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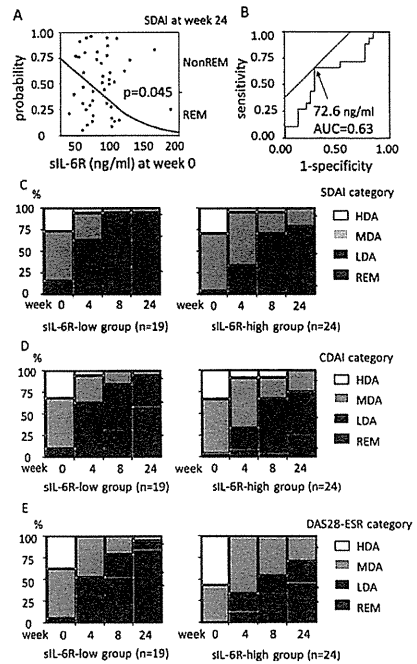

Figure 1 (A) Logistic regression analysis showing the predicted probability of achieving Simplified Disease Activity Index (SDAI) remission at week 24 as a function of sIL-6R at week 0. (B) ROC curve showing a cut-off sIL-6R level of 72.6 ng/mL discriminated between remission and non-remission at week 24, with a sensitivity of 67% and a specificity of 72%. Percentage of patients categorised by SDAI (C), Clinical Disease Activity Index (CDAI) (D), and DAS28-ESR (E) in sIL-6R-low and sIL-6R-high groups at weeks 0, 4, 8, and 24. AUC, area under the curve; CDAI, Clinical Disease Activity Index; DAS28, disease activity score with 28 joint counts; ESR, erythrocyte sedimentation rate; HDA, high disease activity; LDA, low disease activity; MDA, moderate disease activity; REM, remission; ROC, receiver operating characteristic; SDAI, Simplified Disease Activity Index; sIL-6R, soluble interleukin-6 receptor.

week 24 was again significant (84% vs 46%, respectively; p=0.01). Response rate, as assessed by American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) criteria, was numerically better in sIL-6R-low group, although not statistically significant (data not shown).

Baseline levels of sIL-6R predicted clinical remission in RA patients treated with tocilizumab without showing associations with disease activity as shown in previous reports.[6 7] Our results suggest that the amount of target molecule could be considered as one of the predictors when using molecular targeted therapy. This finding could be of help for establishing treatment strategies with tocilizumab, achieving higher remission rate as early as possible along with the treat-to-target recommendation.

Naoshi Nishina,[1] Jun Kikuchi,[1] Misato Hashizume,[2] Keiko Yoshimoto,[1] Hideto Kameda,[1,3] Tsutomu Takeuchi[1]

[1]Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan
[2]Product Research Department, Fuji-Gotemba Research Laboratories, Chugai Pharmaceutical Co., Ltd., Gotemba, Japan
[3]Division of Rheumatology, Department of Internal Medicine, School of Medicine, Toho University, Tokyo, Japan

Correspondence to Dr Naoshi Nishina, Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan; nnishina@a7.keio.jp

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Baseline levels of soluble interleukin-6 receptor predict clinical remission in patients with rheumatoid arthritis treated with tocilizumab: implications for molecular targeted therapy

Interleukin-6 (IL-6) is a monomeric protein that binds to either soluble or membrane-bound IL-6 receptors (IL-6R)[1 2] Tocilizumab, a humanised anti-IL-6R monoclonal antibody, binds to soluble IL-6R (sIL-6R) and membrane-bound IL-6R, blocking signal transduction pathways through competitive inhibition of IL-6 binding.[3] We have previously demonstrated that baseline plasma tumour necrosis factor (TNF) levels are associated with the clinical response to infliximab, anti-TNF monoclonal antibody binding to soluble and membrane-bound TNF.[4] Therefore, it is tempting to speculate that baseline serum levels of sIL-6R, rather than those of IL-6, are associated with clinical response to tocilizumab in patients with rheumatoid arthritis (RA). To test this hypothesis, we analysed serum levels of IL-6 and sIL-6R before tocilizumab treatment in our institution and evaluated their association with clinical remission.

Consecutive patients with RA in our institution who commenced 8 mg/kg tocilizumab treatment every 4 weeks as the first biologic agent between March 2010 and April 2012 were included. At baseline, serum levels of IL-6 and sIL-6R were measured by electrochemiluminescence assay with the Ultra-Sensitive Kit (Meso Scale Discovery, Maryland, USA). In this assay, immunoglobulin inhibiting reagent (Bioreclamation, New York, USA) was used to block heterophilic antibody interference.[5]

The baseline clinical characteristics of the 43 enrolled patients are shown in table 1. Median Simplified Disease Activity Index (SDAI) decreased from 19.78 at baseline to 4.71 at week 24, resulting in SDAI remission in 18 (42%) patients. Median (IQR) IL-6 and sIL-6R at baseline were 4.70 (1.51–8.54) pg/mL and 84.2 (62.2–98.0) ng/mL, respectively. Baseline levels of sIL-6R were not associated with other parameters (data not shown). Univariate logistic regression analysis revealed that the baseline sIL-6R level was an only significant predictor of SDAI remission at week 24 (p=0.045; figure 1A). Multivariate analysis confirmed us that sIL-6R was solely a significant predictor (data not shown). A cut-off sIL-6R level of 72.6 ng/mL discriminated between SDAI remission and non-remission with a sensitivity of 67% and a specificity of 72% (figure 1B), Clinical Disease Activity Index (CDAI) remission with 65% and 69%, and disease activity score with 28 joint counts, erythrocyte sedimentation rate (DAS28-ESR) remission with 59% and 81%, respectively. The numbers of patients with baseline sIL-6R values of ≤72.6 ng/mL (sIL-6R-low) and >72.6 ng/mL (sIL-6R-high) were 19 and 24, respectively. We could not find significant differences in baseline characteristics between the two groups (table 1).

SDAI category changes in sIL-6R-low and sIL-6R-high patients are shown in figure 1C. A significantly higher proportion of patients in sIL-6R-low group achieved SDAI remission compared with those in sIL-6R-high (63% and 25%, respectively; p=0.02 by Fisher's exact test). CDAI and DAS28-ESR category changes are also shown (figure 1D,E). Although the difference in the remission rate of CDAI was marginal between groups (58% in the sIL-6R-low group and 25% in the sIL-6R-high group, respectively; p=0.06), that of DAS28-ESR at

Table 1 Differences in baseline characteristics between sIL-6R-low and sIL-6-high patients

Characteristics	All (n=43)	Baseline sIL-6R		p Value
		sIL-6R-low (n=19)	sIL-6R-high (n=24)	
Age, years	60 (52–67)	57 (45–66)	61 (53–70)	0.33
Gender, female, n (%)	38 (88)	17 (89)	21 (88)	1.00
Disease duration, years	4.5 (2.2–7.9)	5.2 (3.7–7.9)	3 (1.8–8.0)	0.47
Concomitant methotrexate, n (%)	14 (33)	5 (26)	9 (38)	0.52
Methotrexate dose, mg/week	8 (7.1–10)	8 (6–9)	8 (7.75–10)	0.36
Glucocorticoid use, n (%)	14 (33)	5 (26)	9 (38)	0.52
DAS28-ESR	5.1 (4.2–5.9)	4.77 (3.94–6.05)	5.16 (4.46–5.78)	0.51
SDAI	19.78 (14.85–27.99)	19.78 (12.45–27.99)	19.79 (15.32–28.13)	0.59
CDAI	18.3 (13.8–25.2)	18.3 (11.8–26.7)	18.1 (14.2–25.0)	0.73
TJC, 28 joints	5 (3–6)	4 (2–6)	5 (3–7.75)	0.32
SJC, 28 joints	5 (3–8)	5 (3–9)	5 (3–7.5)	0.61
Patient global VAS, mm	45 (23–65)	39 (15–70)	46 (35–64)	0.35
Physician global VAS, mm	41 (26–59)	39 (32–55)	45 (25–60)	0.62
CRP, mg/dL	0.49 (0.13–2.80)	0.45 (0.17–2.21)	0.57 (0.13–3.20)	0.41
ESR, mm/h	46 (19–65)	46 (18–59)	46 (19–69)	0.85
HAQ-DI	1 (0.500–1.250)	1 (0.375–1.500)	1 (0.500–1.125)	0.96
RF value, IU/mL	68 (27–121)	71 (28–106)	65 (17–150)	0.95
Anti-CCP antibody value, IU/mL	141 (17–>300)	94 (19–>300)	238 (16–>300)	0.49
MMP-3 value, ng/mL	104 (60–219)	68 (51.5–260)	113 (60–218)	0.77
IL-6, pg/mL	4.70 (1.51–8.54)	1.82 (1.15–13.2)	5.99 (2.88–8.35)	0.28

Data are shown as median (IQR) or number of patients (%). Statistical differences between median values were analysed by Wilcoxon rank-sum test. Differences in percentage values were analysed by Fisher's exact test.
CCP, cyclic citrullinated peptide; CDAI, clinical disease activity index; CRP, C-reactive protein; DAS28, disease activity score with 28 joint counts; ESR, erythrocyte sedimentation rate; HAQ-DI, health assessment questionnaire–disability index; IL, interleukin; MMP-3 matrix metalloproteinase 3; RF, rheumatoid factor; SDAI, simplified disease activity index; sIL-6R, soluble interleukin-6 receptor; SJC, swollen joint count; TJC, tender joint count; VAS, visual analogue scale.

Baseline tumour necrosis factor alpha levels predict the necessity for dose escalation of infliximab therapy in patients with rheumatoid arthritis

Tsutomu Takeuchi,[1] Nobuyuki Miyasaka,[2] Yoshihiko Tatsuki,[3] Toshiro Yano,[3] Toru Yoshinari,[3] Tohru Abe,[4] Takao Koike[5]

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[1]Division of Rheumatology, Department of Internal Medicine, School of Medicine, Keio University, Shinjuku-ku, Tokyo, Japan
[2]Department of Medicine and Rheumatology, Graduate School of Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan
[3]Mitsubishi Tanabe Pharma Corporation, Osaka, Japan
[4]Saitama Medical Center, Saitama Medical University, Kawagoe, Japan
[5]Department of Medicine II, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Correspondence to
Tsutomu Takeuchi, Division of Rheumatology, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan; tsutake@z5.keio.jp

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ABSTRACT

Objectives To investigate the possible role of baseline plasma tumour necrosis factor alpha levels (baseline-TNF) on the clinical response to infliximab in patients with rheumatoid arthritis (RA).

Methods Patients with RA refractory to methotrexate received 3, 6, or 10 mg/kg of infliximab every 8 weeks, in a randomised, double-blind manner: the RISING study. Clinical response (disease activity score in 28 joints based on C-reactive protein or American College of Rheumatology core set) at week 54 and serum infliximab levels were compared in three patient groups with low, intermediate, or high baseline-TNF (TNF-low, TNF-int, or TNF-high).

Results In TNF-low patients, the clinical response to different doses of infliximab was comparable, whereas TNF-int patients exhibited a dose-dependent trend. In contrast, TNF-high patients (approximately 13% of the total patients) had a clinical response to 10 mg/kg significantly better than the response to 3 and 6 mg/kg of infliximab. In TNF-high patients, the median trough serum levels of infliximab were below the detection limit (<0.1 μg/ml) at 3 and 6 mg/kg but were greater than 2 μg/ml at 10 mg/kg, whereas the levels were approximately 1 μg/ml for each dosage group in TNF-low patients.

Conclusion In patients with RA, baseline-TNF is significantly associated with the clinical response to infliximab in patients with a high baseline-TNF. A higher dose of infliximab may be necessary in these patients, whereas lower doses of infliximab are sufficient for those with a low baseline-TNF. Baseline-TNF may be a useful measure for personalising the treatment of RA using infliximab.

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease that results in joint destruction and disability.[1] Levels of tumour necrosis factor alpha (TNF), an inflammatory cytokine, are elevated in the blood and synovial fluid of patients with RA, and may play a central role in its pathogenesis.[2–7]

Although infliximab, an anti-TNF antibody, exhibits excellent effectiveness in RA,[8–11] insufficient response to the standard treatment of infliximab (3 mg/kg per 8 weeks) has also been observed in some cases in clinical practice. Such patients are usually treated by dose escalation or by shortening the dose interval of the infliximab therapy.[12–15]

The RISING study (NCT00691028) is a randomised, double-blind clinical trial, which has shown that the clinical response to infliximab at a dose of 10 mg/kg is significantly higher than the response to 3 mg/kg infliximab, and that a trough serum level of 1 μg/ml is the threshold for clinical response.[16] However, clinical response to different dose levels of infliximab was significant only for American College of Rheumatology (ACR) improvement criteria and the European League Against Rheumatism (EULAR) responses, and the measurable difference was small.

Several clinical studies have attempted to address whether a higher dose of infliximab provides a better clinical response than standard doses in patients with RA.[8–11 17] However, the results were not consistent among those studies. In addition, a randomised, double-blind study comparing dose escalation and continuation of the standard dose in patients with RA who had an insufficient response to 3 mg/kg of infliximab clearly demonstrated no beneficial response by dose escalation,[18] contrary to our results.

Although the standard dose of infliximab can be efficacious in a large proportion of patients with RA, some patients may require a higher dose of infliximab to achieve clinical response.[19 20] The inconsistent results mentioned above might be explained by the different proportion of patients who might benefit from infliximab dose escalation in each study. Thus the clinical and immunological features of these patients who require higher dose of infliximab are not fully understood.

It is tempting to speculate that the production and resultant plasma levels of TNF, the target molecule of infliximab, exceeds the neutralising capacity of infliximab in insufficient responders who are unable to maintain the threshold serum level of infliximab. Considering that hypothesis, we analysed the RISING study data based on plasma TNF levels. We found that the clinical response of patients with high baseline levels of TNF (baseline-TNF) showed a significant improvement with higher doses of infliximab, whereas patients with low baseline-TNF did not have a better response even with higher doses of infliximab.

METHODS

Patients and study protocol

The study protocol was approved by the local institutional review board and was carried out in accordance with the Helsinki Declaration and good clinical practice. Patient enrollment criteria and study design have previously been described in detail.[16] In the RISING study, 327 patients with active RA, despite receiving previous treatment with methotrexate, were administered 3 mg/kg infliximab at weeks 0, 2 and 6 with methotrexate

(open-label period weeks 0–14). Patients were randomly assigned into three groups using a dynamic allocation procedure based on the clinical response at week 10 and were treated with 3 (n=99), 6 (n=104), or 10 (n=104) mg/kg infliximab, every 8 weeks from weeks 14 to 46, with methotrexate (blinded period weeks 14–54).

Laboratory test values and serum infliximab measurement

Plasma samples for the evaluation of baseline-TNF were collected just before the first infusion of infliximab (week 0). The plasma was separated from EDTA-supplemented blood and was stored at −20 to −80°C. Baseline-TNF levels were measured by ELISA using the QantiGlo ELISA Kit (QTA00B; R&D Systems Inc, Minneapolis, Minnesota, USA) in Mitsubishi Chemical Medience Corporation (Tokyo, Japan). Recombinant human TNF included in the kit was used as reference. Mitsubishi Chemical Medience Corporation has made minor modifications on the assay procedure of the ELISA kit (http://www.rndsystems.com/pdf/qta00b.pdf) and has validated their detection limit. Since the mean relative light units (2 SD) at blank and 0.55 pg/ml were 15.2 (3.4) and 25.2 (5.0) by their modified procedure, respectively, the detection limit was determined as 0.55 pg/ml by adding 2 SD to the relative light units. The coefficient of variation or relative error values of intra-assay or inter-assay TNF were within 3.6% or 6.2%, respectively. They confirmed that freezing and thawing the plasma sample 10 times and storing at −20°C for 22 weeks did not affect the stability of TNF in the plasma.

Sera were collected at weeks 0 and 2 and every 4 weeks thereafter. The serum infliximab level was measured by ELISA using the anti-infliximab monoclonal antibody obtained from Centocor Ortho Biotech Inc. (Horsham, Pennsylvania, USA).[5] Serum infliximab levels below 0.1 μg/ml were undetectable.

Baseline rheumatoid factor (RF) was measured by the latex agglutination test. Antibodies against cyclic citrullinated peptides (CCP) and matrix metalloproteinase-3 (MMP-3) levels were measured by ELISA. The detectable ranges were as follows: RF, 3 IU/ml or greater; anti-CCP antibodies, 0.6 IU/ml or greater to 100 IU/ml or less; and MMP-3, 12.5 ng/ml or greater.

Evaluation of efficacy

The clinical response to infliximab was evaluated using EULAR criteria, and disease activity was assessed using the disease activity score in 28 joints (DAS28) based on C-reactive protein (CRP) and the ACR core set[21] at week 54. The EULAR response was evaluated using the DAS28 based on the erythrocyte sedimentation rate (ESR) assessment method (http://www.reuma-nijmegen.nl/www.das-score.nl/index.html). The cut-off values for DAS28–CRP were as follows, based on a report from Inoue *et al*.[22]: DAS28–CRP remission, less than 2.3; low disease activity (LDA), 2.3 or greater to less than 2.7; moderate disease activity (MDA), 2.7 or greater to 4.1 or less; high disease activity (HDA), greater than 4.1. Joint damage was assessed by the total modified Sharp score (0–390 points).[23]

Analytical method

The clinical responses to infliximab at week 54 were analysed for the full analysis set using the last observation carried forward approach. For the comparison of clinical response among dose groups (3, 6 and 10 mg/kg), logistic regression analysis was performed using the clinical responses at the time of randomisation (week 10) as a covariate.

Because there is no well-established threshold value for an abnormal TNF level in the ELISA kit used, the detection limit, less than 0.55 pg/ml, and a threefold higher level, 1.65 pg/ml, were adopted as cut-off values, and the patients were stratified into the following three patient groups for analysis: low, less than 0.55 pg/ml (TNF-low); intermediate, 0.55 pg/ml or greater to less than 1.65 pg/ml (TNF-int); and high, 1.65 pg/ml or greater (TNF-high).

The association of baseline-TNF with baseline disease activity and the efficacy of infliximab therapy was analysed for the three groups described above (TNF-low, TNF-int and TNF-high patients) employing the Kendall rank correlation coefficient. Baseline disease activity was analysed for the enrolled patients (n=327). Clinical responses (DAS28–CRP or ACR core set) and efficacy on physical function (improved health assessment questionnaire (HAQ) score) at week 54 were analysed using the last observation carried forward approach (n=307). Radiographic progression was analysed in the patients whose radiographic data were obtained at week 54 (n=273). The association between the serum infliximab level and clinical response or disease activity at week 54 was analysed in the patients for whom both serum levels and DAS28–CRP values were obtained at week 54 (n=271).

Primary non-responders were defined as patients who had not achieved LDA throughout the study period (at weeks 2–54), and secondary non-responders as those who had not achieved LDA at week 54 but had achieved it at least once between weeks 2 and 50. Kruskal–Wallis test analysis was performed to compare the rates of TNF-low, TNF-int and TNF-high patients in primary and secondary non-responders versus responders (those who had achieved LDA at week 54).

RESULTS

Patients' background and association between baseline-TNF and parameters of disease activity at baseline

Table 1 shows the baseline demographics and clinical characteristics of the enrolled patients (n=327). The median (IQR), minimum and maximum baseline-TNF values were 0.92 pg/ml (<0.55–1.29 pg/ml), less than 0.55 pg/ml and 9.68 pg/ml, respectively. The proportion of patients with baseline-TNF values less than 0.55 pg/ml (undetectable), 0.55 pg/ml or greater to less than 1.65 pg/ml, and 1.65 pg/ml or greater (TNF-low, TNF-int and TNF-high patients) was 28.7%, 58.4% and 12.8%, respectively. Details on the distribution of baseline-TNF can be found in supplementary figure 1 (available online only). Baseline-TNF was significantly, but slightly, correlated with most parameters of disease activity and HAQ score, but not with radiographic progression (table 2). The significant correlations of baseline-TNF with RF and anti-CCP antibodies were also observed.

Clinical response to infliximab at week 54

Of the enrolled patients, 307 were treated with 3, 6 or 10 mg/kg infliximab during the blinded period, and clinical response was evaluated at week 54 using DAS28–CRP (figure 1). The clinical responses at week 54 were significantly higher in the 10 mg/kg group than in the 3 mg/kg group, and 45.2% of the patients in the 10 mg/kg group achieved DAS28–CRP remission.

Association between baseline-TNF and the efficacy of infliximab therapy at week 54

The EULAR responses and disease activities at week 54 in TNF-low (n=87), TNF-int (n=180), and TNF-high (n=40) patients are shown in figure 2A,B. In TNF-low patients, the clinical response was similar among the 3, 6 and 10 mg/kg groups, showing no dose dependency. In TNF-int patients, the EULAR response rate increased and disease activity decreased as the dose increased, but without significant difference. For TNF-high patients, the clinical response and disease activity were significantly better at 10 mg/kg than at 3 mg/kg and 6 mg/kg. The good response rates were 14%, 31% and 60% in the 3, 6 and 10 mg/kg groups, respectively, and the DAS28–CRP remission rates were 7%, 31% and 50%, respectively. A similar influence by baseline-TNF was also noted on the ACR core set (figure 2C). Significant dose dependency was observed only in TNF-high patients.

