Table 2 continued

Variable	All patients ($n = 52$)	SLE patients		P value
		Without vertebral fractures ($n = 26$)	With vertebral fractures ($n = 26$)	
Treatment				
Ever use of corticosteroids, n (%)	50 (96.2)	26 (100)	24 (92.3)	0.149
Treatment duration of corticosteroids [mean (SD), years]	15.3 (8.2)	13.3 (7.3)[4]	17.6 (8.7)[5]	0.087
Maximal dosage of oral corticosteroids [mean (SD), mg/day]	43.0 (8.2)	45.4 (20.6)[2]	40.6 (19.5)[2]	0.412
Ever use of IV methylprednisolone, n (%)	13 (25.0)	6 (23.1)	7 (26.9)	0.749
Current use of corticosteroids, n (%)	48 (92.3)	26 (100)	22 (84.6)	0.037
Actual prednisone dosage [mean (SD), mg/day]	8.4 (4.8)	7.9 (4.5)	8.9 (5.2)[4]	0.483
History of cyclosporin, n (%)	10 (19.2)	7 (26.9)	4 (15.4)	0.159
Current use of cyclosporin, n (%)	9 (17.3)	5 (19.2)	4 (15.4)	0.714
History of methotrexate, n (%)	1 (1.9)	0 (0)	1 (3.8)	0.313
Current use of methotrexate, n (%)	1 (1.9)	0 (0)	1 (3.8)	0.313
History of cyclophosphamide, n (%)	16 (30.8)	7 (26.9)	9 (34.6)	0.548
History of IV cyclophosphamide, n (%)	9 (17.3)	5 (19.2)	4 (15.4)	0.714
Current use of oral cyclophosphamide, n (%)	1 (1.9)	0 (0)	1 (3.8)	0.313
History of azathioprine, n (%)	7 (13.5)	4 (15.4)	3 (11.5)	0.685
Current use of azathioprine, n (%)	1 (1.9)	1 (3.8)	0 (0)	0.313
History of mizoribine, n (%)	6 (11.5)	3 (11.5)	3 (11.5)	1
History of mycophenolate mofetil (MMF), n (%)	1 (1.9)	1 (3.8)	0 (0)	0.313
History of rituximab, n (%)	2 (3.8)	1 (3.8)	1 (3.8)	1
Current use of major tranquilizer, n (%)	1 (2.0)	0 (0)[1]	1 (3.8)	0.322
Current use of minor tranquilizer, n (%)	14 (26.9)	5 (19.2)	9 (34.6)	0.211
Current use of antispasmodic, n (%)	3 (5.8)	1 (3.8)	2 (7.7)	0.552
Current use of vitamin D supplements, n (%)	32 (61.5)	14 (53.8)	18 (69.2)	0.254
Current hormone replacement therapy, n (%)	2 (3.9)	0 (0)[1]	2 (7.7)	0.157
Current use of vitamin K_2 supplements, n (%)	12 (23.5)	5 (19.2)	7 (28.0)[1]	0.46
Current use of warfarin, n (%)	9 (18.4)	4 (16.0)[1]	5 (20.8)[2]	0.662
Current use of bisphosphonates, n (%)	21 (40.4)	11 (42.3)	10 (38.5)	0.777
Current use of alendronates, n (%)	8 (15.4)	3 (11.5)	5 (19.2)	0.442
Current use of risedronates, n (%)	13 (25.0)	8 (30.8)	5 (19.2)	0.337
Current use of calcium supplements, n (%)	14 (26.9)	6 (23.1)	8 (30.8)	0.532
Current use of calcitonin, n (%)	1 (1.9)	0 (0)	1 (3.8)	0.313
Current use of oral contraceptive, n (%)	1 (2.0)	1 (3.8)	0 (0)[1]	0.322
Current use of vitamin C supplements, n (%)	7 (13.5)	3 (11.5)	4 (15.4)	0.685

Superscript numbers indicate missing data for the corresponding number of SLE patients

Risk factors for vertebral fractures among SLE patients

Table 3 shows the association between selected factors based on univariate analyses and risk of vertebral fracture among SLE patients. Although age, disease duration and CS treatment duration were significantly or marginally different between SLE patients with and without vertebral fractures, only age was included in a multivariate analysis. As these three factors were strongly correlated each other (data not shown), factors in the same equation may be overcontrolling. A history of previous bone fracture was significantly associated with an increased risk of vertebral fractures (adjusted OR = 14.8, 95 % CI = 1.62–134; $P = 0.017$). In contrast, daily use of tea or coffee was marginally associated with a decreased risk (adjusted OR = 0.11, 95 % CI = 0.01–1.01; $P = 0.051$), despite the small number of the cases ($n = 6$). Postmenopausal status (crude OR = 4.67, 95 % CI = 1.34–16.24; $P = 0.015$) was associated with increased risk whereas regular menstruation was associated with decreased risk (crude

Table 3 Association between selected factors and risk of vertebral fracture among systemic lupus erythematosus (SLE) patients

Factors	Crude OR (95 % CI)	*P* value	Adjusted[a] OR (95 % CI)	*P* value
Fish intake 2 + week versus 1 or none	6.25 (0.67–57.9)	0.107	4.04 (0.23–71.2)	0.341
Black tea or coffee daily versus nondaily	0.25 (0.06–1.05)	0.058	0.11 (0.01–1.01)	0.051
Menopausal status post- versus premenopause	4.67 (1.34–16.2)	0.015	0.85 (0.02–38.0)	0.935
Menstruation regular versus irregular	0.22 (0.07–0.75)	0.016	0.06 (0.01–1.74)	0.101
History of previous bone fracture positive versus negative	4.79 (1.14–20.2)	0.033	14.8 (1.62–134)	0.017
Family history of osteoporosis or femoral neck fracture positive versus negative	5.95 (0.64–55.0)	0.116	Not calculable	–

[a] Adjusted for age, fish intake, black tea or coffee intake, menstruation, history of previous bone fracture and family history of osteoporosis or femoral neck fracture

OR = 0.22, 95 % CI = 0.07–0.75; $P = 0.016$). After further adjustment, postmenopausal status and regular menstruation were not significantly associated with the risk of vertebral fractures.

Discussion

This is the first study of prevalence and risk factors of vertebral fracture in female Japanese SLE patients. Only four studies have reported the prevalence of vertebral fractures (20–29 %) in Caucasian SLE patients [10–13]. Bultink et al. [11] showed that vertebral fracture was associated with a history of intravenously administered methylprednisolone and male sex. Almehed et al. [12] showed that high age and low BMD in the total hip but not in the spine were associated with vertebral fractures. In a Chinese population, a high prevalence of asymptomatic vertebral fractures (20.4 %) was also found in women with SLE [24]. We, on the other hand, demonstrated that the prevalence of vertebral fractures was more than double the reported figures (50 % in 52 patients). In this study, the prevalence of osteopenia and osteoporosis among SLE patients was 50 % and 13.8 %, respectively, within the range of prevalence seen in previous studies of SLE patients [5–9]. It is necessary to consider the impact of clinical and environmental factors on the differences of prevalence in Japanese and Caucasians. The incidence of hip fracture is lower [25–27] and of spinal fracture is higher among Asian compared with Caucasian women, as Japanese women have lower BMD at several skeletal sites. Ross et al. [27] reported that age-adjusted prevalence of vertebral fracture has been reported to be higher among native Japanese women than among women of Japanese descent living in Hawaii, and this difference in fracture prevalence might derive from the differences in the prevalence (distributions) of nongenetic risk factors. Kobayashi [28] reported that the calcium content of river water in Japan is lower than that in most European countries. Dietary calcium intake in the Japanese population is less than that in the Hong Kong population (550 mg/day in men and 519 mg/day in women vs. 628 and 569 mg/day for people aged ≥65 years) [29, 30]. Vitamin D deficiency is common among Japanese women, which may affect the outcome of various treatments. Estrogen increases calcium absorption in postmenopausal osteoporosis by increasing serum 1,25-dihydroxyvitamin D. In fact, an additive effect of vitamin D and estrogen on BMD has been suggested in the Japanese population [31]. When reference curves of BMD with aging were compared among Chinese, Japanese, and American Caucasian women, Japanese women had lower BMD at various skeletal sites and a higher rate of BMD decrease with aging than Caucasian women [32]. Although the prevalence of vertebral fracture was significantly higher in Japan compared with Hong Kong, no BMD difference was noted between the two populations. The author explained this finding by the smaller body size of Japanese [33]. In a systematic review, Ruyssen-Witrand et al. [34] showed that vertebral size was an independent risk factor for vertebral fracture.

Most fractures in our study were grade 1 (20–25 % reduction of height) and located in the thoracic spine, confirming previous studies. Almehed et al. [12] reported the fracture prevalence was highest in the midthoracic spine, T6–T8, whereas compression severity was highest in lower lumber spine in SLE patients. In the general population, there are indications of compression fractures being more abundant in the thoracolumbar junction (T11–L2) [12]. The reason fracture location differs between the general population and SLE patients remains unclear.

Our study could not show that low BMD in total hip studies was associated with vertebral fracture ($P = 0.181$ in univariate analysis), although it had the lowest mean BMD in similar studies [10–14]. Our sample size might be too small to detect low BMD as a risk factor of vertebral fracture in patients with SLE. However, three studies

[12–14] reported that there were large proportions of SLE patients with normal BMD and vertebral fractures (30–40 %), which is also consistent with the outcome of our study (37.5 %). BMD is widely recognized as one of the main predictors of bone fractures. Several studies [35–37] have shown that age is a risk factor for fracture independent of bone density. Huang et al. [38] found that age, age at menopause, and years between menarche and menopause significantly influenced vertebral fracture prevalence after adjustment for BMD, suggesting that these variables and BMD both contribute complementary information about fracture risk. Besides BMD, there are other factors influencing the risk of vertebral fracture, such bone dimensions, bone and intervertebral disc quality, bone microarchitecture, spinal loading, and neuronal and muscle function. As some SLE patients are at high risk of thrombosis, impaired microcirculation in bone could damage bone-cell viability and subsequently its possibility of repairing trabecular damage. Mechanisms leading to impaired bone strength could also be initiated by autoantibodies directed against substances necessary for healthy bone remodeling [12]. Factors such as age- and SLE-related changes in bone quality, age-related likelihood of falling, and menopause-related mechanisms may also contribute to fracture risk independent of BMD.

