MS, and Mary Whitman, PhD, (Janssen Services, LLC); Yoshifumi Ukyo and Yoshinori Murakami, PhD (Janssen Pharmaceutical K.K) for assistance in preparing this manuscript.

Contributors All authors contributed to the design and/or conduct of the trial, analysis and/or interpretation of data and manuscript preparation and/or review for critical content. All authors also approved the final manuscript for submission to *ARD*. RC, MP and MW (Janssen Services, LLC) and YU and YM (Janssen Pharmaceutical K.K) provided assistance with preparing this manuscript.

Funding This study was funded by Janssen Pharmaceuticals KK

Competing interests DB is an employee of Janssen Research & Development, LLC and owns stock in Johnson & Johnson. HY has received research grants from Abbott, Bristol Myers Squibb, Chugai Pharmaceutical, Eizai Pharmaceutical, Janssen Pharmaceutical, Mitsubishi Tanabe Pharmaceutical, Otsuka Pharmaceutical, Roche, Takeda Pharmaceutical and Wyeth. KY has received research grants from Astellas Pharmaceutical, Chugai Pharmaceutical, Eizai Pharmaceutical, Immunofuture Inc, Mitsubishi Tanabe Pharma Corporation, Santen Pharmaceutical and Wyeth. MH has received research grants from Abbott, Bristol Myers Squibb, Chugai Pharmaceutical, Eizai Pharmaceutical, Janssen Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, Takeda Pharmaceutical and Wyeth and received consultant fees from Abbott, Bristol Myers Squibb, Chugai Pharmaceutical, Janssen Pharmaceutical and Mitsubishi Tanabe Pharma Corporation. MK has received research grants from Astellas Pharmaceutical, Astra Zeneca, Banyu Pharmaceutical, Daiichi Sankyo Pharmaceutical, Eizai Pharmaceutical, Janssen Pharmaceutical, GlaxoSmithKline, Mitsubishi Tanabe Pharma Corporation, Nippon Boehringer Ingelheim and Novartis. NI has received research grants from Astellas Pharmaceutical, Chugai Pharmaceutical, Eizai Pharmaceutical and Mitsubishi Tanabe Pharma Corporation. NM has received research grants from Abbott, Astellas Pharmaceutical, Banyu Pharmaceutical, Chugai Pharmaceutical, Daiichi Sankyo Pharmaceutical, Eizai Pharmaceutical, Janssen Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, Takeda Pharmaceutical and Teijin Pharmaceutical. TK has received research grants from Abbott, Bristol Myers Squibb, Chugai Pharmaceutical, Eizai Pharmaceutical, Janssen Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, Otsuka Pharmaceutical, Pfizer, Takeda Pharmaceutical and Wyeth. TO is an employee of Janssen Pharmaceutical KK, a wholly owned subsidiary of Johnson & Johnson. TT has received research grants from Abbott, Astra Zeneca, Bristol Myers Squibb, Chugai Pharmaceutical, Eizai Pharmaceutical, Janssen Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, Novartis, Takeda Pharmaceutical and Wyeth. TY is an employee of Mitsubishi Tanabe Pharma Corporation. YT has received research grants from Abbott, Astellas Pharmaceutical, Banyu Pharmaceutical, Chugai Pharmaceutical, Eizai Pharmaceutical, Janssen Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, Pfizer and Takeda Pharmaceutical.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1. **Feldmann M,** Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. *Annu Rev Immunol* 1996;**14**:397–440.
2. **Badolato R,** Oppenheim JJ. Role of cytokines, acute-phase proteins and chemokines in the progression of rheumatoid arthritis. *Semin Arthritis Rheum* 1996;**26**:526–38.
3. **Wolfe F,** Hawley DJ. The longterm outcomes of rheumatoid arthritis: work disability: a prospective 18 year study of 823 patients. *J Rheumatol* 1998;**25**:2108–17.
4. **Young A,** Koduri G. Extra-articular manifestations and complications of rheumatoid arthritis. *Best Pract Res Clin Rheumatol* 2007;**21**:907–27.
5. **Tanaka Y,** Takeuchi T, Inoue E, *et al*. Retrospective clinical study on the notable efficacy and related factors of infliximab therapy in a rheumatoid arthritis management group in Japan: one-year clinical outcomes (RECONFIRM-2). *Mod Rheumatol* 2008;**18**:146–52.
6. **Koike T,** Harigai M, Inokuma S, *et al*. Postmarketing surveillance of safety and effectiveness of etanercept in Japanese patients with rheumatoid arthritis. *Mod Rheumatol* 2011;**21**:343–51.
7. **Shealy D,** Cai A, Staquet K, *et al*. Characterization of golimumab, a human monoclonal antibody specific for human tumor necrosis factor alpha. *MAbs* 2010;**2**:428–39.
8. **Emery P,** Fleischmann RM, Moreland LW, *et al*. Golimumab, a human anti-tumor necrosis factor alpha monoclonal antibody, injected subcutaneously every four weeks in methotrexate-naive patients with active rheumatoid arthritis: twenty-four-week results of a phase III, multicenter, randomized, double-blind, placebo-controlled study of golimumab before methotrexate as first-line therapy for early-onset rheumatoid arthritis. *Arthritis Rheum* 2009;**60**:2272–83.
9. **Keystone EC,** Genovese MC, Klareskog L, *et al*. Golimumab, a human antibody to tumour necrosis factor α given by monthly subcutaneous injections, in active rheumatoid arthritis despite methotrexate therapy: the GO-FORWARD Study. *Ann Rheum Dis* 2009;**68**:789–96.
10. **Schnabel A,** Gross WL. Low-dose methotrexate in rheumatic diseases—efficacy, side effects, and risk factors for side effects. *Semin Arthritis Rheum* 1994;**23**:310–27.
11. **Arnett FC,** Edworthy SM, Bloch DA, *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;**31**:315–24.
12. **Prevoo ML,** van't Hof MA, Kuper HH, *et al*. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. *Arthritis Rheum* 1995;**38**:44–8.
13. **van Riel PL,** van Gestel AM, Scott DL. *EULAR Handbook of Clinical Assessments in Rheumatoid Arthritis*. Alphen Aan Den Rijn, The Netherlands: Van Zuiden Communications, BV, 2000.
14. **van der Heijde DM.** Plain x-rays in rheumatoid arthritis: overview of scoring methods, their reliability and applicability. *Baillieres Clin Rheumatol* 1996;**10**: 435–53.
15. **Zhou H,** Jang H, Fleischmann RM, *et al*. Pharmacokinetics and safety of golimumab, a fully human anti-TNF-alpha monoclonal antibody, in subjects with rheumatoid arthritis. *J Clin Pharmacol* 2007;**47**:383–96.
16. **Tanaka Y,** Harigai M, Takeuchi T, *et al*., for the GO-FORTH Study Group. Golimumab in combination with methotrexate in Japanese patients with active rheumatoid arthritis: results of the GO-FORTH study. *Ann Rheum Dis* 2012;**71**:817–24.
17. **Bruno R,** Washington CB, Lu JF, *et al*. Population pharmacokinetics of trastuzumab in patients with HER2+ metastatic breast cancer. *Cancer Chemother Pharmacol* 2005;**56**:361–9.
18. **Xu Z,** Vu T, Lee H, *et al*. Population pharmacokinetics of golimumab, an anti-tumor necrosis factor-α human monoclonal antibody, in patients with psoriatic arthritis. *J Clin Pharmacol* 2009;**49**:1056–70.
19. **Zhu Y,** Hu C, Lu M, *et al*. Population pharmacokinetic modeling of ustekinumab, a human monoclonal antibody targeting IL-12/23p40, in patients with moderate to severe plaque psoriasis. *J Clin Pharmacol* 2009;**49**:162–75.
20. **Ling J,** Lyn S, Xu Z, *et al*. Lack of racial differences in the pharmacokinetics of subcutaneous golimumab in healthy Japanese and Caucasian male subjects. *J Clin Pharmacol* 2010;**50**:792–802.
21. **Takeuchi T,** Kameda H. The Japanese experience with biologic therapies for rheumatoid arthritis. *Nat Rev Rheumatol* 2010;**6**:644–52.
22. **Nishimoto N,** Hashimoto J, Miyasaka N, *et al*. Study of active controlled monotherapy used for rheumatoid arthritis, an IL-6 inhibitor (SAMURAI): evidence of clinical and radiographic benefit from an x-ray reader blinded randomized controlled trial of tocilizumab. *Ann Rheum Dis* 2007;**66**:1162–7.
23. **Smolen JS,** van der Heijde DM, St Clair EW, *et al*. Predictors of joint damage in patients with early rheumatoid arthritis treated with high-dose methotrexate with or without concomitant infliximab: results from the ASPIRE trial. *Arthritis Rheum* 2006;**54**:702–10.
24. **Emery P,** Fleischmann R, van der Heijde D, *et al*. The effects of golimumab on radiographic progression in rheumatoid arthritis: results of randomized controlled studies of golimumab before methotrexate therapy and golimumab after methotrexate therapy. *Arthritis Rheum* 2011;**63**:1200–10.
25. Simponi (package insert). Japan: Janssen Pharmaceutical KK and Mitsubishi Tanabe Pharma Corporation, 2011.

Inhibition of plasma IL-6 in addition to maintenance of an efficacious trough level of infliximab associated with clinical remission in patients with rheumatoid arthritis: analysis of the RISING Study

Many clinical studies have reported the excellent clinical efficacy of infliximab (IFX), an antitumour necrosis factor α (anti-TNFα) monoclonal antibody, in the treatment of rheumatoid arthritis (RA).[1] IFX is also reported to induce a rapid and marked reduction in circulating interleukin 6 (IL-6) levels, suggesting that its efficacy may result from the suppression of IL-6 as well as TNF.[2–5] In the RISING Study (NCT00691028),[6 7] we observed patients who showed no response to IFX therapy, despite maintaining a serum IFX level higher than the threshold level for clinical response. Here, we examined data on clinical response to better understand the mechanism of action of IFX.

In this study, patients with methotrexate-refractory RA treated with 3 mg/kg of IFX at weeks 0, 2 and 6 were randomly assigned to receive 3, 6 or 10 mg/kg of IFX every 8 weeks from week 14 to 46 in combination with methotrexate. A total of 271 patients were classified into four groups based on serum IFX, which was used as a surrogate marker of TNF suppression since the TNF level could not be accurately measured in the presence of IFX, and plasma IL-6 levels at week 54 (Group 1, IFX-high/IL-6-low; Group 2, IFX-high/IL-6-high; Group 3, IFX-low/IL-6-low; Group 4, IFX-low/IL-6-high), with cut-off values of IFX and IL-6 of 1.0 μg/ml[6 8 9] and 10 pg/ml (lower 80% confidence limit with one tail at baseline), respectively.

The median IL-6 level at baseline was 28.9 pg/ml, which rapidly decreased at week 10 and remained at 2.4 pg/ml at week 54 with a median suppression rate of 87.2%.

Clinical characteristics such as TNF and C reactive protein (CRP) levels and rheumatoid factor (RF) values showed significant differences among groups at baseline (table 1). At week 54, however, significant differences were observed in each of the disease activity indices, with the lowest disease activity in Group 1, intermediate in Groups 2 and 3 and highest in Group 4. In particular, remission rates were significantly higher in Group 1 (figure 1). A difference was also observed in the improvement of physical function. By contrast, the progression of joint damage was inhibited in the majority of patients, with no significant difference between groups.

We speculate that the efficacy of treatment seen in Group 1 resulted from the simultaneous suppression of TNF and IL-6, that in Group 2 from the direct action of TNF neutralisation and that in Group 3 via IL-6 suppression. TNF served as a main modulator of IL-6 in Group 1, as proposed by Feldman *et al*,[2] but exerted only partial modulation in Group 2. RF and

Table 1 Clinical characteristics at baseline and week 54 of the four groups classified by serum infliximab and plasma IL-6 levels at week 54

	Group 1 (n=134) IFX≥1.0 mg/ml, IL-6<10 pg/ml	Group 2 (n=31) IFX≥1.0 mg/ml, IL-6≥10 pg/ml	Group 3 (n=48) IFX<1.0 mg/ml, IL-6<10 pg/ml	Group 4 (n=58) IFX<1.0 mg/ml, IL-6≥10 pg/ml	p Value
At baseline (week 0)					
Plasma tumour necrosis factor level, pg/ml	0.88 (<0.55, 1.22)	0.87 (<0.55, 1.30)	1.01 (<0.55, 1.26)	1.07 (0.68, 1.90)	0.023
Plasma IL-6 level, pg/ml	27.7 (12.3, 61.6)	44.1 (12.1, 95.5)	28.7 (11.2, 54.8)	33.2 (19.5, 72.1)	0.328
DAS28-ESR	6.2 (5.6, 6.8)	6.2 (5.5, 6.7)	6.0 (5.4, 6.8)	6.5 (5.8, 7.0)	0.146
DAS28-CRP	5.5 (5.0, 6.1)	5.4 (4.9, 6.0)	5.1 (4.6, 5.9)	5.7 (5.3, 6.4)	0.045
SDAI	34.6 (27.6, 43.9)	34.0 (27.4, 44.2)	32.4 (25.8, 45.3)	38.3 (31.8, 46.0)	0.199
CDAI	32.0 (25.3, 40.7)	30.2 (24.5, 39.7)	30.4 (24.8, 41.5)	34.9 (28.3, 40.0)	0.395
TJC, 68 joints	15.0 (11.0, 23.0)	13.0 (9.0, 22.0)	15.0 (11.0, 22.0)	16.0 (10.0, 28.0)	0.389
SJC, 66 joints	12.0 (9.0, 17.0)	10.0 (6.0, 14.0)	12.5 (8.0, 16.0)	13.5 (10.0, 18.0)	0.367
CRP, mg/dl	2.0 (1.1, 4.1)	3.0 (1.9, 5.2)	1.7 (0.6, 3.2)	2.9 (2.0, 4.1)	<0.001
ESR, mm/hr	49.5 (32.0, 67.0)	49.0 (43.0, 68.0)	46.5 (33.5, 70.0)	63.5 (38.0, 87.0)	0.057
Patients global VAS, mm	53.0 (34.0, 71.0)	60.0 (36.0, 75.0)	51.0 (34.5, 65.5)	63.5 (46.0, 78.0)	0.067
Physicians global VAS, mm	61.0 (47.0, 75.0)	72.0 (55.0, 85.0)	59.5 (48.0, 76.0)	68.5 (53.0, 79.0)	0.056
TSS, point	40.9 (12.0, 84.9)	33.0 (11.5, 66.5)	20.8 (9.0, 56.5)*	35.5 (14.0, 86.0)	0.139
Estimated yearly progression of TSS, point/year	6.7 (3.2, 11.7)	5.5 (3.3, 12.4)	5.0 (2.5, 8.5)*	7.5 (4.0, 11.4)	0.134
HAQ score, unit	1.06 (0.63, 1.63)	1.25 (0.88, 1.63)	1.19 (0.75, 1.63)	1.38 (0.88, 1.75)	0.266
RF value, IU/ml	78.5 (33.0, 194.0)	81.0 (22.0, 128.0)	165.5 (51.0, 319.5)	130.5 (74.0, 280.0)	0.005
ACPA value, IU/ml	≥100 (24.6, ≥100)	≥100 (28.4, ≥100)	≥100 (32.9, ≥100)	≥100 (47.6, ≥100)	0.128
MMP3 value, ng/ml	191 (101, 388)	271 (183, 523)	174 (71, 364)	257 (109, 415)	0.075
At week 54					
Serum IFX level, μg/ml	4.6 (2.6, 8.3)	2.5 (1.7, 3.5)	<0.1 (<0.1, 0.5)	<0.1 (<0.1, 0.2)	-
Plasma IL-6 level, pg/ml	1.0 (0.6, 1.8)	23.7 (12.9, 42.8)	2.7 (1.1, 6.5)	37.0 (19.2, 68.5)	-
DAS28-ESR	2.6 (2.0, 3.4)	3.4 (2.5, 4.5)	3.6 (2.7, 4.4)	5.1 (4.0, 6.0)	<0.001
DAS28-CRP	1.9 (1.4, 2.7)	2.9 (2.4, 3.7)	2.7 (1.8, 3.7)	4.3 (3.6, 5.3)	<0.001
SDAI	3.6 (1.6, 8.1)	8.8 (4.1, 12.0)	7.0 (2.6, 13.0)	19.0 (10.4, 29.1)	<0.001
CDAI	3.3 (1.4, 8.0)	6.8 (3.8, 10.1)	6.8 (2.3, 12.4)	15.6 (8.0, 25.9)	<0.001
TJC, 68 joints	1.0 (0.0, 3.0)	2.0 (1.0, 5.0)	2.0 (1.0, 5.5)	6.0 (3.0, 15.0)	<0.001
SJC, 66 joints	1.0 (0.0, 2.0)	1.0 (0.0, 4.0)	1.0 (0.0, 4.5)	4.0 (2.0, 8.0)	<0.001
CRP, mg/dl	0.1 (0.0, 0.2)	0.6 (0.2, 2.5)	0.3 (0.1, 0.7)	2.1 (1.0, 4.0)	<0.001
ESR, mm/hr	14.0 (8.0, 24.0)	26.0 (15.0, 41.0)	29.0 (16.5, 44.0)	58.0 (41.0, 84.0)	<0.001
Patients global VAS, mm	9.0 (3.0, 21.0)	16.0 (6.0, 25.0)	13.5 (5.0, 36.0)	29.5 (18.0, 66.0)	<0.001
Physicians global VAS, mm	6.0 (2.0, 12.0)	15.0 (6.0, 26.0)	9.5 (3.0, 23.0)	32.5 (12.0, 53.0)	<0.001
Delta-TSS, point	0.0 (-1.0, 0.5)	0.0 (-1.5, 1.5)	0.0 (-0.5, 1.0)*	0.5 (-0.5, 3.0)	0.056
HAQ score, unit	0.25 (0.00, 0.75)	0.38 (0.13, 1.00)	0.38 (0.00, 0.94)	0.88 (0.38, 1.38)	<0.001
RF value, IU/ml	20.5 (5.0, 60.0)	20.0 (7.0, 44.0)	64.5 (16.5, 170.5)	88.5 (45.0, 238.0)	<0.001
ACPA value, IU/ml	38.2 (10.6, ≥100)	43.3 (10.2, ≥100)	≥100 (23.5, ≥100)	≥100 (31.8, ≥100)	0.009
MMP3 value, ng/ml	69 (37, 113)	185 (114, 387)	55 (38, 106)	220 (131, 390)	<0.001

