Ⅳ．研究成果の刊行物・別刷

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BRIEF REPORT

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Efficacy of arthroscopic synovectomy for the effect attenuation cases of infliximab in rheumatoid arthritis

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Abstract To investigate whether arthroscopic synovectomy is effective for nonresponders to infliximab, anti-tumor necrosis factor-α antibody, for the treatment of rheumatoid arthritis (RA), we assessed seven patients including ten arthroscopic synovectomies in knee joint, in shoulder joint, and in ankle joints. We compared C-reactive protein (CRP) and DAS28 (ESR) before and after surgery at 6 and 50 weeks. After arthroscopic synovectomy, we continued the infliximab treatment with methotrexate in a routine manner. We detected synovium proliferation with vascular increase in patellofemoral joint and around the meniscus and femoral and tibial side of the anterior cruciate ligament in the knee joints. We also found synovial proliferation in rotator interval in the glenohumeral joint and fatty changing in subacromial bursa in the shoulder. In the ankle joint, we found synovial proliferation with white meniscoid between tibiofibular joint to develop impingement. Serum CRP was improved from 3.45±0.4 to 1.12±0.2 at 6 weeks to 1.22±0.4 at 50 weeks after arthroscopic synovectomy. There is no severe side effect of arthroscopic synovectomy during infliximab treatment; however, one patient had slight rash that was improved. DAS28 was improved from 5.58±0.23 to 3.87±0.47 at 6 weeks to 2.58±1.49 at 50 weeks after arthroscopic synovectomy. It is possible that arthroscopic synovectomy can be one of the effective methods to continue with the infliximab treatment when its efficacy decreased or in the nonresponders of infliximab for RA patients.

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Introduction

Even the treatment for rheumatoid arthritis (RA) with anti-tumor necrosis factor-α (TNF-α) therapy such as infliximab, a recombinant IgG1κ monoclonal antibody specific for tumor necrosis factor, including more than 6 mg of methotrexate (MTX) weekly, is the current treatment for RA, there are some cases that fails to control disease activity, prevent structural damage, and maintain quality of life. In patients with RA who have an incomplete response to anti-TNF-α therapy, the method such as the increase of methotrexate (MTX) or infliximab, or also the decrease of the interval period of injection with infliximab, is considerable. In our institute, we treated 35 cases by infliximab with MTX for RA. However, there is no clear strategy to control the disease if infliximab failed to control RA activity. We performed arthroscopic synovectomy in the middle of infliximab infusion interval, for example, 4 weeks after infusion of infliximab. Arthroscopic synovectomy is reported as effective method for early stage of RA [1]. However, degenerative change of RA could not be stopped by arthroscopic synovectomy only in long-term results [2]. We combined anti-TNF-α therapy and arthroscopic synovectomy for the patients who did not respond after around three to five times of infliximab infusion with MTX treatment. Arthroscopic synovectomy is safe and less painful compared with open synovectomy. Therefore, the hospitalization period of patients is relatively short after surgery. In regard to adverse events, infliximab has several adverse events such as high fever, high blood pressure, rash, headache for slight infusion reaction, and tuberculosis for severe adverse event. If we use these drugs too much for RA patients or for long period, it is possible to induce these adverse events, sometimes irreversibly, such as interstitial pneumonia with MTX. To avoid these adverse events, surgical treatment such as arthroscopic synovectomy is one