Corticosteroids are widely used in SLE patients to control disease activity. Longitudinal studies show that the most rapid rate of bone loss occurs within the first 6–12 months of treatment and is similar at both the lumbar spine and femoral neck [39–41]. This bone loss continues at a rate of two to three times greater than normal in patients undergoing long-term CS therapy [42] and is associated with an increased risk of both vertebral and nonvertebral fractures [43]. We found no association between vertebral fractures and other measures of CS because data on cumulative oral intake of CS was not available. On the contrary, there are some studies demonstrating no association between vertebral fractures and CS use [44, 45]. Weinstein et al. [46] demonstrated that osteocyte apoptosis could lead to increased body fragility due to the interruption of the osteocyte network and the need for remodeling. Apoptosis can occur in different cell types in SLE [47]. If apoptosis affects the bones of patients with SLE, this indicates the importance of bone quality rather than quantity in conditions where CS are used or there are other major risk factors for vertebral fractures.

This study showed that history of previous bone fracture at any site was significantly associated with increased risk of vertebral fractures (Table 3). Klotbuecher et al. [48] reported that the strongest associations were observed between prior and subsequent vertebral fractures; women with preexisting vertebral fractures had approximately four times greater risk of subsequent vertebral fractures than those without prior fractures, and most studies reported relative risks of approximately two for other combinations of prior and future fracture sites (hip, spine, wrist, or any site).

We found no association between vertebral fracture and current use of bisphosphonates (alendronates or risedronates). Since first commercial sales in Japan in 2001, we have used bisphosphonates to treat CS-induced osteoporosis in SLE patients. All bisphosphonates approved for osteoporosis treatment show robust efficacy in preventing fractures in registration trials lasting 3–4 years. However, recent information suggests that bisphosphonates should be used cautiously in patients receiving more prolonged CS treatment. Reports of previously unnoticed complications, such as severe suppression of bone turnover (SSBT) and osteonecrosis of the jaw, have emerged with long-term use of bisphosphonates. SSBT, resulting in increased susceptibility to nonspinal fractures that heal poorly, appears to occur earlier when alendronate is coadministered with either glucocorticoids or estrogen [49]. Regarding vertebral fracture, it has been proved that alendronate treatment for >5 years reduced clinically recognized vertebral fractures but not morphometric vertebral fractures without symptoms [Fracture Intervention Trial Long-Term Extension (FLEX) trial] [50].

High caffeine intake has been cited as a risk factor for osteoporosis. Although the mechanisms underlying this association are incompletely understood, caffeine consumption is associated with displacement of milk from the diet [51, 52], inhibition of intestinal calcium absorption [53–56], and increased urinary calcium excretion [53–56]. Some observational studies suggest that the impact of heavy caffeine consumption on calcium metabolism and bone mass or fracture risk may be most detrimental for individuals with very low calcium intake [57–61]. Other studies suggest that caffeine-induced increases in urinary calcium excretion are not associated with impaired calcium absorption and/or bone density among younger women [53, 62], and a small placebo-controlled randomized crossover study demonstrated no significant changes in calcium economy associated with 400 mg/day of caffeine administered for 19 days to 16 women aged 26–35 [63]. Coffee and green tea are the most popular nonalcoholic beverages in Japan. Caffeine is an important component of each of these drinks and is widely used in other foods and medications. The complex pharmacogenetic and physiologic effects of caffeine have prompted a great deal of investigation into the health consequences of caffeine ingestion [64]. In our study, frequent black tea or coffee intake was marginally associated with a decreased risk of vertebral fractures among SLE patients (Table 3). High caffeine intake is reportedly a risk factor for reduced BMD. However, Hagarty et al. [65] reported that older women who

drank tea had higher BMD measurements than did those who did not drink tea because nutrients found in tea, such as flavonoids, may influence BMD, and tea drinking may protect against osteoporosis in older women. As for coffee drinking, Sakamoto et al. [66] reported that coffee did not stimulate bone loss in rats.

In conclusion, the excessive prevalence of vertebral fractures (50 %) in 52 female Japanese SLE patients, especially those with a history of previous bone fracture, was observed in this study, although the strength of this evidence is limited by our small sample size. Therefore, we recommend lateral spine radiograph assessment in female Japanese SLE patients regardless of BMD, CS use, and presence of symptoms, after considering age and menopausal state. Additional studies with larger sample size might be needed to better understand the high prevalence of vertebral fracture occurrence in female Japanese patients with SLE.

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Conflict of interest None.

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Safety and Efficacy of Various Dosages of Ocrelizumab in Japanese Patients with Rheumatoid Arthritis with an Inadequate Response to Methotrexate Therapy: A Placebo-controlled Double-blind Parallel-group Study

MASAYOSHI HARIGAI, YOSHIYA TANAKA, SHINGO MAISAWA and the JA21963 Study Group

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MASAYOSHI HARIGAI, YOSHIYA TANAKA, SHINGO MAISAWA, and the JA21963 Study Group

ABSTRACT. ***Objective.*** To evaluate the safety and efficacy of ocrelizumab (OCR) in Japanese patients with rheumatoid arthritis (RA) with an inadequate response to methotrexate (MTX).

Methods. RA patients with an inadequate response to MTX 6–8 mg/week received an infusion of 50, 200, or 500 mg OCR or placebo on Days 1 and 15 and were observed for 24 weeks. The double-blind period was prematurely terminated because of a possible risk for serious infection from OCR.

Results. A total of 152 patients were randomized into the study. The incidence of infection was 37.7% (43/114) in the OCR groups combined, compared to 18.9% (7/37) in the placebo group. Serious infections occurred in 7 patients in the OCR groups combined; there were no serious infections in the placebo group. Among the serious infections, *Pneumocystis jirovecii* pneumonia occurred in 2 patients in the OCR 200 mg group. The American College of Rheumatology 20% response rates at Week 24 (the primary endpoint) of the OCR 50, 200, and 500 mg groups were 54.1% ($p = 0.0080$), 55.6% ($p = 0.0056$), and 47.2% ($p = 0.044$), respectively, all significantly higher than that of the placebo group (25.0%).

Conclusion. These results suggest inappropriate benefit-risk balance of OCR in this patient population. Because rituximab is not approved for treatment of RA in Japan, it will be necessary to investigate safety and efficacy of other anti-B cell therapies in Japanese patients with RA. (ClinicalTrials.gov NCT00779220). (First Release Jan 15 2012; J Rheumatol 2012;39:486–95; doi:10.3899/jrheum.110994)

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From the Departments of Pharmacovigilance, and Medicine and Rheumatology, Graduate School of Medical and Dental Sciences, and the Clinical Research Center, Tokyo Medical and Dental University, Tokyo; the First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, Kitakyushu; and Chugai Pharmaceutical Co. Ltd., Tokyo, Japan.

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M. Harigai, MD, PhD, Departments of Pharmacovigilance, Medicine and Rheumatology, and the Clinical Research Center, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University; Y. Tanaka, MD, PhD, First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health; S. Maisawa, BSc, Chugai Pharmaceutical Co. Ltd.

Address correspondence to Dr. M. Harigai, Department of Pharmacovigilance, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113-8519, Japan. E-mail: mharigai.mpha@tmd.ac.jp

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The possible involvement of B cells in the pathogenesis and progression of RA, including autoantibody production, autoantigen presentation, T cell activation, and production of proinflammatory cytokines and chemokines, has been suggested[1,2,3,4,5,6]. Based on these reports, clinical trials of rituximab (RTX), a chimeric anti-CD20 monoclonal antibody (mAb) targeting CD20 molecules, were conducted in patients with rheumatoid arthritis (RA)[7,8]. Subsequently, RTX was approved for treatment of RA in Europe and the United States.

Ocrelizumab (OCR) is a humanized mAb that also targets CD20[9,10] and eliminates B cells by inducing antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and apoptosis. While the epitopes recognized by OCR and RTX on the extracellular domain of the CD20 molecule partially overlap, OCR offers some advantages over RTX. First, OCR is expected to be better tolerated over repeated and longterm administration because OCR induced higher ADCC activity and lower CDC activity than RTX *in vitro*; this has clinical relevance because CDC activation has been associated with

the incidence and severity of infusion-related reactions (IRR)[11]. Second, as a humanized mAb, OCR may have lower immunogenicity than RTX, a chimeric mAb.

A 6-month, double-blind, phase I/II study of OCR (the ACTION study) was undertaken in the United States, enrolling patients with RA with an inadequate response to disease-modifying antirheumatic drugs (DMARD). The results of the ACTION study confirmed the clinical usefulness of OCR in combination with methotrexate (MTX)[12]. To investigate the dose-responsive effects of OCR in Japanese patients with RA, we conducted a 24-week, placebo-controlled, double-blind, phase II study of OCR with concomitant MTX treatment in Japanese patients with RA whose response to MTX had proved inadequate.

MATERIALS AND METHODS

Patients. Our study was conducted at 37 sites in Japan with approval from the Institutional Review Board at each participating site. Written informed consent was obtained from each patient participating in the trial. Our study was conducted in accord with the Declaration of Helsinki and the Good Clinical Practice guidelines, and was registered at ClinicalTrials.gov, NCT00779220.

Patients selected were ≥ 20 years old, fulfilled the American College of Rheumatology (ACR) 1987 revised criteria for RA[13], were rheumatoid factor (RF)-positive (> 20 IU/ml), showed an inadequate response to MTX at a dosage of 6–8 mg/week (maximum approved dose in Japan at that time: 8 mg/wk) for at least 12 weeks with a stable dose for the last 4 weeks before study treatment, had not used tocilizumab, infliximab, adalimumab, or leflunomide for at least 8 weeks before study treatment, and had used no other DMARD except MTX for at least 4 weeks before study treatment. Active disease was defined as swollen joint count ≥ 8 (66-joint count), tender joint count ≥ 8 (68-joint count), and either serum C-reactive protein (CRP) ≥ 1.5 mg/dl or erythrocyte sedimentation rate (ESR) ≥ 28 mm/h. Key exclusion criteria were additional autoimmune disorders, previous treatment with cell-depleting agents, neutrophil count < 1500/μl, platelet count < 100,000/μl, IgG or IgM less than the lower limit of normal (LLN), or hemoglobin < 8.5 g/dl.

