- Mantoux tuberculin skin test
- Plain chest X-ray and chest CT scan
- QuantiFERON assay (QFT-3G); at present, it is not known what effect corticosteroids or immunosuppressants have on this test. If necessary), Beta-D-glucan

2. Patients with cardiac dysfunction (particularly heart failure)

- Echocardiography [ejection fraction/fraction shortening (FS/EF)], electrocardiography
- Plain chest X-ray, and brain natriuretic protein (BNP) if necessary

3. Patients with malignant disease; also benign tumors

- Abdominal ultrasonography, CT, and gallium scintigraphy, if necessary
- PET if possible

4. Patients with abnormal findings by routine tests

- Abnormal liver function tests [alanine aminotransferase (ALT) and gamma-glutamyl transpeptidase (γ-GTP)]
- Abnormal complete blood count (CBC) (WBC count and platelet count)

Pretreatment tests

1. Blood tests and urinalysis:
   CBC (WBC count, platelet count, hemoglobin), biochemistry [aspartate aminotransferase (AST), ALT, lactate dehydrogenase (LDH), blood urea nitrogen (BUN), creatinine (Cr), total cholesterol, triglycerides, CRP anti-DNA antibodies, antinuclear antibodies], urinalysis (protein and occult blood), and fecal occult blood
2. Imaging:
   chest and abdominal CT/magnetic resonance imaging (MRI), gallium scintigraphy (PET if possible), and joint assessments by X-rays/contrast-enhanced MRI (particularly of vertebrae and large joints such as the hip joint)
3. Cardiac function tests:
   echocardiography (FS/EF) and electrocardiography
4. Tests for infection (as described above)

Dosage and administration

1. Dose:
   tocilizumab 8 mg/kg for each administration

- Body weight should be measured to calculate the volume of tocilizumab for each administration

2. Preparation of tocilizumab:
   The appropriate dose should be diluted with 50 ml of saline if the patient's weight is $\leq$25 kg, or with 100 ml if the >25 kg
3. Method of administration and dosing interval:
   Secure peripheral intravenous access for infusion, and slowly administer saline-diluted tocilizumab through independent intravenous tubing with a sterile pyrogen-free in-line filter (with pore sizes not greater than 1.2 μm) at a rate of 10 ml/h for the first 15 min. After checking blood pressure, pulse, and other vital signs for abnormalities, increase the infusion rate to 50 ml/h if body weight <25 kg and 100 ml/h if body weight $\geq$25 kg, so that the entire volume is administered over 1 h. The rate of infusion should be adjusted according to occurrence of infusion reactions.
   For systemic JIA, tocilizumab is administered every 2 weeks. Dosing interval can be shortened (up to weekly dosing) or prolonged depending on the degree of JIA activity.
4. Precautions during infusion:
   Treating physicians should remain alert for infusion reactions (e.g., chills, fever, dysphoria, headache) and anaphylactic reactions (e.g., decreased blood pressure, bradycardia, disturbance of consciousness) during infusion
5. Actions to be taken when encountering infusion reactions or anaphylactic symptoms:
   If infusion reactions or anaphylactic symptoms are observed, infusion should be discontinued immediately, and state of consciousness, respiration, blood pressure, and pulse should be checked for abnormalities. Antihistamines (e.g., hydroxyzine, etc.) and corticosteroids (e.g., hydrocortisone sodium, etc.) also should be given intravenously. The patient should be tested for antitocilizumab antibodies [immunoglobulin (Ig)E and IgG antibodies] if possible. Whether to continue or discontinue tocilizumab after an infusion reaction has occurred should be determined cautiously. The treating physician should seek opinions from specialists regarding continuation/withdrawal as well as premedication and adjustment of the infusion method if the decision is to continue.

Evaluation of treatment response

Response to treatment with tocilizumab should be evaluated based on clinical findings (e.g., fever, arthritis) and test findings (e.g., WBC count, CRP, erythrocyte sedimentation rate). However, an objective method of evaluating treatment response has not yet been established. Overall evaluation methods include the American College

of Rheumatology pediatric definition of improvement (ACR Pedi) [12] response and Disease Activity Score 28 (DAS28) [13].

1. Clinical symptoms:
   Changes in general condition, rash, lymphadenopathy, hepatosplenomegaly, and arthritis should be monitored during the treatment. Visual analogue scale (VAS) can be used for physician's global assessment of treatment response. Childhood Health Assessment Questionnaire (CHAQ) [14] is useful to assess changes in patient's activity in daily life.
   Generally, improvement in clinical symptoms (e.g., fever, generalized malaise, arthralgia) occurs immediately after initiating tocilizumab. Even after serum tocilizumab concentration is maintained with continued administration, however, joint symptoms, rash, and malaise may sometimes appear despite normal laboratory test values related to inflammation. In such cases, caution should be taken because those symptoms are suggestive of increased disease activity, such as an increase in blood IL-6 level and/or progression to macrophage activation syndrome.
2. Laboratory tests (e.g., WBC count, hemoglobin, platelet count, CRP, ferritin, urinary β2-microglobulin measured at each tocilizumab infusion):
   Under circumstances in which tocilizumab blood concentration is maintained, there is generally little change in inflammation markers such as CRP, even if disease activity is increased or bacterial infections develop. In such cases, increase in WBC count may occur with either increased disease activity or bacterial infections. It is therefore important to differentiate between increased disease activity and infections by carefully monitoring clinical findings.
3. Imaging:
   Treatment response should be evaluated by obtaining plain X-rays and contrast-enhanced MRI scans of the joints to evaluate arthritis before starting treatment and every 6 months after starting treatment.

Evaluation of safety

1. Laboratory test findings (WBC count, AST, ALT, total cholesterol, triglycerides, anti-DNA antibodies and antinuclear antibodies):
   If abnormal liver function (e.g., abnormalities of ALT and γ-GTP) is observed during the course, the following should be considered as possible causes:

- Adverse reaction to tocilizumab
- Adverse reaction to corticosteroids (e.g., lipid metabolism disorders, fatty liver)
- Adverse reaction to other concomitant drugs
- Viral infection

   If the cause is unknown, it should be decided whether to continue or discontinue treatment with tocilizumab while closely monitoring disease activity.

2. Evaluation of cardiac function [echocardiography (EF/FS)]:
   It is known that administration of TNF-α inhibitors to patients with heart failure further worsens cardiac function, but the effect of inhibition of IL-6 signalling on cardiac function is unclear. Regular electrocardiography and echocardiography are therefore required during treatment with tocilizumab when patients have abnormal cardiac function prior to tocilizumab treatment.

