0.012	NS	13 (19.4)	1.6 (0.8–3.0)	0.177	NS	11 (28.2)	2.5 (1.2–5.3)	0.010	NS	91 (13.4)
HLA-DRB1*04:05	36 (34.0)	1.6 (1.0–2.5)	0.032	NS	32 (47.8)	2.9 (1.7–4.8)	<0.001	<0.001	4 (10.3)	0.4 (0.1–1.0)	0.045	NS	164 (24.2)
HLA-DRB1*09:01	42 (39.6)	1.5 (0.97–2.3)	0.066	NS	24 (35.8)	1.3 (0.7–2.1)	0.385	NS	18 (46.2)	1.9 (1.0–3.7)	0.043	NS	208 (30.7)

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen; JIA, juvenile idiopathic arthritis; NS, not significant; OR, odds ratio; P_c, corrected P-value by multiplying a P-value by the number of alleles tested in each locus.

Table 2 Clinical variables in association with JIA accompanied by uveitis

	JIA patients with uveitis (N=13)	*JIA patients without uveitis (N=93)*	P-*value*	*OR*	*95% CI*
Age at JIA onset (years, mean)	3.2	7.6	0.002	0.7	0.6–0.9
Gender (female, %)	13 (100%)	73 (78.5%)	0.055	ND	ND
Subtype (oligoarticular onset, %)	10 (76.9%)	29 (31.2%)	0.004	7.4	1.9–28.7
ANA (≧1:160, %)	8 (61.5%)	30 (32.3%)	0.061	—	—
RF (≧14.0 ($IU\,ml^{-1}$), %)	1 (7.7%)	56 (60.2%)	0.001	0.1	0.07–0.4
Anti-CCP (≧4.5 ($U\,ml^{-1}$), %)	1 (7.7%)	47 (50.5%)	0.006	0.1	0.01–0.7

Abbreviations: ANA, antinuclear antibody; CCP, cyclic citrullinated peptide; CI, confidence interval; JIA, juvenile idiopathic arthritis; ND, not detectable; OR, odds ratio; RF, rheumatoid factor.

Table 3 Association between HLA and susceptibility to uveitis in patients with JIA

	With uveitis (N=13)	*Without uveitis (N=93)*	*OR*	*95% CI*	*P-value*	P_c
HLA-A*02:06	9 (69.2%)	15 (16.1%)	11.7	3.2–43.0	<0.001	<0.001
HLA-DRB1*04:05	0 (0%)	36 (38.7%)	0.6	0.5–0.7	0.006	NS
HLA-DRB1*09:01	9 (69.2%)	33 (35.5%)	4.1	1.2–14.3	0.020	NS

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen; JIA, juvenile idiopathic arthritis; NS, not significant; OR, odds ratio; P_c, corrected *P*-value by multiplying a *P*-value by the number of alleles tested in each locus; with uveitis, JIA patients with uveitis; without uveitis, JIA patients without uveitis.

Table 4 Two-locus analysis of HLA-A*02:06 and HLA-DRB1*09:01

*HLA-A*02:06*	*HLA-DRB1*09:01*	*JIA-associated uveitis (N=13) (%)*	*Control (N=678) (%)*
(a) Basic data			
Positive (+)	Positive (+)	6 (46.2)	30 (4.4)
Positive (+)	Negative (−)	3 (23.1)	61 (9.0)
Negative (−)	Positive (+)	3 (23.1)	178 (26.3)
Negative (−)	Negative (−)	1 (7.7)	409 (60.3)