Baseline-TNF at clinical response is shown in supplementary table 1 (available online only). Clinical response was significantly influenced by baseline-TNF in the 3 mg/kg group, but not in the 6 and 10 mg/kg groups. Baseline-TNF also influenced the improving physical function, in which the improvement in the HAQ score values and the rates of patients with meaningful improvement (>0.22) in all dosage groups were significantly different in TNF-high patients (supplementary table 2, available online only). Meanwhile, none of the groups showed significant association with radiographic progression.

The proportions of TNF-low, TNF-int and TNF-high patients in responders (n=148) were 34.5%, 56.8% and 8.8%, respectively. On the other hand, those of primary non-responders (n=88) were 21.6%, 62.5% and 15.9%, and those of secondary non-responders (n=71) were 23.9%, 57.7% and 18.3%, respectively. Significant differences were observed between the responders and both of the non-responders (p=0.016 and p=0.032, respectively).

Table 1 Baseline demographics and clinical characteristics of patients enrolled in the RISING study (n=327)

	Baseline value
Age, year	49.9 (12.0)
Gender, female	267 (81.7)*
Disease duration, years	8.2 (8.0)
Steinbrocker stage	
I	32 (9.8)*
II	114 (34.9)*
III	105 (32.1)*
IV	76 (23.2)*
Steinbrocker functional class	
1	58 (17.7)*
2	239 (73.1)*
3	30 (9.2)*
4	0 (0.0)*
Duration of methotrexate use, years	2.7 (2.8)
Methotrexate dose, mg/week	7.8 (1.7)
Glucocorticoid use	223 (68.2)*
DAS28–CRP	5.5 (4.9, 6.2)†
DAS28–ESR	6.2 (5.6, 6.8)†
TJC, 68 joints	15.0 (10.0, 23.0)†
SJC, 66 joints	12.0 (9.0, 17.0)†
CRP, mg/dl	2.4 (1.2, 4.1)†
ESR, mm/h	52 (35, 72)†
Patient pain VAS, mm	54 (39, 72)†
Patient global VAS, mm	54 (36, 72)†
Physician global VAS, mm	64 (50, 78)†
TSS‡	35.5 (11.5, 73.4)†
Estimated yearly progression of TSS, point/year‡	6.1 (3.1, 11.4)†
HAQ	1.1 (0.8, 1.6)†
RF value, IU/ml	90 (36, 223)†
Anti-CCP antibodies value, IU/ml	≥100 (28, ≥100)†
MMP-3 value, ng/ml	219 (105, 419)†

Data in baseline values are mean (SD), *number of patients (%), or †median (IQR).
Health assessment questionnaire (HAQ) score: scores can range from 0 (no difficulty) to 3 (unable to perform this activity).
Total modified Sharp score (TSS): scores can range from 0 to 390 (erosion score 0–230, joint space narrowing score 0–160), with high scores indicating more joint damage.
‡Estimated yearly rate of progression of the TSS was based on the duration of disease and baseline TSS for every patient.
§n=325.
CCP, cyclic citrullinated peptide; CRP, C-reactive protein; DAS 28, disease activity score in 28 joints; ESR, erythrocyte sedimentation rate; MMP-3, matrix metalloproteinase 3; RF, rheumatoid factor; SJC, swollen joint count; TJC, tender joint count; VAS, visual analogue scale.

Association between baseline-TNF and serum infliximab level

Clinical response to infliximab has been shown to be influenced by trough serum infliximab levels, and the threshold level for clinical response is approximately 1 μg/ml.[20 24] In this study, the median (IQR) serum infliximab level at week 54 in patients with EULAR good response, moderate response and no response was 3.1 (1.2–7.1), 1.2 (<0.1–3.6) and less than 0.1 (<0.1–0.4) μg/ml. The median (IQR) level in patients in DAS28–CRP remission, with LDA, with MDA and with HDA at week 54 was 3.4 (1.5–7.5), 2.1 (0.4–4.7), 1.4 (<0.1–4.0) and less than 0.1 (<0.1–0.7) μg/ml, respectively. A significant association was observed between clinical response or disease activity and the serum infliximab level (both p<0.001). In patients with high trough serum infliximab levels, better clinical responses were attained. Meanwhile, in patients with poor clinical response (EULAR no response, high disease activity), the median trough serum level was undetectable (<0.1 μg/ml).

Baseline-TNF greatly affected the serum infliximab levels (figure 3A–C). In TNF-low patients, the median trough serum infliximab levels were equivalent to or greater than the threshold level (1 μg/ml) in all groups (3, 6 and 10 mg/kg). For TNF-int patients, the 3 mg/kg dose did not achieve the threshold level of infliximab but did provide a detectable level (≥0.1 μg/ml). However, in TNF-high patients, the median trough serum infliximab levels were undetectable at both 3 and 6 mg/kg doses, in contrast to the 10 mg/kg dose, at which a high level (>2 μg/ml) was maintained.

The positive rates for anti-infliximab antibodies (human anti-chimeric antibodies; HACA), which is reported to influence the serum infliximab level[25] were 27.3%, 23.1% and 12.5% in the 3, 6 and 10 mg/kg dose levels, respectively. In patients who were negative for HACA, the serum infliximab level was also lower in TNF-high patients than in TNF-low patients, and the median serum infliximab levels at the 3 mg/kg dose at week 54 in TNF-low patients (n=23), TNF-int patients (n=37) and TNF-high patients (n=8) were 1.0, 0.6 and 0.2 μg/ml, respectively.

Association between safety and baseline-TNF or serum infliximab level

The incidences of adverse events (total, serious events, infection, serious infection, or infusion reaction) were almost comparable among patients receiving 3, 6 and 10 mg/kg doses in TNF-low, TNF-int and TNF-high patients. We could find no clear association between the incidence of adverse events and the trough serum level of infliximab either (supplementary table 3, available online only).

DISCUSSION

Although dose escalation or shortening of the administration interval between administrations has been used for patients with RA who show an insufficient response to the standard dose of infliximab (3 mg/kg for 8 weeks after initial induction), the effectiveness of these countermeasures is still controversial.[18–20 26–32] In this report, we hypothesised that the production and resultant plasma level of TNF exceeds the neutralising capacity of infliximab in insufficient responders; and that infliximab is consumed

Table 2 Baseline disease activities in TNF-low, TNF-int and TNF-high patients

	Baseline-TNF			
	TNF-low (n=94)	TNF-int (n=191)	TNF-high (n=42)	p Value (overall)
DAS28-CRP	5.3 (4.9, 5.9)	5.5 (5.0, 6.2)	5.9 (5.2, 6.7)	0.002
DAS28-ESR	5.9 (5.5, 6.4)	6.2 (5.7, 6.8)	6.8 (6.2, 7.3)	<0.001
TJC, 68 joints	14.0 (10.0, 21.0)	15.0 (11.0, 23.0)	16.5 (12.0, 23.0)	0.153
SJC, 66 joints	11.0 (8.0, 16.0)	13.0 (9.0, 17.0)	12.5 (10.0, 18.0)	0.041
Patient pain VAS, mm	49.0 (37.0, 65.0)	58.0 (40.0, 72.0)	68.5 (46.0, 88.0)	<0.001
Patient global VAS, mm	51.0 (36.0, 64.0)	56.0 (36.0, 72.0)	65.0 (43.0, 86.0)	0.016
Physician global VAS, mm	62.0 (50.0, 75.0)	65.0 (49.0, 77.0)	72.0 (51.0, 82.0)	0.081
CRP (mg/dl)	2.0 (1.0, 3.9)	2.4 (1.2, 3.9)	2.8 (1.4, 5.8)	0.028
ESR (mm/h)	46.0 (33.0, 65.0)	53.0 (35.0, 72.0)	67.5 (40.0, 89.0)	0.001
HAQ	1.0 (0.5, 1.4)	1.1 (0.9, 1.6)	1.4 (1.0, 2.1)	0.002
TSS	37.0 (10.5, 76.0)*	36.0 (12.5, 72.3)†	22.5 (13.0, 56.0)	0.960
Estimated yearly progression of TSS, point/year	5.8 (2.8, 12.0)*	6.3 (3.4, 11.3)†	5.5 (2.5, 10.0)	0.833
RF value, IU/ml	85 (27, 215)	81 (37, 167)	238 (126, 554)	0.007
Anti-CCP antibodies value, IU/ml	91 (17, ≥100)	≥100 (30, ≥100)	≥100 (≥100, ≥100)	0.012
MMP-3 value, ng/ml	205 (105, 377)	216 (102, 391)	268 (114, 640)	0.214

Data in baseline value are median (IQR).
Baseline disease activities were evaluated in the patients enrolled in the RISING study (n=327). The associations of baseline-tumour necrosis factor alpha (TNF) with baseline disease activities were analysed in the three groups (TNF-low, TNF-int and TNF-high patients) employing the Kendall rank correlation coefficient.
*n=93.
†n=190.
CCP, cyclic citrullinated peptide; CRP, C-reactive protein; DAS28, disease activity score in 28 joints; ESR, erythrocyte sedimentation rate; HAQ, health assessment questionnaire; MMP-3, matrix metalloproteinase 3; RF, rheumatoid factor; SJC, swollen joint count; TJC, tender joint count; TSS, total modified Sharp score; VAS, visual analogue scale.

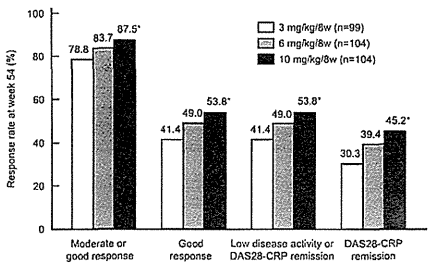

Figure 1 Clinical responses to infliximab therapy in patients with rheumatoid arthritis at week 54. The European League Against Rheumatism response criteria and disease activity were measured by the disease activity score in 28 joints (DAS28)–C-reactive protein (CRP) using the last observation carried forward method. Disease activity was defined as high disease activity, DAS28–CRP greater than 4.1; moderate disease activity, 2.7 or greater to 4.1 or less; low disease activity, 2.3 or greater to less than 2.7; and DAS28–CRP remission, less than 2.3. *p<0.05 versus the 3 mg/kg groups.

to neutralise the TNF, decreasing the serum infliximab level in those patients.

In our study, clinical responses at doses of 3, 6 and 10 mg/kg of infliximab were comparable in TNF-low patients, and the minimum serum infliximab level required for clinical response (approximately 1 μg/ml) was maintained, even in the 3 mg/kg group. Accordingly, the maximal clinical responses can be achieved even with 3 mg/kg therapy in TNF-low patients. In other words, dose escalation does not provide any benefit in this population. In contrast, in TNF-high patients, clinical responses at 3 and 6 mg/kg were significantly decreased in comparison with the response to a 10 mg/kg dose. The trough serum infliximab levels were 'undetectable' at 3 and 6 mg/kg but were greater than 2 μg/ml with a 10 mg/kg dose. As the association between baseline-TNF and the serum infliximab level was also observed in HACA-negative patients, this association was not reflected by the presence of HACA status.

It has been suggested that the threshold serum level for infliximab efficacy on radiographic progression might be lower than that for clinical response.[53] In this study, approximately 90% of the patients whose serum level could be maintained over the threshold for radiographic progression, including even TNF-high patients, showed no radiographic progression. It may explain why baseline-TNF status had no influence on radiographic progression as observed in this study.

The present report has demonstrated that higher doses of infliximab exhibited significantly better clinical and functional response in patients with high baseline-TNF, whereas clinical and functional response was comparable among patients administered 3, 6 and 10 mg/kg of infliximab with low baseline-TNF.

We had reported previously that there was no difference in the safety profile among groups receiving 3, 6 or 10 mg/kg.[16] The rates of adverse events were almost comparable among all dosage groups in TNF-low, TNF-int and TNF-high patients. However, it has been reported that high-dose infliximab therapy might increase the risk of adverse events.[11 54 55] So, the balance between risk and benefit should be considered at the time of infliximab dose escalation, even in TNF-high patients.

There have been several reports on the association between TNF level and the efficacy of infliximab.[56–59] Although the reason for the inconsistent results is unclear, the serum TNF level was measured by bioassay in the report by Marotte *et al*,[57] which is different from this study, in which ELISA was employed. In the studies by Wijbrandts *et al*,[56] clinical response was evaluated at week 16, 2 weeks after infliximab administration when a high serum infliximab level may have been maintained even in patients with a high baseline-TNF. These differences may have led to the different conclusions.

In the RISING study, baseline-TNF was measured in one institution (Mitsubishi Chemical Medience Corporation) concurrently, where minor modifications were made in the original assay procedure and validated for the commercial use of clinical samples.

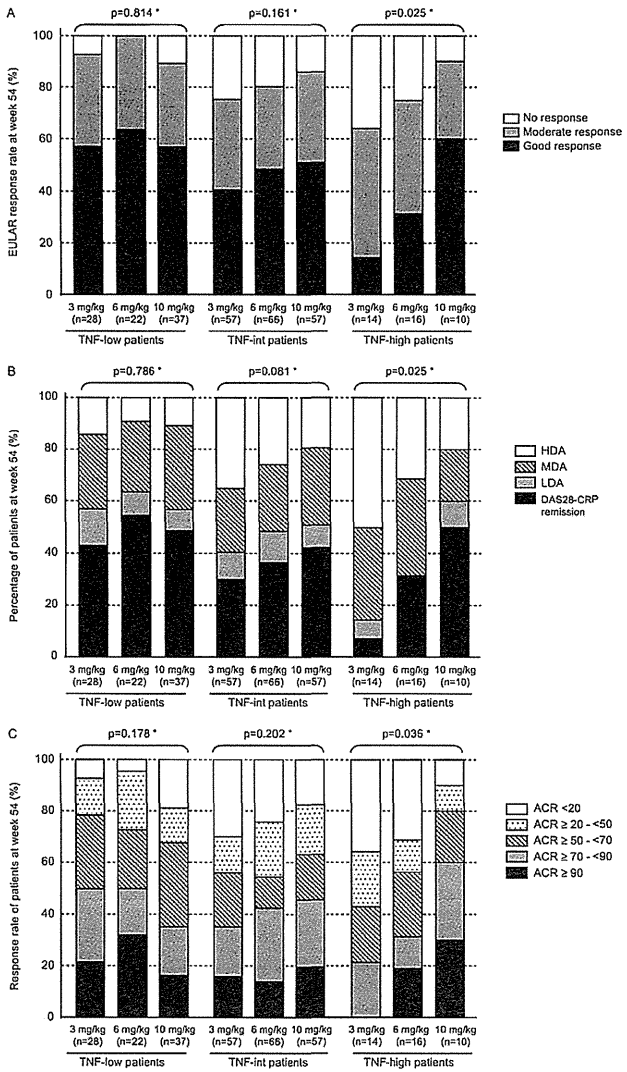

Figure 2 Correlation of plasma tumour necrosis factor alpha (TNF) levels before infliximab therapy (baseline-TNF) with (A) the European League Against Rheumatism (EULAR) response, (B) the disease activity score in 28 joints (DAS28) and (C) American College of Rheumatology (ACR) core set at week 54. Patients were divided into three patients groups by the baseline-TNF level as follows: TNF-low patients, less than 0.55 pg/ml; TNF-int patients, 0.55 pg/ml or greater to less than 1.65 pg/ml; and TNF-high patients, 1.65 pg/ml or greater. EULAR response criteria for disease activity were measured by DAS28–C-reactive protein (CRP). Disease activity was defined as high disease activity (HDA), DAS28–CRP greater than 4.1; moderate disease activity (MDA), 2.7 or greater to 4.1 or less; low disease activity (LDA), 2.3 or greater to less than 2.7; and DAS28–CRP remission, less than 2.3. All clinical responses were evaluated by using the last observation carried forward method. *Kendall rank correlation coefficient.

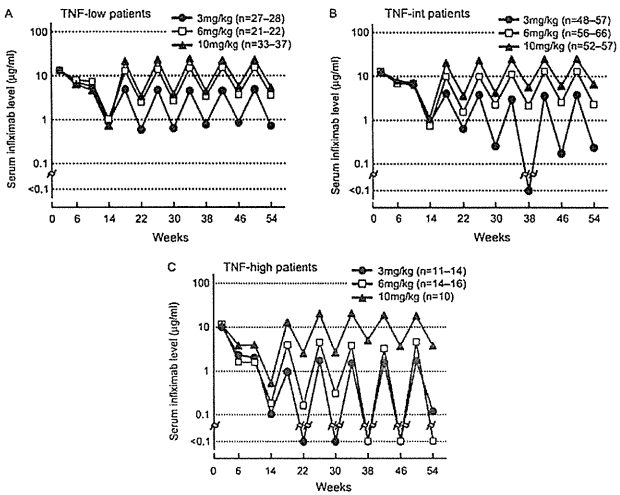

Figure 3 Serum infliximab levels in (A) tumour necrosis factor alpha (TNF)-low, (B) TNF-int and (C) TNF-high patients. Patients were divided into three groups by the plasma TNF level before infliximab therapy (baseline-TNF) as follows: TNF-low patients, less than 0.55 pg/ml; TNF-int patients, 0.55 pg/ml or greater to less than 1.65 pg/ml; and TNF-high patients, 1.65 pg/ml or greater. Serum infliximab levels were measured by ELISA.

It was reported that RF in the samples may cause false-positive results in immunoassays by cross-linking the capture and detection antibodies.[40] In our study, a significant correlation was observed between TNF and RF at baseline (τ value 0.129, p<0.001). However, the maximum baseline RF level was 1560 IU/ml in patients with undetectable baseline-TNF (supplementary figure 2, available online only). Therefore, we considered that RF might interfere with the assay of TNF in patients with greater than 1560 IU/ml, but not in patients with less than 1560 IU/ml. Furthermore, we freshly collected the plasma samples from 39 patients with RA (in which backgrounds were shown in supplementary table 4, available online only), and carried out several tests to evaluate the effects of RF on the ELISA for TNF: 'RF-blocking test', using 40% mouse serum, 20% goat serum and 20% rabbit serum;[41 42] 'IgM-RF adding test', using purified polyclonal IgM–RF (Interference Check RF Plus; Sysmex Corporation, Kobe, Japan); 'mismatch simplex sandwich test',[42] using anti-interleukin 6 antibodies conjugated to horseradish peroxidase from the QantiGlo ELISA Kit for interleukin 6 (Q6000B; R&D Systems Inc) as the detection antibodies. As we did not find a clear signal indicating that RF interacted with our ELISA for TNF, we considered that the TNF levels in our study were not influenced by RF (supplementary tables 5–7, available online only).

The circulating TNF level in patients with RA varied from several pg/ml to greater than 100 pg/ml in different reports.[43–46] Although the patient background should be considered in determining the cause of the variation, the measurement systems used for detecting circulating TNF levels in individual studies may largely account for the variation. In that regard, the cut-off values for baseline-TNF used in this report (0.55 and 1.65 pg/ml) were not absolute, and the titre should be standardised in the future.

So far, some of the patient backgrounds have been investigated for predictors of their clinical response to infliximab. Baseline CRP was reported to be associated with the serum infliximab level,[47] but no consensus has been obtained about the correlation between baseline CRP and clinical response.[36 48] We investigated the influence of baseline CRP on the clinical response as an index of EULAR response as done by baseline-TNF. However, we could find no clear relationship between them (supplementary figure 3A,B, available online only). Given that TNF is one of the key cytokines that induce CRP in various inflammatory diseases such as RA, it is reasonable that baseline-TNF, rather than CRP, would be a good predictor of clinical response to the TNF inhibitor, infliximab.

Many contradictory reports have been published regarding the effectiveness of dose escalation with infliximab.[16–20 26–32] The reason why no consensus has been reached is that dose escalation is significant only in TNF-high patients, so the results of reports have been influenced by the number of patients with high baseline-TNF included in those studies. In the RISING study, TNF-high patients accounted for approximately 13% of the total.

This study may provide insight into our understanding of the relationship between the concentration of circulating cytokines and the dose of antibodies against the cytokines in the treatment of RA. As mentioned by van Vollenhoven,[26] an attempt to apply results obtained under highly controlled circumstances at the group level to the unique individual is necessary. These results may be useful for establishing treatment strategies, such as the appropriate dose of infliximab in accordance with baseline-TNF in patients with RA, not only in daily clinical practice but also in relation to the economics of health care.

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Competing interests TT and NM have received research support and consulting or lecture fees from Mitsubishi Tanabe Pharma Corporation. YT, TY and TY are employees of Mitsubishi Tanabe Pharma Corporation. TA and TK have received consulting and lecture fees from Mitsubishi Tanabe Pharma Corporation.