Study design. This was a placebo-controlled, double-blind, multicenter, phase II study. The overall study design is illustrated in Figure 1. The subjects were randomly allocated into 4 groups, the OCR 50, 200, or 500 mg group, or the placebo group, in equal numbers and then given an infusion of their assigned investigational product on Days 1 and 15. Methylprednisolone 100 mg was given intravenously as premedication 30 min before administration of each investigational product. The use of oral antihistamine and acetaminophen 30 to 60 min before administration of investigational product was also permitted. Patients who were withdrawn from the double-blind period entered the safety followup period and were followed for at least 48 weeks from the first infusion of investigational product. This report includes the initial 24-week results.

All patients received uninterrupted stable dosages of MTX (6–8 mg/wk) and folate (≥ 5 mg/wk) from at least 4 weeks before the initiation of study treatment to the end of the study period. Concomitant use of a stable dosage of oral corticosteroid (prednisolone equivalent dose ≤ 10 mg/day) was permitted if the dosage was unchanged in the last 4 weeks before the study, and the concomitant use of nonsteroidal antiinflammatory drugs was also permitted if the dosage had not been changed within the last 2 weeks. Concomitant use of biological or nonbiological DMARD other than MTX was prohibited. The following rescue treatments were allowed from Week 8 at the investigator's discretion if control of disease activity was judged inadequate: increased MTX up to 8 mg/week, use of nonbiological DMARD, increase of oral corticosteroid, intraarticular administration of corticosteroid, intraarticular administration of hyaluronic acid preparation, and the use of 1 biological DMARD (excluding RTX).

Evaluation. Safety and efficacy were evaluated on Days 1 and 15, and every 4 weeks thereafter from Week 4 to Week 24 in the double-blind treatment period. During the safety followup period, safety and efficacy were evaluated every 12 weeks. The primary efficacy endpoint was the ACR 20% (ACR20) response rate at Week 24[14]. The ACR50 and ACR70 response rates and a reduction in the Disease Activity Score (DAS28-ESR) values[15] and European League Against Rheumatism (EULAR) response rates[16] over time up to Week 24 were calculated as secondary endpoints. The percentage of patients achieving DAS28-ESR remission (DAS28-ESR < 2.6) by Week 24 was investigated as exploratory analyses.

To evaluate safety, all adverse events (AE) that occurred during the study were recorded; their severity was judged using the National Cancer Institute (NCI) Common Toxicity Criteria (CTC) Version 3.0. Serious AE (SAE) were defined using criteria from the International Conference on Harmonization. Serious infections (SI), defined as SAE infections or infections requiring intravenous antibiotic injection, were tabulated. Human anti-human antibody (HAHA) and serum immunoglobulin (IgG, IgM, and IgA) concentrations were also measured. To evaluate pharmacokinetics,

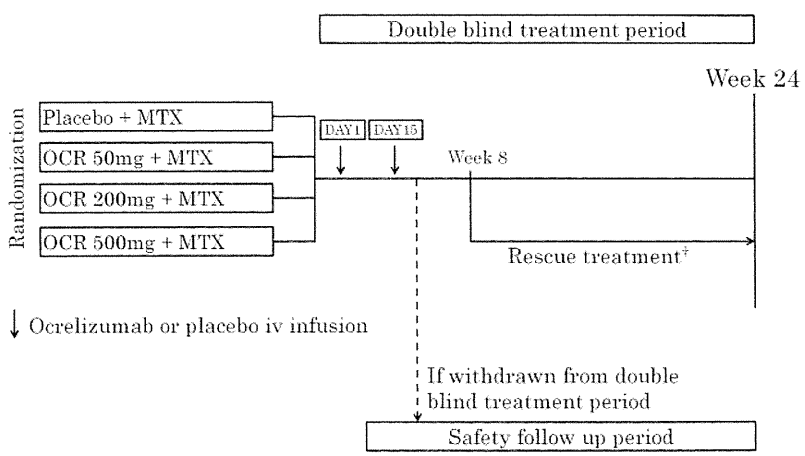

Figure 1. The study design. †Use of additional treatments for RA was permitted after Week 8 if control of disease activity was inadequate, at the discretion of the investigators or subinvestigators. OCR: ocrelizumab; MTX: methotrexate.

OCR concentration in serum was measured, and the number of CD19-positive cells in peripheral blood was measured using flow cytometry.

Statistical analyses. The target sample size was calculated based on the ACR20 response rate in the ACTION study. Using an allocation ratio of 1:2 (placebo vs combined OCR 200 and 500 mg groups), the ACR20 response rate in the combined OCR group of 48.7% and the placebo group of 24.3%, a 2-tailed significance level of 5%, and a power of 80%, the required sample size was calculated by the chi-square test to be 46 patients per group. Allowing for untreated patients, the target group size was set at 50 patients, giving a total target sample size of 200 patients. Calculation of the sample size was performed using nQuery Advisor Version 5.0 (Statistical Solutions Ltd., Farmer's Cross, Ireland).

While our study was in progress, an increased incidence of SI, including opportunistic infections, was reported in multinational clinical trials of OCR that were being conducted at the same time. Based on these safety reports, the enrollment of new patients and the administration of the investigational product in our study were halted, resulting in administration of investigational product to only 151 patients. The double-blind period was prematurely terminated in January 2010 and all patients entered a safety followup period.

The analysis of efficacy was performed using 145 patients (36 patients in the placebo group, 37 in the OCR 50 mg, 36 in the OCR 200 mg, and 36 in the OCR 500 mg), excluding 1 patient in the placebo group, 2 in the OCR 50 mg, and 3 in the OCR 200 mg group who did not receive the second infusion of investigational product because the study was stopped. We recalculated the statistical power and confirmed that it decreased from 80% to 69% with the same assumptions except for the number of patients. The analysis of safety was performed using 151 patients who received investigational products at least once. Safety data were evaluated up to 24 weeks from the first infusion of investigational product regardless of whether patients completed the double-blind period.

The ACR20 response rate at Week 24 (primary endpoint) and ACR50 and ACR70 response rates at Week 24 (secondary endpoints) in each OCR group were compared with the placebo group using the Cochran-Mantel-Haenszel test, accepting a 2-sided significance level of 5%. Based on the predefined analysis plan, descriptive statistics were calculated for the remaining endpoints, but no intergroup comparisons were performed. Adjusted mean changes in DAS28-ESR were based on the analysis of covariance using the baseline value as a covariate.

Efficacy data obtained after the day of rescue treatment or after the day when the decision to withdraw was made were handled as follows: categorical data (ACR responses, EULAR response rates, DAS28-ESR remission) were treated as "no response," continuous data (DAS28-ESR) as "missing data," and the last observation was carried forward.

RESULTS

Baseline characteristics and patient distribution. The mean RA disease duration of the patients in each group was 6.7–10.0 years. The patients had high RA disease activity with a mean DAS28-ESR of 6.3–6.5, a mean serum CRP level of 1.8–3.0 mg/dl, a mean ESR of 53.1–57.0 mm/h, and functional disabilities shown by a mean J-HAQ of 1.3–1.4. The mean MTX dosage was 7.3–7.6 mg/week. In each group, 25.6%–38.9% of the patients had previously received a biological DMARD (Table 1).

Including withdrawals because of the halt in administration of the investigational product, the patients who withdrew from the study before Week 24 numbered 6 in the placebo group, 10 in the OCR 50 mg, 11 in the OCR 200 mg, and 6 in the OCR 500 mg groups. The number of patients who withdrew because of insufficient response was 3 in the placebo group and none in the OCR groups. The proportion of patients receiving rescue treatments up to Week 24 was 32.4% in the placebo group, but lower in the

Table 1. Rheumatoid arthritis (RA) patient demographics and baseline disease characteristics (n = 151).

	Placebo, n = 37	OCR 50 mg, n = 39	OCR 200 mg, n = 39	OCR 500 mg, n = 36
Age, mean (SD), yrs	55.0 (12.1)	54.3 (10.9)	53.1 (10.9)	53.4 (10.3)
No. female (%)	27 (73.0)	30 (76.9)	33 (84.6)	29 (80.6)
RA duration, mean (SD), yrs	8.9 (7.8)	6.7 (7.1)	9.7 (8.1)	10.0 (9.3)
Steinbrocker stage, I/II/III/IV	5/8/9/15	3/15/10/11	3/10/4/22	6/6/13/11
Swollen joint counts (66 joints), mean (SD)	15.2 (6.1)	18.2 (9.7)	15.6 (8.8)	17.4 (9.7)
Tender joint counts (68 joints), mean (SD)	22.2 (11.6)	21.5 (12.3)	19.8 (9.7)	19.0 (9.9)
J-HAQ score, mean (SD)	1.4 (0.6)	1.4 (0.7)	1.3 (0.6)	1.3 (0.7)
CRP, mg/dl, mean (SD)	2.7 (2.7)	2.4 (2.7)	1.8 (1.5)	3.0 (2.8)
ESR, mm/h, mean (SD)	53.1 (29.0)	57.0 (29.0)	54.0 (26.6)	54.7 (31.3)
DAS28-ESR, mean (SD)	6.3 (0.9)	6.5 (0.8)	6.3 (0.8)	6.4 (0.9)
Anti-CCP antibody-positive, no. (%)	35 (94.6)	34 (87.2)	37 (94.9)	32 (88.9)
RF-positive, no. (%)	37 (100)	39 (100)	39 (100)	36 (100)
Corticosteroid use, no. (%)	23 (62.2)	23 (60.0)	28 (71.8)	18 (50.0)
Corticosteroid dose, mg/day, mean (SD)	5.4 (2.2)	5.1 (2.8)	5.2 (2.2)	6.3 (2.4)
MTX dose, mg/wk, mean (SD)	7.4 (0.9)	7.6 (0.8)	7.3 (1.0)	7.6 (0.8)
Previous use of biologics, no. (%)	11 (29.7)	10 (25.6)	11 (28.2)	14 (38.9)
Anti-TNF agent	10 (27.0)	10 (25.6)	10 (25.6)	13 (36.1)
Tocilizumab	2 (5.4)	0 (0.0)	1 (2.6)	3 (8.3)
Abatacept	0 (0.0)	0 (0.0)	1 (2.6)	0 (0.0)
Previous nonbiological DMARD (except for MTX), mean (SD)	1.8 (1.4)	1.4 (1.1)	1.8 (1.6)	1.7 (1.7)

OCR: ocrelizumab; RA: rheumatoid arthritis; TNF: tumor necrosis factor; DMARD: disease-modifying antirheumatic drug; J-HAQ: Japanese version of the Health Assessment Questionnaire; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; DAS28: Disease Activity Score (28 joint count); CCP: cyclic citrullinated protein; RF: rheumatoid factor; MTX: methotrexate.