Summary of adverse reactions in clinical studies of tocilizumab for sJIA

Please consult with the package insert of tocilizumab for its most updated safety profile. Primary or severe adverse reactions in patients with rheumatoid arthritis, Castleman's disease and JIA that were recognized when the agent was approved are shown as "Frequently reported adverse drug reactions"[1] or "clinically significant adverse drug reactions".[2]

Method of corticosteroid tapering

There is no established method of corticosteroid tapering during tocilizumab treatment. Steroid tapering should be carried out cautiously in consultation with specialists.

Suspension or discontinuation of tocilizumab treatment

There is no established method of suspension or discontinuation of tocilizumab treatment. Caution should be taken when stopping tocilizumab treatment or reducing the dose of tocilizumab because, as a consequence, the biological actions of IL-6 may be excessively expressed, which may exacerbate the underlying disease following temporary or

[1] Nasopharyngitis, cholesterol increased, low-density-lipoprotein cholesterol (LDL) increased, triglyceride increased, ALT (glutamyl pyruvic transaminase, GPT) increased, etc.

[2] Anaphylactic shock, anaphylactoid reactions (decrease in blood pressure, dyspnea, loss of consciousness, dizziness, nausea, vomiting, pruritus, flushing, etc.), infections (pneumonia, herpes zoster, infectious gastroenteritis, cellulitis, infectious arthritis, sepsis, nontuberculous mycobacteriosis, tuberculosis, *Pneumocystis jiroveci* pneumonia, etc.), interstitial pneumonia, intestinal perforation, neutropenia, cardiac failure, etc.

permanent discontinuation of tocilizumab. Except for suspension or discontinuation of tocilizumab due to adverse reactions, reduction of dose and/or treatment discontinuation should be carried out cautiously in consultation with specialists.

Other information

Actions to be taken when encountering macrophage activation syndrome [11]

Changes of laboratory examinations and clinical symptoms are not clear in patients treated with tocilizumab. Thus, patients under tocilizumab treatment need to be monitored carefully because clinical findings such as fever, aggravation of the general condition and increased CRP are masked by the effects of tocilizumab. Serum ferritin may not increase markedly if a patient is in the early stage of macrophage activation syndrome.

If abnormalities of 1–5 are observed, the laboratory tests should be repeated after 6–12 h, and if those test findings have worsened further, progression to macrophage activation syndrome should be considered.

1. Change in clinical symptoms (e.g., high fever and worsening malaise during treatment)
2. Decreased platelet count
3. Increased FDP-E fraction and D-dimer
4. Increased urinary β2-microglobulin
5. Markedly increased serum ferritin and increased AST (ALT) and LDH.

Treatment of macrophage activation syndrome as below:

1. Continuous intravenous infusion of heparin (mixed infusion at a dose of 200 units/kg/24 h for maintenance transfusion)
2. Intravenous injection of dexamethasone palmitate (Limethason™) at a dose of 2.5–5.0 mg twice a day
3. Continuous intravenous infusion of cyclosporine (1–2 mg/kg/24 h)

Vaccination while using tocilizumab

At present, the effect of tocilizumab on response to vaccination is unknown. Inactivated vaccines can be administered in sJIA patients receiving tocilizumab as in healthy children. On the other hand, sJIA patients receiving tocilizumab should not be vaccinated with live vaccines because there has been no clinical experience with the vaccination, and it is possible that side effects could occur as a result of vaccination, particularly side effects caused by the vaccine strain.

Guidance on use of tocilizumab for polyarticular juvenile idiopathic arthritis

Eligibility for tocilizumab treatment

Tocilizumab is indicated for patients with refractory polyarticular JIA. Patients are considered to have refractory disease if they meet the following criteria despite receiving treatments according to "Proposal for juvenile idiopathic arthritis guidance on diagnosis and treatment for primary care pediatricians and nonpediatric rheumatologists" [1].

1. To have persistent arthritis and inflammation despite receiving treatments with methotrexate or methotrexate based combination therapies
2. To require prolonged use of corticosteroids and unable to taper off corticosteroids

Note: Tocilizumab is not necessarily indicated for all patients with refractory polyarticular JIA.

Initiation of tocilizumab treatment

For patients with increased disease activity, e.g., experiencing persistent arthritis and continuously showing excessive inflammatory status evidenced by persistently higher CRP and ESR values, tocilizumab treatment should be initiated only after the disease is controlled to some extent by initial induction therapy with methotrexate.

Contraindications and careful administration

1. Contraindications

- Patients with a concurrent serious infection (pneumonia, sepsis etc.)
- Patients with known hypersensitivity to tocilizumab.

2. Careful administration
Pretreatment evaluation for patient eligibility includes screening tests described below. If complications such as infections that are considered recoverable by medications are identified before starting tocilizumab treatment, treatments for such complications need to be prioritized. Tocilizumab treatment should be initiated only after confirming recovery of those complications.

Screening tests

1. Infections
Common infections

- Pneumonia: Plain chest X-ray and chest CT scan
- Blood culture (sepsis or bacteremia)
- Check for dental caries: consultation with oral surgery

- Otitis media, sinusitis etc.
- Urinary tract infection and acute focal bacterial nephritis
- Gallium scintigraphy to exclude deep infection (PET scan if possible)
- HBV, HCV antibody screening tests
- EBV antibody screening tests
  Tuberculosis and latent mycosis
- Family history and tuberculin test history and vaccination history of BCG
- Plain chest X-ray, chest CT
- QuantiFERON assay (QFT-2G) (At present, it is not known what effect corticosteroids or immunosuppressants have on this test)
- Beta-D-glucan

2. Patients with cardiac dysfunction (exclusion of heart failure in particular)

- Echocardiography (FS/EF), electrocardiography
- Plain chest X-ray and BNP

3. Patients with malignant disease (including benign tumors)

- Abdominal ultrasonography, CT and gallium scintigraphy
- PET scan if possible

4. Patients with abnormal findings in routine tests

- Abnormal liver function tests (ALT and γ-GTP)
- Abnormal CBC tests (WBC count and platelet count)