Patient consent Obtained.

Ethics approval The study protocol was approved by the local institutional review board and was carried out in accordance with the Helsinki Declaration and good clinical practice.

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The Multicenter Study of a New Assay for Simultaneous Detection of Multiple Anti-Aminoacyl-tRNA Synthetases in Myositis and Interstitial Pneumonia

Ran Nakashima[1☯], Yoshitaka Imura[1☯], Yuji Hosono[1], Minae Seto[2], Akihiro Murakami[2], Kizuku Watanabe[3], Tomohiro Handa[3], Michiaki Mishima[3], Michito Hirakata[4], Tsutomu Takeuchi[5], Keishi Fujio[6], Kazuhiko Yamamoto[6], Hitoshi Kohsaka[7], Yoshinari Takasaki[8], Noriyuki Enomoto[9], Takafumi Suda[9], Kingo Chida[9], Shu Hisata[10], Toshihiro Nukiwa[10], Tsuneyo Mimori[1]*

1 Department of Rheumatology and Clinical Immunology, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **2** Medical & Biological Laboratories Co., Ltd., Nagoya, Japan, **3** Department of Respiratory Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **4** Medical Education Center/Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan, **5** Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan, **6** Department of Allergy and Rheumatology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan, **7** Department of Medicine and Rheumatology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan, **8** Department of Internal Medicine and Rheumatology, Juntendo University School of Medicine, Tokyo, Japan, **9** Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine, Hamamatsu, Japan, **10** Department of Respiratory Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan

Abstract

Objective: Autoantibodies to aminoacyl-tRNA synthetases (ARSs) are useful in the diagnosis of idiopathic inflammatory myopathy (IIM) with interstitial pneumonia (IP). We developed an enzyme-linked immunosorbent assay (ELISA) system using a mixture of recombinant ARS antigens and tested its utility in a multicenter study. **Methods:** We prepared six recombinant ARSs: GST-Jo-1, His-PL-12, His-EJ and GST-KS expressed in *Escherichia coli*, and His-PL-7 and His-OJ expressed in Hi-5 cells. After confirming their antigenic activity, with the exception of His-OJ, we developed our ELISA system in which the five recombinant ARSs (without His-OJ) were mixed. Efficiency was confirmed using the sera from 526 Japanese patients with connective tissue disease (CTD) (IIM n = 250, systemic lupus erythematosus n = 91, systemic sclerosis n = 70, rheumatoid arthritis n = 75, Sjögren's syndrome n = 27 and other diseases n = 13), 168 with idiopathic interstitial pneumonia (IIP) and 30 healthy controls collected from eight institutes. IIPs were classified into two groups; idiopathic pulmonary fibrosis (IPF) (n = 38) and non-IPF (n = 130). Results were compared with those of RNA immunoprecipitation. **Results:** Sensitivity and specificity of the ELISA were 97.1% and 99.8%, respectively when compared with the RNA immunoprecipitation assay. Anti-ARS antibodies were detected in 30.8% of IIM, 2.5% of non-myositis CTD, and 10.7% of IIP (5.3% of IPF and 12.3% of non-IPF). Anti-ARS-positive non-IPF patients were younger and more frequently treated with glucocorticoids and/or immunosuppressants than anti-ARS-negative patients. **Conclusion:** A newly established ELISA detected anti-ARS antibodies as efficiently as RNA immunoprecipitation. This system will enable easier and wider use in the detection of anti-ARS antibodies in patients with IIM and IIP.

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Competing Interests: Murakami M. and Seto A. are the employees of Medical and Biological Laboratory Co., Ltd. (MBL). This study was performed under collaboration between the Aauthor 's' institutes and MBL. The all authors have declared that they have no other conflicts of interest.

* E-mail: mimorit@kuhp.kyoto-u.ac.jp

☯ These authors contributed equally to this work.

Introduction

A number of autoantibodies can be detected in sera from patients with idiopathic inflammatory myopathy (IIM), some of which are specific to IIM (known as myositis-specific autoantibodies: MSAs). Detection of these autoantibodies is closely associated with IIM clinical manifestations [1,2].

Among MSAs, autoantibodies against aminoacyl-tRNA synthetases (ARSs) are the most frequently detected in adult IIM patients. To date, eight anti-ARS antibodies have been described. Anti-Jo-1 (histidyl-tRNA synthetase) [3,4] is the most common, occurring in approximately 20% of IIM patients [2,5]. Anti-PL-7 (threonyl) [6], anti-PL-12 (alanyl) [7,8], and anti-EJ (glycyl) [9] occur in ~3–4%, and anti-OJ (isoleucyl) [10] and anti-KS (asparaginyl) [11] occur in < 2% of IIM patients. Anti-tyrosyl- and anti-phenylalanyl-tRNA synthetases were also reported in one case each [12,13]. Patients with anti-ARSs show a spectrum of common clinical manifestations known as anti-synthetase syndrome (ASS), including myositis, interstitial pneumonia (IP), non-erosive arthritis, fever, Raynaud's phenomenon, and mechanic's

hands. Of note, the prevalence of IP in anti-ARS-positive patients is as high as 75–95% and IP sometimes precedes myositis [1,14,15]. Yoshifuji *et al.* reported that anti-ARS-positive patients with IP respond better to initial corticosteroid therapy but suffer from a significantly higher recurrence than anti-ARS-negative patients [1]. Therefore, anti-ARS antibodies are useful not only in diagnosing IIM but also in predicting late-onset myopathy in IP-proceeding patients and the clinical course of IP in myositis.

Currently, anti-ARS antibodies are detected using an enzyme-linked immunosorbent assay (ELISA), immunodiffusion or immunoprecipitation, but all of the antibodies are not routinely detected except for anti-Jo-1. To detect anti-ARS antibodies more readily, we established an ELISA system using a mixture of five recombinant ARS antigens: Jo-1, PL-7, PL-12, EJ, and KS. Our intention was to detect these autoantibodies simultaneously as "multiple anti-ARS antibodies". This ELISA system that we developed could be used to detect not only anti-ARS-positive myositis patients but also anti-ARS-positive idiopathic interstitial pneumonia (IIP) patients.

Materials and Methods

Patients

Serum samples were obtained from 694 Japanese adult patients with connective tissue disease (CTD) and IIP who had been followed at eight University Hospitals in Japan and 30 healthy volunteers. Patient diagnoses included IIM (n = 250), systemic lupus erythematosus (SLE) (n = 91), systemic sclerosis (SSc) (n = 70), rheumatoid arthritis (RA) (n = 75), SS (n = 27), other diseases (n = 13), and IIP (n = 168). The diagnoses of IIM, SSc, SLE, and RA were made on the basis of corresponding criteria proposed by Bohan and Peter [16] or the American College of Rheumatology [17,18,19]. IIP was defined as IP of unknown cause in which a patient did not fulfill classification criteria for any specific CTD or vasculitis, or whose lung disease was potentially caused by a drug or occupational-environmental exposure [20]. Patients with IIP were classified into two groups; an idiopathic pulmonary fibrosis (IPF) (n = 38; 12 by histological diagnosis) group and a non-IPF (n = 130; according to the typical radiographic patterns of chest high-resolution computed tomography) group.

All patients and healthy volunteers gave their written informed consent to participate in this study prior to sample collection that was performed in accordance with the Declaration of Helsinki. This study was approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine (Approval number: E544) and also by institutional review boards of all participating centers (Table S1).

Immunoprecipitation

The presence of anti-ARS antibodies was determined by RNA immunoprecipitation (RNA-IP) as previously described [21]. The immunoprecipitated RNA was resolved using urea-polyacrylamide gel electrophoresis and visualized using silver staining. Each anti-ARS antibody was identified according to its mobility and tRNA pattern compared with standard serum.

Construction of expression plasmids for ARS-encoding cDNAs

For the expression and purification of recombinant proteins, full-length cDNAs of PL-12, EJ, PL-7, Jo-1, KS, and OJ (GenBank accession Numbers: D32050, U09587, NM_152295, AY995220, and BC001687, respectively) were first amplified using RT-PCR with HeLa total mRNA as a template. CDNAs for PL-12 and EJ were inserted into pET30a(+) (Novagen, Madison, WI, USA) and expressed as C-terminal His-tagged proteins. CDNAs for Jo-1 and KS were subcloned into pGEX4T-1 and pGEX6P-1 (GE Healthcare UK Ltd, Buckinghamshire, England), respectively, and expressed as N-terminal GST fusion proteins. CDNAs for PL-7 and OJ were engineered with a cMyc-epitope tag and His-tag sequence at their 3′ ends, and inserted into the pFastBacDual vector for baculovirus expression (Invitrogen, Carlsbad, CA, USA). Correct construction of plasmids was confirmed using DNA sequencing.

Expression and purification of recombinant ARSs

Expression and purification of His-tagged recombinant proteins: PL-12 and EJ were expressed in *Escherichia coli* BL-21(DE3) codon plus RIL bacteria (Stratagene, La Jolla, CA, USA). Competent cells were transformed with the vectors and the cells were incubated on Luria-Bertani (LB) agar plates containing 50 μg/mL kanamycin for 15 h at 37°C. A single colony was cultured in LB liquid medium containing kanamycin at 37°C. Addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside to the medium was used to induce expression of recombinant PL-12 and EJ proteins. After a 2-h incubation, cells were harvested using centrifugation and resuspended in ice-cold phosphate buffered saline (PBS) at pH 7.5. The cells were sonicated and soluble cell lysates containing the His-tagged recombinant proteins were separated using centrifugation.

PL-7 and OJ were expressed in baculovirus-infected Hi-5 cells. Each of the expression vectors was transfected into SF-9 cells using Cellfectin (Invitrogen), and the baculovirus stock was prepared from the transfectant culture supernatant. Hi-5 cells infected with baculovirus were incubated for 72 h at 26°C and were harvested using centrifugation, and soluble cell lysates containing recombinant proteins were prepared as described above.

Soluble His-tagged recombinant ARSs were purified using immobilized metal ion affinity chromatography. Cell extracts were applied to TALON® Metal Affinity Resin columns (Clontech, Palo Alto, CA, USA), and the columns were washed with PBS containing 10 mM imidazole. Purified PL-12, EJ, PL-7, and OJ were eluted with PBS containing 50 mM imidazole.

Expression and purification of recombinant GST-ARS fusion proteins: Jo-1 and KS were also expressed in *E. coli* BL-21(DE3) codon plus RIL bacteria in the presence of ampicillin. Transformation, cultivation, induction, and extraction of soluble cell proteins were performed as described for PL-12 and EJ proteins. Soluble GST-Jo-1 and GST-KS fusion proteins were purified on Glutathione Sepharose 4B columns (GE Healthcare UK Ltd.) and eluted with Tris-HCl (pH 8.0) containing 15 mM GSH.

Immunoblotting of recombinant antigens

Purified recombinant ARS antigens were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane as described by Towbin *et al.* [22] with minor modifications. After blocking with 5% skimmed milk, the membrane was incubated for 60 min with serum diluted 1:100 and then incubated for 60 min with a 1:1000 dilution of goat anti-human IgG conjugated to peroxidase (Code No. 208, MBL, Nagoya, Japan). Immunoreactive bands were detected using the Western Blot Detection System WEST-one (iNtRON Biotechnology, Gyeonggi-do, Korea).

ELISA

For detection of each ARS autoantibody, purified recombinant ARSs were individually coated on 96-well microtiter plates (Maxisorp; Nunc, Rochester, NY, USA). PL-12, EJ, PL-7, and Jo-1 were diluted in PBS to a final concentration of 2.5 μg/mL, and KS to 5.0 μg/mL. Each diluent was added at 100 μL/well and incubated overnight at 4°C. The plates were washed twice with PBS, and blocked with PBS containing 1% bovine serum albumin (BSA) and 5% sucrose overnight at 4°C. Sera from patients and normal healthy donors were diluted 1:100 in PBS containing 0.15% Tween 20 (PBS-T), 1% casein enzymatic hydrolysate, and 0.2 mg/mL *E. coli* extract, and 100 μL was applied to each well. After incubation for 60 min at room temperature (RT), the wells were washed four times with PBS-T. Goat anti-human IgG conjugated to peroxidase (Code No. 208, MBL) was diluted 1:7000 in 20 mM HEPES, 135 mM NaCl, 1% BSA, and 0.1% hydroxyphenylacetic acid (peroxidase stabilizer), and 100 μL was added to each well. After incubation for 60 min at RT, the wells were washed four times with PBS-T, and 3,3′,5,5′-tetramethylbenzidine substrate was then added. After a 30-min incubation at RT, the reaction was stopped by adding 100 μL of 0.25 N sulfuric acid and absorbance was read at 450 nm (A_{450}).

For simultaneous detection of five ARS autoantibodies, purified recombinant ARSs were diluted and mixed together in PBS and coated on plates. The final concentrations of PL-12, EJ, PL-7, Jo-1, and KS were 1.25 μg/mL, 0.63 μg/mL, 1.25 μg/mL, 0.63 μg/mL, and 2.5 μg/mL, respectively. The total protein concentration of the mixture was 6.25 μg/mL. ELISA plate preparation and assays were performed as described above. Conversion from A_{450} to a unit value (U/mL) was calculated using the following formula:

$$Unit\ Value(U/mL) = \frac{A_{450}<Sample> - A_{450}<Blank>}{A_{450}<Positive> - A_{450}<Blank>} \times 100$$

A_{450}<Positive> is the absorbance for an anti-Jo-1-positive patient serum that corresponds to a 100 U/mL value. A_{450}<Blank> is the background absorbance of buffer that does not contain serum. A_{450}<Sample> is the absorbance of a tested serum. The cutoff point was defined at 25 U/mL based on the analysis of the receiver operating characteristic curve in this multicenter study.

Statistical analysis

Statistical analyses were performed using StatView version 5.0 software. Clinical information of anti-ARS-negative and positive non-IPF patients was compared using the two-sample t-test or the Fisher's exact test.

Results

Autoantigen preparation

We first prepared six recombinant His-tagged ARS antigens, which were all expressed in *E. coli*. Immunoblot analysis showed that four of them, Jo-1, PL-12, EJ, and KS, were identified by their corresponding autoantibodies as well as by using an ELISA, whereas PL-7 and OJ reacted weakly with their corresponding autoantibodies (data not shown). Because we hypothesized that poor antigenic activity of recombinant PL-7 and OJ was due to a lack of posttranslational modification or proper structural folding, we prepared both fusion proteins expressed in eukaryotic Hi-5 cells using the baculovirus system. We confirmed antigenic activity of the new recombinant PL-7 using an ELISA (Fig.ô 1a) but the activity was lost when examined using immunoblotting (Fig.ô 1c). Recombinant PL-7, denatured using urea or SDS, had weaker antigenic activity than non-denatured PL-7, showing that the 3-dimensional protein structure played an important role in the reaction between the threonyl-tRNA synthetase and the anti-PL-7 antibody (Fig.ô 1a). Because of this antigenic characteristic of PL-7, we decided to prepare other recombinant ARSs, without denaturing reagents, as soluble polypeptides in PBS. Because His-Jo-1 and His-KS were insoluble, they were expressed as GST-recombinant proteins. ELISA revealed that the five newly prepared ARS antigens, His-PL-12, His-EJ, GST-Jo-1, GST-KS, and His-PL-7, displayed suitable antigenic reactivity. Immunoblotting also showed that four of the five ARS antigens, except for His-PL-7, had sufficient antigenic activity (Fig.ô 1b and c).

The recombinant OJ expressed in Hi-5 cells had weak antigenic activity, as confirmed using both immunoblotting and an ELISA (data not shown), suggesting that it is difficult to prepare a recombinant OJ as a single polypeptide that retains antigenic activity.

Establishing an ELISA system for simultaneous detection of five ARS antibodies

To detect multiple ARS antibodies simultaneously, we developed an ELISA system using a mixture of the five recombinant ARSs except for OJ. We tested a variety of antigen mixtures to estimate the most appropriate ratio and concentration to use, and we found that anti-ARS-positive sera showed reactivity with all five different ARSs with the highest sensitivity and specificity occurring at antigen concentrations of 0.63, 1.25, 1.25, 0.63, and 2.5 μg/mL (6.25 μg/mL in total) for histidyl-, threonyl-, alanyl-, glycyl-, and asparaginyl-tRNA synthetases, respectively. To assess potential cross-reactivity, we compared the absorbance values (A_{450}) obtained using an ELISA on every single recombinant ARS with those obtained with the new ELISA using the ARS mixture. When tested using a single-peptide-ELISA, each of the five anti-ARS antibodies showed reactivity with only its corresponding autoantigen. Samples positive for anti-PL-7, PL-12, or KS antibodies showed higher A_{450} values with the new mixed-peptide-ELISA than with the single-peptide ELISA, whereas the samples positive for anti-Jo-1 or EJ antibodies showed no significant difference in A_{450} values obtained with the two ELISAs. Such differences in A_{450} values may be due to different peptide-coating efficiencies because the total peptide concentration was higher in the mixed-peptide-ELISA than in the single-peptide ELISA (data not shown).

Clinical significance of anti-ARS ELISA in CTD

To confirm the efficiency of this newly established ELISA, we screened a total of 694 serum samples from patients with various CTDs and IIP, and 30 healthy controls. The results were compared between the ELISA and the RNA-IP assay (Fig.ô 2). A total of 102 samples were positive for anti-ARS antibodies using the ELISA and all of them, except for one, were identified to have any anti-ARS, other than anti-OJ, using the RNA-IP assay (Tableô 1). The sensitivity and specificity of the new ELISA in the detection of anti-ARS antibodies (including anti-OJ) compared with the RNA-IP technique were 97.1% and 99.8%, respectively (Tableô 1). Anti-ARS antibodies were detected in 30.8% (77/250) of IIM and 2.5% (7/276) of other CTDs (Tableô 2). None of the healthy controls were positive (Fig.ô 2). In IIM, 30.8% (33/107) of polymyositis (PM), 35.5% (33/93) of dermatomyositis (DM), 13.0% (3/23) of amyopathic DM, and 33.3% (1/3) of overlap myositis were positive for anti-ARS antibodies (Tableô 3). Among the 95 anti-ARS-positive IIM patients, 85 (89.4%) had IP, 54 (56.8%) arthralgia/arthritis, 24 (25.3%) had mechanic's hand, 37 (38.9%) had high fever, and 31 (32.6%) had Raynaud's phenomenon, which were consistent with previous reports [15]. The prevalence of these ASS symptoms was significantly higher in the anti-ARS-positive patients than in the negative patients (data

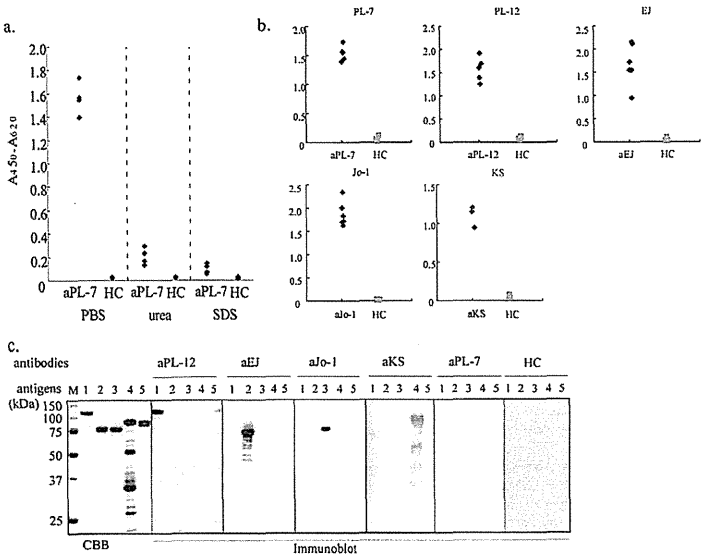

Figure 1. Antigenic activity of recombinant autoantigens a. Antigenic activity of PL-7 in various conditions. Left, purified recombinant PL-7 was eluted and diluted in PBS and coated on ELISA plates. Middle and Right, purified recombinant PL-7 was eluted in PBS and diluted in 8M urea and 2 × SDS sample buffer, respectively, and then coated onto ELISA plates. b. Five recombinant ARS antigens (His-PL-12, His-EJ, GST-Jo-1, GST-KS, and His-PL-7) were prepared as soluble polypeptides in PBS and their antigenic activity was tested in an ELISA using sera from five patients each containing corresponding autoantibodies (only GST-KS was tested using sera from three patients). Six healthy controls were used in each ELISA. c. Purified recombinant ARS antigens were electrophoresed on SDS-PAGE and transferred to a PVDF membrane followed by immunoblot analysis. CBB; Coomassie Brilliant Blue staining of gels, M; molecular weight marker, HC; healthy control, Lane 1; His-PL-12, Lane 2; His-EJ, Lane 3; GST-Jo-1, Lane 4; GST-KS and Lane 5; His-PL-7.
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not shown). There were seven anti-ARS-positive patients with other CTDs; two SSc patients were positive for anti-PL-12, two SLE patients were positive for anti-KS or anti-PL-12, and three RA patients were positive for anti-KS, anti-OJ or anti-PL-12.