OCR groups: 12.8% in the OCR 50 mg, 7.7% in the OCR 200 mg, and 16.7% in the OCR 500 mg groups (Figure 2).

Safety. During the 24-week observation period, the incidence of AE was 59.5% in the placebo group and 79.5% in the OCR 50 mg, 79.5% in the OCR 200 mg, and 61.1% in the OCR 500 mg groups (Table 2). The majority of AE were infections and IRR. An IRR was defined as an AE occurring during or within 24 hours after administration of investigational product.

The proportion of subjects experiencing at least 1 infection was 18.9% (7/37) in the placebo group and 37.7% (43/114) in the OCR groups combined. There were no patients with SI in the placebo group and 7 in the OCR groups combined. There were 4 SI in 2 patients in the OCR 50 mg group, consisting of 1 incident each of herpes zoster, pneumonia, sepsis, and septic shock. Six SI occurred in 4 patients in the OCR 200 mg group, 2 incidents of *Pneumocystis jirovecii* pneumonia (PCP), and 1 each of sepsis, herpes simplex, bacterial pneumonia, and febrile neutropenia. There was 1 SI (epididymitis) in 1 patient in the OCR 500 mg group.

Two incidents of malignant tumors (uterine cancer and ovarian cancer) in 1 patient in the OCR 500 mg group were reported, which were diagnosed 153 days after the first infusion of OCR. There were no intergroup differences in the incidences of other AE.

There were 2 deaths during our study. One was a 61-year-old man who had concurrent depression and hypertension, and a history of cerebral infarction and cerebral hemorrhage. He developed pneumonia and sepsis 67 days after administration of OCR 500 mg followed by septic shock, disseminated intravascular coagulation, and multiorgan failure and died the following day. The other death was a 64-year-old man in the placebo group; he died of acute respiratory failure after withdrawal from the study because of insufficient response. No definitive diagnosis was made and an autopsy was not performed.

The increase in incidences of IRR following the first administration (Day 1) of investigational product was dose-dependent: 0% in the placebo group and 15.4% in the OCR 50 mg, 20.5% in the OCR 200 mg, and 25.0% in the OCR 500 mg groups. Following the second administration (Day 15) of investigational product, the incidence of IRR was markedly decreased in all 3 OCR groups (Table 2, Figure 3), 2.9% in the OCR 50 mg, 6.1% in the OCR 200 mg and 8.8% in the OCR 500 mg groups. All patients, except for 1 in the OCR 500 mg group, who experienced an IRR at the second administration also had an IRR at the first administration. Of the 26 IRR, 4 were moderate (NCI CTC Grade 2) and 22 were mild (NCI CTC Grade 1). One patient in each of the OCR 200 mg and the OCR 500 mg groups withdrew from the study because of IRR.

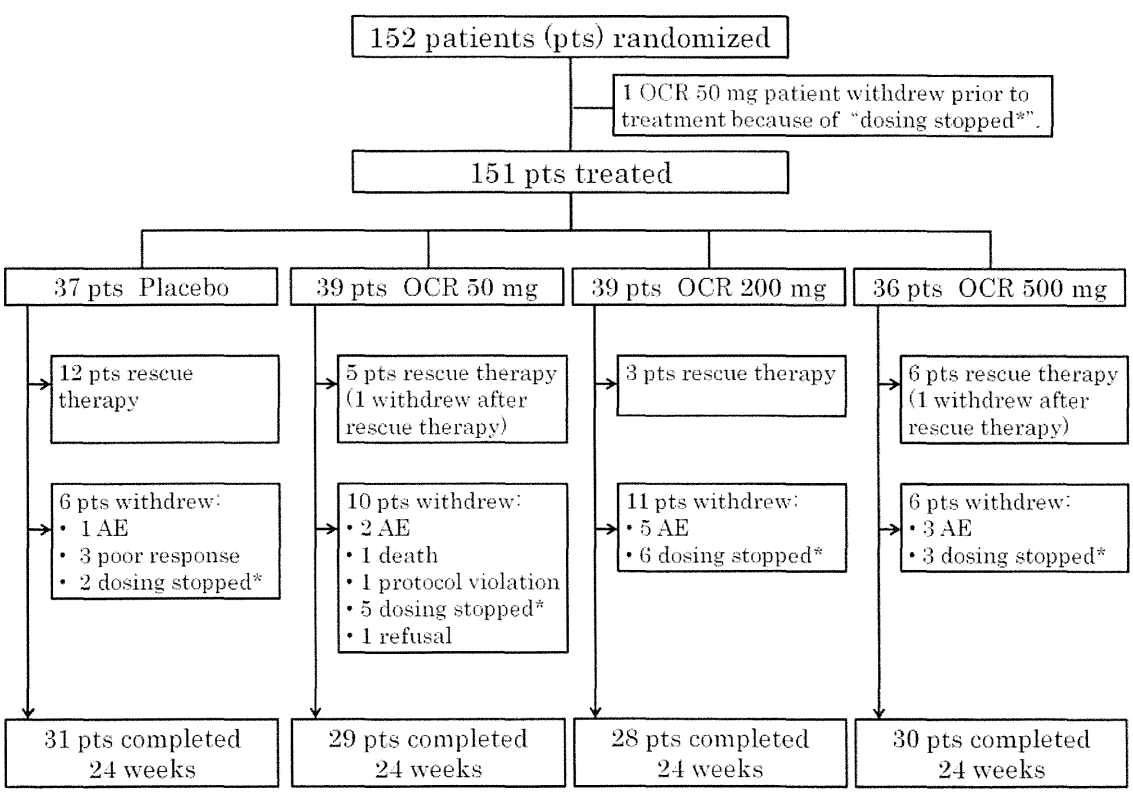

Figure 2. Disposition of patients with RA at Week 24. *Dosing of investigational product was stopped, patients were withdrawn from the study, and enrollment of new patients was halted because of the increased incidence of serious infections, including opportunistic infections, reported in other multinational clinical studies of ocrelizumab (OCR). AE: adverse event.

Table 2. Summary of adverse events (AE) in the safety analysis population of patients with rheumatoid arthritis (n = 151) during the 24-week observation period. Values are the number (%) of patients.

	Placebo, n = 37	OCR 50 mg, n = 39	OCR 200 mg, n = 39	OCR 500 mg, n = 36
Any AE	22 (59.5)	31 (79.5)	31 (79.5)	22 (61.1)
Serious AE	3 (8.1)	2 (5.1)	7 (17.9)	5 (13.9)
AE leading to withdrawal	1 (2.7)	2 (5.1)	5 (12.8)	3 (8.3)
Infection	7 (18.9)	16 (41.0)	16 (41.0)	11 (30.6)
Serious infection	—	2 (5.1)	4 (10.3)	1 (2.8)
Infusion-related reactions	2 (5.4)	6 (15.4)	8 (20.5)	10 (27.8)
Serious infusion-related reactions	—	—	—	1 (2.8)
All AEs affecting ≥ 5% of patients				
Pharyngitis	1 (2.7)	3 (7.7)	1 (2.6)	1 (2.8)
Nasopharyngitis	2 (5.4)	1 (2.6)	3 (7.7)	0
Bronchitis	0	2 (5.1)	3 (7.7)	0
Upper respiratory tract infection	0	1 (2.6)	2 (5.1)	1 (2.8)
Herpes zoster	0	2 (5.1)	1 (2.6)	1 (2.8)
Cystitis	0	0	2 (5.1)	1 (2.8)
P. jirovecii pneumonia	0	0	2 (5.1)	0
Infusion-related reaction	2 (5.4)	6 (15.4)	8 (20.5)	10 (27.8)
Pyrexia	1 (2.7)	2 (5.1)	0	0
Hepatic function abnormal	1 (2.7)	2 (5.1)	2 (5.1)	4 (11.1)
Constipation	1 (2.7)	2 (5.1)	1 (2.6)	0
Stomatitis	2 (5.4)	0	0	0
Upper abdominal pain	0	0	0	2 (5.6)
Urticaria	0	2 (5.1)	0	1 (2.8)
Drug eruption	0	0	2 (5.1)	0
Headache	0	0	1 (2.6)	3 (8.3)
Conjunctivitis	0	2 (5.1)	0	0

OCR: ocrelizumab.

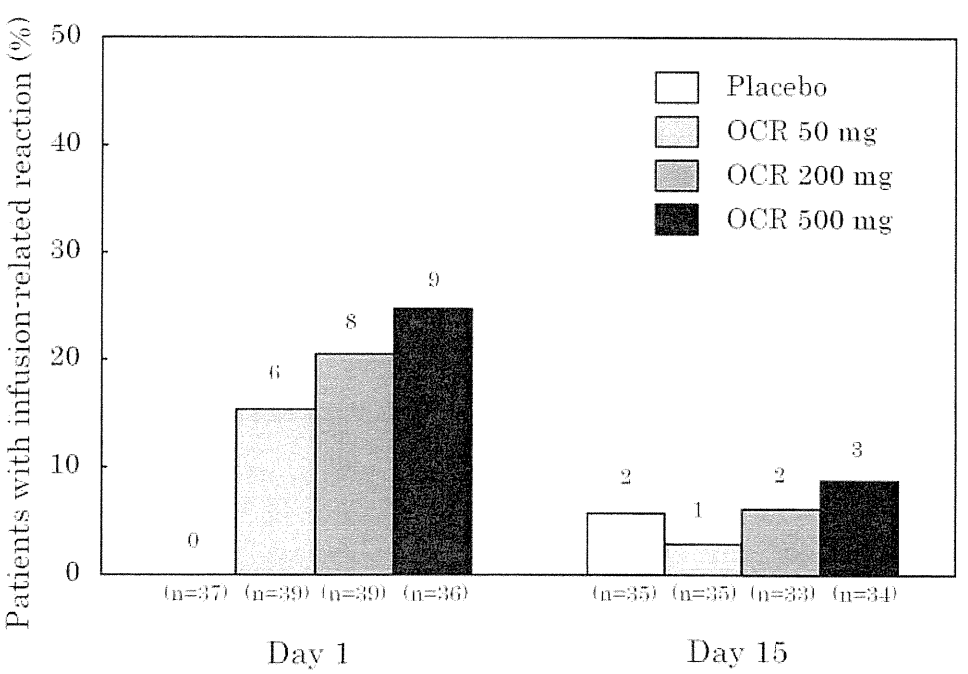

Figure 3. Incidence of infusion-related reactions at Days 1 and 15. Patient numbers shown here represent patients in each group at Days 1 and 15. OCR: ocrelizumab.