Data values are median (IQR). Statistics were analysed using the Kruskal–Wallis test.
*n=46.
ACPA, anticyclic citrullinated peptide antibody; CDAI, clinical disease activity index; CRP, C reactive protein; DAS, disease activity score; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; IFX, infliximab; IL, interleukin; MMP, matrix metalloproteinase; RF, rheumatoid factor; SDAI, simplified disease activity index; SJC, swollen joint count; TJC, tender joint count; TSS, total modified-Sharp score; VAS, visual analogue scale.

anticyclic citrullinated peptide antibody titres were lower in Groups 1 and 2 (IFX-high) than in Groups 3 and 4 (IFX-low). Meanwhile, CRP and matrix metalloproteinase 3 levels were lower in Groups 1 and 3 (IL-6-low) than in Groups 2 and 4 (IL-6-high). However, we found no clear difference in evaluations when using the simplified disease activity index, which includes acute-phase reactants, or the clinical disease activity index, which does not include these reactants. We also analysed the proportion of primary or secondary non-responders in each group, as defined previously,[7] and found no obvious difference indicating a primary or secondary failure pattern.

Several limitations of our study warrant mention. First, we set the cut-off values for IFX and IL-6 levels ourselves because there are no standard reference values. Second, serum IFX level was used as a surrogate marker of TNF suppression, which cannot be accurately measured in the presence of IFX. Third, we had no data on other cytokines, so we could not explore their influence on clinical response.

This study demonstrates that the profound suppression of TNF and IL-6 in IFX treatment results in better clinical response, in addition to clinical remission.

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

[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

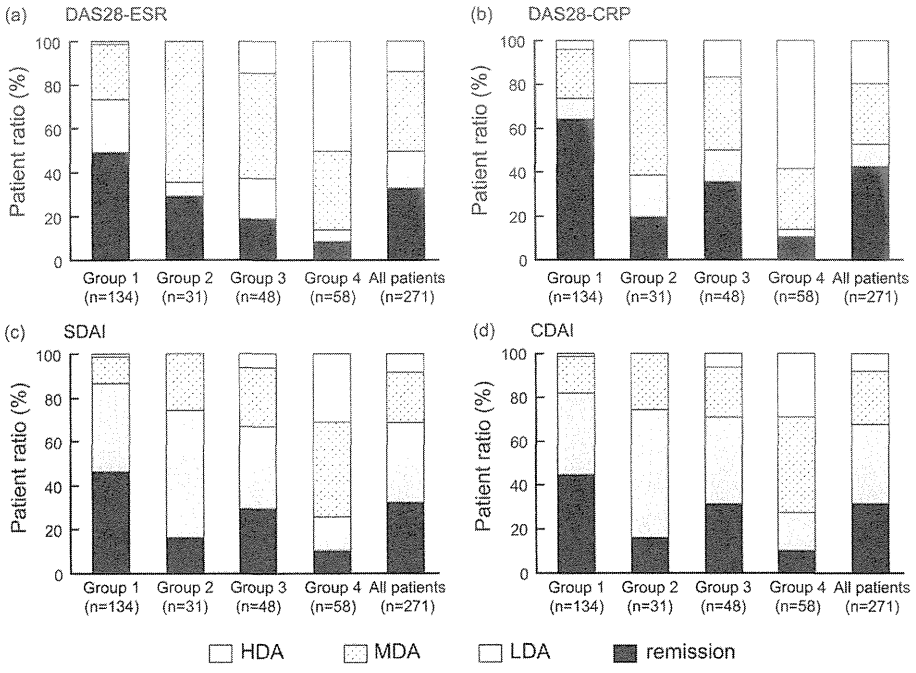

Figure 1 Efficacy of infliximab therapy at week 54 among the four groups classified by serum infliximab and plasma interleukin 6 levels at week 54. Clinical responses were analysed by disease activity score 28 (DAS28)-erythrocyte sedimentation rate (ESR) (A), DAS28-C reactive protein (CRP) (B), simplified disease activity index (SDAI) (C) and clinical disease activity index (CDAI) (D) at week 54. HDA, high disease activity; MDA, moderate disease activity; LDA, low disease activity but no remission. Cut-off values for disease activity in DAS28-CRP were used as reported by Inoue *et al* (HDA: >4.1; MDA: ≥2.7 to ≤4.1; LDA: ≥2.3 to <2.7; remission: <2.3).[10] **Figure 1** Efficacy of infliximab therapy at week 54 among the four

[3]Mitsubishi Tanabe Pharma Corporation, Osaka, Japan
[4]Saitama Medical Center, Saitama Medical University, Kawagoe, Japan
[5]Sapporo Medical Center NTT EC, Chuo-ku, 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

Contributors TT: manuscript preparation, study design, advice for statistics. NM: advice for study design. YT: manuscript preparation, data management. T Yano: statistics. T Yoshinari: data collection. TA: advice for study design. TK: management of the study.

Acknowledgements The authors wish to thank all the investigators who participated in the RISING study.

Funding Mitsubishi Tanabe Pharma Corporation sponsored this clinical trial and was responsible for the collection and analysis of data.

Patients consent Obtained.

Ethics approval This study was conducted with the approval of the local ethics committees of every participant centre.

Competing interests TT has received lecture fees from Abbott Japan, Bristol-Myers Squibb, Chugai Pharmaceutical, Eisai, Janssen Pharmaceutical, Mitsubishi Tanabe Pharma, Novartis, Pfizer Japan and Takeda Pharmaceutical. NM has received grant support from Abbott Japan, Astellas Pharma, Chugai Pharmaceutical, Daiichi Sankyo, Mitsubishi Tanabe Pharma and Takeda Pharmaceutical; and lecture fees from Benesis and Otsuka Pharmaceutical. YT, T Yano and T Yoshinari are employees of Mitsubishi Tanabe Pharma. TA has no conflicts of interest. TK has received lecture fees from Abbott Japan, Bristol-Myers Squibb, Chugai Pharmaceutical, Eisai, Mitsubishi Tanabe Pharma, Otsuka Pharmaceutical, Pfizer and Takeda Pharmaceutical.

Provenance and peer review Not commissioned; externally peer reviewed.

Accepted 25 February 2012

Published Online First 5 May 2012

Ann Rheum Dis 2012;**71**:1583–1585. doi:10.1136/annrheumdis-2011-201069

REFERENCES

1. **Nam JL,** Winthrop KL, van Vollenhoven RF, *et al*. Current evidence for the management of rheumatoid arthritis with biological disease-modifying antirheumatic drugs: a systematic literature review informing the EULAR recommendations for the management of RA. *Ann Rheum Dis* 2010;**69**:976–86.
2. **Feldmann M,** Maini RN. Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned? *Annu Rev Immunol* 2001;**19**:163–96.
3. **Charles P,** Elliott MJ, Davis D, *et al*. Regulation of cytokines, cytokine inhibitors, and acute-phase proteins following anti-TNF-alpha therapy in rheumatoid arthritis. *J Immunol* 1999;**163**:1521–8.
4. **Knudsen LS,** Ostergaard M, Baslund B , *et al*. Plasma IL-6, plasma VEGF, and serum YKL-40: relationship with disease activity and radiographic progression in rheumatoid arthritis patients treated with infliximab and methotrexate. *Scand J Rheumatol* 2006;**35**:489–91.
5. **Dain L,** Braun-Moscovici Y, Baum E, *et al*. Modification of neutrophil function by plasma of rheumatoid arthritis patients treated with infliximab. *Clin Exp Rheumatol* 2006;**24**:38–44.
6. **Takeuchi T,** Miyasaka N, Inoue K, *et al*. Impact of trough serum level on radiographic and clinical response to infliximab plus methotrexate in patients with rheumatoid arthritis: results from the RISING study. *Mod Rheumatol* 2009;**19**:478–87.
7. **Takeuchi T,** Miyasaka N, Tatsuki Y, *et al*. Baseline tumour necrosis factor alpha levels predict the necessity for dose escalation of infliximab therapy in patients with rheumatoid arthritis. *Ann Rheum Dis* 2011;**70**:1208–15.
8. **St Clair EW,** Wagner CL, Fasanmade AA, *et al*. The relationship of serum infliximab concentrations to clinical improvement in rheumatoid arthritis: results from ATTRACT, a multicenter, randomized, double-blind, placebo-controlled trial. *Arthritis Rheum* 2002;**46**:1451–9.
9. **Rahman MU,** Strusberg I, Geusens P, *et al*. Double-blinded infliximab dose escalation in patients with rheumatoid arthritis. *Ann Rheum Dis* 2007;**66**:1233–8.
10. **Inoue E,** Yamanaka H, Hara M, *et al*. Comparison of Disease Activity Score (DAS) 28- erythrocyte sedimentation rate and DAS28- C-reactive protein threshold values. *Ann Rheum Dis* 2007;**66**:407–9.

ARD Online First, published on January 11, 2013 as 10.1136/annrheumdis-2012-202433

OPEN ACCESS

EXTENDED REPORT

Adalimumab, a human anti-TNF monoclonal antibody, outcome study for the prevention of joint damage in Japanese patients with early rheumatoid arthritis: the HOPEFUL 1 study

Tsutomu Takeuchi,[1] Hisashi Yamanaka,[2] Naoki Ishiguro,[3] Nobuyuki Miyasaka,[4] Masaya Mukai,[5] Tsukasa Matsubara,[6] Shoji Uchida,[7] Hideto Akama,[8] Hartmut Kupper,[9] Vipin Arora,[10] Yoshiya Tanaka[11]

▶ Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2012-202433).

For numbered affiliations see end of article

Correspondence to
Professor 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

Received 2 August 2012
Revised 14 November 2012
Accepted 9 December 2012

ABSTRACT

Objectives To evaluate the efficacy and safety of adalimumab+methotrexate (MTX) in Japanese patients with early rheumatoid arthritis (RA) who had not previously received MTX or biologics.

Methods This randomised, double-blind, placebo-controlled, multicentre study evaluated adalimumab 40 mg every other week+MTX 6–8 mg every week versus MTX 6–8 mg every week alone for 26 weeks in patients with RA (≤2-year duration). The primary endpoint was inhibition of radiographic progression (change (Δ) from baseline in modified total Sharp score (mTSS)) at week 26.

Results A total of 171 patients received adalimumab+MTX (mean dose, 6.2±0.8 mg/week) and 163 patients received MTX alone (mean dose, 6.6±0.6 mg/week, p<0.001). The mean RA duration was 0.3 years and 315 (94.3%) had high disease activity (DAS28>5.1). Adalimumab+MTX significantly inhibited radiographic progression at week 26 versus MTX alone (ΔmTSS, 1.5±6.1 vs 2.4±3.2, respectively; p<0.001). Significantly more patients in the adalimumab+MTX group (62.0%) did not show radiographic progression (ΔmTSS≤0.5) versus the MTX alone group (35.4%; p<0.001). Patients treated with adalimumab+MTX were significantly more likely to achieve American College of Rheumatology responses and achieve clinical remission, using various definitions, at 26 weeks versus MTX alone. Combination therapy was well tolerated, and no new safety signals were observed.

Conclusions Adalimumab in combination with low-dose MTX was well tolerated and efficacious in suppressing radiographic progression and improving clinical outcomes in Japanese patients with early RA and high disease activity.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disorder that is associated with joint damage and progressive disability, an increased risk of morbidity related to comorbid conditions, and substantial socioeconomic costs.[1–3] Given the significant impact biologic therapies have had in the treatment of RA, a paradigm shift has emerged toward earlier inclusion of these therapies in the management of RA.[3 4] Furthermore, international guidelines published in 2010 recommend a treat-to-target goal of remission for patients with early RA in order to mitigate radiographic progression and long-term disability.[5] The efficacy and safety of adalimumab, a tumour necrosis factor (TNF)-α inhibitor, administered as monotherapy or in combination with methotrexate (MTX) for the treatment of RA has been well established in clinical trials conducted in Western countries.[6–12] In early RA, the PREMIER and OPTIMA studies demonstrated that initial combination therapy with adalimumab and MTX was superior to MTX alone in inhibiting radiographic progression and improving clinical symptoms.[6 7 12]

Translating efficacy and safety results of RA Western-based studies to an Eastern populace can be potentially misleading given the genetic, medical and environmental differences (eg, body weight) observed between the two populations.[13] A limited number of studies have evaluated the efficacy or effectiveness and safety of adalimumab in Japanese patients. However, these studies either assessed adalimumab monotherapy in moderate-to-severe RA[14] or were retrospective[15] or postmarketing surveillance studies[16] of adalimumab monotherapy or combination therapy in a population with a wide range of RA duration and prior biologic and MTX experience. Thus, a randomised, placebo-controlled study of adalimumab +MTX combination therapy in MTX-naive Japanese patients with early RA was lacking.