Table 1 Comparison of the results between the new ELISA system and RNA-IP.

		RNA-IP		
		+	-	
anti-ARS ELISA	+	101*	1*	102
	-	0* (3)†	622* (619)†	622
	total	101* (104)†	623* (620)†	724

*The results detecting the five anti-ARS antibodies (anti-Jo-1, PL-12, EJ, KS, and PL-7) are described (sensitivity: 100%, specificity: 99.8%).
†Numbers in parenthesis are the results detecting all anti-ARS antibodies (including anti-OJ) (sensitivity: 97.1%, specificity: 99.8%).
doi:10.1371/journal.pone.0085062.t001

Clinical significance of anti-ARS ELISA in IIP

Anti-ARS antibodies were positive in 10.7% (18/168) of IIP patients. Only two patients (5.6%) with IPF were positive for anti-ARS; conversely, 16 patients (12.1%) with non-IPF were positive for anti-ARS antibodies (Table6 2). To investigate whether the anti-ARS-positive IIP were clinically distinct from anti-ARS-negative IIP patients, we compared clinical backgrounds and treatments between anti-ARS-positive and negative non-IPF patients (Table6 4). The anti-ARS-positive patients were significantly younger and a higher proportion was female ($p<0.01$), and they were treated more frequently with glucocorticoids (GC) or the combination of GC and immunosuppressants ($p<0.05$ and $p<0.01$, respectively).

Discussion

Among MSAs/myositis-associated autoantibodies (MAAs), anti-ARSs are the most frequently detected (28–37% [1,23,24]) in adult IIM patients, and anti-ARS-positive patients develop common characteristic symptoms known as ASS. Not only IIM but also apparent IIP patients can be positive for anti-ARS antibodies because IP often precedes myositis [1,14,20,25]. Both myopathy

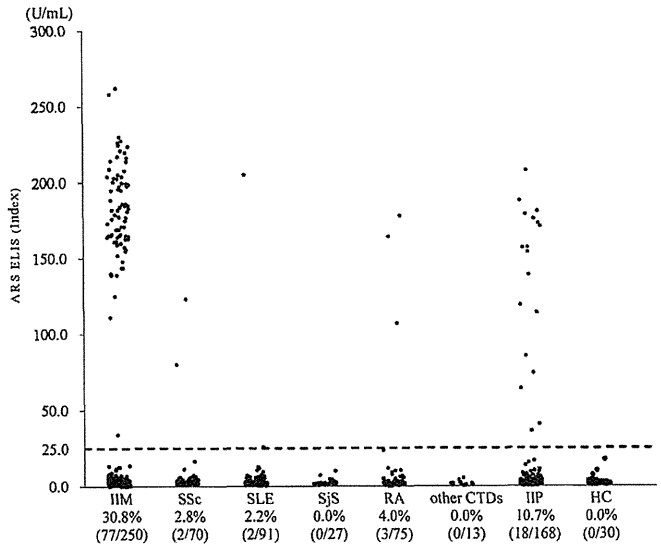

Figure 2. Confirmation of the efficiency of the ELISA system. Using the ELISA system, ARS antibodies were measured in 694 serum samples from patients with various CTDs and IIP, and 30 serum samples from healthy controls. The cutoff value (25 U/mL) is indicated by a horizontal dotted line.
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and IP anti-ARS-positive patients showed a better response to initial GC therapy but it can exacerbate the condition more often in anti-ARS-positive than in anti-ARS-negative patients [1,26]. Therefore, anti-ARS antibodies are useful not only in diagnosis, predicting the clinical course and therapy decisions in IIM, but also in classifying IP patients and predicting late-onset myopathy in IP-preceding patients.

An immunoprecipitation assay has been used to detect each anti-ARS antibody but to date, it can only be performed in a limited number of laboratories. To detect them more easily and routinely, we aimed to establish an ELISA system using the six recombinant ARS antigens to simultaneously detect anti-Jo-1, PL-7, PL-12, EJ, OJ, and KS antibodies. We did not include anti-tyrosyl or phenylalanyl synthetase because they have been reported only in one case each. However, some differences in clinical manifestations and prognoses among patients expressing different ARS antibodies, especially between anti-Jo-1 and non-anti-Jo-1 patients, have been observed [14,15]. However, different treatments for patients expressing different anti-ARSs have not been established. Currently, we treat anti-ARS-positive patients

Table 2 The frequency of each anti-ARS antibody in IIM, other CTD and IIP.

		RNA-IP(%)					
	ARS ELISA	Jo-1	PL-7	PL-12	EJ	KS	OJ
IIM	30.8% (77/250)	13.6	13.2	2.0	6.0	0.0	0.8
other CTDs	2.5% (7/276)	0.0	0.0	1.4	0.0	0.7	0.4
IIP	10.7% (18/168)	3.6	2.4	0.6	1.2	2.4	0.0
IPF	5.3% (2/38)	0.0	0.0	2.6	0.0	2.6	0.0
non-IPF	12.3% (16/130)	4.6	3.1	0.0	1.5	2.3	0.0

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Table 3 The frequency of each anti-ARS antibody in subsets of IIM.

IIM classification	Total	Jo-1	PL-7	PL-12	EJ	KS	n (%)
I polymyositis	107	18	7	3	5	0	33 (30.8)
II dermatomyositis	93	13	10	1	9	0	33 (35.5)
III amyopathic dermatomyositis	23	0	2	0	1	0	3 (13.0)
IV malignancy-associated myositis	7	0	1	0	0	0	1 (14.3)
V juvenile myositis	1	0	0	0	0	0	0 (0)
VI overlap myositis	3	1	0	0	0	0	1 (33.3)
VII unclassified	6	2	3	1	0	0	6(37.5)

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Table 4 Comparison of clinical backgrounds between anti-ARS (+) and (−) non-IPF patients.

	non-IPF n = 130		
	anti-ARS		
	(−) n = 114	(+) n = 16	p-value
age at the onset of the disease (yr) mean	69.6±9.5	56.9±14.5	<0.01
female (n; (ratio%))	39(34.2)	12(75.0)	<0.01
chronic (n; (ratio%))	104(91.2)	13(81.3)	N.S
subacute + acute (n; (ratio%))	5(4.4)	1(6.3)	N.S
acute (n; (ratio%))	2(1.8)	1(6.3)	N.S
glucocorticoids(GC) (n;(%))	49(43)	11(68.8)	<0.05
GC + immunosuppressants(IS) (n;(%))	19(16.7)	8(50.0)	<0.01
only drugs other than IS (n;(%))	8(7.0)	2(12.5)	N.S
PaO_2 at rest (Torr) mean	75.9±14.9	86.5±37.4	N.S
SpO_2 at rest (%) mean	95.7±2.4	97.1±2.1	<0.05
SpO_2 after 6 min walk test (Torr) mean	88.6±5.5	86.9±6.0	N.S
%VC (%) mean	87.7±22.5	77.9±17.4	<0.05
%DLCO (%) mean	51.0±19.5	58.0±23.1	N.S
KL-6 (U/mL) mean	1132±949	1287±693	N.S
SP-D (ng/mL) mean	207±180	180±136	N.S

%VC: % vital capacity, %DLCO: % diffusing capacity of carbon monoxide.
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with expectation of a standard clinical course in which the disease can recur with tapering of GC and in which exacerbation of IP is associated with a poor prognosis [1,14]. Therefore, presently, we are focusing on determining whether a patient with IIM or IIP is anti-ARS positive or not for the first screening when we begin treatment. This is why we decided to use a mixture of ARS antigens and not just single antigens to detect 'multiple anti-ARS antibodies' simultaneously.

We first prepared recombinant ARSs in *E. coli*, but recombinant PL-7 and OJ did not react well with their corresponding autoantibodies either using immunoblotting or an ELISA. For PL-7, structural conformation was important for antigenic activity because the recombinant PL-7 showed good reactivity only when it was expressed in a eukaryotic Hi-5 cell and was not denatured prior to being measured in the ELISA. Conversely, when recombinant PL-7 was denatured with urea or SDS, it was weakly detected with the PL-7 antibody, although its antigenicity was not completely lost. Such antigenic characteristics have also been reported previously by others [27]. This suggests that the synthetase epitope recognized by the anti-PL-7 antibody is in its native tertiary conformation.

In contrast, recombinant OJ (isoleucyl-tRNA synthetase) was not well detected even when it was expressed in Hi-5 cells and analyzed under non-denaturing conditions. This may be due to the unique feature of this isoleucyl-tRNA synthetase, which is a component of the multi-enzyme complex containing nine ARSs with three nonenzymatic factors [28,29]. In screening tests, positivity of anti-OJ in patients' sera was determined by the pattern of immunoprecipitation using HeLa cell extracts as originally described by Targoff *et al.* [28]. But there is a possibility that some 'anti-OJ antibodies' may recognize other components of the multi-enzyme complex rather than isoleucyl-tRNA synthetase itself, or alternatively the structural conformation of the complex may be important for recognition by anti-OJ, as was previously suggested by Targoff *et al.* [10]. They examined 11 patient sera with anti-OJ for evidence of reaction with other components of the complex. Ten out of 11 sera significantly inhibited enzyme activity of isoleucyl-tRNA synthetase, but some of them also significantly inhibited other ARSs such as leucyl-, lysyl-, or arginyl-tRNA synthetases. Moreover, immunoblot analysis of anti-OJ revealed that the majority of the sera could not identify a shared band and only a few sera recognized isoleucyl-tRNA synthetase. These results suggest that most 'anti-OJ sera' may react with multiple synthetases of the multi-enzyme complex or react with conformational epitopes of the complex. For this reason, we considered that it would be difficult to prepare the immunoreactive OJ antigen as a single molecule; therefore, we developed an ELISA system using the other five recombinant ARSs. This may not significantly affect the sensitivity of the ELISA because the prevalence of anti-OJ antibodies in patients is very low among the six anti-ARS antibodies.

The efficiency of this newly established ELISA system was acceptable because the sensitivity and specificity of the system compared with RNA immunoprecipitation were 97.1% and 99.8%, respectively, even if anti-OJ-positive sera was not excluded. The prevalence of anti-ARS in our IIM cohort was comparable with previous reports [1,2]. It was noteworthy that 10.7% of IIP patients, and in particular, 12.1% of non-IPF patients were positive for anti-ARS antibodies and there were some differences between anti-ARS-positive and negative IIP patients in their clinical backgrounds and treatments. Anti-ARS-positive patients were treated significantly more frequently with GC or the combination of GC and immunosuppressants. However, we are not yet ready to recommend immunosuppressive therapy for anti-ARS-positive IIP patients because we have not yet collected enough data on their clinical response and prognosis. Although some of these anti-ARS-positive IIP patients might develop myopathy later, it suggests that the measurement of anti-ARS antibodies may be useful in stratifying patients into disease subsets, which may help in predicting their clinical course.

A line-blot assay for the detection of multiple MSAs/MAAs (EUROLINE Myositis Profile 3) has been used in which anti-Jo-1, PL-7, PL-12, EJ, and OJ are included. This system can detect and discriminate MSAs/MAAs without further anti-ARS tests, but it does not include anti-KS, which has a stronger association with IIP than myositis [30]. To address this point, our system can more efficiently detect anti-ARS and therefore, is the preferred assay to use for IIP patients than the line-blot assay, although our ELISA does not aim to discriminate specificity for each anti-ARS antibody.

In conclusion, our ELISA system using a mixture of five recombinant ARSs shows similar efficiency to RNA immunoprecipitation and makes it possible to more readily detect anti-ARS antibodies in patients with PM/DM and IIP, and can be widely applied in daily practice.

Supporting Information

Table S1 The list of approval by institutional review boards of all participating centers.
(XLSX)

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Author Contributions

Conceived and designed the experiments: TM RN. Performed the experiments: TM RN YI YH MS AM KW TH MM MH TT KF KY HK YT NE TS KC HS NT. Analyzed the data: RN YI. Contributed reagents/materials/analysis tools: TM RN YI YH MS AM KW TH MM MH TT KF KY HK YT NE TS KC HS NT. Wrote the paper: RN.

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ORIGINAL ARTICLE

Efficacy and tolerability of tocilizumab in rheumatoid arthritis patients seen in daily clinical practice in Japan: results from a retrospective study (REACTION study)

Hisashi Yamanaka · Yoshiya Tanaka · Eisuke Inoue · Daisuke Hoshi · Shigeki Momohara · Kentaro Hanami · Naoki Yunoue · Kazuyoshi Saito · Kouichi Amano · Hideto Kameda · Tsutomu Takeuchi

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Abstract Tocilizumab, a humanized monoclonal antibody to the interleukin 6 (IL-6) receptor, was approved for use as rheumatoid arthritis (RA) therapy in Japan in 2008, but its efficacy and tolerability in daily practice has not yet been reported. We report the results of a multicenter retrospective study on the efficacy and safety of tocilizumab involving all patients (n = 229) who were started on tocilizumab therapy at three rheumatology institutes in Japan from April 2008 through to March 2009. Tocilizumab was infused every 4 weeks at a dose of 8 mg/kg according to the drug labeling. Among the 229 patients, 55% concomitantly received methotrexate (MTX) and 63% had previously received anti-tumor necrosis factor (TNF) therapy. Average disease activity score (DAS) 28 of all 229 patients significantly decreased from 5.70 to 3.25 after 24 weeks of therapy. A European League Against Rheumatism (EULAR) good response and DAS28 remission was achieved in 57.4 and 40.7% of the patients, respectively, at 24 weeks. White blood cell counts significantly decreased and liver enzymes and total cholesterol slightly but significantly increased; however, liver enzyme levels did not increase in patients without MTX. Tocilizumab was discontinued in 47 cases (20.5%) due to lack of efficacy (5.2%), adverse events (11.4%), and other reasons (3.9%). The overall retention rate at 24 weeks was 79.5%. Based on these results, we conclude that tocilizumab therapy in daily rheumatology practice appears to be highly efficacious and well tolerated among active RA patients, including the anti-TNF therapy-refractory population. Tocilizumab infusion is therefore applicable not only as an alternative approach for anti-TNF therapy-resistant patients, but also as primary biologic therapy for active RA patients.

Keywords Rheumatoid arthritis · Tocilizumab · IL-6 · Remission · Retrospective study

H. Yamanaka (✉) · E. Inoue · D. Hoshi · S. Momohara
Institute of Rheumatology, Tokyo Women's Medical University, 10-22 Kawada-cho, Shinjuku-ku, Tokyo 162-0054, Japan
e-mail: yamanaka@ior.twmu.ac.jp

Y. Tanaka · K. Hanami · N. Yunoue · K. Saito
The First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health Japan, Kitakyushu, Japan

K. Amano
Division of Rheumatology and Clinical Immunology, Department of Internal Medicine, Saitama Medical Center, Saitama Medical University, Saitama, Japan

H. Kameda · T. Takeuchi
Division of Rheumatology, School of Medicine, Keio University, Tokyo, Japan

Introduction

The introduction of biologic agents into the rheumatoid arthritis (RA) treatment landscape has altered both therapeutic strategies as well as patient outcomes [1–3]. Indeed, the aim of medical treatment of RA patients has shifted from the short-term improvement of the quality of life (QOL) through pain relief to the long-term improvement of QOL by the prevention of joint damage and improved mortality. Tumor necrosis factor (TNF) is considered to be a major cytokine involved in RA pathogenesis, and anti-TNF therapy using monoclonal antibodies or fusion proteins was the first anti-cytokine therapy to be developed for RA [4–6]. Anti-TNF therapy has achieved great success in suppressing rheumatoid inflammation and preventing the progression of joint damage in RA patients [7–9], thereby confirming that TNF indeed appears to be critically involved in the pathogenesis of RA synovitis. However, TNF is not the only cytokine involved in RA pathogenesis, and several candidate cytokines represent potential therapeutic targets for anti-cytokine therapy. Interestingly, the interleukin 1 (IL-1) receptor antagonist anakinra did not demonstrate a significant therapeutic effect in the treatment of RA [10], even though IL-1 is believed to be involved in the pathogenesis of RA synovitis [11], leading to the conclusion that not all cytokines are necessarily successful therapeutic targets for RA anti-cytokine therapy.

Tocilizumab, a monoclonal antibody directed against the IL-6 receptor, was developed in Japan through a collaborative effort of Osaka University and Chugai Pharmaceutical Co. (Tokyo, Japan) [12]. After a series of carefully conducted preclinical studies, tocilizumab was evaluated in RA patients, in whom it demonstrated promising efficacy [13–15]. The efficacy of tocilizumab was assessed in several different patient groups in both Japan and Western countries, and it has been shown to improve the signs and symptoms of RA patients, to suppress the radiological progression of joint damage [16], and to improve the QOL and physical disability [17]. However, the utility of tocilizumab has not been well documented in daily clinical practice outside of the clinical trial setting.

Tocilizumab was first approved as a therapy for RA in Japan in April 2008, and approximately 9,000 Japanese RA patients had received tocilizumab by the end of 2009. Although, as mentioned above, the efficacy and safety of tocilizumab have been well established in several randomized control trials (RCTs), given the differences between the patient populations that participate in RCTs versus those treated in clinical practice, it is also necessary to evaluate the efficacy and safety of this agent in the daily clinical rheumatology practice setting. We have therefore conducted a retrospective study of tocilizumab use in daily rheumatology practice in three rheumatology institutes in Japan and analyzed factors potentially associated with tocilizumab efficacy. Here, we describe the clinical profiles of RA patients seen in daily rheumatology practice during the first 6 months of tocilizumab treatment.

Patients and methods

Patients

The study cohort consisted of all RA patients (n = 229) who fulfilled the classification criteria of the American College of Rheumatology [18] and who had undergone tocilizumab treatment between April 2008 (following formal approval of tocilizumab for RA) up to March 2009 at one of three major rheumatology centers in Japan: (1) the Institute of Rheumatology of the Tokyo Women's Medical University, (2) the First Department of Internal Medicine of the School of Medicine, University of Occupational & Environmental Health Japan, Kitakyushu, or (3) the Division of Rheumatology and Clinical Immunology, Department of Internal Medicine, Saitama Medical Center, Saitama Medical University, Saitama. All data on these patients were evaluated retrospectively.

Demographic data, including disease duration and concomitant therapy, were collected from medical charts. The following parameters were evaluated at 24 weeks after the initial tocilizumab infusion: patient-recorded 28 tender joint counts (TJC), patient-recorded 28 swollen joint count (SJC), patient's global assessment of disease activity [patient's general health (GH)], erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) level.

Tocilizumab therapy

Tocilizumab was infused every 4 weeks at a dose of 8 mg/kg according to the drug labeling and the Japan College of Rheumatology guidelines for tocilizumab therapy [19]. Concomitant use of methotrexate (MTX) was at the discretion of the attending physician.

Therapeutic response

Disease activity was assessed by Disease Activity Score (DAS) 28-ESR and DAS28-CRP calculated using standard formulas [20]. Disability was assessed by the Health Assessment Questionnaire Disability Index (HAQ-DI) using the original HAQ [21] or the Japanese version of HAQ [22]. The primary clinical efficacy endpoint was the decrease in DAS28-ESR from baseline to week 24; secondary endpoints included decreases in DAS28-CRP and HAQ. Response to tocilizumab therapy was also evaluated using the European League Against Rheumatism (EULAR) response criteria [23]. Changes in laboratory data were also evaluated.

Discontinuation of tocilizumab treatment

Cases in which tocilizumab therapy was discontinued were further analyzed and the causes of discontinuation evaluated.

Statistical analysis

Patient baseline characteristics were summarized using mean, standard deviation (SD), median, and percentiles for the overall patient population and for patient subgroups defined by the concomitant use of MTX and prior use of anti-TNF agents. The primary endpoint was assessed by a t test. The secondary endpoints and subgroup differences of

these endpoints were also analyzed by the *t* test. The last observations carried forward (LOCF) method was applied to evaluate efficacy in a valid manner because data could not be obtained from patients who discontinued tocilizumab therapy. Logistic regression was used for the exploratory analysis to identify variables associated with an EULAR good response and remission at 24 weeks as defined by DAS28-ESR. All reported *P* values are two-sided and not adjusted for multiple testing. *P* values <0.05 were considered to indicate statistical significance for the primary endpoint. Data were analyzed with R ver. 2.9.

Results

Demographic data of patients from the three institutes

Baseline characteristics of the 229 patients who received tocilizumab therapy at the three rheumatology institutes in Japan from the time of its formal authorization for use in RA through to the end of March 2009 are shown in Table 1. Overall, 55% of patients concomitantly received MTX, while 63% had previously received anti-TNF therapy prior to tocilizumab; thus, the baseline characteristics of patients who did/did not concomitantly receive MTX and who did/did not receive prior anti-TNF therapy are also shown in Table 1. Prior biologic agents administered before entry into the study included infliximab (85/229 cases; 37%), etanercept (94; 41%), and adalimumab (4; 2%). The baseline data of patients who did and did not previously receive biologic therapy were not very different; however, the data of patients who did and did not receive concomitant MTX substantially differed. Patients who did not receive MTX had a longer disease duration, higher disease activity, and a lower incidence of previous use of anti-TNF agents. It should be noted that the average dose of MTX (8.73 mg/week) was relatively low compared to that administered in Western countries, since there is an official regulation concerning the maximum dose of MTX in Japan [24].