No placebo group patients became HAHA-positive during the study, but 2 patients in the OCR 50 mg group and 1 each in the OCR 200 mg and OCR 500 mg groups were HAHA-positive. An IRR occurred in 1 of the 4 patients who became HAHA-positive; this patient developed the IRR at the first administration prior to the expression of HAHA. No serious AE occurred in any HAHA-positive patient. Two of the 4 HAHA-positive patients achieved ACR20 at Week 24, and 1 achieved ACR70. A comparison of HAHA-positive and HAHA-negative patients showed no consistent difference in serum OCR concentration during the study period. The presence of HAHA did not appear to influence either efficacy or safety outcomes.

Efficacy. The ACR20 response rates at Week 24, the primary endpoint, in the OCR groups were significantly higher than the 25.0% of the placebo group [OCR 50 mg: 54.1% ($p = 0.0080$), OCR 200 mg: 55.6% ($p = 0.0056$), OCR 500 mg: 47.2% ($p = 0.044$)]. The ACR50 responses at Week 24 were 16.7% for the placebo group, 37.8% for the OCR 50 mg, 38.9% for the OCR 200 mg, and 30.6% for the OCR 500 mg groups. The ACR50 response rates in the OCR 50 mg and OCR 200 mg groups were significantly higher than those in the placebo group ($p = 0.038$, $p = 0.031$, respectively). The ACR20, ACR50, and ACR70 response rates over time are shown in Figure 4 A-C. The adjusted means (± SE) of the ΔDAS28-ESR, the good responses rates using the EULAR response criteria, and the DAS28-ESR clinical remission rates (DAS28-ESR < 2.6) of the OCR groups at Week 24 were better than those of the placebo groups (Figure 4D, 4E).

Pharmacodynamics. Although the number of CD19-positive cells increased transiently in the placebo group following intravenous administration of methylprednisolone as premedication on Days 1 and 15, the number remained stable through Week 24. In all 3 OCR-treated groups, the number of CD19-positive cells in peripheral blood decreased rapidly after the first administration of OCR; that effect was maintained throughout the 24-week study period (Figure 5). The proportion of patients in whom the number of CD19-positive cells had recovered to at least LLN (80 cells/μl) or the baseline value, whichever was lower, by Week 24 was 80.6% in the placebo group and 6.9% in the OCR 50 mg, 3.4% in the OCR 200 mg, and 0% in the OCR 500 mg groups.

DISCUSSION

Our double-blind placebo-controlled study demonstrated the safety profile of OCR in Japanese patients with RA. The OCR clinical development program in patients with RA was terminated because the risk of SI outweighed the clinical benefits observed in patients with RA, based on the data from our trial and multinational clinical trials of OCR.

In our study, the majority of AE were IRR and infections, and the incidence of IRR was consistent with results reported for anti-CD20 antibodies[7,8,12]. Characteristic IRR symptoms in the OCR group were hypertension in 7 patients (6.1%), headache in 5 (4.4%), pyrexia in 4 (3.5%), and pruritus in 4 (3.5%); these results did not differ from previous studies of OCR or RTX.

By Week 24, SI had occurred only in the OCR group. In the OCR groups combined, the 7 patients who developed SI and the 107 patients who did not develop SI had comparable baseline white blood cell (WBC), neutrophil, and lymphocyte counts and immunoglobulin (IgG, IgM, and IgA) levels. The WBC, neutrophil, and immunoglobulin levels did not fall below LLN [WBC < 3900/μl; neutrophils < 1500/μl; IgG < 870 mg/dl; IgM < 33 mg/dl (males), < 46 mg/dl (females); IgA < 10 mg/dl] in any of the 7 patients with SI during our study, but the lymphocyte count did fall below 500/μl during the study period in 2 patients with SI. In the OCR groups combined, 2 of the 9 patients (22%) whose lymphocyte counts fell below 500/μl developed SI, while 5 of the 105 patients (4.8%) with lymphocyte count > 500/μl developed SI. Among the SI, PCP occurred in 2 patients in the OCR 200 mg group. At the onset of PCP, both patients exhibited pyrexia, hypoxemia, pulmonary ground-glass opacity, and increased serum ß-D-glucan levels, and 1 patient was positive on the polymerase chain reaction test for *P. jirovecii*. Both patients recovered with methylprednisolone pulse therapy and trimethoprim-sulfamethoxazole. Advanced age, concurrent lung disease, and concomitant corticosteroid use have been reported as risk factors for bacterial pneumonia or SI including PCP during treatment of Japanese RA patients with tumor necrosis factor (TNF) inhibitors[17,18,19,20,21]. In our study, 6 of the 7 patients with SI were using prednisolone concomitantly, but only 3 of the 7 patients with SI were over 60 years of age (3 were in their 40s and 1 in her 20s) and none had concurrent lung disease. Similarly, both patients who developed PCP in our study were taking 7.5 mg/day oral prednisolone, but both were 42 years old and did not have concurrent lung disease. Further, their lymphocyte counts at onset of PCP were decreased to 651 and 890/μl, respectively. These results suggest that risk factors for SI, including PCP, during OCR treatment may differ from those during treatment with TNF inhibitors.

Although there were differences of sample size, patient background, and observation period, the incidence of SI in our study (6.1%) was comparable to the results of other Japanese clinical trials of biologic agents: 7.6% in a 52-week study of tocilizumab in patients with inadequate response to DMARD (SAMURAI study)[22]; 3.3% in a 24-week study of tocilizumab in patients with inadequate response to MTX (SATORI)[23]; 5.2% in a 54-week study of infliximab in patients with inadequate response to MTX (RISING)[24]; and 4.9% in a 24-week study of adalimumab in patients with inadequate response to DMARD (CHANGE)[25]. PCP was observed in 2 patients (1.75%) in our study, but in the SAMURAI, SATORI, and CHANGE studies, no PCP was observed. Further, the incidence of PCP in our study was

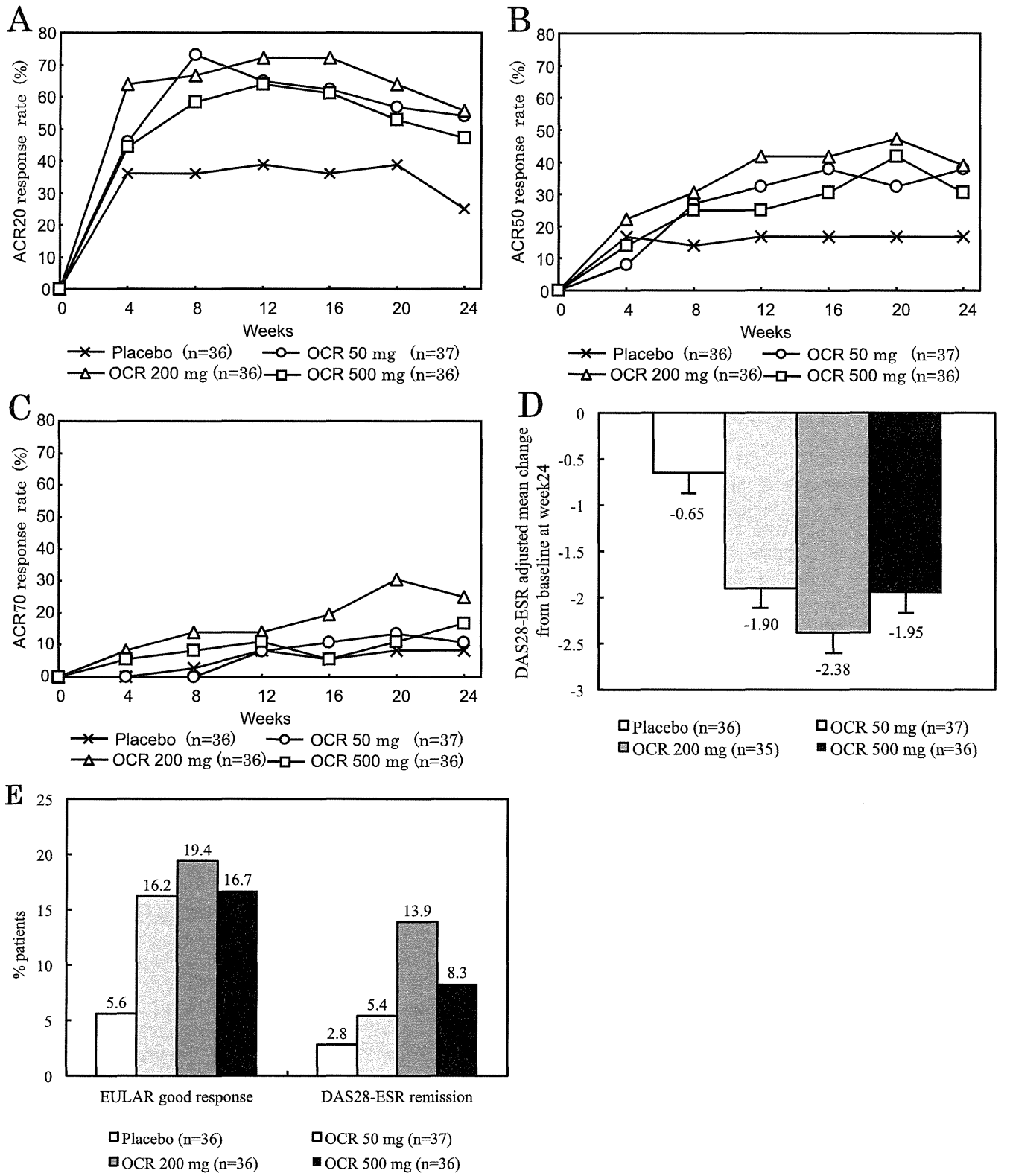

Figure 4. Clinical efficacy of ocrelizumab (OCR). A. ACR20 response rate over time. B. ACR50 response rate over time. C. ACR70 response rate over time. Patients receiving rescue therapy or withdrawing from the study were classified as nonresponders. D. DAS28-ESR mean changes from baseline at Week 24. Error bars represent standard error of the mean. E. The proportion of patients achieving a good response according to the EULAR criteria and remission according to DAS28-ESR. ACR: American College of Rheumatology; DAS28: Disease Activity Score (28 joint count); ESR: erythrocyte sedimentation rate; EULAR: European League Against Rheumatism.