The current study, called adalimumab, a human anti-TNF monoclonal antibody, outcome study for the persistent efficacy under allocation to treatment strategies in early RA, or HOPEFUL 1, was conducted to compare the efficacy and safety of early intervention with adalimumab+MTX versus MTX alone for 26 weeks in inhibiting radiographic progression in MTX-naive Japanese patients with RA.

PATIENTS AND METHODS

Patients aged ≥20 years were evaluated during March 2009 and November 2010 from 94 centres. Eligible patients had RA (1987-revised American College of Rheumatology (ACR) criteria),[17] of ≤2-year duration, a tender joint count ≥10, a swollen joint count ≥8, a C reactive protein (CRP) level ≥1.5 mg/dl or erythrocyte sedimentation rate

To cite: Takeuchi T, Yamanaka H, Ishiguro N, *et al. Ann Rheum Dis* Published Online First: [*please include* Day Month Year] doi:10.1136/annrheumdis-2012-202433

(ESR) ≥28 mm/h, and had ≥1 joint erosion or were rheumatoid factor positive. Patients had not previously received MTX, leflunomide or >2 other disease-modifying antirheumatic drugs (DMARDs). Patients who had previously received cyclophosphamide, cyclosporine, azathioprine, tacrolimus or biologic DMARDs (eg, anti-TNF-α therapy) and patients with a chronic infection, interstitial pneumonia, or a history of tuberculosis or malignancy were excluded from the study.

The phase III trial consisted of a randomised, double-blind, placebo-controlled, 26-week phase followed by a 26-week open-label extension phase (clinicaltrials.gov identifier, NCT00870467; only 26-week double-blind data presented). After a 4-week washout period for patients taking eligible DMARDs and a >2-week screening period for all patients, participants were randomised (1 : 1) to receive subcutaneous adalimumab 40 mg or placebo every other week, both administered in combination with oral MTX 6–8 mg/week (adalimumab +MTX vs MTX alone) for 26 weeks. Treatment with MTX was initiated at 6 mg/week and increased to 8 mg/week in patients who did not experience ≥20% decrease from baseline in tender or swollen joint counts on or after week 8, unless investigators indicated a safety concern. In addition, reduction of the MTX dose to 4 mg/week was permitted at the investigator's discretion. All patients received concomitant oral folic acid 5 mg/week. Patients who experienced a >20% increase from baseline in tender and swollen joint counts at weeks 12, 16 or 20 were to discontinue blinded treatment with adalimumab or placebo and were eligible for open-label rescue treatment with adalimumab 40 mg every other week.

The primary endpoint was inhibition of radiographic progression assessed as the change from baseline (Δ) in modified total Sharp score (mTSS) at week 26. All single-emulsion radiographs of the hands (posteroanterior view) and feet (anteroposterior view) obtained from a patient were scored by two independent readers blinded to patient and treatment, as previously described,[6] with the exception that the triquetrum/pisiform joint was not scored for erosions and the first interphalangeal joint was not scored for joint-space narrowing (range, 0–380) (see online supplementary text for more information).

Secondary efficacy endpoints included ACR responses[18 19] by visit; clinical remission (the 28-joint disease activity score with ESR (DAS28-ESR)<2.6) at week 26;[20 21] and change from baseline in the Health Assessment Questionnaire disability index (HAQ-DI)[22] at week 26. Several additional post hoc analyses were conducted, including assessments of the DAS28-CRP, simplified disease activity index (SDAI)[23] and clinical disease activity index (CDAI) scores[24] over time; clinically relevant radiographic progression (ΔmTSS>3); European League Against Rheumatism responses[25] at week 26; and clinical remission, defined as DAS28-CRP<2.6,[26] SDAI≤3.3,[27 28] CDAI≤2.8[28] or meeting Boolean remission criteria,[27] at week 26. Low, medium and high disease activity was also determined using DAS28-ESR, DAS28-CRP, SDAI and CDAI. Adverse events (AEs) and clinical laboratory parameters were routinely monitored during the study. A 28-day follow-up after the completion of or discontinuation from the study and a 70-day follow-up after the last dose of adalimumab administration were conducted to evaluate safety.

Statistics

The primary endpoint was analysed using the Wilcoxon rank sum test for observed data with a separate supportive analysis using linear extrapolation (LE) to impute missing values. Secondary endpoints were analysed using the Fisher's exact test and Wilcoxon rank sum test for discrete variables and continuous variables, respectively. Non-responder imputation was used for binary variables, and the last-observation-carried-forward approach was applied for continuous variables. The safety population included all randomised patients who received ≥1 dose of study medication and had ≥1 efficacy assessment.

To identify baseline predictors of no radiographic progression (mTSS≤0.5) and clinical remission (DAS28-ESR<2.6),

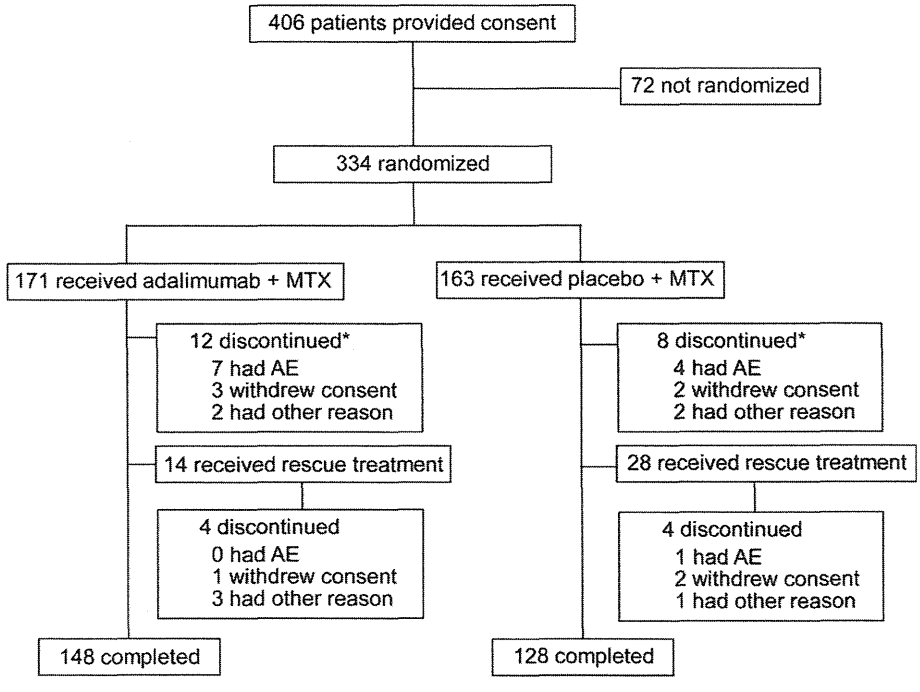

Figure 1 Patient disposition through week 26. *Three adalimumab+MTX patients and one MTX alone patient discontinued from the study by week 26; however, they were included in the efficacy analyses at week 26. AE, adverse event; MTX, methotrexate.

univariate logistic regression analysis was performed, applying 24 baseline demographics and disease characteristics. Significant ($p<0.1$) variables in univariate were included in multivariate models. Last, multivariate models were selected based on model fit statistics (Akaike information criterion and r^2) and clinical significance. Adjusted OR and 95% CIs for selected baseline variables were calculated.

RESULTS

Overall, 334 patients were randomised to treatment and received adalimumab+MTX (n=171) or MTX alone (n=163), and 148 (86.5%) and 128 (78.5%) patients completed the double-blind portion of the study, respectively (figure 1). Demographics and baseline characteristics were well matched between treatment groups (table 1). The mean RA disease duration was 0.3 years, and the majority of patients had ≥1 erosion at baseline and high disease activity. The mean MTX dose during the 26-week study was 6.2±0.8 mg/week in the adalimumab+MTX group and 6.6±0.6 mg/week in the MTX alone group ($p<0.001$). After 26 weeks of treatment, 34.5% (59/171) of adalimumab+MTX patients were receiving MTX 8 mg/week versus 65.0% (106/163) of MTX alone patients ($p<0.001$).

Radiographic progression

Treatment with adalimumab+MTX significantly inhibited radiographic progression (figure 2A) at week 26 versus MTX alone (mean change±SD, 1.5±6.1 vs 2.4±3.2, respectively; $p<0.001$). Results were confirmed by an LE analysis (figure 2A). Changes in radiographic progression during 26 weeks of treatment were also assessed by a cumulative probability plot of ΔmTSS (figure 2B). Fewer adalimumab+MTX patients exhibited radiographic progression (ΔmTSS>0.5), with 62.0% (106/171) of patients showing no radiographic progression versus 35.4% (57/161) of MTX alone patients ($p<0.001$). Furthermore, only 14.0% (24/171) of adalimumab+MTX patients exhibited clinically relevant radiographic progression (ΔmTSS>3) versus 37.3% (60/161) of MTX alone patients ($p<0.001$). In addition, a significantly higher percentage of adalimumab+MTX patients did not experience worsening (≤0.5) in erosion score (73.7% (126/171)) versus MTX alone patients (42.2% (68/161); $p<0.001$). In patients who lacked baseline erosive damage, the continued absence of erosions was reported in more adalimumab+MTX patients versus MTX alone patients (9/9 vs 2/6 patients, respectively; $p=0.01$).

Clinical response

A significantly higher percentage of adalimumab+MTX patients achieved ACR responses versus MTX alone patients at each assessment (figure 3A–C). Significant differences between treatment groups, observed as early as week 2, were maintained through week 26. At week 26, a significantly larger percentage of adalimumab+MTX patients versus MTX alone patients achieved ACR20, ACR50 and ACR70 (figure 3A–C) and ACR90 (12.9% vs 5.5%; $p=0.02$) responses. Significant differences in favour of adalimumab+MTX were also observed from week 2 to 26 for DAS28-ESR, DAS28-CRP, SDAI and CDAI (see online supplementary figure 1A–D). A larger percentage of adalimumab+MTX patients than MTX alone patients demonstrated good or moderate European League Against Rheumatism responses (figure 3D) and were in states of low disease activity or remission after 26 weeks of treatment (figure 3E). Furthermore, a significantly larger percentage of adalimumab+MTX patients versus MTX alone patients satisfied Boolean remission criteria (19.3% vs 8.6%, $p=0.007$). Adalimumab+MTX achieved a 1.8- to 2.2-fold increase in the percentage of patients achieving clinical remission, across all definitions of clinical remission evaluated, versus MTX alone.

Table 1 Demographics and baseline characteristics

Parameter*	Adalimumab+MTX (n=171)	MTX (n=163)
Age±SD (year)	54.0±13.1	54.0±13.2
Females (n (%))	144 (84.2)	128 (78.5)
RA duration±SD (year)	0.3±0.4	0.3±0.4
Weight±SD (kg)	54.4±9.7	56.1±12.3
Previous DMARD use (n (%))	74 (43.3)	87 (53.4)
1 DMARD	57 (33.3)	69 (42.3)
2 DMARDs	17 (9.9)	18 (11.0)
Corticosteroid use at baseline (n (%))	58 (33.9)	49 (30.1)
RF positive (n (%))	146 (85.4)	136 (83.4)
Mean titre±SD (IU/ml)	154.5±202.3	163.7±362.8
Anti-CCP positive (n (%))	145 (84.8)	136 (83.4)
Mean titre±SD (U/ml)	386.2±694.2	241.3±367.2
ESR (mm/h)	59.9±30.1	61.8±29.0
CRP (mg/dl)	2.9±3.0	3.1±3.3
Swollen joint count (n±SD)		
0–28	11.5±4.7	11.8±5.3
0–66	16.5±6.2	17.3±7.7
Tender joint count (n±SD)		
0–28	13.2±5.8	13.2±6.1
0–68	20.7±9.4	21.1±10.2
mTSS	13.6±22.3	13.6±17.4
Erosion score	7.5±11.6	7.3±9.2
Joint space narrowing score	6.2±11.4	6.2±9.4
DAS28-ESR	6.6±0.9	6.6±1.0
DAS28-CRP	5.8±1.0	5.9±1.0
HAQ-DI score	1.1±0.7	1.3±0.8
SDAI score	40.7±12.0	41.4±13.8
CDAI score	37.8±10.9	38.3±12.4
Physician's global assessment of disease activity±SD (mm)	65.8±18.4	66.2±18.8
Patient's global assessment of disease activity±SD (mm)	64.1±24.8	66.4±23.7

*Data are mean±SD unless otherwise indicated.
CCP, cyclic citrullinated peptide; CDAI, clinical disease activity index; CRP, C reactive protein; DAS28-CRP, disease activity score using a 28-joint count and CRP level; DAS28-ESR, disease activity score using a 28-joint count and ESR; DMARD, disease-modifying antirheumatic drug; ESR, erythrocyte sedimentation rate; HAQ-DI, Health Assessment Questionnaire disability index; mTSS, modified total Sharp score; MTX, methotrexate; RA, rheumatoid arthritis; RF, rheumatoid factor; SDAI, simplified disease activity index.

A significantly larger decrease from baseline in mean HAQ-DI score, indicative of an improvement in physical function, was observed for adalimumab+MTX patients versus MTX alone patients at week 26 (−0.6±0.6 vs −0.4±0.6; $p<0.001$). Although the significant difference between the two groups was small (0.2 units), the percentage of patients achieving normal functionality (HAQ-DI score<0.5) after 26 weeks of treatment was also significantly higher with adalimumab+MTX (figure 3F).

Factors associated with the absence of radiographic progression or with clinical remission

Disease activity or function baseline variables generally were associated with the absence of radiographic progression (ΔmTSS≤0.5) and with clinical remission (DAS28-ESR<2.6) in both treatment groups (see online supplementary text and online supplementary table 1).