(b) Stratification analysis in JIA-associated uveitis

Comparison		*Individual association*		*Independent association for A*		*Independent association for B*		*Difference between A and B association*	*Combinatory association*
Factor A	*Factor B*	*Test (1)*	*Test (2)*	*Test (3)*	*Test (4)*	*Test (5)*	*Test (6)*	*Test (7)*	*Test (8)*
*HLA-A*02:06*	*HLA-DRB1*09:01*			++ vs −+	+− vs −−	++ vs +−	−+ vs −−	+− vs −+	++ vs −−
		OR_A	OR_B		$OR_{A,\ non-B}$		$OR_{B,\ non-A}$		$OR_{A,\ B}$
OR		14.5	5.1	20.1	6.9	11.9	4.1	2.9	81.8
95% CI		4.4–48.1	1.5–16.7	2.1–196.5	0.7–66.7	2.8–50.0	1.0–17.4	0.6–14.8	9.5–701.7
P-value		<0.001	0.024	0.002	0.428	<0.001	0.356	1.0	<0.001

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen; JIA, juvenile idiopathic arthritis; OR, odds ratio.

To further investigate the association between HLA and JIA accompanied by uveitis, we compared the frequencies of HLA alleles among JIA patients with or without uveitis. As shown in Table 3, the frequency of HLA-A*02:06 carriers was significantly higher in the JIA patients with uveitis (P_c<0.001, OR 11.7, 95% CI 3.2–43.0). The frequency of HLA-DRB1*09:01 carriers was also higher in patients with uveitis, although this was not statistically significant after correction for multiple testing (P=0.020, P_c=not significant) (Table 3). No other HLA-A, -B or -DRB1 alleles, including HLA-B*27 (B27), DRB1*11 (DR11) and DRB1*13 (DR13), showed any significant associations with JIA accompanied by uveitis.[3,5,8]

To investigate the contribution of HLA-A*02:06 and HLA-DRB1*09:01 with susceptibility to JIA accompanied by uveitis, we performed a two-locus analysis according to Svejgaard and Ryder (Table 4).[12] This suggested a synergistic interaction of these two HLA alleles in susceptibility to this form of JIA. Thus, the odds risks conferred by A*02:06 in the presence or absence of DRB1*09:01 were 20.1 (test (3)) and 6.9 (test (4)), respectively; for DRB1*09:01 in the presence or absence of A*02:06, these values were 11.9 (test (5)) and 4.1 (test (6)), respectively (Table 4). In addition, the odds risk conferred by the presence of both HLA alleles was 81.8 (test (8)), which was much higher than A*02:06 alone (test (4)) or DRB1*09:01 alone (test (6)), further supporting a synergistic interaction.

Finally, we performed multiple logistic regression analysis to evaluate risk factors for JIA accompanied by uveitis, and demonstrated that HLA-A*02:06 was the most significant of these (P=0.014, OR 7.8, 95% CI 1.5–40.5).

DISCUSSION

It is well known that genetic factors influence susceptibility to autoimmune disorders, such as rheumatoid arthritis and JIA.[14–16] HLA is one of the most important genetic factors for these kinds of diseases; there are also ethnic differences in the contribution of HLA to disease susceptibility. In the present study, we evaluated associations between HLA and susceptibility to articular JIA and its clinical subtypes, polyarticular and oligoarticular JIA in Japanese children (Table 1). We found that HLA-DRB1*04:05 was significantly associated with polyarticular JIA, whereas HLA-A*02:06 and HLA-DRB1*09:01 tended to be associated with oligoarticular JIA. These findings imply the existence of genetic differences between the polyarticular and oligoarticular JIA.

HLA-DRB1*04:05 encodes an amino-acid sequence in the DRβ chain, which is well known as an epitope shared between the HLA-DR molecules associated with susceptibility to rheumatoid arthritis in many ethnic groups[17,18] including Japanese,[19] as well as polyarticular JIA in Taiwanese.[20] Although there is debate about whether rheumatoid arthritis and polyarticular JIA could be similar clinical conditions,[21] HLA-DRB1*04:05 may contribute to the pathogenesis of chronic arthritis at different ages and in different ethnic groups. Further studies are required to determine the molecular mechanisms by which HLA-DRB1*04:05 contributes to pathogenesis.

Herein, we found an association of both HLA-A*02:06 and DRB1*09:01 with susceptibility to JIA accompanied by uveitis (Tables 3 and 4). In the clinical setting, the frequency of occurrence