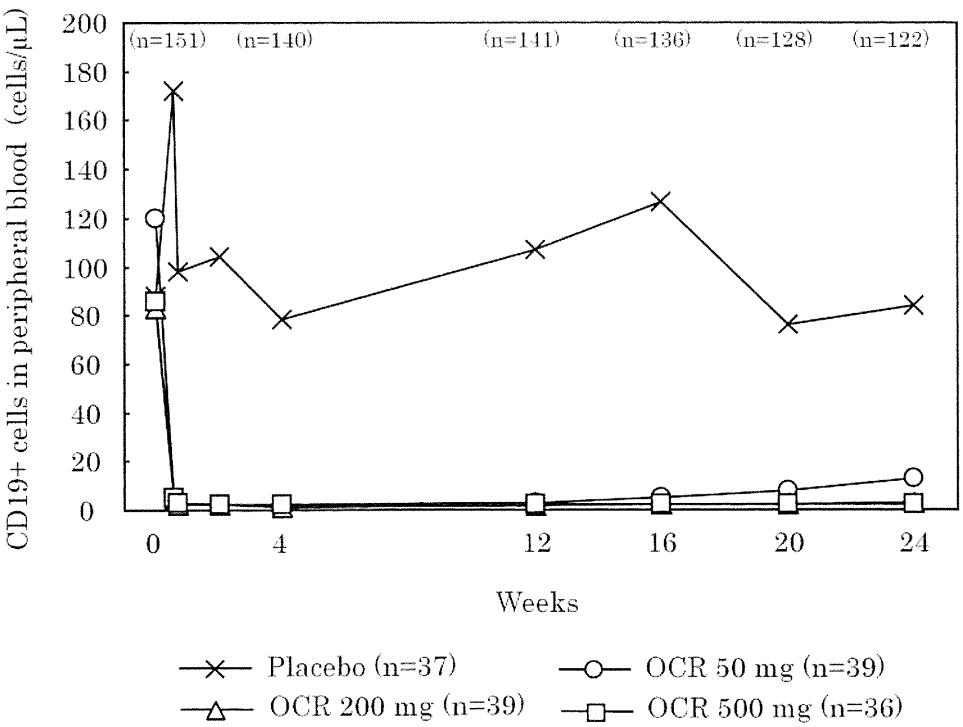

Figure 5. Median peripheral blood CD19-positive B cell counts over time. The lower limit of normal (LLN) was 80 cells/μl. The patient numbers shown with each investigational group represent the number of patients in that group at Time 0. Numbers shown above the different timepoints represent total number of patients in the study at that timepoint. OCR: ocrelizumab.

higher than those in the Japanese postmarketing surveillance data of biologic agents (tocilizumab, infliximab, and etanercept), that is, 0.2% to 0.4%[17,18,19,26]. These results suggest that treatment with OCR in Japanese patients with RA may have higher risk for PCP than treatment with the other biologic agents.

A possible association of efficacy with B cell depletion was reported in patients with RA treated with OCR in the ACTION study[12], which ascertained that B cell depletion was maintained until Week 24 in groups that received ≥ 200 mg OCR, but was not maintained in groups with lower dosages. Significant improvements of signs and symptoms of RA shown by relatively stringent response criteria, including ACR70 response, DAS28-ESR clinical remission, and EULAR good response, were also obtained only in groups that received ≥ 200 mg OCR[12]. The clinical response to RTX has been reported to be determined by the level of B cell depletion rather than by the dose of the drug[27]. In our study, the percentages of patients with peripheral blood B cell count at Week 24 that was above LLN or baseline values were 6.9%, 3.4%, and 0% in the OCR 50, 200, and 500 mg groups, respectively. The OCR 200 mg group showed higher clinical responses than the other 2 OCR groups in every efficacy criterion used in our study. In addition, the peripheral B cell count recovered to at least the LLN (80 cells/μl) or the baseline value in 4 patients in the OCR groups combined, but these patients showed sustained efficacy through Week 24. It is difficult to draw firm conclusions because of the small number of patients with B cell recovery and the limited study period (24 weeks), but these results suggest that peripheral B cell count alone may not account for maintenance of efficacy in patients with RA treated with OCR.

As a limitation of our study, we note the dosage of MTX. The mean MTX dosage in each group was 7.3–7.6 mg/week, which was lower compared to clinical trials of OCR for RA conducted in some Western countries. The approved maximum dose of MTX was 8 mg/week in Japan when this trial was implemented and we had to design the trial under this restriction. This should be taken into account when interpreting our results.

Serious infections, including PCP, occurred only in the combined OCR groups in our study, possibly indicating an elevated risk for SI from OCR use in Japanese patients with RA. Treatment with OCR resulted in better clinical responses than treatment with the placebo in Japanese RA patients with an inadequate response to MTX, about 30% of whom had been previously treated with a biological DMARD. Although we should take into account the small sample size and the premature termination of the study, these results would suggest an inappropriate benefit-risk balance for OCR in this patient population. Because of the lack of

approval for RTX for RA in Japan and the recommended use of the drug for patients with RA who have failed TNF inhibitor therapy in Western countries, it will be necessary to investigate the safety and efficacy of other anti-B cell therapies in Japanese patients with RA.

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APPENDIX

List of study collaborators. Primary investigators of the JA21963 study group: Kazuhide Tanimura (Hokkaido Medical Center for Rheumatic Diseases), Hiroki Takahashi (Sapporo Medical University), Yukitomo Urata (Seihoku Central Hospital), Yasuhiko Hirabayashi (Hikarigaoka Spellman Hospital), Tomonori Ishii, Hiroshi Fujii (Tohoku University Hospital), Takayuki Sumida (Tsukuba University Hospital), Chihiro Terai (Jichi Medical University Saitama Medical Center), Ryutaro Matsumura (National Hospital Organization Chiba-East Hospital), Makoto Sueishi (National Hospital Organization Shimoshizu Hospital), Kazuhiko Yamamoto (The University of Tokyo Hospital), Akio Yamada, Daitaro Kurosaka (Jikei University School of Medicine), Akio Mimori (International Medical Center of Japan), Yusuke Miwa (Showa University Hospital), Masataka Kuwana (Keio University Hospital), Shinichi Kawai (Toho University Omori Medical Center), Yoshiaki Ishigatsubo (Yokohama City University Hospital), Kazunori Sugimoto (Fukui General Clinic), Noriyoshi Ogawa (Hamamatsu University School of Medicine), Toshiaki Miyamoto (Seirei Hamamatsu General Hospital), Shigenori Tamaki, Motokazu Kai (National Hospital Organization Mie Chuou Medical Center), Daisuke Kawabata (Kyoto University Hospital), Toshio Tanaka (Osaka University Hospital), Masaaki Inaba (Osaka City University Hospital), Shunichi Kumagai, Akio Morinobu, Yasushi Miura (Kobe University Hospital), Hajime Sano (Hyogo College of Medicine), Naoki Kashihara, Yoshitaka Morita (Kawasaki Medical School Hospital), Kazuhiko Ezawa (Kurashiki Kosai Hospital), Yuji Yamanishi, Masanori Kawashima (Hiroshima City Hospital), Seizo Yamana, Mitsuhiro Iwahashi (Higashihiroshima Memorial Hospital), Hiroaki Dobashi (Kagawa University), Kiyoshi Takasugi (Dohgo Spa Hospital), Takahiko Horiuchi (Kyusyu University Hospital), Eiichi Suematsu (National Hospital Organization Kyushu Medical Center), Takaaki Fukuda (Kurume University Medical Center), Katsumi Eguchi, Atsushi Kawakami (Nagasaki University Hospital).

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PLoS GENETICS

A Genome-Wide Association Study Identified *AFF1* as a Susceptibility Locus for Systemic Lupus Eyrthematosus in Japanese

Yukinori Okada[1,2,3☯], Kenichi Shimane[1,2☯], Yuta Kochi[1,2☯*], Tomoko Tahira[4], Akari Suzuki[1], Koichiro Higasa[3], Atsushi Takahashi[3], Tetsuya Horita[5], Tatsuya Atsumi[5], Tomonori Ishii[6], Akiko Okamoto[2], Keishi Fujio[2], Michito Hirakata[7], Hirofumi Amano[8], Yuya Kondo[9], Satoshi Ito[9], Kazuki Takada[10], Akio Mimori[11], Kazuyoshi Saito[12], Makoto Kamachi[13], Yasushi Kawaguchi[14], Katsunori Ikari[14], Osman Wael Mohammed[15], Koichi Matsuda[15], Chikashi Terao[16,17], Koichiro Ohmura[16], Keiko Myouzen[1], Naoya Hosono[18], Tatsuhiko Tsunoda[19], Norihiro Nishimoto[20], Tsuneyo Mimori[16], Fumihiko Matsuda[17], Yoshiya Tanaka[12], Takayuki Sumida[9], Hisashi Yamanaka[14], Yoshinari Takasaki[8], Takao Koike[5], Takahiko Horiuchi[21], Kenshi Hayashi[4], Michiaki Kubo[18], Naoyuki Kamatani[3], Ryo Yamada[1,17], Yusuke Nakamura[15], Kazuhiko Yamamoto[1,2]

1 Laboratory for Autoimmune Diseases, Center for Genomic Medicine (CGM), RIKEN, Yokohama, Japan, **2** Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan, **3** Laboratory for Statistical Analysis, CGM, RIKEN, Yokohama, Japan, **4** Division of Genome Analysis, Research Center for Genetic Information, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan, **5** Department of Medicine II, Hokkaido University Graduate School of Medicine, Sapporo, Japan, **6** Department of Hematology and Rheumatology, Tohoku University Graduate School of Medicine, Sendai, Japan, **7** Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan, **8** Department of Internal Medicine and Rheumatology, Juntendo University School of Medicine, Tokyo, Japan, **9** Division of Clinical Immunology, Doctoral Program in Clinical Sciences, Graduate School of Comprehensive Human Science, University of Tsukuba, Tsukuba, Japan, **10** Departments of Medicine and Rheumatology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan, **11** Division of Rheumatic Diseases, National Center for Global Health and Medicine, Tokyo, Japan, **12** First Department of Internal Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan, **13** Department of Immunology and Rheumatology, Unit of Translational Medicine, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan, **14** Institute of Rheumatology, Tokyo Women's Medical University, Tokyo, Japan, **15** Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan, **16** Department of Rheumatology and Clinical immunology, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **17** Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan, **18** Laboratory for Genotyping Development, CGM, RIKEN, Yokohama, Japan, **19** Laboratory for Medical Informatics, CGM, RIKEN, Yokohama, Japan, **20** Laboratory of Immune Regulation, Wakayama Medical University, Wakayama, Japan, **21** Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan

Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease that causes multiple organ damage. Although recent genome-wide association studies (GWAS) have contributed to discovery of SLE susceptibility genes, few studies has been performed in Asian populations. Here, we report a GWAS for SLE examining 891 SLE cases and 3,384 controls and multi-stage replication studies examining 1,387 SLE cases and 28,564 controls in Japanese subjects. Considering that expression quantitative trait loci (eQTLs) have been implicated in genetic risks for autoimmune diseases, we integrated an eQTL study into the results of the GWAS. We observed enrichments of cis-eQTL positive loci among the known SLE susceptibility loci (30.8%) compared to the genome-wide SNPs (6.9%). In addition, we identified a novel association of a variant in the AF4/FMR2 family, member 1 (*AFF1*) gene at 4q21 with SLE susceptibility (rs340630; $P = 8.3 \times 10^{-9}$, odds ratio = 1.21). The risk A allele of rs340630 demonstrated a cis-eQTL effect on the *AFF1* transcript with enhanced expression levels ($P < 0.05$). As *AFF1* transcripts were prominently expressed in $CD4^+$ and $CD19^+$ peripheral blood lymphocytes, up-regulation of *AFF1* may cause the abnormality in these lymphocytes, leading to disease onset.