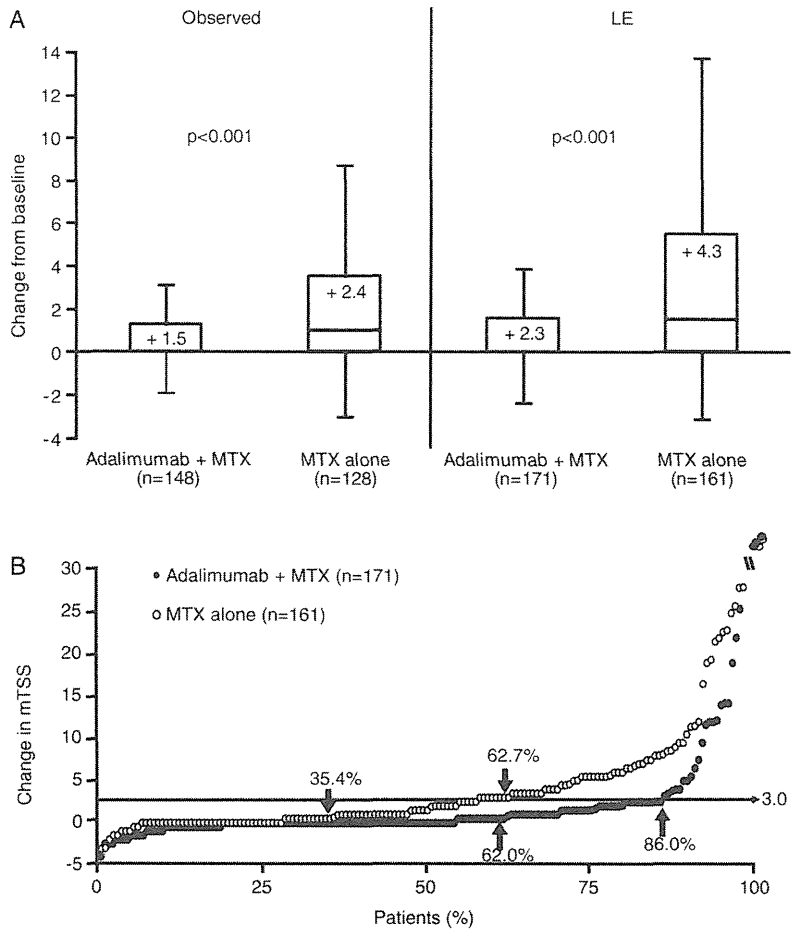

Figure 2 (A) Box plot of change from baseline in mTSS at week 26 with adalimumab+MTX versus MTX alone and (B) cumulative probability plot of mean change from baseline to week 26 in mTSS score (LE). Thickened horizontal lines in (A) indicate median values, the boxes mark the interval between the 25th and 75th percentiles, whiskers indicate the IQR and mean values are reported in the boxes. No radiographic progression (change from baseline in mTSS≤0.5) was reported in 62.0% (106/171) of adalimumab+MTX patients versus 35.4% (57/161) of MTX alone patients (p<0.001). No clinically relevant radiographic progression (change from baseline mTSS≤3) was reported in 86.0% (147/171) of adalimumab+MTX patients versus 62.7% (101/161) of MTX alone patients (p<0.001) (B). LE, linear extrapolation; mTSS, modified total Sharp score; MTX, methotrexate. p Value determined using Wilcoxon rank sum test.

Safety

The mean treatment duration during the double-blind phase was 168.7±36.6 days for adalimumab+MTX patients (mean cumulative adalimumab dose, 477.4±104.5 mg) and 162.8±38.6 days for MTX alone patients. Overall, there were 376 and 302 AEs reported in the adalimumab+MTX group and the MTX alone group, respectively. There were no significant differences in the percentage of patients with AEs in the adalimumab+MTX group (80.7% (138/171)) versus the MTX alone group (71.8% (117/163)), and the incidence of severe AEs was rare (table 2). No significant differences in the incidence of AEs of interest were observed between the two groups, with the exception of injection-site reactions, which were reported in 10.5% of adalimumab+MTX patients and 3.7% of MTX alone patients (p=0.02; table 2). Serious infections were observed in two adalimumab+MTX patients (one case each of pneumonia and infectious enteritis) and one MTX alone patient (*Pneumocystis jiroveci* pneumonia), occurring at rates of 2.5 and 1.4 events per 100 patient-years, respectively. There were no reports of demyelination, tuberculosis or malignancy during the study. One death, due to worsening of interstitial lung disease, occurred in the MTX alone group.

DISCUSSION

The HOPEFUL 1 study was designed to evaluate the efficacy and safety of adalimumab in combination with MTX in Japanese patients with early RA. This is the first description of a clinical trial of anti-TNF therapy+MTX versus MTX alone in MTX-naive Japanese patients with early RA and high disease activity. It is also the first randomised trial evaluating the efficacy of anti-TNF therapy+low-dose MTX versus low-dose MTX alone for the inhibition of radiographic progression in any patient population. This study extends observations from Western studies of adalimumab by demonstrating the superiority of adalimumab+MTX to MTX alone for the inhibition of radiographic progression and improvement in clinical outcomes in Japanese patients with early RA. Moreover, the combination of adalimumab+MTX significantly improved a wide array of clinical and functional disease activity measures and responses versus MTX alone, with improvements observed as early as the first assessment (week 2) and maintained through the 26-week double-blind trial.

Following 26 weeks of treatment, the mean ΔmTSS (primary endpoint) in adalimumab+MTX patients (1.48) in the current study was significantly smaller than observed in MTX alone patients (2.38). In addition, a similar trend in inhibition of radiographic progression in patients with early RA was observed in the OPTIMA study, with a smaller mean ΔmTSS in adalimumab+MTX patients (0.15) versus MTX alone patients (0.96; p<0.001).[12] The difference between the two treatment groups (0.8) at week 26 was similar to the difference observed in the current study (0.9 (observed)).[12] Furthermore, baseline characteristics, including RA duration, in the two studies were generally similar, but the OPTIMA study had a lower percentage of previous DMARD use.

A similar trend in inhibition of radiographic progression in the current study was observed in the PREMIER study, with a

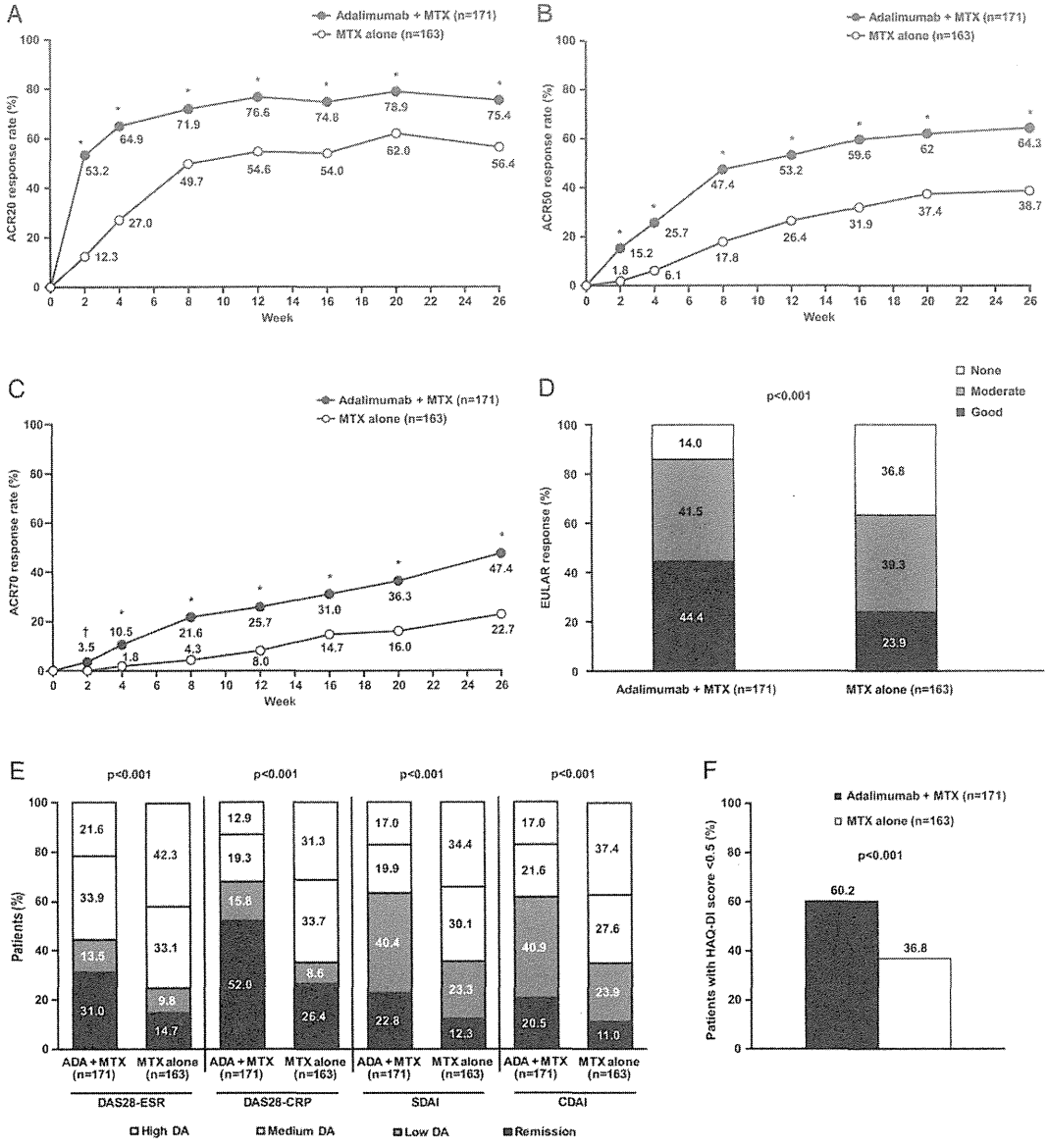

Figure 3 Percentage of patients with an (A) ACR20 response, (B) ACR50 response or (C) ACR70 response over time; (D) the percentage of patients with a EULAR response at week 26; (E) the percentage of patients with low, medium or high disease activity at week 26; and (F) the percentage of patients achieving functional remission (HAQ-DI score<0.5) at week 26. The following values were used to identify remission, low, medium and high disease activity for each clinical assessment in (E): DAS28-ESR or DAS-CRP (<2.6, ≥2.6–<3.2; ≥3.2–≤5.1, >5.1, respectively), SDAI (≤3.3, >3.3–≤11.0, >11.0–≤26.0, >26.0, respectively), and CDAI (≤2.8, >2.8–≤10.0, >10.0–≤22.0, >22.0, respectively). *p<0.001 versus MTX alone. †p=0.03 versus MTX alone. ACR, American College of Rheumatology; ADA, adalimumab; CDAI, clinical disease activity index; DA, disease activity; DAS28-CRP, disease activity score using a 28-joint count and C reactive protein level; DAS28-ESR, disease activity score using a 28-joint count and erythrocyte sedimentation rate; EULAR, European League Against Rheumatism; HAQ-DI, Health Assessment Questionnaire disability index; MTX, methotrexate; SDAI, simplified disease activity index.

smaller mean ΔmTSS in adalimumab+MTX patients (0.8) versus MTX alone patients (3.5; p<0.001). However, the mean difference in radiographic progression between the two treatments groups, although statistically significant, was smaller in the current study (0.9 (observed); 2.0 (LE)) than in the PREMIER study (2.7).

In the current study, the SD for the mean ΔmTSS at week 26 was generally high. When the median ΔmTSS was compared using observed data, results were in good agreement between the PREMIER study (0.0 (adalimumab+MTX) vs 1.3 (MTX alone); data on file) and the current study (0.0 (adalimumab +MTX) vs 1.0 (MTX alone)). Alternatively, the smaller difference in improvement observed in the current study may also be related to the mTSS scoring method used, but this seems unlikely because only two joints assessed in PREMIER were omitted from scoring in the present analysis. The mean duration of RA was also shorter in the current study (0.3 years) versus the PREMIER study (0.7–0.8 years), although the percentage of patients who had previously taken DMARDs was higher (43.3–53.4% vs 31.5–32.5%). There were also slight differences in mean baseline tender and swollen joint counts and CRP levels, which were higher in the PREMIER study and considered

Table 2 Adverse events (AEs)

Parameter	Patients (n (%)) Adalimumab+MTX (n=171)	MTX (n=163)
Any AE	138 (80.7)	117 (71.8)
Severe AE	1 (0.6)	1 (0.6)
Serious AE	7 (4.1)	4 (2.4)
Infectious AE	59 (34.5)	48 (29.4)
Serious infection	2 (1.2)	1 (0.6)
AEs leading to study drug discontinuation	7 (4.1)	6 (3.7)
AEs of interest		
Elevated liver function test level	32 (18.7)†	21 (12.9)†
Injection-site reaction	18 (10.5)*	6 (3.7)
Haematological event	7 (4.1)	8 (4.9)
Allergic reaction	1 (0.6)	2 (1.2)
Interstitial lung disease	1 (0.6)	1 (0.6)
Lupus-like syndrome	0	1 (0.6)
Opportunistic infection	0	1 (0.6)

*p=0.02 versus MTX.
†≥94% of events were mild in severity.
MTX, methotrexate.

related to the longer duration of RA at baseline versus the current study. Furthermore, the MTX dose of 6–8 mg/week, although consistent with the dosage commonly administered in Japan at the time the study was conducted, was substantially lower than that commonly administered in Western countries (eg, 15–20 mg/week). In the PREMIER study, MTX was initiated at 7.5 mg/week, increased to 15 mg/week during weeks 4–8, and increased to 20 mg/week starting at week 9. In addition, the mean MTX dose during the 26 weeks of the current study was significantly lower in the adalimumab+MTX group (6.2±0.8 mg/week) versus the MTX alone group (6.6±0.6 mg/week; $p<0.001$), thereby potentially impacting the ΔmTSS and thus the maximal difference observed between the two treatment groups. Therefore, these multiple differences may have contributed to the small difference in radiographic outcomes between the current study and the PREMIER study. Whether the difference in radiographic outcomes can be explained by differences between Japanese and Western populations remains unclear, although this seems unlikely. Longer-term studies may help elucidate potential differences in outcomes.

Since this study was conducted, the maximum approved MTX dosage in Japan has been increased from 8 to 16 mg/week in patients with RA. Therefore, this study provides important information on the efficacy of low-dose MTX and anti-TNF therapy versus low-dose MTX alone for the inhibition of radiographic progression. Data suggest that patients with early RA who may not tolerate higher doses of MTX will likely benefit from adalimumab+low-dose MTX combination therapy.

Given the lower MTX dose prescribed, one could question whether we might only be seeing natural progression in the MTX only arm. It is ethically difficult to include a true placebo arm in clinical trials of ≥6 months duration for early active RA, particularly when MTX is recommended as first-line therapy to achieve clinical remission/low disease activity. Although an important question to ponder, a placebo arm in long-term clinical trials in early active RA appears to be unrealistic, and further research using highly sensitive and reproducible imaging techniques during a short-term placebo-treatment period in early active RA is warranted.

It is also important to note that the current patient population had severe baseline symptoms, including baseline erosions, despite only several months since RA onset. This scenario is becoming increasingly less common in Western populations due to treat-to-target recommendations and earlier intervention. In Japan, general practitioners are still seeing many early RA patients and referrals to rheumatologists are often delayed. In addition, the diagnosis of RA in this trial was based upon 1987 classification criteria. Thus, these factors may have played a role in the conundrum of more severe baseline clinical symptoms yet shorter mean disease duration.

The clinical results of the current study are supported by the HARMONY study, which retrospectively determined the effectiveness and safety of adalimumab 40 mg every other week with or without MTX (mean dose, 8.5 mg/week) in Japanese patients with RA (mean RA duration, 9.0±9.5 years) with or without prior biologic treatment.[15] Although patients in the HARMONY study had more established disease and the study design was retrospective, adalimumab+MTX patients (n=143) had an improvement from baseline in DAS28-ESR score at week 24 (baseline, 5.3; week 24, 3.3), which was within the range but slightly smaller than the improvement observed in the current study at week 26 (baseline, 6.6; week 26, 3.7; see online supplementary figure 1A). Clinical remission rates for adalimumab+MTX patients were also comparable between the HARMONY study (week 24, 35.0%) and the current study (week 26, 31.0%).