Pre-treatment tests

1. Blood tests and urinalysis
   CBC (WBC count, platelet count and hemoglobin), biochemistry (AST, ALT, LDH, BUN, Cr, total cholesterol, triglycerides, CRP, anti-DNA antibodies, antinuclear antibodies) and urinalysis (protein and occult blood), fecal occult bloods.
2. Imaging
   Chest and abdominal CT/MRI, gallium scintigraphy (PET scan if possible) and joint assessments by X-rays/MRI.
3. Cardiac function tests
   Echocardiography (FS/EF) and electrocardiography
4. Tests for infection (as described above)

Dosage and administration

1. Dose
   Tocilizumab dose: 8 mg/kg for each administration
   Body weight should be measured to calculate the volume of tocilizumab for each administration.
2. Preparation of tocilizumab
   The appropriated dose of tocilizumab should be diluted with 50 ml of saline if the patient's body weight is 25 kg or less, or 100 ml if the weight is over 25 kg.
3. Method of administration and dosing interval
   Secure peripheral intravenous access for infusion, and slowly administer the saline-diluted tocilizumab through independent intravenous tubing with a sterile pyrogen-free in-line filter (with pore sizes not greater than 1.2 microns) at a rate of 10 ml/h for the first 15 min. After checking blood pressure, pulse and other vital signs for abnormalities, increase the infusion rate to 50 ml/h if body weight is less than 25 kg and 100 ml/h if body weight is 25 kg or greater, so that the entire volume is administered over 1 h. The rate of infusion should be adjusted according to occurrence of infusion reactions.
   For polyarticular JIA, tocilizumab is administered every 4 weeks.
4. Precautions during infusion
   Treating physicians should remain alert for infusion reactions (e.g., chills, fever, dysphoria, and headache) and anaphylactic reactions (e.g., decreased blood pressure, bradycardia, and disturbance of consciousness) during infusion.
5. Actions to be taken if encounter infusion reactions or anaphylactic symptoms
   If infusion reactions or anaphylactic symptoms are observed, infusion of tocilizumab should be discontinued immediately, and state of consciousness, respiration, blood pressure and pulse should be checked for abnormalities. Antihistamines (e.g., hydroxyzine, etc.) and corticosteroids (e.g., hydrocortisone sodium, etc.) should also be given intravenously. The patient should be tested for anti-tocilizumab antibodies (IgE and IgG antibodies) if possible. Whether continue or discontinue tocilizumab after experiencing infusion reactions should be determined cautiously. Treating physician should seek opinions from specialists regarding continuation/withdrawal as well as pre-medication and adjustment of infusion method if decide to continue.

Evaluation of treatment response

Response to treatment with tocilizumab in articular JIA should be evaluated based on changes in arthritis (e.g., clinical symptoms and radiographic findings) and inflammation (e.g., CRP and rheumatoid factor). ACR Pedi [12] response and DAS28 [13] are used for global assessment.

1. Clinical symptoms
   Physician's global assessment of treatment response is measured by VAS in conjunction with clinical

assessment of arthritis. CHAQ [14] is useful to assess changes in patient's activity of daily life.
Generally, improvement in clinical symptoms (e.g., fever, generalized malaise, arthralgia) occurs immediately after initiating tocilizumab. Even after serum tocilizumab concentration is maintained with continued administration, however, joints symptoms and malaise may sometimes come out despite normal laboratory test values related to inflammation. In such cases, caution should be taken because those symptoms are suggestive of increased disease activity, such as an increase in blood IL-6.

2. Laboratory test findings (e.g., WBC count, hemoglobin, platelet count, CRP, SAA, ferritin, urinary β2-microglobulin: to measure at each tocilizumab infusion)
Under circumstances where the tocilizumab blood concentration is maintained, there is generally little change in inflammation markers such as CRP even if disease activity is increased or bacterial infections develop. In such cases, increase in the WBC count may occur with increased disease activity or bacterial infections. It is therefore important to make differentiation between increased disease activity and infections by careful monitoring of clinical findings.
3. Imaging
Treatment response should be evaluated by obtaining plain X-rays and contrast-enhanced MRI scans of the joints to evaluate arthritis before starting treatment and every 6 months after starting treatment.

Evaluation for safety

1. Laboratory test findings (WBC count, AST, ALT, total cholesterol, triglycerides, anti-DNA antibodies and antinuclear antibodies)
If abnormal liver function (e.g., abnormalities of ALT and γ-GTP) is observed during the course, the following should be considered as possible causes.

- Adverse reaction to tocilizumab
- Adverse reaction to corticosteroids (e.g., lipid metabolism disorders, fatty liver)
- Adverse reaction to other concomitant drugs
- Viral infection

If the cause is unknown, it should be decided whether to continue or discontinue treatment with tocilizumab while closely monitoring disease activity.

2. Evaluation of cardiac function (echocardiography [EF/FS])
It is known that administration of TNF-α inhibitors to patients with heart failure further worsens cardiac function, but the effect of inhibition of IL-6 signalling on cardiac function is unclear. Regular electrocardiography and echocardiography are therefore required during treatment with tocilizumab when patients have abnormal cardiac functions prior to tocilizumab treatment.

Summary of adverse reactions in clinical studies of tocilizumab for polyarticular juvenile idiopathic arthritis

Please consult with the package insert of tocilizumab for its most updated safety profile.

Method of steroid tapering and discontinuation of methotrexate

There is no established method of corticosteroid tapering or discontinuation of methotrexate during tocilizumab treatment, but both should be carried out cautiously in consultation with specialists.

Suspension or discontinuation of tocilizumab treatment

There is no established method of suspension or discontinuation of tocilizumab treatment. Caution should be taken when stopping or reducing tocilizumab because the biological actions of IL-6 may be excessively expressed, which may exacerbate the underlying disease following temporary or permanent tocilizumab discontinuation. Except for suspension or discontinuation of tocilizumab due to adverse reactions, dose reduction/or treatment discontinuation should be carried out cautiously in consultation with specialists. In polyarticular JIA, no clear findings have been obtained in relation to suspending or discontinuing tocilizumab due to improvement in disease activity, because there has been no clinical experience with this.

Other information

Vaccination while using tocilizumab

This part is same as sJIA. Please see "Vaccination while using tocilizumab" under the heading "Guidance on tocilizumab for systemic juvenile idiopathic arthritis".

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patent for juvenile arthritis. Syuji Takei received speaking fee from Chugai Pharmaceutical. Yasuhiko Itoh received consulting fee from Chugai Pharmaceutical.