Table 1 Arthroscopic synovectomy (ASS) during infliximab treatment for RA

Case	Age (years)	Stage	Class	Operated joints
1. M.S.	61	II	II	Left-knee ASS
2. K.M.	62	IV	III	Right-shoulder ASS
3. Y.Y.	69	III	III	Left-ankle ASS
4. N.O.	49	II	II	Right-knee ASS, left-elbow ASS, left-wrist ASS, left-ankle ASS
5. T.I.	68	II	II	Right-knee ASS
6. S.N.	58	II	II	Right-shoulder ASS
7. T.H.	67	II	III	Left-knee ASS

of the options to treat RA with infliximab simultaneously. Efficacy of infliximab was already reported in long-term period, and it also prevented joint destruction by using sharp score [3]. However, there is no report of synovial finding in weak response to infliximab by arthroscopy during anti-TNF-α therapy. Here is the first report that synovial proliferation in the knee, the shoulder, and the ankle joints was less responsive to infliximab treatment. We investigated the combination of infliximab and arthroscopic synovectomy and also of DAS28 change to assess the efficacy of arthroscopic synovectomy in the short period for RA patients who had not responded well to infliximab.

Patients and methods

We performed arthroscopic synovectomy in 7 patients out of 35 infliximab and MTX cases for the treatment of RA. Infliximab (3 mg/kg) (Centcore, USA, Tanabe Co.) was administered at the rate of 60 ml/h for 30 min and 125 ml/h by infusion during 1-day admission. The patients include one male and six females from 49 to 68 years old, with average of 62 years old. Three patients underwent arthroscopic synovectomy after administration of infliximab for four times, two patients after five times, and two patients after six times. All patients initially responded to infliximab and MTX, but gradually, the effect decreased; the average of C-reactive protein (CRP) was 3.45±0.4 (2.7–5.6) mg/dl at the surgery. The indication for operation was that after treatment of infliximab, CRP was more than 2.5 mg/dl, and the numbers of arthritis joints were limited to within five joints of relatively large joints such as knee, shoulders, including ankles and wrists. The infliximab treatment included a diagnosis of RA based on the ACR (formerly, the American Rheumatism Association) criteria [4] and categorization by Steinbrocker et al. [5]. Five patients were categorized as stage II RA, one patient as stage III, and one patient as stage III. Four patients were class II, and three patients were class III. Three patients were given 6 mg of MTX during infliximab treatment, and

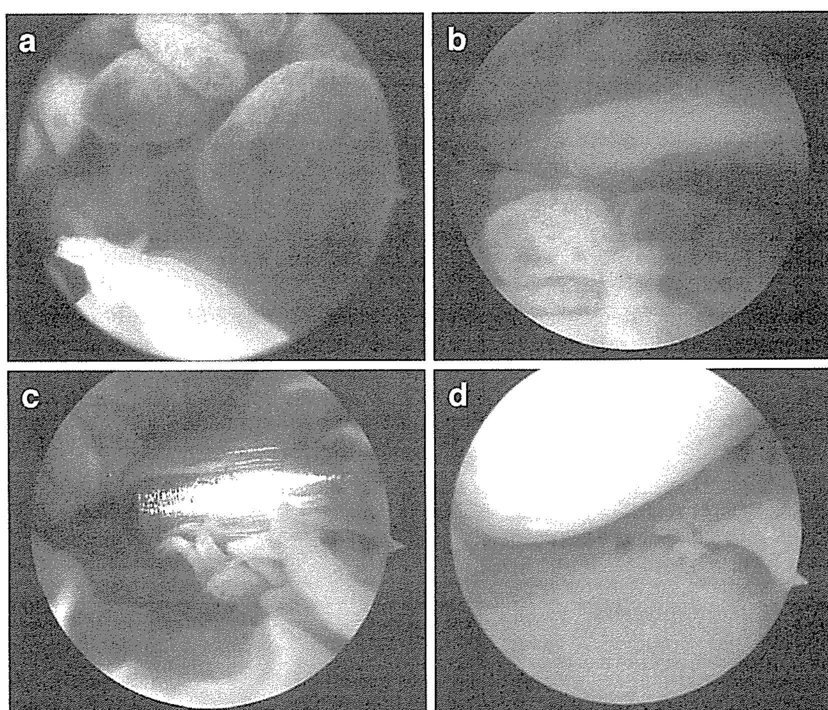

Fig. 1 Arthroscopic finding and synovectomy during infliximab treatment for RA. **a** Patellofemoral joint, **b** around medial disc, **c** shaving synovium around anterior cruciate ligament, **d** the view cleaned-up after arthroscopic synovectomy