The safety profile of the current study was generally consistent with those in previous clinical studies of adalimumab in patients with RA conducted in Japan.[14–16] There were no reports of demyelination, tuberculosis or malignancy, and there were no statistically significant differences in the incidence of serious AEs, serious infections, opportunistic infections or lupus-like reactions between adalimumab+MTX patients versus MTX alone patients. There was a significantly higher incidence of injection-site reactions for adalimumab+MTX patients versus MTX alone patients, but the incidence (10.5%) was similar to that reported for the 167 adalimumab±MTX patients in the HARMONY study (12.0%). The incidence of injection-site reactions in both of these studies was lower than the 30.8% reported for the 91 adalimumab monotherapy patients (40 mg every other week) in the CHANGE study,[14] possibly related to the immunosuppressive effects of concomitant MTX in the current study and in some of the patients in the HARMONY study.

In the multivariate regression analyses (see online supplementary table 1), lower baseline CRP level was identified as a predictor of radiographic non-progression in adalimumab+MTX patients, whereas normal baseline CRP level (≤0.3 mg/dl) appeared to have an increased likelihood of radiographic non-progression. However, no baseline predictors appeared to predict both the lack of progression and clinical remission. Furthermore, baseline mTSS was not an independent predictor for either treatment group in this study.

Overall, adalimumab+MTX was well tolerated in Japanese patients with early RA with no new safety signals and with a safety and tolerability profile similar to that observed in Western populations. Administration of adalimumab in combination with MTX was efficacious in improving radiographic and clinical responses in MTX-naive patients with early RA, high disease activity and poor prognostic factors (eg, rheumatoid factor positive or with baseline erosive damage) through week 26. Given its radiographic, clinical and functional superiority versus MTX monotherapy, consideration should be given to administration

of anti-TNF-α and MTX combination therapy in patients with early RA and high disease activity.

Author affiliations
[1]Division of Rheumatology, Department of Internal Medicine, School of Medicine, Keio University, Shinjuku-ku, Tokyo, Japan
[2]Institute of Rheumatology, Tokyo Women's Medical University, Shinjuku-ku, Tokyo, Japan
[3]Department of Orthopedic Surgery, Nagoya University Graduate School and School of Medicine, Showa-ku, Nagoya, Japan
[4]Department of Medicine and Rheumatology, Graduate School of Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan
[5]Division of Rheumatology and Hematology, Department of Medicine, Sapporo City General Hospital, Chuo-ku, Sapporo, Japan
[6]Matsubara Mayflower Hospital, Katou-shi, Hyogo, Japan
[7]Uchida Clinic of Rheumatic Diseases, Sumida-ku, Tokyo, Japan
[8]Eisai Co, Ltd., Bunkyo-ku, Tokyo, Japan
[9]Abbott GmbH & Co KG, Ludwigshafen, Germany
[10]Abbott Laboratories, Abbott Park, Illinois, USA
[11]The First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, Japan, Yahatanishi-ku, Kitakyushu, Japan

Acknowledgements The authors would like to thank all the patients, investigators and support staff who participated in the study, Sourav Santra, PhD, formally of Abbott, who provided statistical support, and Mary Beth C. Moncrief, PhD, of MedThink SciCom, for editorial assistance in the writing of this manuscript; this assistance was funded by Abbott.

Contributors All the authors evaluated the study results, interpreted the data and suggested additional analyses. All authors contributed to the development and critical review of manuscript and approved the final version.

Funding This study was supported by Abbott Japan Co (Tokyo, Japan) and Eisai Co (Tokyo, Japan).

Competing interests TT has received consulting fees, speaking fees, honoraria and/or research grant support from Abbott Japan Co; Astellas Pharma Inc; Astra-Zeneca K.K.; Bristol-Myers Squibb; Chugai Pharmaceutical Co; Daiichi-Sankyo Co; Eisai Co; Janssen Pharmaceutical K.K.; Mitsubishi Tanabe Pharma Corporation; Pfizer Japan Inc; and Takeda Pharmaceutical Co. HY has received research grants from Abbott Japan Co; Bristol-Myers Squibb; Chugai Pharmaceutical Co; Eisai Co; Janssen Pharmaceutical K.K.; Mitsubishi Tanabe Pharma Corporation; Otsuka Pharmaceutical Co; Pfizer Japan Inc; Takeda Industrial Pharmaceutical Co; and UCB Japan Co, and speakers honoraria/consulting fees from Abbott Japan Co; Bristol-Myers Squibb; Chugai Pharmaceutical Co; Eisai Co; Janssen Pharmaceutical K.K.; Mitsubishi Tanabe Pharma Corporation; Otsuka Pharmaceutical Co; Pfizer Japan Inc; Takeda Pharmaceutical Co; and UCB Japan Co. NI has received research grants from Astellas Pharmaceutical; Chugai Pharmaceutical Co; Eisai Co; and Mitsubishi Tanabe Pharmaceutical Co. NM has received research grants from Abbott Japan Co; Astellas Pharmaceutical; Banyu Pharmaceutical; Chugai Pharmaceutical Co; Daiichi Sankyo Pharmaceutical Co; Eisai Co; Janssen Pharmaceuticals; Mitsubishi Tanabe Pharma Corporation; Takeda Pharmaceutical Co; and Teijin Limited. MM has received research grants from Abbott Japan Co; Eli Lilly Japan K.K.; GlaxoSmithKline K.K.; Pfizer Japan Inc; Bristol-Myers Squibb; and Otsuka Pharmaceutical Co, and received compensation for work on this manuscript from Abbott Japan Co. TM has received research grants from Chugai Pharmaceutical Co; Bristol-Myers Squibb; Nippon Kayaku Co; Otsuka Pharmaceutical Co; Takeda Pharmaceutical Co; Eli Lilly Japan K.K.; Eli Lilly and Company; Astellas Pharma Inc; Pfizer Japan Inc; AstraZeneca K.K.; and Santen Pharmaceutical Co, and received compensation for work on this manuscript from Abbott Japan Co. SU has received research grants from Abbott Japan Co, and received compensation for work on this manuscript from Abbott Japan Co. HA is an employee of Eisai Co, Tokyo, Japan. HK is an employee of Abbott GmbH and Co KG, Ludwigshafen, Germany, and may hold Abbott stock or options. VA is an employee of Abbott Laboratories, Abbott Park, Illinois, USA, and may hold Abbott stock or options. YT has received consulting fees, speaking fees and/or honoraria from Mitsubishi Tanabe Pharma Corporation; Abbott Japan Co; Eisai Co; Chugai Pharmaceutical Co; Janssen Pharmaceutical K.K.; Santen Pharmaceutical Co; Pfizer Japan Inc; Astellas Pharma Inc; Daiichi-Sankyo Co; GlaxoSmithKline K.K.; Astra-Zeneca; Otsuka Pharmaceutical Co; Actelion Pharmaceuticals Japan; Eli Lilly Japan K.K.; Nippon Kayaku Co; UCB Japan Co; Quintiles Transnational Japan Co; Ono Pharmaceutical Co; and Novartis Pharma K.K. YT has received research grants from Bristol-Myers Squibb; MSD K.K.; Chugai Pharmaceutical Co; Mitsubishi Tanabe Pharma Corporation; Astellas Pharma Inc; Abbott Japan Co; Eisai Co; and Janssen Pharmaceutical K.K.

Patient consent Obtained.

Ethics approval An institutional review board approved the study at each site.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1 Filipovic I, Walker D, Forster F, *et al*. Quantifying the economic burden of productivity loss in rheumatoid arthritis. *Rheumatology (Oxford)* 2011;50:1083–90.
2 Scott DL, Wolfe F, Huizinga TWJ. Rheumatoid arthritis. *Lancet* 2010;376:1094–108.
3 Takeuchi T. Revolutionary change in rheumatoid arthritis management with biological therapy. *Keio J Med* 2011;60:75–81.
4 Saag KG, Teng GG, Patkar NM, *et al*. American College of Rheumatology 2008 recommendations for the use of nonbiologic and biologic disease-modifying antirheumatic drugs in rheumatoid arthritis. *Arthritis Rheum* 2008;59:762–84.
5 Smolen JS, Aletaha D, Bijlsma JW, *et al*. For the T2T Expert Committee. Treating rheumatoid arthritis to target: recommendations of an international task force. *Ann Rheum Dis* 2010;69:631–7.
6 Breedveld FC, Weisman MH, Kavanaugh AF, *et al*. For the PREMIER Investigators. The PREMIER study: a multicenter, randomized, double-blind clinical trial of combination therapy with adalimumab plus methotrexate versus methotrexate alone or adalimumab alone in patients with early, aggressive rheumatoid arthritis who had not had previous methotrexate treatment. *Arthritis Rheum* 2006;54:26–37.
7 van der Heijde D, Breedveld FC, Kavanaugh A, *et al*. Disease activity, physical function, and radiographic progression after longterm therapy with adalimumab plus methotrexate: 5-year results of PREMIER. *J Rheumatol* 2010;37:2237–46.
8 Weinblatt ME, Keystone EC, Furst DE, *et al*. Adalimumab, a fully human anti-tumor necrosis factor α monoclonal antibody, for the treatment of rheumatoid arthritis in patients taking concomitant methotrexate: the ARMadalimumab trial. *Arthritis Rheum* 2003;48:35–45.
9 van de Putte LBA, Atkins C, Malaise M, *et al*. Efficacy and safety of adalimumab as monotherapy in patients with rheumatoid arthritis for whom previous disease modifying antirheumatic drug treatment has failed. *Ann Rheum Dis* 2004;63:508–16.
10 Keystone EC, Kavanaugh AF, Sharp JT, *et al*. Radiographic, clinical, and functional outcomes of treatment with adalimumab (a human anti-tumor necrosis factor monoclonal antibody) in patients with active rheumatoid arthritis receiving concomitant methotrexate therapy: a randomized, placebo-controlled, 52-week trial. *Arthritis Rheum* 2004;50:1400–11.
11 Furst DE, Schiff MH, Fleischmann RM, *et al*. Adalimumab, a fully human anti tumor necrosis factor-alpha monoclonal antibody, and concomitant standard antirheumatic therapy for the treatment of rheumatoid arthritis: results of STAR (Safety Trial of Adalimumab in Rheumatoid Arthritis). *J Rheumatol* 2003;30:2563–71.
12 Kavanaugh A, Fleischmann RM, Emery P, *et al*. Clinical, functional and radiographic consequences of achieving stable low disease activity and remission with adalimumab plus methotrexate or methotrexate alone in early rheumatoid arthritis: 26-week results from the randomised, controlled OPTIMA study. *Ann Rheum Dis* 2013;72:64–71.
13 Takeuchi T, Kameda H. The Japanese experience with biologic therapies for rheumatoid arthritis. *Nat Rev Rheumatol* 2010;6:644–52.
14 Miyasaka N, The CHANGE Study Investigators. Clinical investigation in highly disease-affected rheumatoid arthritis patients in Japan with adalimumab applying standard and general evaluation: the CHANGE study. *Mod Rheumatol* 2008;18:252–62.
15 Takeuchi T, Tanaka Y, Kaneko Y, *et al*. Effectiveness and safety of adalimumab in Japanese patients with rheumatoid arthritis: retrospective analyses of data collected during the first year of adalimumab treatment in routine clinical practice (HARMONY study). *Mod Rheumatol* 2012;22:327–38.
16 Koike T, Harigai M, Ishiguro N, *et al*. Safety and effectiveness of adalimumab in Japanese rheumatoid arthritis patients: postmarketing surveillance report of the first 3,000 patients. *Mod Rheumatol* 2012;22:498–508.
17 Arnett FC, Edworthy SM, Bloch DA, *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31:315–24.
18 Felson DT, Anderson JJ, Boers M, *et al*. American College of Rheumatology. Preliminary definition of improvement in rheumatoid arthritis. *Arthritis Rheum* 1995;38:727–35.
19 Felson DT, Anderson JJ, Lange ML, *et al*. Should improvement in rheumatoid arthritis clinical trials be defined as fifty percent or seventy percent improvement in core set measures, rather than twenty percent? *Arthritis Rheum* 1998;41:1564–70.
20 Fransen J, Creemers MCW, van Riel PLCM. Remission in rheumatoid arthritis: agreement of the disease activity score (DAS28) with the ARA preliminary remission criteria. *Rheumatology (Oxford)* 2004;43:1252–5.
21 Prevoo ML, van't Hof MA, Kuper HH, *et al*. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective

longitudinal study of patients with rheumatoid arthritis. *Arthritis Rheum* 1995;38:44–8.
22 Fries JF, Spitz P, Kraines RG, *et al*. Measurement of patient outcome in arthritis. *Arthritis Rheum* 1980;23:137–45.
23 Smolen JS, Breedveld FC, Schiff MH, *et al*. A simplified disease activity index for rheumatoid arthritis for use in clinical practice. *Rheumatology (Oxford)* 2003;42:244–57.
24 Aletaha D, Nell VPK, Stamm T, *et al*. Acute phase reactants add little to composite disease activity indices for rheumatoid arthritis: validation of a clinical activity score. *Arthritis Res Ther* 2005;7:R796–806.
25 van Gestel AM, Prevoo MLL, van't Hof MA, *et al*. Development and validation of the European League Against Rheumatism response criteria for rheumatoid arthritis. Comparison with the preliminary American College of Rheumatology and the World Health Organization/International League Against Rheumatism criteria. *Arthritis Rheum* 1996;39:34–40.
26 Wells G, Becker J-C, Teng J, *et al*. Validation of the 28-joint Disease Activity Score (DAS28) and European League Against Rheumatism response criteria based on C-reactive protein against disease progression in patients with rheumatoid arthritis, and comparison with the DAS28 based on erythrocyte sedimentation rate. *Ann Rheum Dis* 2009;68:954–60.
27 Felson DT, Smolen JS, Wells G, *et al*. American College of Rheumatology/European League Against Rheumatism provisional definition of remission in rheumatoid arthritis for clinical trials. *Ann Rheum Dis* 2011;70:404–13.
28 Aletaha D, Smolen J. The Simplified Disease Activity Index (SDAI) and the Clinical Disease Activity Index (CDAI): a review of their usefulness and validity in rheumatoid arthritis. *Clin Exp Rheumatol* 2005;23(5 suppl 39):S100–8.