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Exonic deletion of *CASP10* in a patient presenting with systemic juvenile idiopathic arthritis, but not with autoimmune lymphoproliferative syndrome type IIa

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Summary

Systemic 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 defined. To detect copy number variations, we performed single nucleotide polymorphism (SNP) array analysis in 50 patients with s-JIA. We found a 13-kb intragenic deletion of *CASP10* in one patient. RT-PCR of the mRNA extracted from the patient's lymphoblastoid cells revealed that *CASP10* mRNA was truncated. Sequencing the mRNA revealed that this deletion resulted in a frame shift with an early stop codon. *CASP10* is known as a causative gene for autoimmune lymphoproliferative syndrome (ALPS) type IIa, another childhood syndrome of lymphadenopathy and splenomegaly associated with autoimmune haemolytic anaemia and thrombocytopenia. TCR $\alpha\beta^+$ CD4/CD8 double-negative T cells in the peripheral blood as a diagnostic marker of ALPS were not high in this patient and lymphocyte apoptosis induced by anti-Fas antibody was normal, denying ALPS in the patient. The father and a sister of the patient showing no symptoms of ALPS or s-JIA, also had the same deletion. Furthermore, we found no other mutations of *CASP10* in the other 49 s-JIA patients. These data suggest that the pathogenic significance of *CASP10* mutations should be carefully evaluated in s-JIA or even ALPS type IIa in further studies.

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Introduction

Systemic juvenile idiopathic arthritis (s-JIA) (OMIM #604302) is a rare inflammatory disease classified as a subtype of chronic childhood arthritis. The annual UK incidence of JIA is ten cases per 100 000 children under 16 years of age (Symmons *et al.*, 1996), and approximately 11% of patients with JIA suffer from s-JIA (Symmons *et al.*, 1996). s-JIA is a clinically heterogenous febrile disease, manifested by spiking fever, erythematous skin rash, pericarditis and hepatosplenomegaly. Abnormalities in the innate immunity [cytokines such as interleukin (IL)-1, IL-6 and neutrophils and monocytes/macrophages] play a major role in the pathogenesis of s-JIA, being distinguished from other JIA subtypes. One of the major features of s-JIA is its progression to macrophage activation syndrome. On the basis of these features, consensus is emerging that s-JIA should be classified as an autoinflammatory syndrome rather than a classic autoimmune disease (Vastert *et al.*, 2009).

To date, two genetic factors, *HLA* and *PTPN22*, have been confirmed as JIA susceptibility genes in multiple populations (Hinks *et al.*, 2009). For example, HLA-DR1 and HLA-DR4 have been reported to increase risk for polyarticular JIA in many populations (Glass & Giannini, 1999). However, as seen in these reports, the associations are mainly seen in polyarticular JIA but not in s-JIA. There is some evidence which show other genes, such as *MIF*, *IL6*, *IL10*, *TNF*, *MUNC13-4* and *PRF1* being associated with s-JIA in different populations and subtypes (Fishman *et al.*, 1998; Donn *et al.*, 2001, 2002; Thomson & Donn, 2002; De Benedetti *et al.*, 2003; Zhang *et al.*, 2008; Vastert *et al.*, 2010). However, these genes account for only a small part of the total genetic contribution to JIA. Therefore, the genetic background underlying the s-JIA remains poorly defined.

Autoimmune lymphoproliferative syndrome (ALPS) is a rare childhood syndrome characterized by chronic massive, nonmalignant lymphadenopathy and splenomegaly, expansion of TCR $\alpha\beta^+$ double-negative T cells

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.

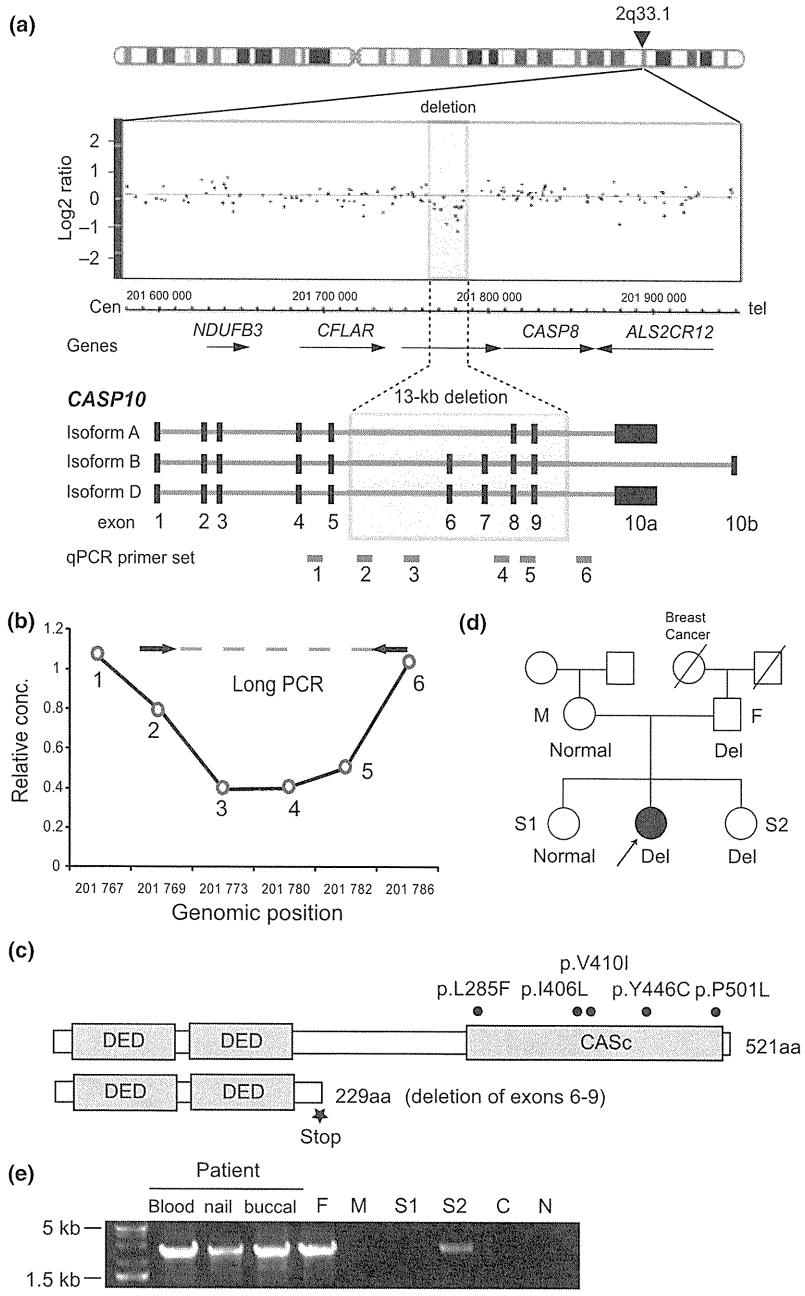


Figure 1. Characterization of the 2q33.1 microdeletion. (a) Result of Genome-wide human SNP array 6.0. The position (*x*-axis matching the genomic location of chromosome 2) and log2 ratio (*y*-axis) of each SNP probes are indicated. The 13-kb deletion is within *CASP10* encompassing exons 6–9. (b) Breakpoint 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