Efficacy of tocilizumab

Clinical efficacy was evaluated by DAS28-ESR, DAS28-CRP, EULAR response criteria, and HAQ-DI. Among all 229 patients, DAS28-ESR decreased from 5.70 ± 1.24 (mean ± SD) to 3.25 ± 1.62, DAS28-CRP decreased from 4.96 ± 1.19 to 0.87 ± 1.41, and HAQ-DI decreased from 1.58 ± 0.79 to 1.33 ± 0.87 at week 24 of treatment, demonstrating a significant improvement in these three clinical parameters ($P < 0.0001$). Figure 1 illustrates the decrease of the DAS28-ESR and its components (TJC, SJC, ESR, and GH) together with CRP. Although the decreases were significant for all clinical markers, the decreases in CRP and ESR were the most striking. The decrease in GH was statistically significant, but to a lesser degree than that of the other parameters.

The efficacy of tocilizumab was assessed in patients who did and did not receive concomitant MTX (Fig. 2). The mean DAS28-ESR and HAQ-DI values for patients with concomitant MTX use ($n = 127$) were 5.51 ± 1.16 and 1.44 ± 0.74, respectively, at baseline and 2.89 ± 1.50 and 1.14 ± 0.78, respectively, at 24 weeks. However, the DAS28 and HAQ-DI values of patients who did not receive concomitant MTX ($n = 102$) were 5.94 ± 1.30 and 1.75 ± 0.82, respectively, at baseline and 3.71 ± 1.67 and 1.58 ± 0.92, respectively, at 24 weeks (Fig. 2b, e). At all time points after week 4, DAS28-ESR, DAS28-CRP, and HAQ-DI values were significantly lower in patients who concomitantly received MTX ($P < 0.0001$).

The efficacy of tocilizumab therapy was also compared between patients who did and did not previously receive anti-TNF therapy. Mean DAS28-ESR and HAQ-DI values in patients who previously received anti-TNF therapy ($n = 144$) were 5.72 ± 1.25 and 1.54 ± 0.75, respectively, at baseline and 3.21 ± 1.60 and 1.30 ± 0.83, respectively, at 24 weeks; in contrast, patients who did not previously receive anti-TNF therapy ($n = 85$) had values of 5.66 ± 1.22 and 1.64 ± 0.85, respectively, at baseline and 3.33 ± 1.67 and 1.39 ± 0.94, respectively, at 24 weeks (Fig. 2c, f). At all time points, no differences were observed between patients who did and did not previously receive anti-TNF therapy.

The proportions of patients who had high disease activity, moderate disease activity, low disease activity, and remission as assessed by the DAS28-ESR were 67.9, 30.4, 0.9, and 0.9%, respectively, at baseline, and 14.6, 31.4, 13.3, and 40.7%, respectively, at 24 weeks (Fig. 3). EULAR responses at 24 weeks were good in 57.4% of patients, moderate in 30.6% of patients, and absent in 12.0% of patients, as assessed by the DAS28-ESR.

Changes of categorized disease activity were compared between patients who did and did not receive concomitant MTX. Superior efficacy was observed in patients who received concomitant MTX; remission rates by the DAS28 (<2.6) were 40.7% in the total patient population, 49.2% in patients who received concomitant MTX, and 30.0% in patients who did not receive concomitant MTX. In contrast, while changes in categorized disease activity were also compared between patients who did and did not previously receive anti-TNF therapy, no differences were noted.

Similarly, response to tocilizumab as determined by the EULAR response criteria was compared between patients who did and did not receive concomitant MTX and between patients who did and did not previously receive

Table 1 Baseline characteristics of the RA patients who received tocilizumab and were enrolled in this retrospective study

	All cases ($n = 229$)							Concomitant methotrexate				Prior use of anti-TNF			
								With MTX ($n = 127$)		Without MTX ($n = 102$)		With anti-TNF ($n = 144$)		Without anti-TNF ($n = 85$)	
	Mean	SD	Median	25 percentile	75 percentile	Minimum	Maximum	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Female (%)	84.0							82.0		87.0		81.0		89.0	
Age (years old)	58.7	13.6	61.0	52.0	68.0	15.0	88.0	55.5	14.0	62.8	11.9	56.5	14.3	62.4	11.4
RA duration (years)	12.4	11.1	10.0	4.0	18.0	0.1	72.0	10.3	8.5	15.0	13.2	12.1	10.4	12.9	12.2
Biologics—history (%)	63.0							72.0		51.0		100.0		0.0	
Prior use of infliximab (%)	37.0														
Prior use of etanercept (%)	41.0														
Prior use of adalimumab (%)	2.0														
MTX use (%)	55.0							100.0		0.0		64.0		41.0	
MTX dose (mg/week)	8.7	3.1	8.0	6.0	10.0	3.0	15.0	8.7	3.1			8.7	3.1	8.8	2.9
Other DMARD use (%)	34.0														
Corticosteroid use (%)	76.0							75.0		76.0				72.0	
Prednisolone dose (mg/day)	5.3	3.2	5.0	3.0	6.0	0.5	20.0	5.3	2.6	5.4	3.8	78.0	2.9	5.1	3.7
Swollen Joint Count (/28)	7.7	5.7	7.0	3.0	11.0	0.0	28.0	7.1	5.1	8.6	6.3	8.0	5.9	7.4	5.5
Tender Joint Count (/28)	7.9	6.5	6.0	3.0	12.0	0.0	28.0	6.7	5.4	9.3	7.3	8.0	6.7	7.6	6.1
CRP (mg/dl)	3.2	3.0	2.4	0.9	4.5	0.0	18.0	3.2	2.8	3.1	3.3	3.2	3.0	3.1	3.1
ESR (mm/1 h)	62.6	29.8	63.0	41.5	83.5	3.0	149.0	61.4	28.5	64.1	31.4	62.1	28.5	63.5	32.1
General Health (mm)	56.5	23.5	54.0	41.5	75.3	0.0	100.0	53.2	23.8	60.5	22.6	56.6	22.8	56.1	24.8
DAS28-ESR	5.7	1.2	5.6	4.9	6.6	1.6	9.0	5.5	1.2	5.9	1.3	5.7	1.3	5.7	1.2
DAS28-CRP	5.0	1.2	4.8	4.1	5.8	1.7	8.3	4.8	1.1	5.2	1.3	5.0	1.2	4.9	1.2
DAS28 remission (%)	1.0							1.0		1.0		1.0		1.0	
DAS28 low (%)	1.0							2.0		0.0		1.0		1.0	
DAS28 moderate (%)	30.0							35.0		24.0		31.0		29.0	
DAS28 high (%)	68.0							62.0		75.0		68.0		68.0	
HAQ-DI	1.6	0.8	1.5	1.0	2.2	0.0	3.0	1.4	0.7	1.7	0.8	1.5	0.7	1.6	0.8

RA Rheumatoid arthritis, *SD* standard deviation, *MTX* methotrexate, *TNF* tumor necrosis factor, *DMARD* disease-modifying antirheumatic drug, *CRP* C-reactive protein, *ESR* erythrocyte sedimentation rate, *DAS28* Daily Activity Score based on 28 joints, *HAQ-DI* Health Assessment Questionnaire Disability Index

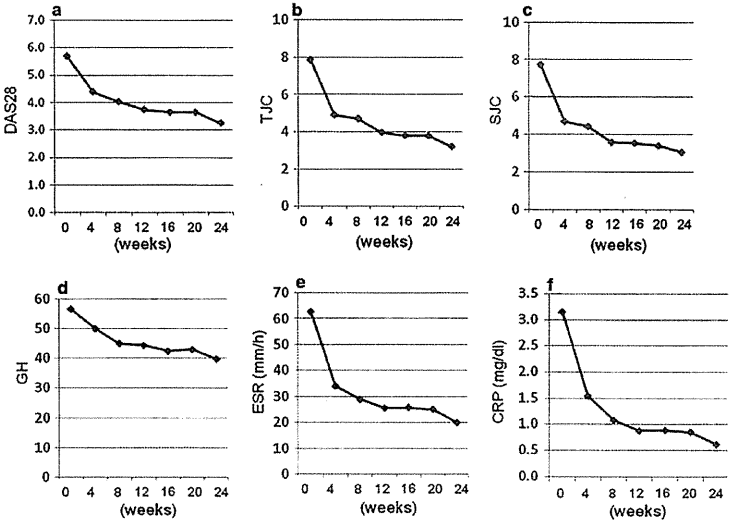

Fig. 1 Efficacy of tocilizumab infusion in inhibiting the signs and symptoms of rheumatoid arthritis (RA) patients seen in daily clinical practice. Values of the Daily Activity Score based on 28 joints (*DAS28*; **a**) and its components [tender joint count (*TJC*; **b**), swollen joint count (*SJC*; **c**), good health (*GH*; **d**), and erythrocyte sedimentation rate (*ESR*; **e**)] together with C-reactive protein (*CRP*) levels (**f**) from baseline to 24 weeks after the initiation of tocilizumab therapy are shown. Data were analyzed by the last observations carried forward (LOCF) method. Values were significantly lower than baseline values at all time points after 4 weeks of treatment ($P < 0.00001$)

anti-TNF therapy. While higher efficacy was observed in patients who received concomitant MTX, prior use of anti-TNF agents did not appear to influence the EULAR response.

Demographic factors related to clinical efficacy of tocilizumab

Logistic regression analysis of factors associated with a good EULAR response at 24 weeks ($n = 120$) versus moderate or no response ($n = 89$) was conducted using gender, age, disease duration, prior use of anti-TNF agent, MTX dose, prednisolone dose, HAQ at baseline and DAS28 at baseline as explanatory variables. The analysis revealed that younger age was independently associated with a good EULAR response.

Similarly, the same analysis of factors associated with remission at 24 weeks ($n = 92$) versus no remission ($n = 137$) indicated that younger age and lower DAS28 values were independently associated with remission. No other factors appeared to have a statistically significant predictive value for remission.

Laboratory data after initiation of tocilizumab

Laboratory data were regularly monitored throughout the 24 weeks of tocilizumab therapy (Table 2). In terms of those laboratory values used to measure disease activity, CRP levels, ESR, and matrix metalloproteinase-3 (MMP-3) levels had significantly decreased by 24 weeks, while hemoglobin (Hb) levels had significantly increased.

With respect to laboratory parameters used to monitor safety, total cholesterol (TC) levels and liver enzyme [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] levels slightly but significantly increased, while white blood cell (WBC) counts significantly decreased. However, liver enzyme levels did not increase significantly in patients who did not concomitantly receive MTX. No clinically relevant safety findings were reported even in patients with elevated liver enzyme levels.

Discontinuation of tocilizumab therapy

A Kaplan–Meier plot of tocilizumab discontinuation is shown in Fig. 4. Tocilizumab was discontinued during the

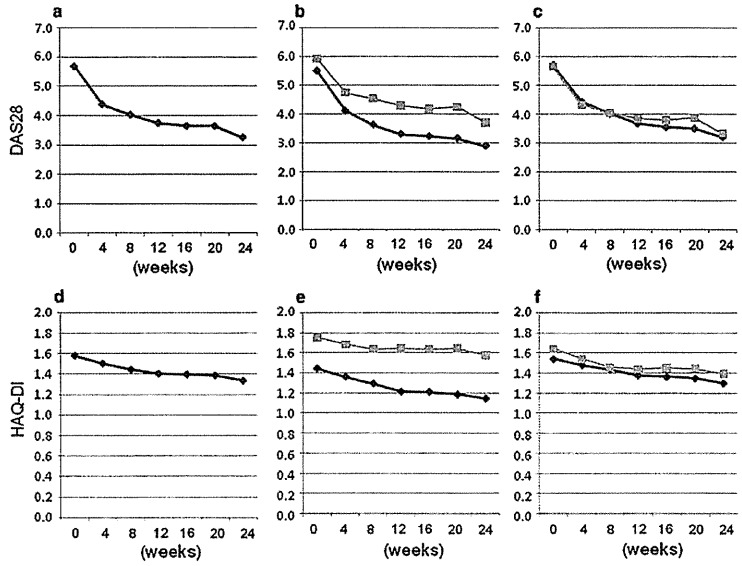

Fig. 2 Efficacy of tocilizumab infusion in inhibiting the signs and symptoms of RA patients in daily clinical practice. Values of DAS28 (**a**–**c**) and HAQ-DI (**d**–**f**) from baseline to 24 weeks after initiation of tocilizumab therapy are shown: **a**, **d** all 229 cases, **b**, **e** patients who concomitantly received methotrexate (MTX; *filled diamond*, $n = 127$) or did not receive MTX (*filled square*, $n = 102$), **c**, **f** patients who previously received anti-tumor necrosis factor (TNF) therapy (*filled diamond*, $n = 144$) or did not previously receive anti-TNF therapy (*filled square*, $n = 85$). Data were analyzed by the LOCF method. **a**, **d** At all time points after 4 weeks, values are significantly decreased from baseline ($P < 0.00001$), **b**, **e** at all time points after 4 weeks, values of patients who concomitantly received MTX are significantly lower than those of patients who did not ($P < 0.00001$), **c**, **f** at all timepoints after 4 weeks, values did not differ between patients who did and did not previously receive anti-TNF therapy

24-week observation period in 47 (20.5%) of the 229 patients due to a lack of efficacy (12 patients, 5.2%), adverse events (26 patients, 11.4%), and other reasons (9 patients, 3.9%)—and continued in 79.5% of patients. The retention rate at 24 weeks was 80.8% in patients who concomitantly received MTX, 74.4% in those who did not concomitantly receive MTX ($P = 0.197$ by log-rank test), 77.6% in those who previously received anti-TNF therapy, and 78.5% in those who did not previously receive anti-TNF therapy ($P = 0.892$ by log-rank test). Adverse events responsible for the discontinuation of tocilizumab included six cases of pneumonia, three cases of cardiac failure and concomitant disease aggravation, two cases of hepatic failure (liver enzyme elevation), interstitial pneumonia, skin ulcer and cerebral hemorrhage, and one case of anaphylaxis, chest pain, myocardial infarction, breast cancer, leucopenia, and phlegmon (Table 3).

Discussion

This study was conducted to determine the efficacy of tocilizumab therapy and to identify other factors associated with the effectiveness of tocilizumab therapy in Japanese RA patients receiving treatment in a university hospital outpatient setting at three rheumatic disease institutions. A study of the efficacy of tocilizumab in daily practice in a study group in the Kyushu area demonstrated the significant efficacy of this new biologic [25]. Many investigator-oriented studies of tocilizumab are currently being conducted in Japan, and more data should be available in the near future.

During the study period, only three anti-TNF therapies (infliximab, etanercept, and adalimumab) were available to treat RA in Japan; no other biologic agents, including abatacept and rituximab, was available. Tocilizumab was

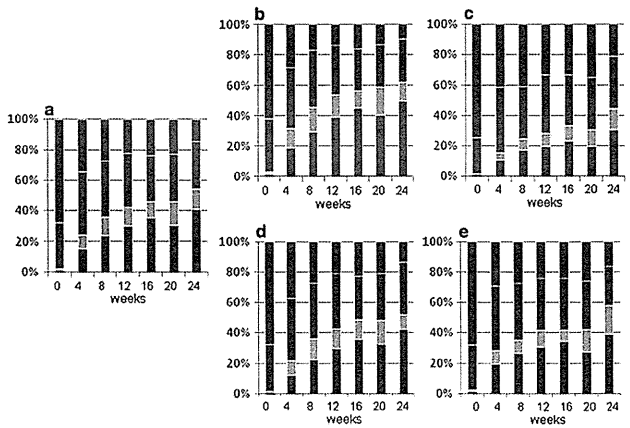

Fig. 3 Efficacy of tocilizumab infusion in inhibiting the signs and symptoms of RA patients seen in daily clinical practice. Disease activity as assessed by DAS28 values at baseline and after 24 weeks of tocilizumab therapy is shown: **a** all 229 cases, **b** patients who concomitantly received MTX ($n = 127$), **c** patients who did not concomitantly receive MTX ($n = 102$), **d** patients who previously received anti-TNF therapy ($n = 144$), **e** patients who did not previously receive anti-TNF therapy ($n = 85$). Disease activity was categorized as high (DAS28 > 5.1), moderate ($3.2 \leq$ DAS28 ≤ 5.1), low ($2.6 \leq$ DAS28 < 3.2) and remission (DAS28 < 2.6) from the top column. Data were analyzed by the LOCF method

first approved in Japan in April 2008, and results from clinical studies on its efficacy and safety in RA patients seen in daily rheumatology practice outside the clinical trial setting are quite important for evaluation purposes.

All of the patients enrolled in this study were essentially MTX-resistant, two-thirds were anti-TNF therapy-resistant, and the average DAS28 at baseline was 5.70. Consequently, these patients had severe RA. Based on our results, tocilizumab therapy was highly efficacious in these RA patients seen in daily clinical practice, and the overall remission rate at 24 weeks was as high as 40.7%.

It is difficult to compare efficacy in clinical studies with strict inclusion and exclusion criteria to that observed in daily clinical practice. The Japan College of Rheumatology provides a guideline for tocilizumab use [19] to which most rheumatologists comply; however, the regulations of this guideline are much less stringent compared to the inclusion and exclusion criteria of clinical studies. Even after these differences are taken into consideration, the efficacy of tocilizumab demonstrated in our study is comparable to or even higher than those reported in clinical studies. The remission rates in our study were 40.7% in the overall study population, 49.2% in patients who concomitantly received MTX, and 30.0% in patients who did not concomitantly receive MTX, while remission rates in previously reported clinical studies ranged from 27% in the OPTION study [17] to 59% in the SAMURAI study [10]. The tocilizumab-induced remission rate in the SAMURAI study was remarkably high, most likely because this study was conducted in early RA patients whose average disease duration was 2 years [10]. In contrast, the average disease duration of patients in our study was 12.4 years. Since tocilizumab directly reduces acute-phase reactants, such as CRP and ESR [26], it is not surprising that decreases in the ESR and CRP levels were among the most prominently changed DAS28 components, as shown in Fig. 1. However, other factors, including TJC, SJC, and GH, were also significantly decreased; thus, the higher remission rates observed in this study must not have been solely dependent on the potent suppression of acute-phase reactants by tocilizumab.