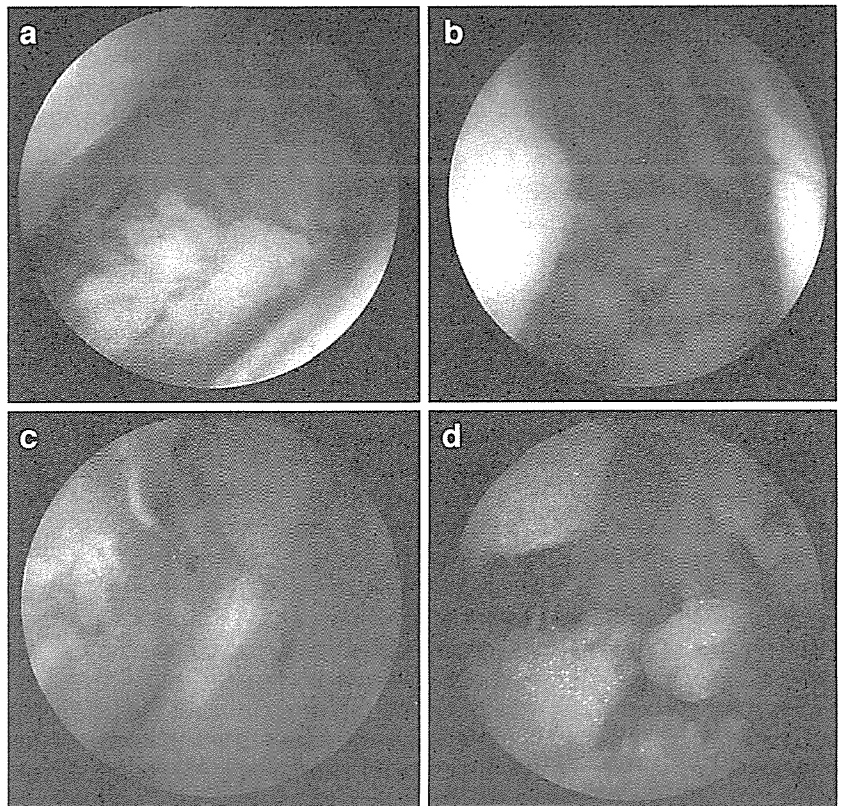

Fig. 2 Arthroscopic finding in shoulder arthroscopy. **a** Synovial proliferation with white fibrous tissue at rotator interval, **b** around the AIGHL, **c** the anterior limb was floated with synovium over the LHB, **d** subacromial bursa

four patients were given 8 mg of MTX (Table 1). Predonin (5 mg/day) was administered in three patients. Prednisolone sodium succinate (20 mg) was used as steroid cover after surgery in two patients. At arthroscopic synovectomy, general anesthesia was administered in the shoulder arthroscopic synovectomy, and lumber anesthesia was administered in the knee and the ankle joints. We used 4.0-mm arthroscope (Smith & Nephew, USA) for the knee and the shoulder joints and 2.7-mm arthroscope (Smith & Nephew) for the ankle joints. We also used shaver apparatus (Smith & Nephew) and VAPR (Johnson & Johnson, USA) to remove synovium for arthroscopic synovectomy.

We observed synovium in articular joints directly by arthroscopy in knee, shoulder, and ankle to analyze how and where synovium proliferate in joints during infliximab treatment. Furthermore, we investigated the change of CRP (mg/dl), DAS28 (CRP), ACR 20, ACR50, ACR70 before and after arthroscopic synovectomy. For data analysis, we used Wilcoxon test of nonparametric statistic test to compare the CRP, DAS28, ACR20, ACR50, ACR70 before and 6 and 50 weeks after surgery.