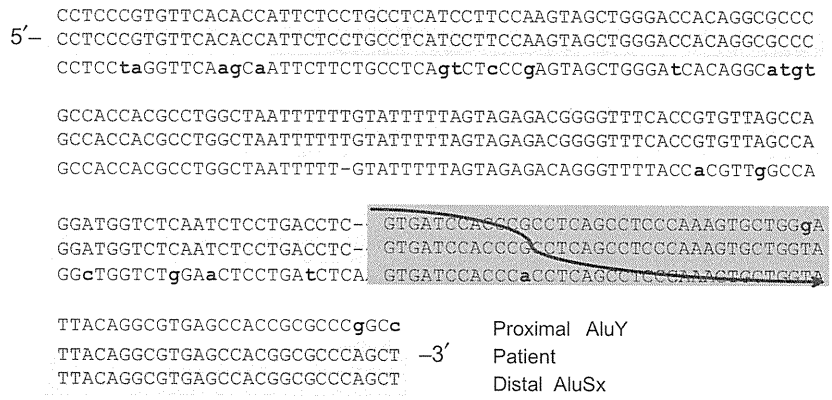


Figure 2. Result of deletion breakpoint sequence. The top, middle and bottom nucleotide strands show the proximal, recombined and distal sequences respectively. Matched sequences are shown as uppercase letters and unmatched ones as lowercase letters. Pale grey boxes show the same sequences and darker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

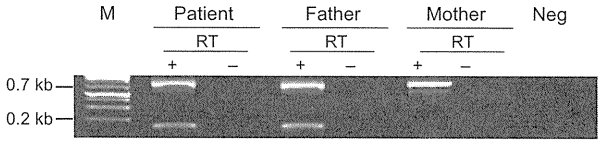
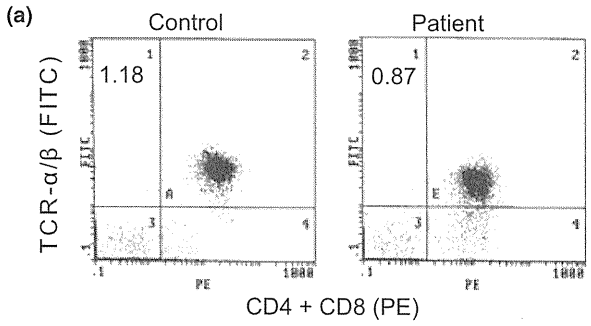


Figure 3. Result of *CASP10* RT-PCR. The lymphoblastoid cell lines which were not treated with cycloheximide were used. Forward primer was designed at exon 5 and reverse primer was designed at exon 10. The predicted size of the PCR product was 742 bp for normal cDNA and 145 bp for the deletion product.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



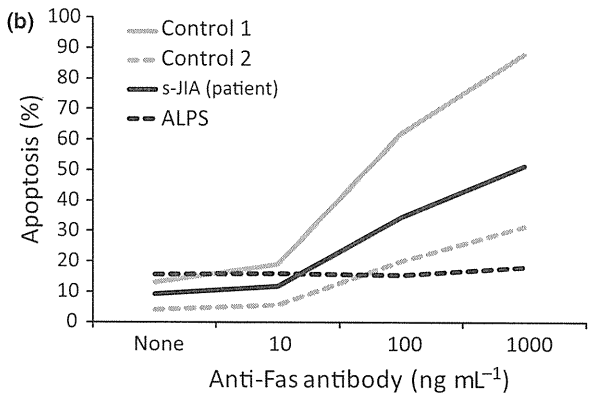


Figure 4. (a) Fluorescence-activated cell sorting (FACS) analysis of TCR αβ$^+$ CD4/CD8 double-negative T cells. TCR αβ$^+$ CD4/CD8 double-negative T cells in the peripheral blood were not high in this patient. (b) Fas-induced T-cell apoptosis assay. Apoptosis of activated T cells were induced by anti-Fas 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.

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Conflict of Interest

Authors declare no conflict of interest in this study.

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Influence of polymorphisms within the methotrexate pathway genes on the toxicity and efficacy of methotrexate in patients with juvenile idiopathic arthritis

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Methotrexate (MTX), which causes adverse effects, such as liver and/or renal dysfunction, is the most common disease-modifying antirheumatic drug used for the treatment of rheumatoid arthritis and articular-type juvenile idiopathic arthritis (JIA).
- Pharmacogenetic studies analysing the MTX pathway genes would aid in the development of more personalized therapy.
- Results regarding the influence of gene polymorphisms on the toxicity and efficacy of MTX are conflicting, and there are marked differences between racial groups in pharmacogenetics.

WHAT THIS STUDY ADDS

- The non-TT genotype at γ-glutamyl hydrolase (*GGH*) T16C is associated with a high risk of liver dysfunction due to MTX, even after adjustment for duration of MTX treatment.
- Longer time interval from disease onset to MTX treatment and rheumatoid factor positivity are associated with lower efficacy of MTX in Japanese patients, as reported previously in Caucasian patients with JIA.

AIMS

We investigated whether several polymorphisms within the methotrexate (MTX) pathway genes were related to the toxicity and efficacy of MTX in 92 Japanese patients with articular-type juvenile idiopathic arthritis (JIA).

METHODS

Eight gene polymorphisms within the MTX pathway genes, namely, *RFC*, *BCRP*, *MTHFR* (two), *FPGS*, γ-glutamyl hydrolase (*GGH*; two) and *ATIC*, were genotyped using TaqMan assays. Liver dysfunction was defined as an increase in alanine transaminase to five times the normal upper limit. Non-responders to MTX were defined as patients refractory to MTX and were therefore treated with biologics.