Concomitant use of MTX resulted in a rapid and sustained response to tocilizumab, even though the average MTX dose was relatively low (average at baseline: 8.7 mg/week) compared to that used in Western countries. Government regulations have limited the maximum dose of MTX to 8 mg/week in Japan; however, many rheumatologists prescribe higher MTX doses off-label [24, 27]. It is remarkable that such low-dose MTX potentiates tocilizumab action in severe RA patients. The differences in baseline characteristics between patients who did and did not concomitantly receive MTX may have accounted for this result, since patients who received MTX had a longer disease duration, higher disease activity, and lower usage of anti-TNF therapies. It is likely that tocilizumab was prescribed in combination with MTX in patients who had a lower risk of adverse events and as monotherapy in patients with a higher risk. Due to the observational nature of this study, safety issues associated with combined tocilizumab/MTX were not sufficiently evaluated; however, considering the high retention rate (Fig. 4) and laboratory profiles (Table 2) associated with tocilizumab therapy with or without MTX, the addition of MTX does not appear to significantly increase the risk of adverse events. As the

Table 2 Laboratory data at baseline and 24 weeks

Laboratory tests	Week	All cases				Concomitant methotrexate								Prior use of anti-TNF							
						With MTX				Without MTX				With anti-TNF				Without anti-TNF			
		n	Mean	SD	*P*	*n*	Mean	SD	*P*	*n*	Mean	SD	*P*	*n*	Mean	SD	*P*	*n*	Mean	SD	*P*
WBC ($/mm^3$)	0	208	7,707.2	2,701.3	<0.0001	111	7,557.7	2438.6	<0.0001	97	7,878.4	2977.5	<0.0001	132	7,697.0	2,558.6	<0.0001	76	7,725.0	2,950.4	0.00033
	24	208	6,270.2	2,649.5		111	5,969.4	2693.2		97	6,614.4	2569.3		132	6,122.7	2,567.2		76	6,532.3	2,785.4	
Hb (g/dl)	0	206	11.1	1.7	<0.0001	111	11.3	1.7	<0.0001	95	10.9	1.7	<0.0001	131	11.2	1.6	<0.0001	75	11.0	1.8	<0.0001
	24	206	12.0	1.9		111	12.3	1.8		95	11.7	1.9		131	12.2	1.9		75	11.8	1.8	
AST (IU/l)	0	211	22.7	14.3	<0.0001	113	21.2	8.2	<0.0001	98	24.4	18.9	0.2183274	134	23.3	17.0	0.00821	77	21.7	7.6	<0.0001
	24	211	27.5	13.9		113	28.1	14.0		98	26.7	13.9		134	27.5	15.6		77	27.3	10.3	
ALT (IU/l)	0	211	18.5	15.0	<0.0001	113	18.1	9.9	<0.0001	98	18.9	19.3	0.1307027	134	19.6	17.1	0.00324	77	16.5	10.0	<0.0001
	24	211	26.1	24.4		113	29.6	28.8		98	22.2	17.5		134	27.3	27.9		77	24.1	16.7	
TC (mg/dl)	0	166	189.0	38.2	<0.0001	86	188.8	35.7	<0.0001	80	189.1	41.0	<0.0001	98	190.4	37.8	<0.0001	68	186.8	39.0	<0.0001
	24	166	204.3	39.7		86	206.5	32.7		80	202.0	46.1		98	206.5	37.7		68	201.2	42.4	
ESR (mm/1 h)	0	227	62.6	29.8	<0.0001	126	61.4	28.5	<0.0001	101	64.1	31.4	<0.0001	143	62.1	28.5	<0.0001	84	63.5	32.1	<0.0001
	24	227	24.2	28.1		126	19.6	25.2		101	29.9	30.4		143	23.4	26.9		84	25.5	30.1	
CRP (mg/dl)	0	227	3.2	3.0	<0.0001	127	3.2	2.8	<0.0001	100	3.1	3.3	<0.0001	143	3.2	3.0	<0.0001	84	3.1	3.0	<0.0001
	24	227	0.9	2.1		127	0.8	1.8		100	1.1	2.5		143	0.9	1.9		84	1.0	2.4	
MMP-3 (ng/ml)	0	190	332.9	366.2	<0.0001	98	346.0	434.5	<0.0001	92	319.0	277.4	<0.0001	119	342.8	307.8	<0.0001	71	317.5	449.4	0.00148
	24	190	195.7	289.9		98	191.1	363.0		92	200.5	184.6		119	180.3	180.3		71	226.0	413.1	

P value between 0 week and 24 weeks

WBC White blood cells, *Hb* hemoglobin, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *TC* total cholesterol, *MMP-3* matrix metalloproteinase-3

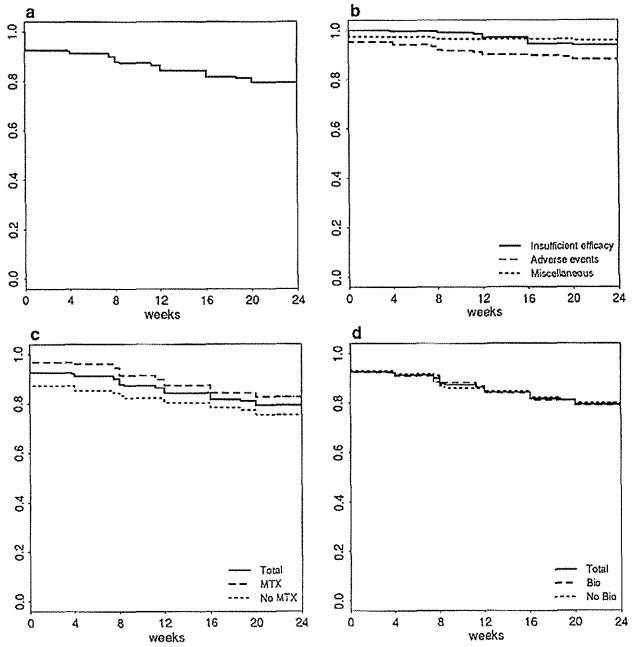

Fig. 4 Kaplan–Meier curve of the retention rate of tocilizumab therapy. **a** Tocilizumab retention rate in all 229 cases. **b** Tocilizumab retention rates by cause of discontinuation. **c** Tocilizumab retention rates in patients who did ($n = 127$) and did not ($n = 102$) receive concomitant MTX. **d** Tocilizumab retention rates in patients who did ($n = 144$) and did not ($n = 85$) previously receive anti-TNF therapy

addition of MTX to the therapeutic regimen may enhance the efficacy of tocilizumab, concomitant use of MTX is recommended with tocilizumab therapy in patients in whom safety is not a concern.

An interesting finding was the lack of any differences in tocilizumab efficacy in patients who did or did not previously receive anti-TNF therapy, suggesting that clinical response to tocilizumab therapy is independent of the prior use of anti-TNF agents. This result is inconsistent with the report of Nakashima et al. [25], in which the authors reported a higher efficacy of tocilizumab in biologic naïve patients. It is highly possible that the definition of MTX-refractory or anti-TNF refractory differs between rheumatologists. Although the baseline DAS28-ESR values in our study (5.7) and that of Nalashima et al. (5.5) were comparable, the average dose of MTX at baseline was higher in our study (8.7 ± 3.1 vs. 6.8 ± 2.1 mg/week). This higher dose means that the definition of MTX-refractory was more stringent in our study than in that of Nakashima et al. and that our RA patients had more active disease. It is reasonable to assume that the severity of RA in patients would affect the clinical outcome after tocilizumab therapy.

Clinical data on rituximab and abatacept demonstrate that both of these agents reduce disease activity in patients with inadequate responses to anti-TNF therapy. However, the remission rates of these non-TNF biologic agents in anti-TNF-naïve or -refractory populations differ. For abatacept, the remission rate in MTX-resistant patients (AIM study) was 14.8% [28], while that in anti-TNF therapy-refractory patients (ATTAIN study) was 10.0% [29]. In the case of rituximab, the American College of Rheumatology (ACR) 70% rate in MTX-resistant patients (DANCER study) was 20% [30], and that in anti-TNF therapy-refractory patients (REFLEX study) was 12% [31]. In contrast to rituximab and abatacept, remission rates observed in tocilizumab clinical studies were quite similar between MTX-resistant patients (OPTION, 27% [17]; TOWARD, 30% [32]) and anti-TNF therapy-refractory patients (RADIATE, 30% [33]). Although it may not be

Table 3 Cause of tocilizumab discontinuation

Cause of discontinuation	Number of cases/events
Lack of efficacy	12 cases (5.2%)
Adverse events	26 cases (11.4%)
Pneumonia	6
Heart failure	3
Worsening comorbidity	3
Liver damage	2
Interstitial pneumonia	2
Skin ulcer	2
Cerebellar hemorrhage	2
Anaphylaxis	1
Chest pain	1
Myocardial infarction	1
Leukopenia	1
Breast cancer	1
Phlegmon	1
Miscellaneous	9 cases (3.9%)
Unknown	4
Moving	3
Patient's will	2
Total	47 cases (20.5%)

appropriate to compare the results of clinical trials conducted in these different patient population, it is interesting to realize that the results from tocilizumab clinical studies have been fairly consistent despite the patient population.

TNF is widely accepted as an important cytokine [4], and it is known to induce IL-6 activity [34, 35]; however, IL-6 activity is not totally dependent on TNF stimulation [1, 36, 37]. The results of our study also suggest that tocilizumab has mechanisms of action that are unique from those of anti-TNF agents. As such, TNF represents an important therapeutic option for anti-TNF agent non-responders as well as disease-modifying antirheumatic drug (DMARD) non-responders.

In an attempt to identify predisposing factors for tocilizumab efficacy, we analyzed factors associated with remission using the demographic characteristics of our RA patients as the explanatory variables for logistic regression. The results of this analysis revealed that a low DAS28 at baseline and a younger age were associated with the induction of remission. Although patients at an earlier disease stage have been reported to be more sensitive to anti-rheumatic treatments [38], disease duration in our study did not appear to be correlated with remission. Since this study was conducted as a retrospective analysis of data collected in daily practice, many confounding factors could have potentially influenced the data. However, considering the high remission rate observed in the SAMURAI study (59%) [16], which was conducted in early RA patients in Japan, tocilizumab may prove to be most efficacious in younger patients and/or in those with early-stage disease.

Since IL-6 has pleiotropic actions, some of which are inconsistent with TNF actions, the inhibition of IL-6 action by tocilizumab may cause unexpected adverse reactions. Thus, the safety profile associated with tocilizumab needs to be carefully evaluated. With respect to laboratory parameters, decreased WBC counts and increased liver enzyme levels and/or serum cholesterol levels have been reported to be associated with tocilizumab use [15, 39]. However, in our study, although significant WBC count decreases and AST/ALT and TC increases were observed, these changes were tolerable (Table 2). Only two patients discontinued tocilizumab due to hepatic disorders (Table 3), and the liver enzyme levels returned to normal after the discontinuation of tocilizumab therapy. Interestingly, liver enzyme levels significantly increased in patients who concomitantly received MTX, but not in those who did not receive MTX, which is consistent with the findings of previous studies [16, 17, 32, 33]. Among 47 patients who discontinued tocilizumab, 12 did so due to insufficient efficacy, while 26 discontinued because of adverse events. Among the latter, six patients discontinued due to pneumonia. Pneumonia was the most frequent adverse event observed in previous Japanese post-marketing surveillance (PMS) evaluation of infliximab [40] and etanercept [27], which is consistent with the results of our study. However, it should be noted that susceptibility to infection is an important concern not only in association with anti-TNF therapies but also with the anti–IL-6 activity of tocilizumab.

The safety profile of tocilizumab therapy was recently extensively investigated in Japan using an all-case registered PMS study conducted by Chugai Pharmaceutical under the auspices of the regulatory authority of the Japanese government, with effective suggestions from the subcommittee of the Japan College of Rheumatology. A total of 9,000 cases were registered from April 2008 to December 2009, and an interim analysis is currently being conducted. The final analysis is expected to describe the safety profile of and risk factors associated with tocilizumab therapy on a large scale.

In conclusion, this REACTION study confirmed the efficacy of tocilizumab in Japanese patients with RA. Tocilizumab was also well tolerated, and the retention rate at 24 weeks was 79.5%. The promising efficacy of tocilizumab in improving measures of disease activity and preventing progression of this disabling disease has allowed this agent to become one of the critical advances in the management of RA in recent years. The present data should facilitate a more efficacious use of this expensive biological agent in daily rheumatology practice, not only in Japan but also in many countries throughout the world.

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Conflict of interest None of authors are industry employees. HY has received lecture fees from Abbott, Bristol-Meyers, Chugai Pharma, Eisai Pharma, F. Hoffmann-La Roche Ltd, Mitsubishi-Tanabe Pharma, Takeda Pharmaceutical Co Ltd, and Wyeth. YT has received consultant fees from Mitsubishi-Tanabe Pharma and Pfizer Inc, and lecture fees from Mitsubishi-Tanabe Pharma, Takeda Pharmaceutical Co Ltd, Abbott, Eisai Pharma, and Chugai Pharma. TT has received lecture fees from Abbott, Brystol-Meyers, Chugai Pharma, Eisai Pharma, Mitsubishi-Tanabe Pharma, Takeda Pharmaceutical Co Ltd., Janssen Pharmaceutical KK, and Wyeth.

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Meta-analysis identifies nine new loci associated with rheumatoid arthritis in the Japanese population

Yukinori Okada[1–3,40], Chikashi Terao[4,5,40], Katsunori Ikari[6,40], Yuta Kochi[1,2,40], Koichiro Ohmura[5], Akari Suzuki[1], Takahisa Kawaguchi[4], Eli A Stahl[7,8], Fina A S Kurreeman[7–9], Nao Nishida[10], Hiroko Ohmiya[3], Keiko Myouzen[1], Meiko Takahashi[4], Tetsuji Sawada[11], Yuichi Nishioka[12], Masao Yukioka[13], Tsukasa Matsubara[14], Shigeyuki Wakitani[15], Ryota Teshima[16], Shigeto Tohma[17], Kiyoshi Takasugi[18], Kota Shimada[17], Akira Murasawa[19], Shigeru Honjo[20], Keitaro Matsuo[21], Hideo Tanaka[21], Kazuo Tajima[22], Taku Suzuki[6,23], Takuji Iwamoto[6,23], Yoshiya Kawamura[24], Hisashi Tanii[25], Yuji Okazaki[26], Tsukasa Sasaki[27], Peter K Gregersen[28], Leonid Padyukov[29], Jane Worthington[30], Katherine A Siminovitch[31], Mark Lathrop[32,33], Atsuo Taniguchi[6], Atsushi Takahashi[3], Katsushi Tokunaga[10], Michiaki Kubo[34], Yusuke Nakamura[35], Naoyuki Kamatani[36], Tsuneyo Mimori[5], Robert M Plenge[7,8], Hisashi Yamanaka[6], Shigeki Momohara[6,41], Ryo Yamada[37,41], Fumihiko Matsuda[4,38,39,41] & Kazuhiko Yamamoto[1,2,41]

Rheumatoid arthritis is a common autoimmune disease characterized by chronic inflammation. We report a meta-analysis of genome-wide association studies (GWAS) in a Japanese population including 4,074 individuals with rheumatoid arthritis (cases) and 16,891 controls, followed by a replication in 5,277 rheumatoid arthritis cases and 21,684 controls. Our study identified nine loci newly associated with rheumatoid arthritis at a threshold of $P < 5.0 \times 10^{-8}$, including *B3GNT2, ANXA3, CSF2, CD83, NFKBIE, ARID5B, PDE2A-ARAP1, PLD4* and *PTPN2*. *ANXA3* was also associated with susceptibility to systemic lupus erythematosus ($P = 0.0040$), and *B3GNT2* and *ARID5B* were associated with Graves' disease ($P = 3.5 \times 10^{-4}$ and 2.9×10^{-4}, respectively). We conducted a multi-ancestry comparative analysis with a previous meta-analysis in individuals of European descent (5,539 rheumatoid arthritis cases and 20,169 controls). This provided evidence of shared genetic risks of rheumatoid arthritis between the populations.

Rheumatoid arthritis is a complex autoimmune disease characterized by inflammation and the destruction of synovial joints and affects up to 1% of the population worldwide. To date, more than 35 rheumatoid arthritis susceptibility loci, including *HLA-DRB1*, *PTPN22*, *PADI4*, *STAT4*, *TNFAIP3* and *CCR6*, among others, have been identified by GWAS in multiple populations[1–12] and by several meta-analyses of the original GWAS[13–16]. In particular, each meta-analysis of these GWAS uncovered a number of loci that were not identified in the single GWAS, leading to recognition of the enormous power of the meta-analysis approach for detecting causal genes in disease. However, these previous meta-analyses have been performed solely in European populations[13–16] and not in Asian ones. As multi-ancestry studies on validated rheumatoid arthritis susceptibility loci showed the existence of both population-specific and shared genetic components of rheumatoid arthritis[10,17], additional studies in Asian populations might provide useful insight into the underlying genetic architecture of rheumatoid arthritis, which would otherwise be difficult to capture using the studies in a single population. Here, we report a meta-analysis of GWAS and a replication study for rheumatoid arthritis in a Japanese population that was conducted by the Genetics and Allied research in Rheumatic diseases NETworking (GARNET) consortium[10,12]. We subsequently performed a multi-ancestry comparative analysis that incorporated results from a previously conducted meta-analysis of individuals of European ancestry[15].

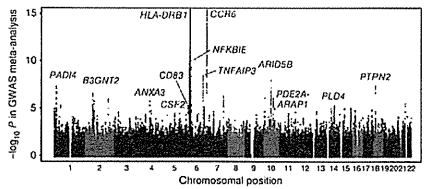

Figure 1 Manhattan plots of the GWAS meta-analysis for rheumatoid arthritis in the Japanese population. The genetic loci that satisfied the genome-wide significance threshold of $P < 5.0 \times 10^{-8}$ (gray line) in the meta-analysis or in the combined study of the meta-analysis and the replication study are presented. The *y* axis shows the $-\log_{10} P$ values of the SNPs in the meta-analysis. The SNPs for which the *P* values were smaller than 1.0×10^{-15} are indicated at the upper limit of the plot.

A full list of author affiliations appears at the end of the paper.

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Table 1 Results of the GWAS meta-analysis and the replication studies for rheumatoid arthritis

						Associations in Japanese										Associations in Europeans[c]		
						GWAS meta-analysis			Replication study			Combined study			GWAS meta-analysis			
															Allele 1 Freq.			
rsID[a]	Chr.	Position (bp)	Cytoband	Gene(s)	Allele 1/2	RA	Control	OR (95% CI)[b]	*P*	OR (95% CI)[b]	*P*	OR (95% CI)[b]	*P*		RA	Control	OR (95% CI)[b]	*P*
SNPs with significant associations ($P < 5.0 \times 10^{-8}$ in the combined study)																		
rs11900673	2	62305165	2p15	*B3GNT2*	T/C	0.31	0.28	1.15 (1.08–1.21)	3.5×10^{-6}	1.09 (1.04–1.14)	6.0×10^{-4}	1.11 (1.07–1.15)	1.1×10^{-8}		0.13	0.13	1.05 (0.98–1.13)	0.17
rs2867461	4	79732239	4q21	*ANXA3*	A/G	0.46	0.44	1.13 (1.08–1.19)	4.7×10^{-6}	1.12 (1.08–1.17)	1.2×10^{-7}	1.13 (1.09–1.17)	1.2×10^{-12}		0.37	0.37	0.98 (0.92–1.04)	0.52
rs657075	5	131458017	5q31	*CSF2*	A/G	0.38	0.36	1.12 (1.06–1.18)	3.2×10^{-5}	1.11 (1.06–1.16)	3.8×10^{-6}	1.12 (1.08–1.15)	2.8×10^{-10}		0.10	0.10	1.04 (0.95–1.13)	0.37
rs12529614	6	14204637	6p23	*CD83*	C/T	0.16	0.14	1.19 (1.10–1.27)	6.8×10^{-6}	1.11 (1.05–1.18)	6.0×10^{-4}	1.14 (1.09–1.19)	2.0×10^{-8}		0.055	0.053	1.11 (0.99–1.24)	0.074
rs2233434	6	44340898	6p21.1	*NFKBIE*	G/A	0.24	0.21	1.23 (1.16–1.31)	9.2×10^{-11}	1.17 (1.11–1.23)	2.2×10^{-9}	1.19 (1.15–1.24)	5.8×10^{-19}		0.059	0.040	1.57 (1.11–2.21)	0.0099
rs10821944	10	63455095	10q21	*ARID5B*	G/T	0.39	0.36	1.17 (1.11–1.23)	1.0×10^{-8}	1.15 (1.10–1.20)	3.0×10^{-10}	1.16 (1.12–1.20)	5.5×10^{-18}		0.29	0.26	1.11 (1.05–1.17)	1.9×10^{-4}
rs3781913	11	72061144	11q13	*PDE2A-ARAP1*	T/G	0.71	0.69	1.11 (1.05–1.17)	3.2×10^{-4}	1.13 (1.08–1.18)	6.7×10^{-7}	1.12 (1.08–1.16)	5.8×10^{-10}		0.45	0.43	1.04 (0.99–1.09)	0.13
rs2841277	14	104462050	14q32	*PLD4*	T/C	0.72	0.69	1.11 (1.05–1.18)	2.8×10^{-4}	1.18 (1.13–1.24)	7.0×10^{-12}	1.15 (1.11–1.19)	1.9×10^{-14}		0.47	0.46	1.02 (0.96–1.09)	0.54
rs2847297	18	12787694	18p11	*PTPN2*	G/A	0.37	0.33	1.16 (1.11–1.23)	3.5×10^{-8}	1.06 (1.01–1.11)	0.013	1.10 (1.07–1.14)	2.2×10^{-8}		0.36	0.34	1.10 (1.05–1.15)	9.2×10^{-5}
SNPs with suggestive associations ($5.0 \times 10^{-8} \leq P < 5.0 \times 10^{-6}$ in the combined study)																		
rs4937362	11	127997949	11q24	*ETS1-FLI1*	T/C	0.71	0.68	1.13 (1.07–1.19)	2.0×10^{-5}	1.07 (1.02–1.12)	0.0061	1.09 (1.05–1.13)	7.5×10^{-7}		0.46	0.44	1.06 (1.01–1.11)	0.015
rs3783637	14	54417868	14q22	*GCH1*	C/T	0.76	0.74	1.13 (1.07–1.20)	6.5×10^{-5}	1.07 (1.02–1.13)	0.0062	1.10 (1.06–1.14)	2.0×10^{-6}		0.88	0.88	0.99 (0.88–1.11)	0.87
rs1957895	14	60978085	14q23	*PRKCH*	G/T	0.40	0.39	1.12 (1.06–1.18)	4.1×10^{-5}	1.07 (1.02–1.12)	0.0022	1.09 (1.05–1.13)	3.6×10^{-7}		0.093	0.089	1.01 (0.95–1.07)	0.73
rs6496667	15	88694672	15q26	*ZNF774*	A/C	0.38	0.35	1.13 (1.07–1.19)	4.7×10^{-5}	1.07 (1.02–1.11)	0.0050	1.09 (1.05–1.13)	1.4×10^{-6}		0.21	0.20	1.07 (1.01–1.13)	0.021
rs74044928	16	23796341	16p12	*PRKCB1*	T/C	0.65	0.62	1.13 (1.07–1.19)	1.5×10^{-5}	1.05 (1.01–1.10)	0.026	1.08 (1.05–1.12)	4.0×10^{-6}		0.75	0.75	1.01 (0.94–1.09)	0.79
rs2280381	16	84576134	16q24	*IRF8*	T/C	0.86	0.84	1.16 (1.08–1.25)	1.0×10^{-4}	1.09 (1.03–1.15)	0.0049	1.12 (1.07–1.17)	2.4×10^{-6}		0.62	0.60	1.05 (0.99–1.11)	0.081
SNPs in previously reported rheumatoid arthritis susceptibility loci ($P < 5.0 \times 10^{-8}$ in the GWAS)																		
rs756449	1	17547439	1p36	*PADI4*	T/C	0.44	0.40	1.17 (1.11–1.24)	4.6×10^{-8}	–	–	–	–		0.38	0.37	1.09 (1.03–1.05)	0.0022
rs2157337	6	32609122	6p21.3	*HLA-DRB1*	C/T	0.59	0.44	1.99 (1.86–2.11)	2.6×10^{-118}	–	–	–	–		0.69	0.46	2.50 (2.39–2.62)	$<1.0 \times 10^{-200}$
rs6932056	6	138284130	6q23	*TNFAIP3*	C/T	0.092	0.073	1.35 (1.23–1.49)	3.2×10^{-9}	–	–	–	–		0.044	0.034	1.41 (1.24–1.60)	1.3×10^{-7}
rs1571878	6	167460832	6q27	*CCR6*	C/T	0.54	0.48	1.31 (1.24–1.39)	3.2×10^{-19}	–	–	–	–		0.47	0.43	1.13 (1.08–1.19)	5.9×10^{-7}

Chr., chromosome; Freq., frequency; RA, rheumatoid arthritis; OR, odds ratio; CI, confidence interval.
[a]SNPs with $P < 5.0 \times 10^{-6}$ in the combined study of the GWAS meta-analysis and the replication study or SNPs with $P < 5.0 \times 10^{-6}$ on the GWAS meta-analysis are annotated according to forward strand and NCBI Build 36.3. Full results of the replication study are provided in Supplementary Table 3. [b]Odds ratio of Allele 1. [c]Associations in the previous meta-analysis in European populations[15].