Results

In the knee arthroscopic finding, we detected high vascular synovium in patellofemoral joint (Fig. 1a) and between the disc and tibial cartilage (Fig. 1b). We shaved and removed the synovium (Fig. 1c), and the joint cavity was made clear and clean after arthroscopic synovectomy (Fig. 1d). In the

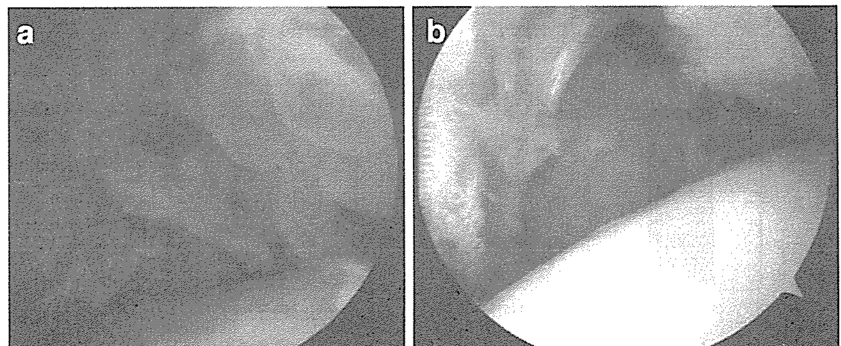

Fig. 3 Arthroscopic finding in ankle arthroscopy. **a** Synovium proliferation between tibiofibular joint, **b** after arthroscopic synovectomy

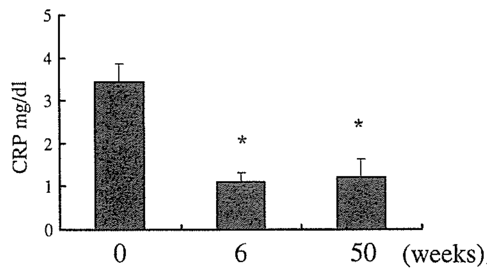

Fig. 4 Serum CRP changes after 0, 6, and 50 weeks by arthroscopic synovectomy

shoulder arthroscopy, we found synovial proliferation with white fibrous tissue at rotator interval (Fig. 2a) and around the anteroinferior glenohumeral ligament (AIGHL) (Fig. 2b). The anterior limb was floated with synovium over the long head of biceps tendon (LHB) (Fig. 2c). The difference between the knee and the shoulder synovium is that shoulder synovium has white fibrous tissue to induce the joint impingement in the glenohumeral joint. In subacromial bursa, the fatty tissue was increased in bursal synovium (Fig. 2d), and we removed those tissues by shaver and VAPR without bony resection for arthroscopic debridement. In ankle arthroscopic finding, we detected synovium proliferation between tibiofibular joint that invades the erosion of tibia (Fig. 3a), and we removed those tissues clearly to see joint cartilage not to induce impingement (Fig. 3b). The average of CRP at preoperation, 3.45±0.4 (2.7–5.6) mg/dl, was changed to 1.12±0.2 (0.6–1.8) mg/dl at 6 weeks later and improved to 1.22±0.4 (0.8–1.9) mg/dl at 50 weeks later. Therefore, the CRP was improved after 6 weeks and continued until 50 weeks by arthroscopic synovectyomy during infliximab treatment (Fig. 4). DAS28 was calculated to be 5.58±0.23 at 0 week, 3.1874±0.47 at 6 weeks, and 2.576±1.49 at 50 weeks after arthroscopic synovectomy (Fig. 5). Therefore, arthroscopic synovectyomy was clinically effective for the patients who tolerated the effect of infliximab. ACR20 was 86%, ACR50 was 57%, and ACR70 was 29% at 6 weeks, and ACR20 was 71%, ACR50 was 42%, and ACR70 was 29% at 50 weeks. Therefore, the efficacy of infliximab and MTX was clinically enhanced by arthroscopic synovectomy.