RESULTS

The non-TT genotype at *GGH* T16C was associated with a high risk of liver dysfunction ($P = 0.028$, odds ratio = 6.90, 95% confidence interval 1.38–34.5), even after adjustment for the duration of MTX treatment. A longer interval from disease onset to treatment (8.5 and 21.3 months, $P = 0.029$) and rheumatoid factor positivity ($P = 0.026$, odds ratio = 2.87, 95% confidence interval 1.11–7.39) were associated with lower efficacy of MTX.

CONCLUSIONS

The non-TT genotype at *GGH* T16C was associated with a high risk of liver dysfunction, presumably because the C allele of *GGH* C16T may reduce the activity of GGH. The time interval before MTX treatment and rheumatoid factor positivity were associated with the efficacy of MTX treatment. The pharmacogenetics of the MTX pathway genes affects the toxicity and efficacy of MTX in Japanese JIA patients.

Introduction

Juvenile idiopathic arthritis (JIA) is one of the most common forms of paediatric chronic arthritis, with an incidence of approximately 9.7 per 100 000 children (aged 15 years and under) in Japan [1, 2]. Methotrexate (MTX) is the most common disease-modifying antirheumatic drug used for the treatment of articular-type JIA, namely the polyarticular- and oligoarticular-onset types of JIA [2]. Methotrexate is effective in about 75% of cases of the articular-type JIA, but causes adverse effects, such as liver and/or renal dysfunction [2, 3]. The effects of polymorphisms within the MTX pathway genes on the toxicity and efficacy of MTX in patients with rheumatoid arthritis (RA) and JIA have been studied [4–6].

The influence of polymorphisms within the MTX pathway genes encoding solute carrier family 19 member 1 (SLC19A1), also known as reduced folate carrier (RFC), 5,10-methylenetetrahydrofolate reductase (MTHFR), folypolyglutamate synthetase (FPGS), γ-glutamyl hydrolase (GGH), 5-aminomidazole-4-carboxamide ribonucleotide transformylase (ATIC) and breast cancer resistance protein (BCRP/ABCG2) on the toxicity and efficacy of MTX in patients with RA, JIA and other diseases has been studied [4–9]. However, results regarding the influence of these polymorphisms on the toxicity and efficacy of MTX are conflicting, and there are marked differences in pharmacogenetics between racial groups [10]. Therefore, we investigated whether polymorphisms within the MTX pathway genes were related to the toxicity and efficacy of MTX in 92 patients with articular-type JIA in Japan.

Patients and methods

Study population

Patients were eligible if they met the International League of Association for Rheumatology classification criteria for articular-type JIA [11]. A total of 92 children (74 girls and 18 boys; 12 with seronegative polyarticular onset, 46 with seropositive polyarticular onset and 34 with oligoarticular onset) in this study were treated at the Yokohama City University Hospital between December 2007 and December 2009.

All 92 patients had been treated with MTX for at least 3 months without biologics. Initially, MTX was administered orally at a dosage of 4–5 mg m^{-2} per week. Then the dosage was adjusted depending on tolerability and response (maximal dosage, 10 mg m^{-2} $week^{-1}$) [2]. Prednisolone was used concomitantly with MTX in 89 patients (96.7%). Folic acid supplementation was performed in nine patients (9.9%). Clinical data were collected from a patient's medical record without any knowledge of the individual's polymorphisms.

The study was performed in accordance with the Declaration of Helsinki, and approval for it was obtained from the Yokohama City University School of Medicine Ethics Committee. Each patient or his/her guardians gave written informed consent to participate in this study.

Definitions of toxicity and efficacy

For the evaluation of toxicity, liver dysfunction was defined as an increase in serum alanine transaminase (ALT) level to five times the normal upper limit before the addition of biologics.

Responders to MTX were defined as follows: (i) patients in whom the medication was terminated because they had remission of symptoms; (ii) patients who continued the treatment with stable doses of MTX; and (iii) patients who continued MTX treatment with the concomitant use of acceptable doses of prednisolone, without the addition of biologics, such as anti-tumour necrosis factor therapy [12] and anti-interleukin-6 receptor antibody therapy [13, 14].

Non-responders to MTX were defined as patients who were refractory to MTX and thus treated with biologics. Treatment with biologics was conducted according to the following criteria: (i) patients with a history of treatment with nonsteroidal anti-inflammatory drugs and MTX; and (ii) patients who had the active disease for at least 3 months after MTX treatment (up to 10 mg m^{-2} $week^{-1}$). Active disease was characterized by five or more swollen joints and three or more joints with limited range of movement accompanied by pain and/or tenderness, or the use of high doses of corticosteroids (>0.25 mg kg^{-1} daily), with accompanying unacceptable side-effects [12, 13].

Clinical predictors

Clinical predictors that may influence a patient's disease state and the toxicity and efficacy of MTX were selected on the basis of previous reports [5, 6, 15, 16]. The following factors were included: sex; age at disease onset; duration of MTX treatment; time interval from disease onset to MTX treatment; rheumatoid factor (RF) status; anti-cyclic citrullinated peptide (anti-CCP) status; and concomitant use of prednisolone and folic acid.

Genetic predictors

Genomic DNA was isolated from peripheral blood using the QIAamp DNA Mini kit (Qiagen K.K., Tokyo, Japan).

The following eight single nucleotide polymorphisms (SNPs) within the MTX pathway genes encoding RFC, MTHFR, FPGS, GGH, ATIC and BCRP were selected according to previous reports [4–9]. Genotyping for the SNPs of *RFC* G80A (rs1051266), *MTHFR* A1298C (rs1801131), *MTHFR* C677T (rs1801133), *FPGS* A1994G (rs10106), *GGH* C452T (rs11545078), *GGH* T16C (rs1800909), *ATIC* C347G (rs2372536) and *BCRP* C421A (rs2231142) was performed using the TaqMan assay (Applied Biosystems, Foster City, CA, USA). TaqMan SNP Genotyping Assays were used for *MTHFR* A1298C and *MTHFR* C677T, and Custom TaqMan SNP Genotyping Assays were used for *RFC* G80A, *FPGS*

A1994G, *GGH* C452T, *GGH* T16C, *ATIC* C347G and *BCRP* C421A [9] (see Supplementary data 1). These SNPs were analysed in real-time PCRs by the AB7500 Real Time PCR system (Applied Biosystems), in the conditions recommended by the manufacturer. Allele discrimination was performed using SDS software version 1.4 (Applied Biosystems).