The meta-analysis included 4,074 rheumatoid arthritis cases (with 81.4% and 80.4% of the subjects being positive for antibody to cyclic citrullinated peptide (anti-CCP) and rheumatoid factor, respectively) and 16,891 controls from three GWAS of Japanese subjects (from the BioBank Japan Project[10,18], Kyoto University[12] and the Institute of Rheumatology Rheumatoid Arthritis (IORRA)[19]; **Supplementary Table 1**). After the application of stringent quality control criteria, including principal-component analysis (PCA; **Supplementary Fig. 1**) for each GWAS, the meta-analysis was conducted by evaluating ~2.0 million autosomal SNPs with minor allele frequencies (MAFs) ≥0.01, which were obtained through whole-genome imputation of genotypes on the basis of the HapMap Phase 2 East Asian panels (Japanese in Tokyo (JPT) and Han Chinese in Beijing (CHB)). The inflation factor of the test statistics in the meta-analysis λ_{GC} was as low as 1.036, suggesting no substantial effects of population structure (**Supplementary Table 2**). The quantile-quantile plot of *P* values showed a marked discrepancy in the values in its tail from those anticipated under the null hypothesis that there is no association—even after removal of the SNPs located in the human leukocyte antigen (HLA) region, the major rheumatoid arthritis susceptibility locus—thereby showing the presence of significant associations in the meta-analysis (**Supplementary Fig. 2**).

We identified seven loci in the current meta-analysis that satisfied the genome-wide significance threshold of $P < 5.0 \times 10^{-8}$. These included previously known rheumatoid arthritis susceptibility loci, such as *PADI4* at 1p36, *HLA-DRB1* at 6p21.3, *TNFAIP3* at 6q23 and *CCR6* at 6q27 (refs. 1,3,6,10,15) (the smallest $P = 2.6 \times 10^{-118}$ was found at the *HLA-DRB1* locus; **Fig. 1** and **Table 1**). To our knowledge, the other three loci identified, *NFKBIE* at 6p21.1, *ARID5B* at 10q21 and *PTPN2* at 18p11, are newly associated ($P = 9.2 \times 10^{-11}$, 1.0×10^{-8} and 3.5×10^{-8}, respectively).

To validate the associations identified in the meta-analysis, we conducted a replication study of two independent Japanese rheumatoid arthritis case-control cohorts (cohort 1: 3,830 rheumatoid arthritis cases and 17,920 controls, cohort 2: 1,447 rheumatoid arthritis cases and 3,764 controls; **Supplementary Table 1**). To increase the number of subjects and enhance statistical power, genotype data obtained from other GWAS projects conducted for non-autoimmune diseases in Japanese using Illumina platforms were used for the replication control panels. For each of the 46 loci that exhibited $P < 5.0 \times 10^{-4}$ in

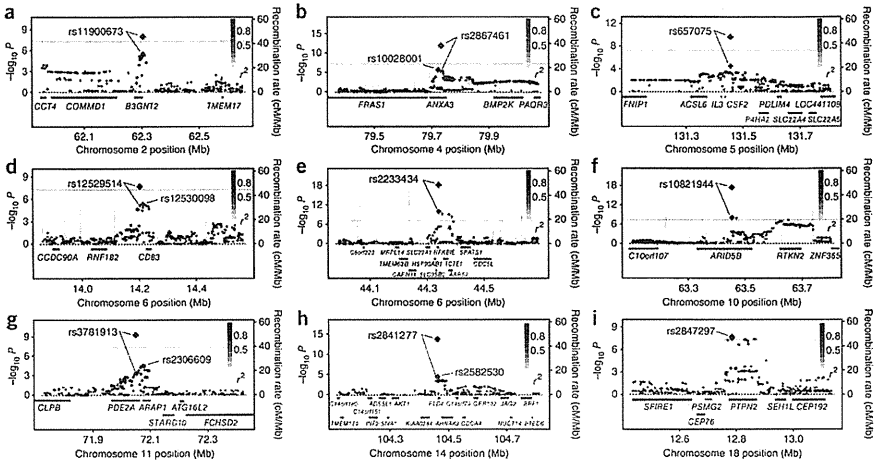

Figure 2 Regional plots of the loci newly associated with rheumatoid arthritis at the genome-wide significance threshold of $P < 5.0 \times 10^{-8}$ in the combined study of the meta-analysis and the replication study. (a–i) Regional plots are shown at *B3GNT2* (a), *ANXA3* (b), *CSF2* (c), *CD83* (d), *NFKBIE* (e), *ARID5B* (f), *PDE2A-ARAP1* (g), *PLD4* (h) and *PTPN2* (i). Diamonds represent the $-\log_{10} P$ values of the SNPs, and the red diamonds represent the $-\log_{10} P$ values of the SNPs in the meta-analysis. Red color for the smaller circles represents the r^2 value with the most significantly associated SNP (larger red circle). The purple circle represents the *P* value in the combined study. The blue line shows the recombination rates given by the HapMap Phase 2 east Asian populations (release 22). RefSeq genes at the loci are indicated below. Genes nearest to the marker SNPs at the loci are colored blue (**Supplementary Note**), and genes implicated in eQTL analysis are colored red (**Supplementary Table 4**). At 11q13, two genes (*PDE2A* and *ARAP1*) that are nearest to the SNP selected for the replication study and the most significant SNP in the meta-analysis are highlighted. The plots were drawn using SNP Annotation and Proxy Search (SNAP) version 2.2.

the meta-analysis and had not been reported as rheumatoid arthritis susceptibility loci[1–16], we selected a marker SNP for the replication study (Online Methods and **Supplementary Table 3**).

In the combined analyses of the meta-analysis and the replication study, including a total of 9,351 rheumatoid arthritis cases and 38,575 controls, we identified six newly associated loci, in addition to the *NFKBIE*, *ARID5B* and *PTPN2* loci, that satisfied the significance threshold of $P < 5.0 \times 10^{-8}$, including *B3GNT2* at 2p15, *ANXA3* at 4q21, *CSF2* at 5q31, *CD83* at 6p23, *PDE2A-ARAP1* at 11q13 and *PLD4* at 14q32 (**Figs. 1** and **2** and **Table 1**). Of these loci, *NFKBIE* had the smallest *P* value (5.8×10^{-19}). Although association with rheumatoid arthritis has been described for the *CSF2* and *PTPN2* loci[11,15,16,20,21], ours is the first report to our knowledge validating these associations with a threshold of $P < 5.0 \times 10^{-8}$. Suggestive associations were also observed in *ETS1-FLI1* at 11q24, *GCH1* at 14q22, *PRKCH* at 14q23, *ZNF774* at 15q26, *PRKCB1* at 16p12 and *IRF8* at 16q24 ($5.0 \times 10^{-8} \leq P < 5.0 \times 10^{-6}$). A summary of the genes in the newly associated loci and the results of *cis* expression quantitative trait locus (*cis* eQTL) analysis of the marker SNPs are provided (**Supplementary Table 4** and **Supplementary Note**).

Previous studies have reported associations of rheumatoid arthritis susceptibility loci with other autoimmune diseases[4,10,15,16]. Therefore, we assessed the association of these newly identified susceptibility loci with systemic lupus erythematosus (SLE) by examining the results of an SLE GWAS in the Japanese population (891 cases and 3,384 controls)[22] and in Graves' disease by genotyping 1,783 cases[10] (the controls from the SLE analysis were used for testing for Graves' disease). We observed significant associations of the *ANXA3* locus with SLE and of the *B3GNT2* and *ARID5B* loci with Graves' disease, which showed the same directional effects of the alleles as in rheumatoid arthritis ($P < 0.05/9 = 0.0056$, Bonferroni correction of the number of loci; **Supplementary Table 5**). It should be noted that relatively small sample sizes in the SLE and Graves' disease cohorts might yield limited statistical power, and further evaluations enrolling larger numbers of subjects would be desirable.

To highlight genetic backgrounds of rheumatoid arthritis that are common and divergent in different ancestry groups, we conducted a multi-ancestry comparative analysis of the present study in Japanese and a previous GWAS meta-analysis in Europeans that included 5,539 rheumatoid arthritis cases and 20,169 controls[15] (**Fig. 3a–c**). First, we compared associations in the reported[1–16] or newly identified rheumatoid arthritis susceptibility loci (**Fig. 3a** and **Supplementary Table 6**). Of the 46 rheumatoid arthritis risk variants evaluated, 6 were monomorphic in Japanese, and all were polymorphic in Europeans. We observed significant associations at 22 loci in Japanese and at 36 loci in Europeans (false discovery rate (FDR) < 0.05, $P < 0.0030$), with 14 loci being shared between the populations. Of the newly associated rheumatoid arthritis susceptibility loci identified in our Japanese meta-analysis, significant associations were also observed in the European meta-analysis at the *ARID5B* and *PTPN2* loci ($P = 1.9 \times 10^{-4}$ and 9.2×10^{-5}, respectively; **Table 1**). Significant positive correlation of odds ratios was observed between the studies ($r = 0.822$, $P = 8.1 \times 10^{-11}$; Fig. 3b), suggesting that a substantial proportion of genetic factors are shared between

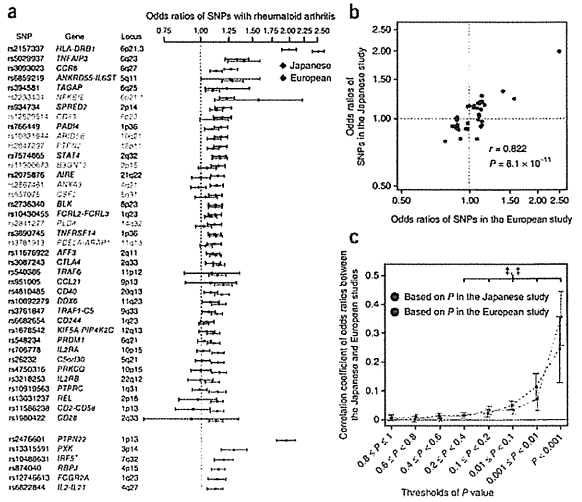

Figure 3 Overlap of the associations with rheumatoid arthritis between Japanese and European populations. (a) Forest plots of SNPs in the rheumatoid arthritis susceptibility loci (**Supplementary Table 6**). We selected the genetic loci that have been validated to be associated with rheumatoid arthritis susceptibility by showing associations in the reports of multiple cohorts or satisfying the genome-wide significant threshold ($P < 5.0 \times 10^{-8}$) in previous studies, including in the meta-analysis and replication phases[1–16]. For each of the loci, the most significant SNP among those reported in the previous or present study were selected[1–16]. SNPs in the newly identified rheumatoid arthritis susceptibility loci are colored green. Odds ratios and 95% confidence interval (CI) values are based on rheumatoid arthritis risk alleles, and the SNPs are ordered according to the odds ratios in the Japanese study. Several SNPs were monomorphic in the Japanese population. The odds ratios of these SNPs in the European study are presented below. The asterisk indicates that an association of another variant at the *IRF5* locus was reported in the Japanese population[24]. (b) Correlation of the odds ratios of the SNPs in the validated rheumatoid arthritis susceptibility loci between the two populations. SNPs that were polymorphic in both populations were used; odds ratios were based on the minor allele in the Japanese population. (c) Correlation of the odds ratios of the genome-wide SNPs, excluding the rheumatoid arthritis susceptibility loci. Correlations were evaluated for sets of SNPs stratified by the thresholds based on the meta-analysis *P* values in each population after pruning of the SNPs by LD ($r^2 < 0.3$). Correlation coefficient and 95% CI are indicated on the *y* axis. Significant correlation of the odds ratios was observed (‡, $P < 0.005$), even for the SNPs that showed moderate associations with rheumatoid arthritis (meta-analysis $P < 0.4$ in each population).

the two ancestry groups[17]. When the rheumatoid arthritis cases of the Japanese GWAS meta-analysis were stratified into anti-CCP–positive or rheumatoid factor–positive cases ($n = 3,209$) and controls ($n = 16,891$), similar results were observed (data not shown). Nevertheless, most of the SNPs assessed here are not necessarily causal variants, and further fine mapping of the loci is warranted to precisely evaluate the shared genetic predisposition between the populations.

Next, we compared regional associations within each of the loci and identified unique patterns in the *ARID5B* locus at 10q21 (**Supplementary Fig. 3**). In Japanese, three peaks of association were observed ($P = 1.0 \times 10^{-8}$ at rs10821944, $P = 5.7 \times 10^{-8}$ at rs10740069 and $P = 8.5 \times 10^{-6}$ at rs224311). These three variants were in weak linkage disequilibrium (LD) in Japanese ($r^2 < 0.10$), indicating independent associations with each of the other SNPs that satisfied a region-wide significance threshold of $P < 3.5 \times 10^{-5}$ (conditional $P = 4.3 \times 10^{-6}$, 1.7×10^{-5} and 1.8×10^{-5}, respectively) (**Supplementary Fig. 3**). In contrast, there was only one peak of association in Europeans ($P = 1.2 \times 10^{-6}$ at rs12764378; $r^2 = 0.59$ with rs10821944 in Europeans), and no additional association was observed in conditional analysis with rs12764378 (the smallest conditional $P = 2.2 \times 10^{-4}$), suggesting that the number of independent associations may be different at this locus in the two populations.

Finally, we conducted polygenic assessment for common variants showing modest associations to rheumatoid arthritis (those not meeting the genome-wide association threshold). This approach has been recognized to be a means to explain a substantial proportion of genetic risk[23]. For the SNPs that were shared between the two meta-analyses but not included in the validated rheumatoid arthritis susceptibility loci, we adopted LD pruning of the SNPs ($r^2 < 0.3$). We then evaluated the correlation of odds ratios of the SNPs between the two meta-analyses and observed a significant positive correlation ($r = 0.023$, $P < 1.0 \times 10^{-300}$). When the SNPs were stratified according to the *P* values in each meta-analysis, significant positive correlations of odds ratios were observed for the SNPs, even for those showing modest association ($P < 0.4$ in the meta-analysis of Japanese or Europeans; $r = 0.014–0.36$ for each *P* value range, $P < 0.005$ for each correlation test) (**Fig. 3c**). Correlations (r) of odds ratios observed herein suggest substantial overlap of the genetic risk of rheumatoid arthritis between the two populations, not only in the validated rheumatoid arthritis susceptibility loci but also at the loci showing nonsignificant associations. This suggests the usefulness of a meta-analysis approach involving multiple ancestry groups in identifying additional susceptibility loci.

In summary, we identified multiple new loci associated with rheumatoid arthritis through a large-scale meta-analysis of GWAS in Japanese. Multi-ancestry comparative analysis provided evidence of significant overlap in the genetic risks of rheumatoid arthritis between Japanese and Europeans. Thus, findings from the present study should contribute to the further understanding of the etiology of rheumatoid arthritis.

URLs. GARNET consortium, http://www.twmu.ac.jp/IOR/garnet/home.html; The BioBank Japan Project (in Japanese), http://biobankjp.org/; International HapMap Project, http://www.hapmap.org/; PLINK, http://pngu.mgh.harvard.edu/~purcell/plink/; EIGENSTRAT, http://genepath.med.harvard.edu/~reich/Software.htm; MACH and mach2dat, http://www.sph.umich.edu/csg/abecasis/MACH/index.

html; R statistical software, http://cran.r-project.org/; SNAP, http://www.broadinstitute.org/mpg/snap/index.php; NCBI GEO database, http://www.ncbi.nlm.nih.gov/geo/.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

Y. Okada, C.T., K.I., Y. Kochi and K.O. designed the study and drafted the manuscript. Y. Okada, C.T., K.I., T.K., H.O., N.N., M.T., M.I., K. Tokunaga and M.K. managed genotyping and manipulation of GWAS data. Y. Okada, Y. Kochi, C.T. and K.I. managed genotyping of replication cohorts. Y. Okada, T.K., H.O., E.A.S., A. Takahashi and R.Y. performed statistical analysis. Y. Kochi, A.S., K. Myouzen, T. Sawada, Y. Nishoka, M.Y., T. Matsubara, S.W., R.T. and S.T. collected samples and managed phenotype data for the rheumatoid arthritis cohorts from the BioBank Japan Project and CGM, RIKEN. C.T., K.O., T.K., M.T., K. Takasugi, K.S., A.M., S.H., K. Matsuo, H. Tanaka, K. Tajima and M.L. collected samples and managed phenotype data for the rheumatoid arthritis cohorts from Kyoto University. K.I., T. Suzuki, T.I., Y. Kawamura, H. Tanii, Y. Okazaki and T. Sakaki collected samples and managed phenotype data for the rheumatoid arthritis cohorts from IORRA. Y. Kochi managed the data for the SLE and Graves' disease cohorts. A.S., C.T. and K.I. analyzed the sera of subjects with rheumatoid arthritis. E.A.S., F.A.S.K., P.K.G., J.W., K.A.S., L.P. and R.M.P. managed the data for the rheumatoid arthritis cohorts in European populations. A. Taniguchi, A. Takahashi, K. Tokunaga, M.K., Y. Nakamura, N.K., T. Minori, R.M.P., H.Y., S.M., R.Y., F.M. and K.Y. supervised the overall study.

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The authors declare no competing financial interests.