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* E-mail: ykochi@src.riken.jp

☯ These authors contributed equally to this work.

Author Summary

Although recent genome-wide association study (GWAS) approaches have successfully contributed to disease gene discovery, many susceptibility loci are known to be still uncaptured due to strict significance threshold for multiple hypothesis testing. Therefore, prioritization of GWAS results by incorporating additional information is recommended. Systemic lupus erythematosus (SLE) is an autoimmune disease that causes multiple organ damage. Considering that abnormalities in B cell activity play essential roles in SLE, prioritization based on an expression quantitative trait loci (eQTLs) study for B cells would be a promising approach. In this study, we report a GWAS and multi-stage replication studies for SLE examining 2,278 SLE cases and 31,948 controls in Japanese subjects. We integrated eQTL study into the results of the GWAS and identified *AFF1* as a novel SLE susceptibility loci. We also confirmed cis-regulatory effect of the locus on the *AFF1* transcript. Our study would be one of the initial successes for detecting novel genetic locus using the eQTL study, and it should contribute to our understanding of the genetic loci being uncaptured by standard GWAS approaches.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoantibody production, complement activation, and multi-organ damage [1]. Familial aggregation demonstrates that both genetic and environmental factors play a role in pathogenesis of SLE [2]. Genetic studies using candidate gene-approaches, and recently, genome-wide association studies (GWAS), have uncovered more than 25 SLE susceptibility genes, including *HLA-DRB1*, *IRF5*, *STAT4*, *ITGAM*, *BLK*, *TNFAIP3*, and others [3–18]. However, most of these studies were conducted in European populations [3–13,15,17], and few studies have been conducted in Asian populations [14,16,18]. Since the epidemiology of SLE has demonstrated that the prevalence of disease substantially differs among populations, genetic backgrounds of SLE should be also heterogeneous across populations [19,20]. Therefore, additional studies in Asians might provide novel insights. It is of note that GWAS for SLE in Chinese populations identified novel loci that had not been detected in Europeans, such as *ETS1*, *IKZF1*, and *WDFY4* [14,16].

Another issue raised by the previous GWASs for complex diseases is that many susceptibility loci still remained uncaptured, owing to its strict significance threshold for multiple hypothesis testing [21]. In SLE, for example, the 26 risk loci identified by the previous GWAS explained only an estimated 8% of the total genetic susceptibility to the disease [15]. Therefore, it is still important to examine the sub-loci of GWAS, in order to reveal the entire picture of genetic etiology. To effectively explore these uncaptured loci, prioritization of GWAS results by incorporating additional information implicated in the disease pathophysiology is recommended [22,23]. Considering that abnormalities in B cell activity play essential roles in SLE [1] and that expression quantitative trait loci (eQTL) have been implicated to comprise approximately a half of genetic risks for autoimmune diseases [24], prioritization based on an eQTL study for B cells would be a promising approach for SLE [25]. Moreover, an eQTL itself assures the presence of functional variant(s) that regulate gene expression. Thus, eQTL increases the prior probability of the presence of disease-causal variant(s) in the locus more effectively and unbiasedly, compared to other knowledge-based prioritizations such as gene pathway analysis [24].

Here, we report a GWAS and multi-stage replication studies for SLE examining 2,278 SLE cases and 31,948 controls in Japanese subjects. We integrated eQTL study into the results of the GWAS, which effectively enabled to detect a novel SLE susceptibility locus.

Results

GWAS for SLE

In the GWAS, 891 SLE cases and 3,384 controls in Japanese subjects were genotyped over 550,000 single nucleotide polymorphism (SNP) markers (Table S1, S2 and Figure 1). We applied stringent quality control (QC) criteria and evaluated associations of 430,797 autosomal SNPs, as previously described [26]. No substantial population stratification was demonstrated through principal component analysis (Figure S1) or a Quantile–Quantile plot of *P*-values (inflation factor, λ_{GC}, = 1.088, Figure S2), suggesting homogenous ancestries of our study population [27].

We identified significant associations in six chromosomal loci that satisfied the genome-wide significance threshold of $P<5.0\times10^{-8}$ (Table 1 and Figure 2A). These loci have been reported to be associated with SLE susceptibility (*STAT4*, *TNFAIP3*, *HIP1*, *BLK*, *ETS1*, and the HLA region) [3–18]. We also observed significant replications in 17 of the previously reported SLE susceptibility loci [3–18] ($\alpha = 0.01$; Table 2). Of these, significant replications were enriched in the loci identified through the studies in Asian populations (80%; 8 of the 10 loci), including *RASGRP3*, *IKZF1*, *HIP1*, *WDFY4*, intergenic region at 11q23, *ETS1*, *SLC15A4*, *ELF1*, and *HIC2-UBE2L3* [14,16,18], compared to those in European populations (56.3%; 9 of the 16 loci) [3–13,15,17].

Incorporation of eQTL study into GWAS results

For the selection of SNPs incorporated in the replication studies of the potential association signals, we evaluated cis-eQTL effects of the SNPs using publically available gene expression data [28], and prioritized the results of the GWAS. After applying QC criteria, we evaluated the expression levels of 19,047 probes assayed in lymphoblastoid B cell lines from Phase II HapMap East-Asian individuals [29] using Illumina's human whole-genome expression array (WG-6 version 1) [28]. For each of the SNPs included in our GWAS, probes located within ±300 kbp regions were focused on as cis-eQTLs (average 4.93 probes per SNP). We denoted the SNPs which exhibited significant associations with expression levels of any of the corresponding cis-eQTLs as eQTL positive (false discovery rate (FDR) *Q*-values<0.2). We observed enrichments of eQTL positive loci among the SLE susceptibility loci (30.8%; 8 of the 26 evaluated loci) including a well-known eQTL gene of *BLK* [11,25] (Table 2), compared to the genome-wide SNPs (6.9%) and compared even to the SNPs in the vicinity of expressed loci (among the SNPs located within ±10 kbp of probes used for the expression analysis, 13.1% were eQTL positive; Table S3).

By prioritizing the results of the GWAS using the eQTL study, we selected 57 SNPs from 1,207 SNPs that satisfied $P<1.0\times10^{-3}$ in the GWAS. We subsequently referred the associations of the selected SNPs using the results of the concurrent genome-wide scan for SLE in an independent Japanese population (Tahira T et al. Presented at the 59th Annual Meeting of the American Society of Human Genetics, October 21, 2009). In the scan, 447 SLE cases and 680 controls of Japanese origin were evaluated using a pooled DNA approach [30]. We selected SNPs if any association signals were observed in the neighboring SNPs of the

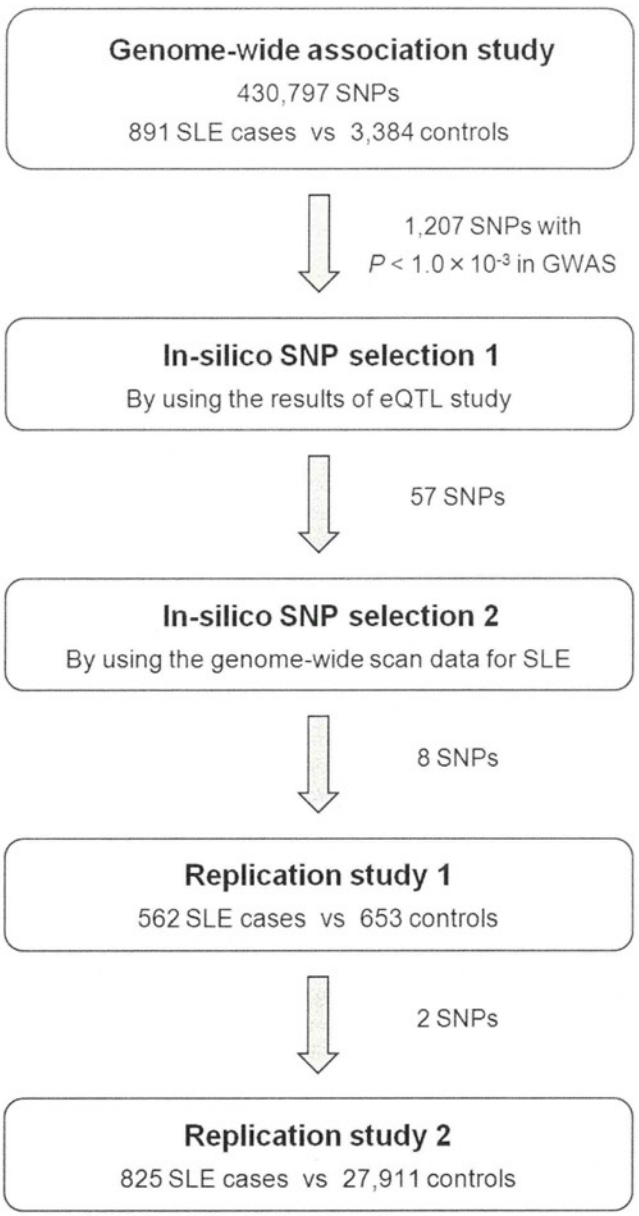

Figure 1. Design of the GWAS and multi-stage replication studies for SLE in Japanese subjects. A total of 2,278 SLE cases and 31,948 controls were enrolled. The clinical characteristics of the subjects are summarized in Table S1 and S2. Details of the genome-wide scan data for SLE referenced in the *in silico* SNP selection 2 are described elsewhere (Tahira T et al. Presented at the 59th Annual Meeting of the American Society of Human Genetics, October 21, 2009).
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pooled analysis. As a result, 8 SNPs remained for further investigation (Table S4).