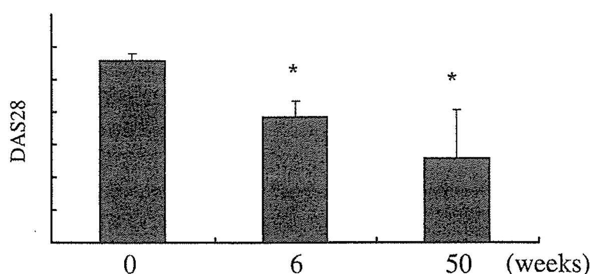

Fig. 5 DAS28 after 0, 6, and 50 weeks by arthroscopic synovectomy

Discussion

Surgical treatment for RA is one the options to be considered if the medical treatment does not respond well. Synovium produces many cytokines and chemokines to develop joint cartilage destruction [6]. Synovectomy is a surgical method used to reduce the cytokines and chemokines especially in joint fluid [7]. There are the two ways to do synovectomy for RA joints, one is arthroscopic synovectomy and the other is open synovectomy. The clinical outcome was much the same in both groups, but there was gradual deterioration, especially after 8 years [8]. RA patients have skin problems such as thin skin that is easily wounded and infected. Arthroscopic synovectomy takes precaution not to get wide wound to induce infection. The patients of infliximab easily get infected, because the function of macrophages was decreased by blocking TNF-α on the surface of macrophages. Therefore, we prefer arthroscopic surgery as a less-invasive method compared with open surgery. As regard to shoulder joint, there is no detailed paper about shoulder arthroscopic synovectomy for RA. We were the first to report shoulder arthroscopic finding and synovectomy especially during infliximab treatment. However, there is a paper that describes infliximab treatment as a reasonable approach for treating early glenohumeral osteoarthritis that had failed to respond to any operative treatment, in which the humeral head and glenoid remain concentric, and where there is still a visible joint space on an axillaries radiograph [9]. We focused our debridement on subacromial bursa so as not to induce impinge pain. After surgery, the range of motion was improved especially in the internal rotation (average from L5 to Th12). We found villous and high vascularity synovitis in arthroscopic finding of effect attenuation cases of infliximab similar to virgins for biological agents.

Synovectomy of the knee in early inflammatory arthritis appears to be successful in decreasing swelling and pain, when the underlying disorders are unresponsive to aggressive medical therapy. Indications for synovectomy may be debated, but generally, the criteria of failure of appropriate medical management for a period of 6 to 12 months in the absence of significant radiographic changes may be accepted as an appropriate indication for consideration of synovectomy. Traditional open synovectomy has been associated with loss of motion in the knee joint suffering from inflammatory arthritis. Multiple reports of the outcome of arthroscopic synovectomy appear to indicate that loss of motion is less of a problem than is seen following an open synovectomy and that the outcome of arthroscopic synovectomy in palliation of the arthritic syndrome is equally effective. A review by Doets et al. [10] concluded that although arthroscopic synovectomy in the setting of RA produced fair or good results in 50% of the cases, one half of the 83 patients in this series had undergone total replacement at a mean interval of 4 years after synovectomy. Matsui et al. [8] similarly reported gradual deterioration in clinical function among patients who had undergone arthroscopic synovectomy for the treatment of RA of the knee. However, arthroscopic synovectomy was associated with less loss of motion and more rapid return of function than open

synovectomy. The advantages of arthroscopic synovectomy therefore appear to be shorter hospitalization and more rapid rehabilitation with less risk of loss of motion than that associated with open synovectomy. Arthroscopic synovectomy in RA may be associated with moderate blood loss. No suction drain is needed in the joint because of less blood loss. Most patients are hospitalized 3–5 days following synovectomy. Although only synovectomy may transiently reduce the swelling associated with inflammatory arthritis of the knee, it does not appear that synovectomy significantly alters the progression of degenerative change in the articular cartilage of the joint [11, 12]. However, it is generally accepted that effects of synovectomy will be kept for about 5 years, and total synovectomy reduces disease activity of the refractory RA following remission [13]. Thus, it is possible to say that improvement of disease activity of given cases might be due to the effects of synovectomy itself. But if we combine anti-TNF-α therapy such as infliximab and arthroscopic synovectomy, the efficacy may be more continuous than synovectomy only. In our data, DAS28 was decreased at 50 weeks after surgery. This clinical improvement may be useful for the continued use of infliximab without adverse events.