Statistical analysis

For continuous predictors, such as age and duration of MTX treatment, Student's unpaired *t*-test was used to assess the association between clinical predictors and the toxicity and efficacy. For categorical predictors, such as genetic predictors and sex, a χ^2 test and Fisher's exact test were used to assess the association between predictors and the toxicity and efficacy. Possible confounding effects among the predictors were adjusted using a multiple logistic regression model.

Haplotype phases and haplotype frequencies were estimated using the Expectation-Maximization algorithm (minimum haplotype frequency >0.05). All statistical analyses were carried out using the SAS system version 9 (SAS Institute Inc., Cary, NC, USA).

Results

Distribution of the polymorphisms within the MTX pathway genes

The genotype frequencies for the eight SNPs under study were in Hardy–Weinberg equilibrium ($P > 0.05$). Each result was consistent with the findings of a previous report (see Supplementary data 2) [17].

The toxicity of MTX

Of 92 patients, 10 developed liver dysfunction. Methotrexate treatment of longer duration was a risk factor for liver dysfunction (104.3 months with liver dysfunction, 53.6 months without, $P = 0.005$). No other clinical variables were associated with liver dysfunction (Table 1). None of the patients with folic acid supplementation had liver dysfunction. However, this correlation of folic acid supplementation preventing liver dysfunction was not statistically significant, presumably because of the small study population.

Regarding the association between liver dysfunction and genetic predictors, the TT genotype at *GGH* T16C was a low risk factor for liver dysfunction [$P = 0.031$, odds ratio (OR) = 0.20, 95% confidence interval (CI) 0.03–0.98; Table 2 and Supplementary data 3]. In contrast, the non-TT genotype at *GGH* T16C was a high risk factor for liver dysfunction ($P = 0.031$, OR = 5.10, 95% CI 1.02–25.6), which is of significant clinical interest. This association was statistically significant even after adjustment for duration of MTX treatment ($P = 0.028$, OR = 6.90, 95% CI 1.38–34.5). None of the other SNPs was associated with liver dysfunction.

The *MTHFR* haplotypes and *GGH* haplotypes showed no significant association with liver dysfunction (data not shown).

The efficacy of MTX

Of 92 patients, 67 were non-responders to MTX. Delayed MTX treatment from disease onset (21.3 months with non-responders *vs.* 8.5 months with responders, $P = 0.029$) and RF positivity ($P = 0.026$, OR = 2.87, 95% CI 1.11–7.39) were risk factors for lower efficacy of MTX (Table 3). No other clinical variables were associated with efficacy.

Regarding the association between the efficacy of MTX and genetic predictors, there was no gene polymorphism significantly associated with efficacy (Table 4). The *MTHFR* haplotypes and *GGH* haplotypes showed no significant association with efficacy (data not shown).

In 64 patients treated with MTX within 1 year of disease onset, the CC genotype at *ATIC* C347G tended to be associated with lower efficacy. However, this was not statistically significantly after adjustment for the time interval and RF ($P = 0.106$, OR = 0.38, 95% CI 0.12–1.23) (Table 5).

Discussion

Several studies have shown the influence of polymorphisms within the MTX pathway genes on the toxicity and

Table 1

Association between clinical predictors and liver dysfunction

	ALT >5.0 times normal ($n = 10$)	ALT ≤5.0 times normal ($n = 82$)	*P* value
Age at onset (years, mean)	9.5	7.4	0.138
Sex (male)	20.0%	19.5%	0.971
Time interval from onset to treatment (months, mean)	17.7	17.9	0.987
Prednisolone	90.0%	97.6%	0.204
Folic acid	0.0%	11.0%	0.270
Duration of MTX treatment (months, mean)	104.3	52.6	0.005
MTX efficacy	30.0%	26.8%	0.832

ALT, alanine transaminase.

Table 2

Association between genetic predictors and liver dysfunction

Genotype	Allele model* OR†	*P* value	Dominant model* OR†	*P* value	Recessive model* OR†	*P* value
RFC G80A	1.51	0.414	0.21	0.121	0.59	0.627
BCRP C421A	1.05	0.930	0.80	0.840	0.99	0.988
MTHFR C677T	1.45	0.451	1.12	0.896	2.28	0.214
MTHFR A1298C	0.89	0.852	1.08	0.539	0.74	0.655
FPGS A1994G	0.54	0.249	4.88	0.068	0.70	0.600
GGH T16C	0.42	0.118	0.83	0.475	0.20	0.031
GGH C452T	0.61	0.506	–	–	0.61	0.502
ATIC C347G	1.40	0.560	0.48	0.814	1.17	0.336

M, major allele; and m, minor allele. Major alleles are the A allele at *RFC* G80A, C allele at *BRCP* C421A, C allele at *MTHFR* C677T, A allele at *MTHFR* A1298C, G allele at *FPGS* A1994G, T allele at *GGH* T16C, C allele at *GGH* C452T and C allele at *ATIC* C347G. *Allele model: M *vs.* m; dominant model, (MM or Mm) *vs.* mm; recessive model, MM *vs.* (Mm or mm). †Non-adjusted odds ratio.

Table 3

Association between clinical predictors and methotrexate efficacy

	Responder ($n = 25$)	Non-responder ($n = 67$)	*P* value
Age at onset (years, mean)	6.6	7.9	0.180
Sex (male)	12.0%	22.4%	0.264
Time interval from onset to treatment (months, mean)	8.5	21.3	0.029
Prednisolone	96.0%	97.0%	0.807
Folic acid	4.0%	11.9%	0.254
C-reactive protein at start of treatment (mg dl^{-1}, mean)	2.8	3.3	0.685
Anti-cyclic citrullinated peptide [>4.5 (U ml^{-1})]	32.0%	55.2%	0.062
Rheumatoid factor [>14 (IU ml^{-1})]	40.0%	65.7%	0.026