OPEN ACCESS Freely available online PLoS one

ACPA-Negative RA Consists of Two Genetically Distinct Subsets Based on RF Positivity in Japanese

Chikashi Terao[1,2]*, Koichiro Ohmura[1]*, Katsunori Ikari[3], Yuta Kochi[4], Etsuko Maruya[5], Masaki Katayama[1], Kimiko Yurugi[6], Kota Shimada[7], Akira Murasawa[8], Shigeru Honjo[9], Kiyoshi Takasugi[10], Keitaro Matsuo[11], Kazuo Tajima[11], Akari Suzuki[4], Kazuhiko Yamamoto[12], Shigeki Momohara[3], Hisashi Yamanaka[3], Ryo Yamada[2], Hiroo Saji[5], Fumihiko Matsuda[2,13,14], Tsuneyo Mimori[1]

1 Department of Rheumatology and Clinical Immunology, Kyoto University Graduate School of Medicine, Kyoto, Japan, **2** Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan, **3** Institute of Rheumatology, Tokyo Women's Medical University, Tokyo, Japan, **4** Laboratory for Autoimmune Diseases, Center for Genomic Medicine, RIKEN, Yokohama, Japan, **5** HLA Laboratory, Kyoto, Japan, **6** Department of Transfusion Medicine and Cell Therapy, Kyoto University Hospital, Kyoto, Japan, **7** Department of Rheumatology, Sagamihara National Hospital, National Hospital Organization, Sagamihara, Japan, **8** Department of Rheumatology, Niigata Rheumatic Center, Niigata, Japan, **9** Rheumatoid Arthritis Center, Saiseikai Takaoka Hospital, Toyama, Japan, **10** Department of Internal Medicine, Center for Rheumatic Diseases, Dohgo Spa Hospital, Matsuyama, Japan, **11** Aichi Cancer Center Hospital and Research Institute, Nagoya, Japan, **12** Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan, **13** CREST program, Japan Science and Technology Agency, Kawaguchi, Saitama, Japan, **14** Institut National de la Sante et de la Recherche Medicale (INSERM) Unite U852, Kyoto University Graduate School of Medicine, Kyoto, Japan

Abstract

HLA-DRB1, especially the shared epitope (SE), is strongly associated with rheumatoid arthritis (RA). However, recent studies have shown that SE is at most weakly associated with RA without anti-citrullinated peptide/protein antibody (ACPA). We have recently reported that ACPA-negative RA is associated with specific HLA-DRB1 alleles and diplotypes. Here, we attempted to detect genetically different subsets of ACPA-negative RA by classifying ACPA-negative RA patients into two groups based on their positivity for rheumatoid factor (RF). HLA-DRB1 genotyping data for totally 954 ACPA-negative RA patients and 2,008 healthy individuals in two independent sets were used. HLA-DRB1 allele and diplotype frequencies were compared among the ACPA-negative RF-positive RA patients, ACPA-negative RF-negative RA patients, and controls in each set. Combined results were also analyzed. A similar analysis was performed in 685 ACPA-positive RA patients classified according to their RF positivity. As a result, HLA-DRB1*04:05 and *09:01 showed strong associations with ACPA-negative RF-positive RA in the combined analysis ($p = 8.8 \times 10^{-6}$ and 0.0011, OR: 1.57 (1.28–1.91) and 1.37 (1.13–1.65), respectively). We also found that HLA-DR14 and the HLA-DR8 homozygote were associated with ACPA-negative RF-negative RA ($p = 0.00022$ and 0.00013, OR: 1.52 (1.21–1.89) and 3.08 (1.68–5.64), respectively). These association tendencies were found in each set. On the contrary, we could not detect any significant differences between ACPA-positive RA subsets. As a conclusion, ACPA-negative RA includes two genetically distinct subsets according to RF positivity in Japan, which display different associations with HLA-DRB1. ACPA-negative RF-positive RA is strongly associated with HLA-DRB1*04:05 and *09:01. ACPA-negative RF-negative RA is associated with DR14 and the HLA-DR8 homozygote.

Citation: Terao C, Ohmura K, Ikari K, Kochi Y, Maruya E, et al. (2012) ACPA-Negative RA Consists of Two Genetically Distinct Subsets Based on RF Positivity in Japanese. PLoS ONE 7(7): e40067. doi:10.1371/journal.pone.0040067

Editor: Pierre Bobé, Institut Jacques Monod, France

Received March 10, 2012; **Accepted** May 31, 2012; **Published** July 6, 2012

Funding: This study was supported by Grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, as well as by research grants from the Japan Rheumatism Foundation, the Waksman Foundation, and the Mitsubishi Pharma Research Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding received for this study.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: a0001101@kuhp.kyoto-u.ac.jp (CT); ohmurako@kuhp.kyoto-u.ac.jp (KO)

Introduction

Rheumatoid arthritis (RA) is the most common cause of chronic arthritis worldwide and results in severe joint destruction [1]. Genetic and environmental factors have been shown to be associated with its onset [2–3]. Among the susceptibility genes to RA, HLA-DRB1 has been shown to be the strongest genetic determinant of RA susceptibility, and its association with RA susceptibility has been repeatedly shown to be independent of ethnicity [4–5]. A common amino acid sequence extending from the 70th to 74th in the HLA-DRβ chain, which is known as the "shared epitope (SE)", is considered to be the reason for the association between HLA-DRB1 and RA, and the association between the SE and RA has been reported to be ethnicity-independent [6–8]. However, recent studies have shown that the SE is strongly associated with RA patients who have anti-citrullinated peptide/protein antibodies (ACPA), which is a highly specific marker of RA [9], but that it is not or only weakly associated with RA without ACPA [7,10–11]. Among the various HLA-DRB1 alleles, HLA-DR3 [12] and HLA-DR13 [13] were reported to be associated with ACPA-negative RA in populations of European descent, but these results were not confirmed in a

meta-analysis of a large Caucasian cohort [8]. In Asian populations, we recently reported that DRB1*12:01 is a HLA-DRB1 susceptibility allele for ACPA-negative RA in Japanese populations and that DRB1*04:05, the most common SE allele in Japanese, and *14:03 showed moderate associations with ACPA-negative RA susceptibility [14]. We also reported that DRB1*15:02 and *13:02 displayed protective associations with ACPA-negative RA and that being homozygous for HLA-DR8 was associated with ACPA-negative RA susceptibility. While a very small Japanese study suggested that HLA-DRB1*09:01 is associated with ACPA-negative RA [15], our study did not detect a significant association between them. These findings suggest that ACPA-negative RA is genetically different from ACPA-positive RA in terms of its associations with HLA-DRB1 alleles. While some specific alleles and diplotypes seem to be associated with ACPA-negative RA, the genetic characteristics of ACPA-negative RA have not been fully elucidated. Recently, UK group reported that SE is associated with ACPA-negative RF-positive RA in UK population [16]. However, whether this is true to other population is uncertain. Moreover, the associations of other alleles than SE with subgroups of ACPA-negative RA have never been reported. Here, we show that when we classified ACPA-negative RA into two subsets based on rheumatoid factor (RF) positivity, we were able to clearly distinguish them from each other according to their associations with HLA-DRB1 alleles, not only with SE, but with other alleles. We also compared ACPA-positive RA patients based on their RF positivity to examine whether we can apply this classification to ACPA-positive RA.

Results

HLA-DRB1 Alleles Associated with ACPA-negative RF-positive RA

We compared 179 ACPA-negative RF-positive RA with 1508 controls in collection 1 for their frequency of HLA-DRB1 alleles, followed by comparison of 267 ACPA-negative RF-positive RA with 500 controls in collection 2. Significant association was evaluated in the combined analysis. Regarding HLA-DRB1 alleles that were previously shown to be associated with ACPA-negative RA, we found that all of the alleles, namely, HLA-DRB1*12:01, *04:05, *13:02, *14:03, and *15:02 showed association tendency with ACPA-negative RF-positive RA in the combined study (Table 1). Interestingly, HLA-DRB1*04:05 ($p = 8.8 \times 10^{-6}$, odds ratio (OR): 1.57) showed the strongest association, while its association with entire ACPA-negative RA was moderate in the previous study. When we analyzed the associations of the SE, we found that it displayed a significant association ($p = 0.00013$, OR: 1.37). HLA-DRB1*04:05 was responsible for most of the association of SE because none of the other SE alleles showed significant associations with ACPA-negative RF-positive RA. We also found that HLA-DRB1*09:01, which was not associated with ACPA-negative RA as a single allele, was found to be significantly associated with ACPA-negative RF-positive RA ($p = 0.0011$, OR: 1.37). Importantly, these association tendencies written above were observed in both collections (Table 1). Logistic regression analysis was carried out to examine whether the susceptibility associations were dependent on a lack of protective alleles or vice versa. As a result, it was demonstrated that HLA-DRB1*04:05, *09:01, and *12:01 showed significant associations ($p<0.0005$), while the associations of HLA-DRB1*14:03, *13:02, and *15:02 were moderate to suggestive (Table S1). Next, we analyzed the dosage effects of the alleles and found that the association between HLA-DRB1*09:01 and ACPA-negative RF-positive RA showed a clear dosage effect (Figure S1). HLA-DRB1*12:01 also showed a dosage effect (data not shown due to small number). HLA-DRB1*04:05 did not show a dosage effect, suggesting that the effect of HLA-DRB1*04:05 on the predisposition to ACPA-negative RF-positive RA is a dominant effect.

HLA-DRB1 Alleles Associated with ACPA-negative RF-negative RA

Next we compared 274 ACPA-negative RF-negative RA with 1,508 controls, followed by comparison between 234 ACPA-negative RF-negative RA and 500 controls. Interestingly, we did not observe association of HLA-DRB1*04:05 and *09:01 with ACPA-negative RF-negative RA, while HLA-DRB1*12:01, *13:02, *14:03, and *15:02 were moderately associated with ACPA-negative RF-negative RA (Table 2). The SE was not associated with ACPA-negative RF-negative RA. DR14 was found to be significantly associated with ACPA-negative RF-negative RA and HLA-DRB1*14:03 and *14:06 comprised the association of HLA-DR14 (Table S2). These association tendencies in ACPA-negative RF-negative RA were observed in both sets (Table 2). Logistic regression analysis confirmed that none of the associations were mutually dependent and that the association of DR14 remained significant ($p = 0.00069$, Table S3). DR14 could not be evaluated the dosage effect because neither the cases nor controls included DRB1*14:03 or *14:06 homozygotes or the DRB1*14:03 and *14:06 diplotype.

HLA Diplotype Analysis: DR8 Homozygote and *12:01/*09:01 Diplotype

As we previously showed that the DR8 homozygote was significantly associated with susceptibility to ACPA-negative RA, we analyzed its associations with ACPA-negative RF-positive RA and RF-negative RA. As a result, we found that the HLA-DR8 homozygote is exclusively associated with ACPA-negative RF-negative RA in the combined study ($p = 0.00013$, OR: 3.08 for ACPA-negative RF-negative RA, Table 2; $p = 0.86$, OR: 1.08 for ACPA-negative RF-positive RA, Table 1). The effect of DR8 on the susceptibility to ACPA-negative RF-negative RA was not dose-dependent (OR: 1.04 for HLA-DR8 heterozygote).

We also found that the combination of HLA-DRB1*12:01 and *09:01, the diplotype that was most strongly associated with susceptibility to ACPA-negative RA in the previous study, was especially strongly associated with ACPA-negative RF-positive RA ($p = 5.0 \times 10^{-6}$, OR: 4.97 for ACPA-negative RF-positive RA; $p = 0.040$, OR: 2.46 for ACPA-negative RF-negative RA).

We found that the similar associations were seen between the alleles/diplotypes and ACPA-negative RF-positive erosive RA and ACPA-negative RF-negative erosive RA (except for that between HLA-DRB1*12:01 and the ACPA-negative RF-negative subset), even though the number of patients was limited (Table S4).

Comparison between ACPA-negative RF-positive RA and ACPA-negative RF-negative RA

To compare the usage of HLA-DRB1 allele between ACPA-negative RF-positive RA and ACPA-negative RF-negative RA, we directly compared the allele and diplotype frequencies between the two groups (Table 3). As expected, HLA-DRB1*09:01 and *04:05 showed significant differences in their frequencies between the two subsets ($p = 0.0018$ and 0.0034, respectively). The SE was more common in the ACPA-negative RF-positive RA patients ($p = 0.0047$), whereas DR14 was more prevalent in the ACPA-negative RF-negative RA patients ($p = 0.028$). The DR8 homozygote was more frequently seen in the ACPA-negative RF-negative RA patients than in the ACPA-negative RF-positive RA patients

Table 1. Association of HLA-DRB1 alleles with ACPA-negative RF-positive RA.

HLA-DRB1 allele	1st set				2nd set				combined analysis			
	$ACPA (-)RF(+)RA	$control	p	OR	$ACPA (-)RF(+)RA	$control	p	OR	$ACPA (-)RF(+)RA	$control	p	OR
*04:05	65 (18.2%)	340 (11.3%)	0.00015	1.75 (1.30–2.34)	88 (16.5%)	129 (12.9%)	0.055	1.33 (0.99–1.79)	153 (17.2%)	469 (11.7%)	8.8×10^{-6}	1.57 (1.28–1.91)
*09:01	70 (19.6%)	432 (14.3%)	0.0086	1.45 (1.10–1.92)	99 (18.5%)	154 (15.4%)	0.11	1.25 (0.95–1.65)	169 (18.9%)	586 (14.6%)	0.0011	1.37 (1.13–1.65)
*12:01	13 (3.6%)	91 (3%)	0.53	1.21 (0.67–2.19)	35 (6.6%)	37 (3.7%)	0.012	1.83 (1.14–2.93)	48 (5.4%)	128 (3.2%)	0.0014	1.73 (1.23–2.43)
*13:02	21 (5.9%)	273 (9.1%)	0.043	0.63 (0.40–0.99)	18 (3.4%)	52 (5.2%)	0.10	0.64 (0.37–1.1)	39 (4.4%)	325 (8.1%)	0.00013	0.52 (0.37–0.73)
*14:03	7 (2.0%)	39 (1.3%)	0.31	1.52 (0.68–3.43)	13 (2.4%)	14 (1.4%)	0.14	1.76 (0.82–3.77)	20 (2.2%)	53 (1.3%)	0.040	1.71 (1.02–2.88)
*15:02	43 (12.0%)	369 (12.2%)	0.90	0.98 (0.70–1.37)	37 (6.9%)	113 (11.3%)	0.0060	0.58 (0.4–0.86)	80 (9.0%)	482 (12.0%)	0.010	0.72 (0.56–0.93)
SE	106 (29.6%)	677 (22.4%)	0.0024	1.45 (1.14–1.85)	150 (28.1%)	233 (23.3%)	0.039	1.29 (1.01–1.63)	256 (28.7%)	910 (22.7%)	0.00013	1.37 (1.17–1.62)
DR14	29 (8.1%)	253 (8.4%)	0.85	0.96 (0.64–1.44)	48 (9.0%)	73 (7.3%)	0.24	1.25 (0.86–1.83)	78 (8.7%)	326 (8.1%)	0.55	1.08 (0.83–1.40)
Diplotype												
DR8/DR8	3 (1.7%)	17 (1.1%)	0.46	1.49 (0.28–5.24)	3 (1.1%)	8 (1.6%)	0.76	0.70 (0.12–2.94)	6 (1.3%)	25 (1.2%)	0.86	1.08 (0.44–2.65)
*12:01/*09:01	5 (2.8%)	10 (0.66%)	0.0041	4.30 (1.45–12.74)	9 (3.3%)	3 (0.60%)	0.0051	5.76 (1.42–33.42)	14 (3.1%)	13 (0.6%)	5.0×10^{-6}	4.97 (2.32–10.66)

OR: odds ratio.
SE: shared epitope: HLA-DRB1*01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *04:10, *04:13, *04:16, *10:01, *14:02, and *14:06.
doi:10.1371/journal.pone.0040067.t001

(p = 0.021). When we applied logistic regression analysis to the HLA-DRB1*09:01, *04:05, and HLA-DR14, their associations were revealed to be significant and do not depend on each other (p = 0.00067 and 0.00072, respectively, Table S5), except for that of DR14 (p = 0.30).

Comparison between ACPA-positive RF-positive RA and ACPA-positive RF-negative RA

Next, we analyzed whether these allele usage differences are also seen in ACPA-positive RA. We collected data about the HLA-DRB1 genotypes of 154 ACPA-positive RF-negative RA patients and 531 ACPA-positive RF-positive RA patients. As the SE and HLA-DRB1*09:01 were found to be associated with ACPA-positive RA, we analyzed the differences in the frequencies of these alleles [17]. In comparison with the healthy controls, SE and HLA-DRB1*09:01 were associated with a predisposition to ACPA-positive RF-positive RA as well as ACPA-positive RF-negative RA and displayed comparable odds ratios in logistic regression analysis (Table 4). No HLA-DRB1 alleles showed a strong specific association with a particular subset. When we directly compared the two subsets of ACPA-positive RA, no alleles displayed significant associations (Figure 1, Table S6). However, whether the two subsets of ACPA-positive RA share most of HLA-DRB1 susceptibility associations is inconclusive due to the small number of RF-negative subset.