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ORIGINAL INVESTIGATION

No association between complement factor H gene polymorphism and exudative age-related macular degeneration in Japanese

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Abstract Age-related macular degeneration (ARMD) is the leading cause of blindness in the elderly population not only Western but also Asian industrial countries. In Caucasian, a polymorphism of the complement factor H gene (*CFH*), the C allele of rs1061170 (Y402H), was established as the first strong genetic factor for excursively exudative type of ARMD. In this study, we performed an extensive sequencing of the 22 exons in the *CFH* gene by recruiting 146 exudative ARMD patients and 105 normal controls of Japanese origin and identified 61 polymorphisms. We found that the frequency of the C allele of rs1061170 (Y402H) is much lower (0.04) in Japanese controls than in Caucasians (0.45). No case disease susceptibility to exudative ARMD was noted for rs1061170 (Y402H) ($\chi^2 = 3.19$, $P_{\text{corr}} = 0.423$), or other 12 single nucleotide polymorphisms (SNPs) whose frequency is greater than 0.05. When haplotypes were inferred for 13 SNPs (these 12 SNPs with a frequency greater than 0.05 and rs1061170), three haplotypes whose pattern was similar to those in Caucasians were identified but with substantial difference in frequency. Again we failed to identify genetic association between Japanese exudative ARMD and any of the haplotypes including the J1 haplotype which was shown to be susceptible to ARMD in Caucasians ($\chi^2 = 3.92$, $P_{\text{corr}} = 0.157$). *CFH* does not appear to be a primary hereditary contributor to ARMD in Japanese. The absence of *CFH* contribution to ARMD in Japanese may correlate with the findings in ethnic differences of ARMD phenotypes.

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This work was accomplished by equal contribution of two groups organized by the last two authors.

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Introduction

Age-related macular degeneration (ARMD) is the leading cause of blindness in the elderly population, and is a worldwide public health issue (Bird 2003). The early stages of ARMD are characterized by drusen, extracellular deposits of proteins, lipids and cellular debris located between Bruch's membrane and the retinal pigment epithelium (RPE). After progression to later stages, exudation with choroidal neovascularization (CNV) or geographic atrophy develops, causing photoreceptor dysfunction in the macula. Along with dietary and environmental risk factors for ARMD, twin studies and segregation analyses have identified heredity as a primary contributor to ARMD susceptibility (Meyers et al. 1995; Weeks et al. 2001, 2004; Seddon et al. 2005). Indeed, ABCA4 (*ABCA4*

[MIM*601691]) and Apolipoprotein E (*APOE* [MIM*107741]) have been reported as genetic risk factors (Klaver et al. 1998; Allikmets 2000).