Replication studies and identification of *AFF1*

Then, we performed two-stage replication studies using independent SLE cohorts for Japanese subjects (cohort 1 with 562 SLE cases and 653 controls, and cohort 2 with 825 SLE cases and 27,911 controls). First, we evaluated the selected 8 SNPs in the replication study 1. In the replication study 2, 2 SNPs that satisfied $P<1.0\times10^{-6}$ in the combined study of GWAS and replication study 1 were further evaluated (Figure 1). Among the evaluated SNPs, we observed significant replications in the SNP located in the genomic region of the AF4/FMR2 family, member 1 gene (*AFF1*) at 4q21 (rs340630; $P=4.6\times10^{-5}$ and $P=0.0094$ in the two individual cohorts, respectively; Table 3, Table S5, and Figure 2B). The combined study for the GWAS ($P=1.5\times10^{-4}$) and the replication studies demonstrated significant associations of rs340630 that satisfied the genome-wide significance threshold ($P=8.3\times10^{-9}$, OR = 1.21, 95% CI 1.14–2.30).

Cis-eQTL effect of rs340630 on *AFF1* transcripts

Since the landmark SNP in the *AFF1* locus, rs340630, was prioritized through the eQTL study as an eQTL positive SNP (Table 3), we further validated its cis-eQTL effect using Epstein-Barr virus (EBV)-transfected B cell lines established from Japanese individuals (Pharma SNP Consortium (PSC) cells, $n=62$). The correlation between rs340630 genotypes and the expression levels of *AFF1* was significant in the PSC cells stimulated with phorbol myristate acetate (PMA) ($R^2=0.074$, $P=0.033$; Figure 3A). The expression levels increased with the number of SLE-risk (A) alleles. To further confirm this cis-regulatory effect, we performed allele-specific transcript quantification (ASTQ) of *AFF1*. The transcript levels of each allele were quantified by qPCR using an allele specific probe for a SNP in the 5′-untranslated region (rs340638), which was in absolute LD with rs340630 ($r^2=1.0$, $D'=1.0$). We examined PSC-cells ($n=17$) that were heterozygous for both rs340630 and rs340638. The mean ratio of each transcript (A over G allele; the A allele comprises a haplotype with the risk (A) allele of rs340630) were significantly increased to 1.07 compared to the ratio of the amount of DNA (1.00, $P=0.012$) (Figure 3B). These results suggest that rs340630, or SNP(s) in LD with it, are a regulatory variant predisposing SLE susceptibility through increased expression levels of *AFF1*.

Expression of *AFF1* in $CD4^+$ and $CD19^+$ peripheral blood lymphocytes

AFF1 is known to be involved in cytogenetic translocations of acute lymphoblastic leukemia (ALL) [31]. Its fusion protein with the mixed-lineage leukemia gene (*MLL*) is implicated in the regulation of transcription and the cell cycle of lymphocytes [31]. To investigate the expression pattern of *AFF1* in normal tissues, we evaluated the transcript levels of *AFF1* in a panel of various tissues. We observed prominent expression of *AFF1* in $CD4^+$ and $CD19^+$ peripheral blood lymphocytes, implying an important role for *AFF1* in helper-T-cells and B-cells (Figure 3C).

Discussion

Through a GWAS and multi-staged replication studies consisting of 2,278 SLE cases and 31,948 controls in Japanese subjects, our study identified that the *AFF1* locus was significantly associated with SLE susceptibility.

As well as the identification of the novel SLE susceptibility locus, we observed significant replications of associations in the previously reported susceptibility loci. The replications were especially enriched in the loci identified through the studies in Asian populations, compared to those in European populations. Considering the ethnical heterogeneities in the epidemiology of SLE [19,20], these observations suggest the similarities in the genetic backgrounds of SLE shared within Asian populations, and also the existence of the both common and divergent genetic backgrounds encompassed between European and Asian populations.

Table 1. Results of a genome-wide association study for Japanese patients with SLE.

rsID[a]	Chr	Position (bp)	Cytoband	Gene	Allele[b]	No. subjects		Allele 1 freq.		OR (95%CI)	*P*
					1/2	Case	Control	Case	Control		
rs10168266	2	191,644,049	2q32	*STAT4*	T/C	891	3,384	0.37	0.27	1.59 (1.42–1.78)	2.7×10^{-16}
rs9501626	6	32,508,322	6p21	HLA region	A/C	891	3,381	0.20	0.12	1.86 (1.62–2.13)	1.0×10^{-18}
rs2230926	6	138,237,759	6q23	*TNFAIP3*	G/T	891	3,377	0.11	0.069	1.75 (1.47–2.08)	1.9×10^{-10}
rs6964720	7	75,018,280	7q11	*HIP1*	G/A	891	3,384	0.25	0.19	1.43 (1.27–1.63)	1.3×10^{-8}
rs2254546	8	11,381,089	8p23	*BLK*	G/A	891	3,384	0.78	0.72	1.42 (1.61–1.25)	4.1×10^{-8}
rs6590330	11	127,816,269	11q24	*ETS1*	A/G	891	3,368	0.48	0.39	1.44 (1.30–1.60)	1.3×10^{-11}

[a]SNPs that satisfied the threshold of $P<5.0\times10^{-8}$ were indicated.
[b]Based on forward strand of NCBI Build 36.3.
SLE, systemic lupus erythematosus; OR, odds ratio.
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To effectively detect the novel SLE susceptibility locus, we integrated cis-eQTL effects of the SNPs and prioritized the results of the GWAS. In addition to identifying a novel locus for SLE-susceptibility, our study demonstrated approximately 30% of confirmed SLE-susceptibility loci were comprised of cis-eQTLs. We also confirmed cis-regulatory effect of the landmark SNP in the *AFF1* locus, rs340630, on *AFF1* transcripts, which had been prioritized through the eQTL study. These results would suggest that accumulation of quantitative changes in gene expression would accelerate the disease onset of SLE. It would also demonstrate the validity of applying eQTL study in the search of the susceptible genes for SLE or other autoimmune diseases, as previously suggested in the study for celiac disease [24]. To our knowledge, this is one of the initial studies to successfully discover a new locus by prioritizing GWAS results using eQTLs, and should contribute to the approaches assessing genetic loci still being uncaptured by recent large-scaled GWASs due to stringent significance threshold for multiple hypothesis testing [21].

We observed prominent expression levels of *AFF1* in $CD4^+$ and $CD19^+$ peripheral blood lymphocytes, which would imply an important role for *AFF1* in helper-T-cells and B-cells. In fact, *AFF1* is essential for normal lymphocyte development, as demonstrated in mice deficient for *AFF1*; severe reduction were observed in the thymic double positive CD4/CD8 population and the bone marrow pre-B and mature B-cell numbers [32]. The risk A allele of rs340630 demonstrated a cis-eQTL effect on the *AFF1* transcript with enhanced expression levels. As the *AFF1* locus was also demonstrated as an eQTL in primary liver cells [33], the cis-regulatory effect may hold in primary cells as well as lymphoblastoid cells used in the present study. However, because the mechanism of transcriptional regulation is substantially different among cell types [34], cell-type specific analyses including those for primary T-cells and B-cells are needed for understanding the precise role of *AFF1* variant in primary lymphocytes. Although further functional investigation is necessary, our observation suggested that *AFF1* is involved in the etiology of SLE through the regulation of development and activity of lymphocytes. It is of note that *AFF3*, which also belongs to the AF4/FMR2 family, is associated with susceptibility to autoimmune diseases [35].

One of our study's limitations is the selection of SNPs for the replication study using the results of the pooled DNA approach [30], which used a different genotyping platform from that of the present GWAS. Moreover, the association signals based on Silhouette scores in pooled analysis would be less reliable compared to those based on individual genotyping. Since direct comparisons of the association signals of the same single SNPs between the studies would be difficult due to these issues, we adopted the complementary approach that referred the association signals of the multiple SNPs in the pooled analysis for each of the single SNPs in the GWAS, taking account of LD and physical distances between the SNPs. However, there would exist a possibility that the variant(s) truly associated with SLE was left not to be examined in the replication study. It should be noted that only 1 SNP among the 8 selected SNPs yielded the significant association with SLE, although further enrichments of the significant associations might be anticipated. To elucidate effectiveness and limitation of our approach, further assessments of the studies on the remaining loci would be desirable. It should also be noted that the control-case ratio of the subjects were relatively high in the replication study 2 (=33.8), and this disproportionate ratio could have induced potential bias on the results of the association analysis of the SNPs. However, considering the homogeneous ancestries of the Japanese population [27] and that principal component analysis did not demonstrate significant population stratification in the control subjects of the replication study 2 (data not shown), the bias owing to population stratification might not be substantial.

In summary, through a GWAS and multi-staged replication studies in a Japanese population integrating eQTL study, our study identified *AFF1* as a novel susceptibility locus for SLE.

Materials and Methods

Subjects

We enrolled 2,278 systemic lupus erythematosus (SLE) cases and 31,948 controls. SLE cases enrolled in the genome-wide association study (GWAS) ($n=891$) or part of the 2nd replication study ($n=83$) were collected from 12 medical institutes in Japan under the support of the autoimmune disease study group of Research in Intractable Diseases, Japanese Ministry of Health, Labor and Welfare: Hokkaido University Graduate School of Medicine, Tohoku University Graduate School of Medicine, the University of Tokyo, Keio University School of Medicine, Juntendo University School of Medicine, University of Occupational and Environmental Health, University of Tsukuba, Tokyo Medical and Dental University, National Center for Global Health and Medicine, Nagasaki University, Wakayama Medical University, and Jichi Medical University. SLE cases ($n=562$) and controls ($n=653$) enrolled in the 1st replication study were collected from Kyushu University. Some of the SLE cases ($n=742$) and controls ($n=27,911$) enrolled in the 2nd replication study were collected from Kyoto University, Tokyo Women's