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[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]Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan. [5]Department of Rheumatology and Clinical Immunology, Graduate School of Medicine, Kyoto University, Kyoto, Japan. [6]Institute of Rheumatology, Tokyo Women's Medical University, Tokyo, Japan. [7]Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA. [8]Broad Institute, Cambridge, Massachusetts, USA. [9]Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands. [10]Department of Human Genetics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan. [11]Department of Rheumatology, Tokyo Medical University Hospital, Tokyo, Japan. [12]Yamanashi Prefectural Central Hospital, Yamanashi, Japan. [13]Department of Orthopaedic Surgery, Yukioka Hospital, Osaka, Japan. [14]Matsubara Mayflower Hospital, Hyogo, Japan. [15]Osaka Minami National Hospital, Osaka, Japan. [16]Department of Orthopedic Surgery, Tottori University, Tottori, Japan. [17]Department of Rheumatology, National Hospital Organization, Sagamihara Hospital, Kanagawa, Japan. [18]Center for Rheumatic Diseases, Dohgo Spa Hospital, Ehime, Japan. [19]Department of Rheumatology, Niigata Rheumatic Center, Niigata, Japan. [20]Saiseikai Takaoka Hospital, Toyama, Japan. [21]Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Aichi, Japan. [22]Aichi Cancer Center Hospital and Research Institute, Aichi, Japan. [23]Department of Orthopaedic Surgery, Keio University, Tokyo, Japan. [24]Yokohama Clinic, Warakukai Medical Corporation, Yokohama, Japan. [25]Department of Psychiatry, Mie University School of Medicine, Mie, Japan. [26]Metropolitan Matsuzawa Hospital, Tokyo, Japan. [27]Graduate School of Education, University of Tokyo, Tokyo, Japan. [28]The Feinstein Institute for Medical Research, North Shore–Long Island Jewish Health System, Manhasset, New York, USA. [29]Rheumatology Unit, Department of Medicine in Solna, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden. [30]Arthritis Research Campaign–Epidemiology Unit, The University of Manchester, Manchester Academic Health Science Centre, Manchester, UK. [31]Division of Medicine, University of Toronto, Mount Sinai Hospital and University Health Network, Toronto, Ontario, Canada. [32]Commisariat a l'Energie Atomique (CEA), Institut Genomique, Centre National de Genotypage, Evry, France. [33]Fondation Jean Dausset, Centre d'Etude du Polymorphisme Humain, Paris, France. [34]Laboratory for Genotyping Development, CGM, RIKEN, Yokohama, Japan. [35]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan. [36]Laboratory for International Alliance, CGM, RIKEN, Yokohama, Japan. [37]Unit of Statistical Genetics, Center for Genomic Medicine Graduate School of Medicine Kyoto University, Kyoto, Japan. [38]Core Research for Evolutional Science and Technology (CREST) Program, Japan Science and Technology Agency, Kawaguchi, Japan. [39]Institut National de la Santé et de la Recherche Médicale (INSERM), Unité U852, Kyoto University Graduate School of Medicine, Kyoto, Japan. [40]These authors contributed equally to this work. [41]These authors jointly directed this work. Correspondence should be addressed to Y.K. (ykochi@src.riken.jp) or K.O. (ohmurako@kuhp.kyoto-u.ac.jp).

ONLINE METHODS

Subjects. The Japanese participants in the meta-analysis (4,074 rheumatoid arthritis cases and 16,891 controls) and the replication study (5,277 rheumatoid arthritis cases and 21,684 controls) were obtained through the collaborations of the GARNET consortium (**Supplementary Table 1**)[10,12]. The meta-analysis was conducted on three independent GWAS (from the BioBank Japan Project[18] with 2,414 rheumatoid arthritis cases and 14,245 controls[10], Kyoto University with 1,237 rheumatoid arthritis cases and 2,087 controls[12] and IORRA[19] with 423 rheumatoid arthritis cases and 559 controls). The replication study consisted of two independent cohorts (cohort 1 included 3,830 rheumatoid arthritis cases and 17,920 controls, and cohort 2 included 1,447 rheumatoid arthritis cases and 3,764 controls). We employed a case-control cohort of SLE (891 cases and 3,384 controls)[22] and 1,783 cases with Graves' disease[10]. Details of 5,539 rheumatoid arthritis cases and 20,169 controls included in the meta-analysis in European populations were described elsewhere[15]. All participants provided written informed consent for participation in the study, as approved by the ethical committees of the institutional review boards. Detailed descriptions of the participating subjects are provided (**Supplementary Note**).

Genotyping and quality control in the GWAS. Genotyping platforms and quality control criteria for the GWAS, including cutoff values for sample call rates, SNP call rates, MAF and Hardy-Weinberg P values, are given (**Supplementary Table 2**). For the subjects enrolled in each of three GWAS, we excluded closely related subjects with first- or second-degree kinship, which was estimated using PLINK version 1.06 (see URLs). We also excluded the subjects determined to be ancestry outliers from East Asian populations using PCA performed by EIGENSTRAT version 2.0 (see URLs) along with HapMap Phase 2 panels (release 24; **Supplementary Fig. 1**). Genotype imputation was performed on the basis of the HapMap Phase 2 East Asian populations, using MACH version 1.0.16 (see URLs) in a two-step procedure as described elsewhere[25]. We excluded imputed SNPs with MAF < 0.01 or *Rsq* < 0.5 from each of the GWAS. Associations of the SNPs with rheumatoid arthritis were assessed by logistic regression models assuming additive effects of the allele dosages of the SNPs using mach2dat software (see URLs).

Meta-analysis. We included 1,948,139 autosomal SNPs that satisfied quality control criteria in all three GWAS (**Supplementary Table 2**). SNP information was based on a forward strand of the NCBI build 36.3 reference sequence. The meta-analysis was performed using an inverse variance method assuming a fixed-effects model from the study-specific effect sizes (logarithm of odds ratio) and the standard errors of the coded alleles of the SNPs determined with the Java source code implemented by the authors[25]. Genomic control corrections[26] were carried out on test statistics of the GWAS using the study-specific inflation factor (λ_{GC}) and was applied or reapplied to the results of our current meta-analysis (**Supplementary Fig. 2**).

Replication study. We selected a SNP for the replication study from each of the loci that exhibited $P < 5.0 \times 10^{-4}$ in the meta-analysis that had not previously been reported as rheumatoid arthritis susceptibility loci[1–16] (**Supplementary Table 3**). For control subjects, we used genotype data obtained from additional GWAS for non-autoimmune diseases or healthy controls, genotyped using Illumina HumanHap550 BeadChips or HumanHap610-Quad BeadChips, and the cases for rheumatoid arthritis and Graves' disease were genotyped with the TaqMan genotyping system (Applied Biosystems; **Supplementary Table 1**). Selection of the SNP was conducted according to the following criteria: if the SNP with the most significant association in the locus was genotyped in the replication control panel, then that SNP was selected; otherwise, a tag SNP in the replication control panel with the strongest LD was selected (mean $r^2 = 0.89$). For the three SNPs that yielded low call rates (<90%), we alternatively selected proxy SNPs with the second strongest LD. As a result, average genotyping call rates of the SNPs were 99.9% and 99.0% for the controls and cases, respectively. We then evaluated concordance rates between the assayed genotypes by applying these two different methods to samples from 376 subjects who were randomly selected. This procedure yielded high concordance rates of ≥99.9%. Associations of the SNPs were evaluated using logistic regression assuming an additive-effects model of genotypes in R statistical software version 2.11.0 (see URLs). The combined study of the meta-analysis and replication study was performed using an inverse variance method assuming a fixed-effects model[25].

***Cis* eQTL analysis.** For each marker SNP of the newly identified rheumatoid arthritis susceptibility locus, correlations between SNP genotypes and expression levels of genes located 300 kb upstream or downstream of the SNP measured in B-lymphoblastoid cell lines (GSE6536) were evaluated using data from the HapMap Phase 2 east Asian populations[27].

Multi-ancestry analysis of the meta-analyses in Japanese and Europeans. We evaluated the associations of the variants in the validated rheumatoid arthritis susceptibility loci by comparing the results from the current meta-analysis in Japanese with those from a previous meta-analysis in Europeans[15]. We assessed two variants in the *IRF5* locus, where different causal variants were identified in the two populations[24]. For the conditional analysis of the regional associations in the *ARID5B* locus (**Supplementary Fig. 3**), we repeated the meta-analysis at that locus by incorporating genotypes of the referenced SNP(s) as additional covariate(s). For comparison of the odds ratios of the SNPs, we first selected SNPs that were shared between the meta-analyses in Japanese and Europeans. Next, we removed the SNPs located more than 1 Mb away from each of the marker SNPs in the validated rheumatoid arthritis susceptibility loci, except for in the HLA region, where we removed the SNPs located between 24,000,000 bp to 36,000,000 bp on chromosome 6 because of the existence of long-range haplotypes with rheumatoid arthritis susceptibility in this region[28]. LD pruning of the SNPs was conducted for the SNP pairs that were in LD ($r^2 \geq 0.3$) in both HapMap Phase 2 East Asian and Utah residents of Northern and Western European ancestry (CEU) populations (release 24). Correlations of the odds ratios were evaluated using R statistical software version 2.11.0.

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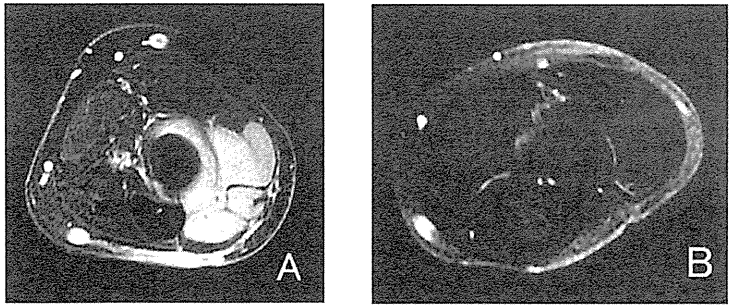
Clinical Images: Solitary extranodal malignant lymphoma of the forearm in rheumatoid arthritis



The patient, a 57-year-old woman with longstanding rheumatoid arthritis (RA), presented with a nontender nodule on her left forearm that had progressively increased in size over several months. Her RA was in complete remission after 18 months of etanercept treatment, in combination with oral pulse methotrexate at a dosage of 10 mg/week. Magnetic resonance imaging (MRI) of her left forearm revealed a lesion with high signal intensity in the extensor muscles on T2-weighted, fat-suppressed images, suggesting myositis or extranodal lymphoma (**A**). An open biopsy was performed, and histopathologic examination revealed a diffuse large B cell lymphoma. A positron emission tomography/computed tomography scan obtained 2 weeks later did not show any lymphoma in other parts of the body, and the histologic abnormality was not found in the bone marrow biopsy specimen. Despite the absence of clear evidence of an association between anti–tumor necrosis factor therapy and increased risk of lymphoma, etanercept and methotrexate were immediately discontinued. An MRI performed 1 month after discontinuation of etanercept and methotrexate showed complete regression of the lymphoma (**B**). The patient is currently receiving careful clinical monitoring with no antineoplastic treatment.

Takuji Iwamoto, MD, PhD
Yohei Seto, MD, PhD
Katsunori Ikari, MD, PhD
Hisashi Yamanaka, MD, PhD
Shigeki Momohara, MD, PhD
Tokyo Women's Medical University
Tokyo, Japan

PADI4 polymorphism predisposes male smokers to rheumatoid arthritis

Yuta Kochi,[1] Mohamed M Thabet,[2] Akari Suzuki,[1] Yukinori Okada,[3] Nina A Daha,[2] René E M Toes,[2] Tom W J Huizinga,[2] Keiko Myouzen,[1] Michiaki Kubo,[1] Ryo Yamada,[4] Yusuke Nakamura,[5] Kazuhiko Yamamoto[3]

► Supplementary tables are published online only. To view these files please visit the journal online (http://ard.bmj.com)

[1] Center for Genomic Medicine (CGM), RIKEN, Yokohama, Japan
[2] Leiden University Medical Center, Leiden, The Netherlands
[3] Graduate School of Medicine, the University of Tokyo, Tokyo, Japan
[4] Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan
[5] Institute of Medical Science, the University of Tokyo, Tokyo, Japan

Correspondence to
Dr Yuta Kochi, Laboratory for Autoimmune Diseases, CGM, RIKEN, 7-3-1 Hongo, Bunkyo-Ku, Tokyo 113-0033, Japan; ykochi@src.riken.jp

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ABSTRACT

Objective To elucidate the differential role of peptidyl arginine deiminase 4 (*PADI4*) polymorphism in rheumatoid arthritis (RA) between Asian and European populations, possible gene–environmental interactions among the *PADI4* polymorphism, sex and smoking status were analysed.

Methods Three independent sets of case–control samples were genotyped for single-nucleotide polymorphisms in *PADI4*; Japanese samples (first set, 1019 RA patients, 907 controls; second set, 999 RA patients, 1128 controls) using TaqMan assays and Dutch samples (635 RA patients, 391 controls) using Sequenom MassARRAY platform. The association of *PADI4* with RA susceptibility was evaluated by smoking status and sex in contingency tables and logistic regression models.

Results In the first set of Japanese samples, *PADI4* polymorphism (rs1748033) showed a greater risk in men (OR_{allele} 1.39; 95% CI 1.10 to 1.76; p_{trend}=0.0054) than in women and in ever-smokers (OR_{allele} 1.25; 95% CI 1.02 to 1.53; p_{trend}=0.032) than in never-smokers. Moreover, the highest risk was seen in male ever-smokers (OR_{allele} 1.46; 95% CI 1.12 to 1.90; p_{trend}=0.0047). Similar trends were observed in the second set of Japanese samples as well as in Dutch samples.

Conclusion *PADI4* polymorphism highly predisposes male smokers to RA, and the genetic heterogeneity observed between Asian and European populations may be partly explained by differences in smoking prevalence among men.

Rheumatoid arthritis (RA) is a multigenic disease caused by interactions between genetic predispositions and environmental factors that result in abnormal immune response and joint destruction. The *HLA-DRB1* region is considered to be the major genetic determinant of RA susceptibility, but recent genetic studies have revealed multiple non-human leucocyte antigen susceptibility genes for RA.[1] Among these, the peptidyl arginine deiminase 4 (*PADI4*) gene, which encodes a post-translational modification enzyme that converts arginine to citrulline residues in proteins, is thought to have significant relevance in RA pathogenesis as anti-citrullinated protein antibodies (ACPA) are specifically observed in the sera of patients.[2 3]

The association of the *PADI4* polymorphism with RA susceptibility was first reported in a Japanese population[2] and has been replicated in several Asian populations.[4 5] Conversely, inconsistent results have been observed in populations of European ancestry.[6–8] A meta-analysis confirmed the association in Asian populations, but not in European populations.[6 7] The genetic heterogeneity observed between different populations could be partly explained by the difference of disease severity between the study populations, as the *PADI4* polymorphism was reported to influence erosive joint status.[9] However, it could also be explained by unknown gene–gene or gene–environmental interactions with *PADI4*, and the higher magnitude of risk with *PADI4* in Asian populations suggests the presence of these interacting factors.

Smoking is one of the well-established environmental factors in RA,[10] and several studies have described associations with the appearance of ACPA in RA patients.[3] Klareskog *et al*[11] first reported that citrullinated proteins were detected in bronchoalveolar lavage cells from smokers but not in those from non-smokers. A later study by Makrygiannakis *et al*[12] showed that a significantly increased *PADI2* expression and a higher trend of *PADI4* expression were observed in bronchoalveolar lavage cells from smokers compared with non-smokers. These lines of evidence suggest that the upregulated expression of PADI enzymes provoked by smoking may promote the citrullination of proteins in the lung, leading to citrulline autoimmunity in RA.[3]

The present study examined possible interactions between *PADI4* polymorphism, sex and smoking status, and discusses the resulting influence on the genetic heterogeneity in *PADI4* observed between Asian and European populations.

METHODS

Subjects

Japanese RA patients (first set n=1019, second set n=999) were provided by the Leading Project for Personalized Medicine in the Ministry of Education, Culture, Sports, Science and Technology, Japan (BioBank Japan).[13] Unrelated Japanese controls (first set n=907, second set n=1128) were recruited through Midousuji Rotary club and several medical institutes in Japan. These Japanese case–control sets were independent from that used in the previous study.[2] Dutch cohorts and RA patients were previously described.[14] RA patients (n=635) were part of the Leiden Early Arthritis Clinic, which comprises an inception cohort of patients with recent-onset arthritis (duration of symptoms <2 years). Those patients were diagnosed with RA within the first year after their initial visit. All individuals with RA met the 1987 revised criteria of the American College of Rheumatology for RA.[10] The characteristics of the cohorts are described in detail in supplementary table 1 (available online only). All subjects entered into this study provided informed consent prior to participation in the study, and all study protocols were preapproved by the ethics committees of each institute.

Smoking status

Smoking status was determined for each individual on the basis of self-reported information. An ever-smoker was defined as a person who had smoked tobacco, cigarettes or pipes at any stage in their life, whereas a never-smoker was defined as someone who had never smoked any of these. Smoking status was available for all the samples in the first Japanese case–control set, cases in the second Japanese set and a part of the Dutch RA patients (52.9%), but not for the control subjects in the second Japanese and Dutch sets.

SNP genotyping

The four exonic single-nucleotide polymorphisms (SNP) comprising two major transcripts of *PADI4* (rs11203366=padi4_89, rs11203367=padi4_90, rs874881=padi4_92 and rs1748033=padi4_104) were genotyped.[2] Two of these SNP (rs11203367 and rs1748033) tag the three haplotypes (two common haplotypes and one rare haplotype, see supplementary table 2, available online only) and provide full information for *PADI4*. These were also tested in the Dutch population. In the Japanese population SNP were genotyped using predesigned TaqMan SNP genotyping assays (Applied Biosystems, Carlsbad, California, USA). Fluorescence was detected using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). In the Dutch population SNP were genotyped using time-of-flight mass spectrometry-based Sequenom MassARRAY Platform (Sequenom, San Diego, California, USA). Genotyping assessment was made for over 95% of samples, for all of the polymorphisms genotyped. All SNP were in Hardy–Weinberg equilibrium in control subjects according to χ^2 statistics (p>0.01).

Statistical analysis

The case–control association of each SNP was tested with the Cochran Armitage trend test and the χ^2 test. Genotype and

Table 1 Association of the *PADI4* polymorphism and RA stratified with sex and smoking status in a Japanese population*

		Sum		MAF			
	Set	Case	Contol	Case	Contol	Per allele OR	p Value for trend test
rs11203367							
All	1st	1019	907	0.43	0.40	1.14 (1.00 to 1.29)	0.045
	2nd	996	1124	0.42	0.40	1.09 (0.96 to 1.23)	0.16
Men	1st	190	672	0.48	0.39	1.44 (1.14 to 1.81)	0.0022
	2nd	185	448	0.44	0.40	1.18 (0.92 to 1.51)	0.19
Women	1st	829	235	0.42	0.41	1.02 (0.82 to 1.25)	0.84
	2nd	811	676	0.41	0.39	1.07 (0.92 to 1.24)	0.31
Ever-smoker	1st	337	488	0.47	0.39	1.35 (1.11 to 1.65)	0.0024
	2nd	302	1124	0.43	0.40	1.15 (0.95 to 1.38)	0.12
Never-smoker	1st	682	418	0.41	0.40	1.03 (0.86 to 1.23)	0.71
	2nd	694	1124	0.41	0.40	1.06 (0.92 to 1.21)	0.36
Male ever-smoker	1st	155	451	0.50	0.39	1.61 (1.24 to 2.09)	0.00031
	2nd	145	448	0.46	0.40	1.25 (0.96 to 1.63)	0.10
Male never-smoker	1st	35	221	0.39	0.40	0.92 (0.54 to 1.54)	0.77
	2nd	40	448	0.39	0.40	0.95 (0.59 to 1.52)	0.84
Female ever-smoker	1st	182	37	0.44	0.47	0.86 (0.52 to 1.42)	0.56
	2nd	157	676	0.41	0.39	1.05 (0.82 to 1.35)	0.64
Female never-smoker	1st	647	197	0.41	0.40	1.04 (0.83 to 1.32)	0.68
	2nd	654	676	0.41	0.39	1.08 (0.92 to 1.26)	0.31
rs1748033							
All	1st	1018	904	0.37	0.35	1.12 (0.98 to 1.27)	0.089
	2nd	996	1125	0.36	0.34	1.08 (0.95 to 1.22)	0.20
Men	1st	190	669	0.42	0.34	1.39 (1.10 to 1.76)	0.0054
	2nd	185	448	0.40	0.34	1.25 (0.97 to 1.60)	0.08
Women	1st	828	235	0.36	0.36	1.00 (0.81 to 1.24)	0.96
	2nd	811	677	0.35	0.34	1.05 (0.90 to 1.22)	0.50
Ever-smoker	1st	336	485	0.40	0.35	1.25 (1.02 to 1.53)	0.032
	2nd	302	1125	0.38	0.34	1.19 (0.99 to 1.43)	0.055
Never-smoker	1st	682	418	0.36	0.34	1.07 (0.89 to 1.28)	0.47
	2nd	694	1125	0.35	0.34	1.03 (0.90 to 1.19)	0.59
Male ever-smoker	1st	155	448	0.44	0.34	1.46 (1.12 to 1.90)	0.0047
	2nd	145	448	0.41	0.34	1.34 (1.02 to 1.75)	0.039
Male never-smoker	1st	35	221	0.36	0.34	1.09 (0.64 to 1.85)	0.75
	2nd	40	448	0.34	0.34	0.96 (0.59 to 1.56)	0.90
Female ever-smoker	1st	181	37	0.37	0.41	0.87 (0.52 to 1.45)	0.60
	2nd	157	677	0.36	0.34	1.06 (0.82 to 1.37)	0.60
Female never-smoker	1st	647	197	0.36	0.35	1.03 (0.81 to 1.30)	0.79
	2nd	654	677	0.35	0.34	1.04 (0.89 to 1.22)	0.55

*rs112033673 (T/C, T is the minor allele) and rs174803 (T/C, T is the minor allele) were genotyped for the test. Both case and control subjects were stratified with smoking status in the first set, whereas only case subjects were stratified with smoking status in the second set.
MAF, minor allele frequency; *PADI4*, peptidyl arginine deiminase 4; RA, rheumatoid arthritis.