Discussion

In this study, we demonstrated that classifying Japanese ACPA-negative RA patients based on their RF positivity successfully divided them into two genetically different subsets, which displayed different associations with HLA-DRB1. We showed that HLA-DRB1*09:01 and *04:05, strong susceptibility alleles to ACPA-positive RA, were also associated with ACPA-negative RF-positive subset, and that DR14 and the DR8 homozygote were associated only with the ACPA-negative RF-negative subset (Figure 1). Since the titer of RF fluctuates along with disease activity much more than that of ACPA, we were very careful to take the maximum RF titer when multiple titers were available for a particular patient, in order to prevent the RF positive subset from being contaminated with RF negative RA patients. The Recent UK population study reported the association of SE with ACPA-negative RF-positive RA [16]. Our study not only confirmed this association in Japanese RA, but also showed that the association of SE with ACPA-negative RF-positive RA is mainly due to the effect of HLA-DRB1*04:05 and that HLA-DRB1*09:01, HLA-DR14, and homozygote of HLA-DR8 are specifically associated with subsets of ACPA-negative RA.

These above-mentioned association tendencies were observed in the first set and successfully replicated in the second set, indicating that we can avoid population stratification or sampling bias. The effect sizes (odds ratio) of the alleles were comparable in each cohort (Tables 1 and 2) and the associations in the combined analysis reached significant level, although the p-values in each set did not reach the significance level due to the limited number of samples they contained. These data indicate that our results are reliable, at least in Japanese populations, although further replication studies including other populations are favorable. In the current study, we used logistic regression analysis to confirm independency of associated alleles in each comparison. When we used relative predispositional effects (RPE) method [18] to stratify associated alleles, we obtained the similar results to those we obtained by logistic regression analysis (data not shown).

Table 2. Association of HLA-DRB1 alleles with ACPA-negative RF-negative RA.

	1st set				2nd set				combined analysis			
HLA-DRB1 allele	[$]ACPA(-)RF(-)RA	[$]control	*p*	OR	[$]ACPA(-)RF(-)RA	[$]control	*p*	OR	[$]ACPA(-)RF(-)RA	[$]control	*p*	OR
*04:05	69 (12.6%)	340 (11.3%)	0.37	1.13 (0.86–1.49)	57 (12.2%)	129 (12.9%)	0.70	0.94 (0.67–1.31)	126 (12.4%)	469 (11.7%)	0.52	1.07 (0.87–1.32)
*09:01	74 (13.5%)	432 (14.3%)	0.61	0.93 (0.72–1.22)	65 (13.9%)	154 (15.4%)	0.45	0.89 (0.65–1.21)	139 (13.7%)	586 (14.6%)	0.46	0.93 (0.76–1.13)
*12:01	28 (5.1%)	91 (3.0%)	0.012	1.73 (1.12–2.67)	27 (5.8%)	37 (3.7%)	0.070	1.59 (0.96–2.65)	55 (5.4%)	128 (3.2%)	0.00071	1.74 (1.26–2.40)
*13:02	28 (5.1%)	273 (9.1%)	0.0023	0.54 (0.36–0.81)	34 (7.3%)	52 (5.2%)	0.070	1.59 (0.96–2.65)	62 (6.1%)	325 (8.1%)	0.033	0.74 (0.56–0.98)
*14:03	12 (2.2%)	39 (1.3%)	0.10	1.71 (0.89–3.29)	10 (2.1%)	14 (1.4%)	0.30	1.54 (0.68–3.49)	22 (2.2%)	53 (1.3%)	0.047	1.65 (1.00–2.73)
*15:02	51 (9.3%)	369 (12.2%)	0.051	0.74 (0.54–1.00)	36 (7.7%)	113 (11.3%)	0.033	0.65 (0.44–0.97)	87 (8.6%)	482 (12.0%)	0.0020	0.69 (0.54–0.87)
SE	131 (23.9%)	677 (22.4%)	0.45	1.09 (0.88–1.34)	103 (22%)	233 (23.3%)	0.58	0.93 (0.71–1.21)	234 (23.0%)	910 (22.7%)	0.80	1.02 (0.87–1.2)
DR14	69 (12.6%)	253 (8.4%)	0.0016	1.57 (1.19–2.09)	51 (10.9%)	73 (7.3%)	0.021	1.55 (1.07–2.26)	120 (11.8%)	326 (8.1%)	0.00022	1.52 (1.21–1.89)
Diplotype												
DR8/DR8	12 (4.4%)	17 (1.1%)	9.1×10^{-5}	4.02 (1.90–8.51)	7 (3.0%)	8 (1.6%)	0.21	1.90 (0.68–5.29)	19 (3.7%)	25 (1.2%)	0.00013	3.08 (1.68–5.64)
*12:01/*09:01	4 (1.5%)	10 (0.66%)	0.25	2.22 (0.50–7.76)	4 (1.7%)	3 (0.60%)	0.22	2.88 (0.48–19.80)	8 (1.6%)	13 (0.6%)	0.040	2.46 (1.01–5.96)

doi:10.1371/journal.pone.0040067.t002

In our previous study [14], HLA-DRB1*09:01 was not significantly associated with ACPA-negative RA, in spite of the association it displayed in combination with HLA-DRB1*12:01. In the current study, we showed that HLA-DRB1*09:01 displayed a strong dose-dependent association with ACPA-negative RF-positive RA, but not with ACPA-negative RF-negative RA. These findings were confirmed by a direct comparison between the two subsets. A small study in Japan suggested that HLA-DRB1*09:01 is associated with ACPA-negative RA [15]. Our results suggest that their study mainly included ACPA-negative RF-positive RA patients. HLA-DRB1*09:01 was shown to reduce the ACPA titer in Japanese ACPA-positive RA patients [19–20]. Therefore, HLA-DRB1*09:01 might increase the titer of RF and decrease that of ACPA, although our study also showed that HLA-DRB1*09:01 is associated with ACPA-positive RF-negative RA.

HLA-DRB1*04:05, which is a major component of the SE in Asians [17], was shown to be significantly associated with ACPA-negative RA in our previous study. The current study showed that it is only associated with ACPA-negative RF-positive RA. This predisposition was also confirmed by direct comparison of the two subsets. As we could not detect a dosage effect of HLA-DRB1*04:05, its susceptibility effect might occur in a dominant manner. It is interesting that of the many SE alleles only HLA-DRB1*04:05 is associated with ACPA-negative RF-positive RA. This does not seem to be due to the relatively low frequencies of the other SE alleles (Table 1). Therefore, the common amino acid sequence that extends from the 70^{th} to the 74^{th} amino acid of the HLA-DRβ chain might not be important for the development of ACPA-negative RF-positive RA. As immunization of citrullinated peptide induced arthritis in HLA-DR4 transgenic mice [21] and citrullinated peptides were shown to have higher affinity to HLA-DR4 [22], high affinity of SE to citrullinated antigen is hypothesized to be the link between SE and RA development. Our findings may raise possibility of another mechanism of SE in developing arthritis.

It is quite interesting that HLA-DRB1*04:05 and *09:01, strongly associated alleles with ACPA-positive RA, are associated with ACPA-negative RF-positive RA. Although there are genetic similarities between ACPA-negative RF-positive RA and ACPA-positive RA, they should be considered to be different subsets as SE alleles other than HLA-DRB1*04:05 are not associated with ACPA-negative RF-positive RA and the HLA-DRB1*09:01 and *12:01 diplotype is strongly associated with ACPA-negative RF-positive RA.

When we analyzed the HLA-DR14 serotype, it showed a strong association with ACPA-negative RF-negative RA, largely due to HLA-DRB1*14:03 and *14:06. When we compared the frequency of DR14 in each ACPA-negative subset after stratifying the data according to the presence of HLA-DRB1*09:01 and *04:05, DR14 did not display a significant effect. In this sense, the specific association of DR14 with ACPA-negative RF-negative RA needs to be confirmed.

The HLA-DR8 homozygote displayed an association with ACPA-negative RA in our previous study [14]. The current study demonstrated that its association is specific to ACPA-negative RF-negative RA. As the number of HLA-DR8 homozygote is limited, further replication is necessary for this association. No association between the HLA-DR8 and 14 diplotype and susceptibility to ACPA-negative RF-negative RA was found (data not shown).

It is interesting that HLA-DR14 and HLA-DR8, associated serotype with ACPA-negative RF-negative RA, were reported association with psoriatic arthritis [23]. HLA-DR14 is often linked with HLA-Cw*06, susceptibility serotype to psoriasis arthritis in European [24]. HLA-Cw*06 is rare in Japanese (<1%) and the

Table 3. Direct comparison of HLA-DRB1 allele frequency between ACPA-negative RF-positive RA and ACPA-negative RF-negative RA.

HLA-DRB1	ACPA(−)RF(+)RA Number of allele (%)	ACPA(−)RF(−)RA Number of allele (%)	p	OR (95%CI)
*09:01	169 (18.9%)	139 (13.7%)	0.0018	1.47 (1.15–1.88)
*04:05	153 (17.2%)	126 (12.4%)	0.0034	1.46 (1.13–1.89)
*08:02	24 (2.7%)	52 (5.1%)	0.0068	0.51 (0.31–0.84)
*14:06	8 (0.9%)	21 (2.1%)	0.037	0.43 (0.19–0.97)
SE	256 (28.7%)	234 (23.0%)	0.0047	1.35 (1.09–1.65)
DR14	78 (8.7%)	120 (11.8%)	0.028	0.72 (0.53–0.97)
DR8/DR8	6 (1.3%)	19 (3.7%)	0.021	0.35 (0.14–0.89)

doi:10.1371/journal.pone.0040067.t003

strong association between HLA-Cw*06 and HLA-DR14 is not observed in Japan (<10%). While psoriatic arthritis is not reported to be associated with these serotypes in Japan, association between these serotypes and arthritis is interesting.

It could be argued that ACPA-negative RA includes some non-RA arthritic diseases such as psoriasis, seronegative spondyloarthropathy and other collagen vascular diseases. Thus, we analyzed the associations between the above-mentioned alleles and diplotypes with ACPA-negative RA displaying bone erosion to examine whether the same association patterns were present in this strictly defined cohort. The typical bone erosions of RA are rarely seen in other arthritic disorders. As a result, we found the same associations. Therefore, we are convinced that our findings were not caused by the contamination of our study population by patients with other diseases. Since RF sometimes normalizes after treatment, the RF-negative RA patients whose RF titers were not measured at multiple points might not have been RF-negative. So, we re-analyzed our data by excluding the RA patients for whom consecutive RF titers were not available. As a result, we found the same tendency of associations for each allele and diplotype in each subset (data not shown), indicating that these subsets are stable.

Analysis using ACPA-positive RF-positive RA and ACPA-positive RF-negative RA patients compared with healthy controls did not result in distinct differences in HLA-DRB1 association. The SE is associated with both ACPA-positive RF-positive and RF-negative RA. HLA-DRB1*09:01 was found to be associated with both subsets after stratifying the patients according to their SE alleles. We also did not detect an association between HLA-DR14 or the HLA-DR8 homozygote and either subset. While 154 ACPA-positive RF-negative RA patients in our study are too small in number to detect the difference in HLA-DRB1 alleles with weak effect size between the two ACPA-positive subsets, these results suggest that there are no big differences in the HLA usage of the two subsets in ACPA-positive RA. To confirm our results and to detect possible different frequency of other HLA-DRB1 alleles in the two ACPA-positive subsets, replication study is necessary.

Table 4. Logistic regression analysis of HLA-DRB1 alleles with ACPA-positive RF-positive RA and ACPA-positive RF-negative RA.

	ACPA(+)RF(+)RA		ACPA(+)RF(−)RA	
HLA-DRB1	p*	OR (95%CI)*	p*	OR (95%CI)*
SE	$<2\times10^{-16}$	3.21 (2.72–3.78)	$<2\times10^{-16}$	3.03 (2.33–3.94)
*09:01	2.4×10^{-9}	1.83 (1.5–2.25)	0.0035	1.67 (1.17–2.37)

*p-values and odds ratios in logistic regression analysis using SE and HLA-DRB1*09:01.

doi:10.1371/journal.pone.0040067.t004

In the current study, we performed multiple comparisons in each subset and between subsets. The associations should be evaluated in the combined analysis with significant level corrected by Bonferronii's method and independency of each association should be evaluated by logistic regression analysis or RPE method. In this sense, p-values around cut-off level in the combined analysis should be taken with caution and the associations should be confirmed by independent study.

We have shown that ACPA-negative RA includes two genetically distinct subsets in Japanese population: RF-positive and RF-negative RA. This is the first report in Asians to show that these subsets are genetically distinct. We have to clarify the clinical difference between these two subsets. We also have to clarify whether non-HLA genes display different associations with each subset. So far, many genome wide association studies (GWAS) of RA and ACPA-positive RA have been performed, and more than twenty genes or loci have been shown to be susceptibility loci [25–38]. However, no GWAS studies have detected susceptibility genes for ACPA-negative RA with genome-wide significance [39]. This is probably due to the relatively small number of patients studied, but it might be overcome by stratifying ACPA-negative RA patients into RF-positive and RF-negative subsets. Since RA susceptibility genes usually cross ethnic boundaries [40], global collaboration might result in a fruitful dissection of these minor subsets.

Materials and Methods

Ethics Statement

This study was approved by the local ethical committees at each institution, namely, Kyoto University Graduate School and Faculty of Medicine, Ethics Committee, Tokyo Women's Medical University Genome Ethics Committee, and the ethics committee of RIKEN, and written informed consent was obtained from all patients.

Study Subjects

DNA samples were collected from ACPA-negative RA patients at Kyoto University Hospital, Tokyo Women's Medical University [41], and RIKEN with the support of BioBank Japan. All patients were Japanese and had been diagnosed by rheumatologists

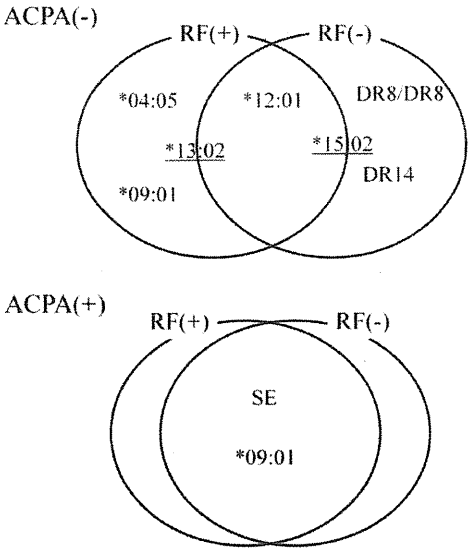

Figure 1. Summary of the HLA-DRB1 alleles associated with ACPA-negative RA and ACPA-positive RA. The relationships between the RF-positive and RF-negative subsets of ACPA-negative and ACPA-positive RA in terms of their associations with HLA-DRB1 alleles are illustrated. While the two subsets of ACPA-positive RA seem to share most associations with HLA-DRB1, the two ACPA-negative RA subsets possess specific alleles and HLA-DRB1 diplotypes. The underlined alleles are protective alleles.
doi:10.1371/journal.pone.0040067.g001

according to the 1987 American College of Rheumatology revised criteria for RA [42]. The control DNA samples were collected at Aichi Cancer Center Hospital, the DNA banks of the Pharma SNP Consortium [43], and HLA laboratory. A more detailed description of the collection procedure was given in a previous study [14]. We performed association studies using similar study design of the two collections to our previous study; namely, collection 1 for 456 ACPA-negative RA and 1508 healthy subjects, and collection 2 for 501 ACPA-negative RA and 500 healthy people. RF data were available for 453 out of 456 cases in collection 1 and all of 501 cases in collection 2. 179 patients were RF-positive in collection 1 and 267 patients were RF-positive in collection 2. We also collected DNA samples from 531 ACPA-positive RF-positive RA patients at Kyoto University Hospital and 154 ACPA-positive RF-negative RA patients at Kyoto University and Tokyo Women's Medical University.