Table 4

Association between genetic predictors and methotrexate efficacy

Genotype	Allele model* OR†	*P* value	Dominant model* OR†	*P* value	Recessive model* OR†	*P* value
RFC G80A	1.01	0.979	1.32	0.572	1.61	0.435
BCRP C421A	1.28	0.496	0.24	0.151	0.99	0.979
MTHFR C677T	0.75	0.399	0.79	0.708	0.42	0.115
MTHFR A1298C	1.05	0.918	0.36	0.282	0.87	0.775
FPGS A1994G	0.95	0.900	1.37	0.726	1.01	0.984
GGH T16C	1.01	0.986	2.83	0.294	1.24	0.654
GGH C452T	1.15	0.805	–	–	1.15	0.805
ATIC C347G	0.65	0.237	1.08	0.931	0.50	0.139

*Allele model: M *vs.* m.; dominant model, (MM or Mm) *vs.* mm; recessive model, MM *vs.* (Mm or mm). †Non-adjusted odds ratio.

efficacy of MTX in patients with RA [4, 8, 9]. However, results are conflicting, and there are marked differences between racial groups in pharmacogenetic studies [10]. We could find only two studies on the pharmacogenetics of MTX in patients with JIA in Caucasian patients [5, 6], but not one in an Asian population. This is the first reported study on pharmacogenetics of MTX in patients with JIA in an Asian population.

First, we found that the non-TT genotype at *GGH* T16C was associated with a high risk of liver dysfunction. This should be taken into consideration in treating patients carrying the non-TT genotype at *GGH* T16C with MTX in order to prevent liver dysfunction.

Once inside the cell, MTX undergoes FPGS-catalysed polyglutamation by the addition of two to seven glutamic acid groups. The polyglutamated form is not

Table 5

Association between *ATIC* 347CC genotype and methotrexate efficacy in patients with the early phase of juvenile idiopathic arthritis

(a)	OR†	95% Confidence interval	*P* value
ATIC 347CC genotype	0.32	0.11–0.93	0.033

(b)	OR‡	95% Confidence interval	*P* value
ATIC 347CC genotype	0.38	0.12–1.23	0.106
Rheumatoid factor [>14 (IU ml^{-1})]	0.22	0.07–0.72	0.012
Time interval*	0.85	0.70–1.04	0.12

*Time interval, time interval from disease onset to methotrexate treatment. †Non-adjusted odds ratio. ‡Adjusted odds ratio.

readily transported across the cell membrane, and thus, the intracellular half-life of MTX is increased. This polyglutamation process is reversed via GGH-catalysed removal of the glutamic acid groups. Therefore, the amount of intracellular MTX-polyglutamates (MTX-PGs) depends on the net rate of polyglutamation determined by the opposing activities of FPGS and GGH [8].

It was reported that *GGH* T16C, which results in a Cys6Arg substitution, was associated with the efficacy of MTX in patients with RA. The variant C allele may cause a loss of GGH activity, resulting in decreased efflux of MTX and thus increased intracellular MTX-PG levels [8]. This result was consistent with ours. Although we did not address the MTX-PG levels in hepatic cells, it is possible that the C allele at *GGH* T16C was associated with reduced GGH activity and thereby increased the MTX-PG levels in hepatic cells. As a result, the risk of liver dysfunction rises. The AA genotype at *FPGS* A1994G tended to be associated with liver dysfunction ($P = 0.068$, OR = 4.88, 95% CI 0.78–30.9). Future research using large study populations to address the effects of the combination of *GGH* and *FPGS* polymorphisms on MTX toxicity is needed.

The MTX dosage was probably associated with the toxicity and efficacy of the drug. In this cohort study, some patients underwent MTX treatment at other hospitals and had liver dysfunction before being referred to our institution. For these patients, we did not have access to previous medical records concerning the exact dosage of MTX at the time of liver dysfunction. As a general rule, non-responders to MTX received higher dosages of MTX (up to 10 mg m^{-2}) before the introduction of biologics than the responders. We therefore used MTX efficacy as the clinical predictor instead of MTX dosage. The MTX efficacy tended to be associated with liver dysfunction ($P = 0.083$), although the effect of MTX dosage on the toxicity and efficacy of this drug should be evaluated directly in the future.

Second, we found that the longer time interval from disease onset to MTX treatment and RF positivity were associated with lower efficacy of MTX. This was consistent with previous research results. Time to treatment was reported as an important factor in the response to MTX in patients with JIA [6], and RF positivity was associated with worse disease activity [18, 19].

Paediatric rheumatologists have recently been able to use MTX for patients with earlier phases of JIA, because MTX has become well known as a first-line drug in the treatment of RA and JIA [2, 3]. Therefore, we analysed the subgroup of early JIA patients. In those who were treated with MTX within 1 year of disease onset, the CC genotype at *ATIC* C347G tended to be associated with the lower efficacy of MTX. Methotrexate-polyglutamates inhibit ATIC, the last enzyme in the *de novo* purine synthesis pathway. Methotrexate achieves part of its anti-inflammatory effect through inhibition of ATIC, which results in the release of the anti-inflammatory agent, adenosine [9].

It was reported that RA patients with the G allele at *ATIC* C347G, resulting in a Thr116Ser substitution, were likely to have a good response to MTX [9]. Although the effect of the C347G polymorphism on ATIC enzyme activity is unknown, *ATIC* C347G may be in linkage disequilibrium with an unknown functional variant, which is associated with the activity of the purine synthesis pathway and with the level of adenosine production. Future basic and clinical prospective studies on a large number of patients are needed to elucidate this association.

There are some limitations to the present study. The incidence of RF positivity in the patient population studied was higher than generally seen (~10%) [18], presumably because our institution is one of the very few paediatric rheumatology centres in Japan, and many severe cases with RF positive are referred to our institution for highly specialized treatment with biologics [13, 20]. The efficacy rate of MTX in this study (28%) was significantly lower than those in previous Japanese reports [2, 3]. This may be due to the use of a new second-line choice of biologics, as well as the characteristics of our institution and the lower limit of the maximal MTX dosage (10 mg m^{-2}) for the treatment of JIA in Japan [2].

In summary, we found an association between the non-TT genotype at *GGH* T16C and liver dysfunction due to MTX. We also found an influence of time interval from disease onset to MTX treatment on the efficacy of MTX in Japanese patients with JIA. Our study showed the importance of early use of MTX for patients with JIA as well as the possibility of more personalized therapy for patients with JIA based on pharmacogenetic study of the MTX pathway genes.

Competing Interests

There are no competing interests to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplementary data 1
TaqMan® SNP Genotyping Assays.
Supplementary data 2
Distribustion of gene polymorpshisms under the study.
Supplementary data 3
Distribustion of gene polymorpshisms in patiets with or without liver dysfunction.

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