ACPA Detection

The MESACUP CCP ELISA kit (Medical and Biological Laboratories Co., Ltd, Nagoya, Japan) was used to detect 2nd generation ACPA in each RA patient, according to the manufacturer's instructions. A cut-off value of 4.5 U/ml was used to define ACPA positivity.

RF Detection

The serum RF concentrations of samples in collection 1 were quantified using a latex agglutination turbidimetric immunoassay. An ELISA assay was used to determine the RF levels of samples in collection 2. When multiple values for RF had been obtained at different visits, we used the maximum RF value for each patient. The cut off values of each detection kit in each hospital were employed.

HLA-DRB1 Genotyping

The HLA-DRB1 typing methods were previously described [14]. Briefly, the WAKFlow system or the AlleleSEQR HLA-DRB1 typing kit (Abbott, Tokyo, Japan) was used for the HLA-DRB1 typing. The following HLA-DRB1 alleles were classified as belonging to the SE: DRB1*01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *04:10, *04:13, *04:16, *10:01, *14:02, and *14:06.

Statistical Analysis

The frequency of each allele or diplotype was compared among the ACPA-negative RF-positive RA, ACPA-negative RF-negative RA patients, and the healthy controls in each set and combined set using the chi-square test or Fisher's exact test. The same analyses were performed in ACPA-positive RA patients classified according to their RF possession. Ninety-five percent confidence intervals (CI) for the OR were also calculated. Logistic regression analysis was used to evaluate the effects of each allele by adjusting for the influence of strongly-associated alleles. Single alleles were regarded as significant when they showed p-values of less than 0.0026 in a combined study, which is obtained by Bonferroni's correction. For diplotype analyses, we regarded 0.025 as the cut off level for significance because we performed just two tests. All statistical analyses were performed using the R statistic system (http://www.R-project.org) or SPSS (version 18).

Supporting Information

Figure S1 Dosage effects of HLA-DRB1*04:05 and *09:01 alleles on ACPA-negative RF-positive RA susceptibility. Each column represents the odds ratio for developing ACPA-negative RF-positive RA associated with possessing one (red column) or two (green column) alleles of HLA-DRB1*04:05 or *09:01.
(TIF)

Table S1 Logistic regression analysis of associated alleles with ACPA-negative RF-positive RA. *p-values and odds ratios in logistic regression analysis using the six alleles listed above.
(DOC)

Table S2 Association between HLA-DR14 and ACPA-negative RF-negative RA.
(DOC)

Table S3 Logistic regression analysis of associated alleles with ACPA-negative RF-negative RA. *p-values and odds ratios in logistic regression analysis using HLA-DR14 and three HLA-DRB1 alleles listed above.
(DOC)

Table S4 Association of HLA-DRB1 with ACPA-negative RA erosive subsets. [a]Total allele number is 268. [b]Total allele number is 212.
(DOC)

Table S5 Logistic regression analysis of assoicated alleles with ACPA-negative RF-positive RA, compared with ACPA-negative RF-negative RA. *p-values and odds ratios in logistic regression analysis using HLA-DRB1*09:01, *04:05, and HLA-DR14. [a]HLA-DRB1 alleles which showed $p<0.05$ in Table 3 were used for analysis.
(DOC)

Table S6 Comparison between ACPA-positive RF-positive RA and ACPA-positive RF-negative RA. [a] Alleles with frequency more than 1% in any groups are shown.
(DOC)

Acknowledgments

We would like to thank Dr. Naoichiro Yukawa, Dr. Hajime Yoshifuji, Dr. Daisuke Kawabata, Dr. Takaki Nojima, Dr. Takashi Usui, and Dr. Takao Fujii of Kyoto University for collecting the DNA samples. We would also like to thank Mr. Taishi Shigeki for developing the clinical database software used at Dohgo Spa hospital. We would like to thank Dr. Yasuo Miura and Dr. Taira Maekawa of Kyoto University for their support of HLA-DRB1 genotyping. Moreover, we wish to thank all of the doctors and medical staff who collected the samples. This study was performed with the support of the Genetics and Allied research in Rheumatic diseases Networking (GARNET) consortium.

Author Contributions

Conceived and designed the experiments: CT KO KI YK RY FM TM. Performed the experiments: CT KI YK EM K. Yurugi MK AS HS. Analyzed the data: CT. Contributed reagents/materials/analysis tools: KI EM KS AM SH K. Takasugi KM K. Tajima SM HY K. Yamamoto HS TM. Wrote the paper: CT KO.

References

1. Firestein GS (2003) Evolving concepts of rheumatoid arthritis. Nature 423: 356–361.
2. MacGregor AJ, Snieder H, Rigby AS, Koskenvuo M, Kaprio J, et al. (2000) Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. Arthritis Rheum 43: 30–37.
3. Kallberg H, Ding B, Padyukov L, Bengtsson C, Ronnelid J, et al. (2011) Smoking is a major preventable risk factor for rheumatoid arthritis: estimations of risks after various exposures to cigarette smoke. Ann Rheum Dis 70: 508–511.
4. Deighton CM, Walker DJ, Griffiths ID, Roberts DF (1989) The contribution of HLA to rheumatoid arthritis. Clin Genet 36: 178–182.
5. Gorman JD, Lum RF, Chen JJ, Suarez-Almazor ME, Thomson G, et al. (2004) Impact of shared epitope genotype and ethnicity on erosive disease: a meta-analysis of 3,240 rheumatoid arthritis patients. Arthritis Rheum 50: 400–412.
6. Gregersen PK, Silver J, Winchester RJ (1987) The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum 30: 1205–1213.
7. Ohmura K, Terao C, Maruya E, Katayama M, Matoba K, et al. (2010) Anti-citrullinated peptide antibody-negative RA is a genetically distinct subset: a definitive study using only bone-erosive ACPA-negative rheumatoid arthritis. Rheumatology (Oxford) 49: 2298–2304.
8. van der Woude D, Lie BA, Lundstrom E, Balsa A, Feitsma AL, et al. (2010) Protection against anti-citrullinated protein antibody-positive rheumatoid arthritis is predominantly associated with HLA-DRB1*1301: a meta-analysis of HLA-DRB1 associations with anti-citrullinated protein antibody-positive and anti-citrullinated protein antibody-negative rheumatoid arthritis in four European populations. Arthritis Rheum 62: 1236–1245.
9. Schellekens GA, Visser H, de Jong BA, van den Hoogen FH, Hazes JM, et al. (2000) The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. Arthritis Rheum 43: 155–163.
10. Morgan AW, Thomson W, Martin SG, Carter AM, Erlich HA, et al. (2009) Reevaluation of the interaction between HLA-DRB1 shared epitope alleles, PTPN22, and smoking in determining susceptibility to autoantibody-positive and autoantibody-negative rheumatoid arthritis in a large UK Caucasian population. Arthritis Rheum 60: 2565–2576.
11. Huizinga TW, Amos CI, van der Helm-van Mil AH, Chen W, van Gaalen FA, et al. (2005) Refining the complex rheumatoid arthritis phenotype based on specificity of the HLA-DRB1 shared epitope for antibodies to citrullinated proteins. Arthritis Rheum 52: 3433–3438.
12. Verpoort KN, van Gaalen FA, van der Helm-van Mil AH, Schreuder GM, Breedveld FC, et al. (2005) Association of HLA-DR3 with anti-cyclic citrullinated peptide antibody-negative rheumatoid arthritis. Arthritis Rheum 52: 3058–3062.
13. Lundstrom E, Kallberg H, Smolnikova M, Ding B, Ronnelid J, et al. (2009) Opposing effects of HLA-DRB1*13 alleles on the risk of developing anti-citrullinated protein antibody-positive and anti-citrullinated protein antibody-negative rheumatoid arthritis. Arthritis Rheum 60: 924–930.
14. Terao C, Ohmura K, Kochi Y, Ikari K, Maruya E, et al. (2011) A large-scale association study identified multiple HLA-DRB1 alleles associated with ACPA-negative rheumatoid arthritis in Japanese subjects. Ann Rheum Dis 70(12): 2134–2139.
15. Furuya T, Hakoda M, Ichikawa N, Higami K, Nanke Y, et al. (2007) Differential association of HLA-DRB1 alleles in Japanese patients with early rheumatoid arthritis in relationship to autoantibodies to cyclic citrullinated peptide. Clin Exp Rheumatol 25: 219–224.
16. Mackie SL, Taylor JC, Martin SG, Wordsworth P, Steer S, et al. (2012) A spectrum of susceptibility to rheumatoid arthritis within HLA-DRB1: stratification by autoantibody status in a large UK population. Genes Immun 13: 120–128.
17. Lee HS, Lee KW, Song GG, Kim HA, Kim SY, et al. (2004) Increased susceptibility to rheumatoid arthritis in Koreans heterozygous for HLA-DRB1*0405 and *0901. Arthritis Rheum 50: 3468–3475.
18. Payami H, Joe S, Farid NR, Stenszky V, Chan SH, et al. (1989) Relative predispositional effects (RPEs) of marker alleles with disease: HLA-DR alleles and Graves disease. Am J Hum Genet 45: 541–546.
19. Okada Y, Suzuki A, Yamada R, Kochi Y, Shimane K, et al. (2010) HLA-DRB1*0901 lowers anti-cyclic citrullinated peptide antibody levels in Japanese patients with rheumatoid arthritis. Annals of the Rheumatic Diseases 69: 1569–1570.
20. Terao C, Ikari K, Ohmura K, Suzuki T, Iwamoto T, et al. (2012) Quantitative effect of HLA-DRB1 alleles to ACPA levels in Japanese rheumatoid arthritis: no strong genetic impact of shared epitope to ACPA levels after stratification of HLA-DRB1*09:01. Annals of the Rheumatic Diseases 71: 1095–1097.
21. Hill JA, Bell DA, Brintnell W, Yue D, Wehrli B, et al. (2008) Arthritis induced by posttranslationally modified (citrullinated) fibrinogen in DR4-IE transgenic mice. Journal of Experimental Medicine 205: 967–979.
22. Hill JA, Southwood S, Sette A, Jevnikar AM, Bell DA, et al. (2003) Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. Journal of Immunology 171: 538–541.
23. Queiro-Silva R, Torre-Alonso JC, Tinture-Eguren T, Lopez-Lagunas I (2004) The effect of HLA-DR antigens on the susceptibility to, and clinical expression of psoriatic arthritis. Scand J Rheumatol 33: 318–322.
24. Ho PY, Barton A, Worthington J, Plant D, Griffiths CE, et al. (2008) Investigating the role of the HLA-Cw*06 and HLA-DRB1 genes in susceptibility to psoriatic arthritis: comparison with psoriasis and undifferentiated inflammatory arthritis. Ann Rheum Dis 67: 677–682.
25. Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, et al. (2003) Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. Nat Genet 34: 395–402.
26. Kochi Y, Yamada R, Suzuki A, Harley JB, Shirasawa S, et al. (2005) A functional variant in FCRL3, encoding Fc receptor-like 3, is associated with rheumatoid arthritis and several autoimmunities. Nat Genet 37: 478–485.
27. Suzuki A, Yamada R, Kochi Y, Sawada T, Okada Y, et al. (2008) Functional SNPs in CD244 increase the risk of rheumatoid arthritis in a Japanese population. Nat Genet 40: 1224–1229.
28. Kochi Y, Okada Y, Suzuki A, Ikari K, Terao C, et al. (2010) A regulatory variant in CCR6 is associated with rheumatoid arthritis susceptibility. Nat Genet 42: 515–519.
29. Terao C, Ohmura K, Katayama M, Takahashi M, Kokubo M, et al. (2011) Myelin basic protein as a novel genetic risk factor in rheumatoid arthritis-a genome-wide study combined with immunological analyses. PLoS One 6: e20457.
30. Terao C, Yamada R, Ohmura K, Takahashi M, Kawaguchi T, et al. (2011) The human AIRE gene at chromosome 21q22 is a genetic determinant for the predisposition to rheumatoid arthritis in Japanese population. Hum Mol Genet 20: 2680–2685.
31. Plenge RM, Seielstad M, Padyukov L, Lee AT, Remmers EF, et al. (2007) TRAF1-C5 as a risk locus for rheumatoid arthritis-a genomewide study. N Engl J Med 357: 1199–1209.
32. Remmers EF, Plenge RM, Lee AT, Graham RR, Hom G, et al. (2007) STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. N Engl J Med 357: 977–986.
33. Plenge RM, Cotsapas C, Davies L, Price AL, de Bakker PI, et al. (2007) Two independent alleles at 6q23 associated with risk of rheumatoid arthritis. Nat Genet 39: 1477–1482.
34. Wellcome Trust Case Control Consortium (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447: 661–678.
35. Thomson W, Barton A, Ke X, Eyre S, Hinks A, et al. (2007) Rheumatoid arthritis association at 6q23. Nat Genet 39: 1431–1433.
36. Raychaudhuri S, Remmers EF, Lee AT, Hackett R, Guiducci C, et al. (2008) Common variants at CD40 and other loci confer risk of rheumatoid arthritis. Nat Genet 40: 1216–1223.
37. Gregersen PK, Amos CI, Lee AT, Lu Y, Remmers EF, et al. (2009) REL, encoding a member of the NF-kappaB family of transcription factors, is a newly defined risk locus for rheumatoid arthritis. Nat Genet 41: 820–823.
38. Raychaudhuri S, Thomson BP, Remmers EF, Eyre S, Hinks A, et al. (2009) Genetic variants at CD28, PRDM1 and CD2/CD58 are associated with rheumatoid arthritis risk. Nat Genet 41: 1313–1318.
39. Padyukov L, Seielstad M, Ong RT, Ding B, Ronnelid J, et al. (2011) A genome-wide association study suggests contrasting associations in ACPA-positive versus ACPA-negative rheumatoid arthritis. Ann Rheum Dis 70: 259–265.
40. Okada Y, Terao C, Ikari K, Kochi Y, Ohmura K, et al. (2012) Meta-analysis identifies nine new loci associated with rheumatoid arthritis in the Japanese population. Nature Genetics 44: 511–516.

41. Matsuda Y, Singh G, Yamanaka H, Tanaka E, Urano W, et al. (2003) Validation of a Japanese version of the Stanford Health Assessment Questionnaire in 3,763 patients with rheumatoid arthritis. Arthritis Rheum 49: 784–788.
42. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, et al. (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31: 315–324.
43. Kamatani N, Sekine A, Kitamoto T, Iida A, Saito S, et al. (2004) Large-scale single-nucleotide polymorphism (SNP) and haplotype analyses, using dense SNP Maps, of 199 drug-related genes in 752 subjects: the analysis of the association between uncommon SNPs within haplotype blocks and the haplotypes constructed with haplotype-tagging SNPs. Am J Hum Genet 75: 190–203.