The complement system plays a fundamental role in inflammation and innate immunity. In ARMD pathogenesis, the inflammatory process has been suggested to play a role. Among the molecules involved in the complement system, complement factor H protein is a critical regulator of complement alternative pathway activation (Turnberg and Botto 2003; Rodriguez de Cordoba et al. 2004). Indeed, the characteristic extracellular deposits of early ARMD (drusen) are rich in complement factor H protein (Hageman et al. 2001; Umeda 2005). Genetic variants of *CFH* are known to increase the risk of immunity/inflammatory disorders, such as atypical hemolytic uremic syndrome (HUS [MIM235400]) (Perez-Caballero et al. 2001; Rodriguez de Cordoba et al. 2004). Moreover, multiple studies on European descendants recently reported that a missense single nucleotide polymorphism (SNP), rs1061170 (Y402H), in *CFH* as well as those in LD with rs1061170, are associated with development of ARMD (Conley et al. 2005; Edwards et al. 2005; Hageman et al. 2005; Haines et al. 2005; Klein et al. 2005; Rivera et al. 2005; Souied et al. 2005; Zareparsi et al. 2005). In order to test whether *CFH* is a major genetic determinant of ARMD in Japanese, we performed a systematic SNP genotyping study of *CFH* using DNAs from Japanese ARMD patients.

Methods

Subjects ascertainment

A total of 146 unrelated CNV positive ARMD cases (mean age 68.0 years; deviation 7.65 years; male:female 78.7:21.3) and 105 subjects without any clinical feature of ARMD as controls (mean age 60.2 years; deviation 16.2 years; male:female 49.6:50.4) were recruited. Among the ARMD cases, only 6.84% cases showed soft drusen as well as CNV. All the cases and the controls received a full examination by experienced macula specialists, including slit-lamp biomicroscopy, dilated funduscopy and contact lens observation of the macula. In addition, the cases received fluorescein angiography with a scanning laser ophthalmoscope (SLO, Rodenstock, Germany), and certificated classic and/or occult pattern of CNV. Patients with geographic atrophy, which is a clinically rare type of Japanese ARMD (Oshima et al. 2001), were not included in the current study. Secondary choroidal neovascular diseases, including angioid streaks, degenerative myopia, idiopathic CNV and presumed ocular histoplasmosis syndrome, were all excluded with clinical presentation including angiographic manifestation. Cicatricial macular lesion, which is occasionally diagnosed incorrectly as ARMD, was also excluded. All participants were enrolled under protocols approved by the Institutional Review Board.

Mutation screening and genotyping

Genomic DNAs were extracted from peripheral blood leukocytes of the volunteers using a DNA extraction Kit (QIAamp DNA Blood Kit, QIAGEN, Hilden, Germany). Polymerase chain reaction was carried out using 15 ng of genomic DNA. A total of 502 chromosomes, 210 chromosomes from controls and 292 chromosomes from ARMD cases, were amplified for the 22 exons including 5′- and 3′-untranslated region (UTR) and adjacent intronic regions of *CFH* gene. Oligonucleotide primers for PCR and sequencing were designed based on Gene ID: 3075, NT_004487.17 and NM_00186.2. The PCR products were used as the templates for direct sequencing with the fluorescent dideoxynucleotides (BigDye Terminator Ready Reaction Mix, Applied Biosystems) on an ABI 3730 automated sequencer (Applied Biosystems) using standard conditions. Segregating sites were identified and genotypes were confirmed directly from electrophorograms using Genalys (http://www.software.cng.fr/docs/genalys.html).

Statistical methods

Associations were tested for allele frequency of individual SNP as well as LD, and haplotypes were inferred for frequent SNPs with a minor allele frequency greater than 0.05 with rs1061170 using Haploview ver 3.2 (http://www.broad.mit.edu/mpg/haploview/) [exact probability test for contingency table tests, D′ for pairwise LD index (Barrett et al. 2005) and *P* value correction by permutation test (no. of iterations = 10,000)]. Logistic regression analysis was performed for gender and age correction by Statistica (StatSoft, Oklahoma).

Results

In 105 Japanese controls, 41 polymorphisms (38 SNPs, 3 insertion/deletions) were identified, of which 23 (20 SNPs, 3 insertion/deletions) were novel and were not registered in dbSNP (build 123). Among the 38 SNPs identified, 14 were located in exons

(8 nonsynonymous and 6 synonymous). All identified SNPs were in the Hardy–Weinberg equilibrium ($P > 0.2$). Among them, 26 SNPs were relatively uncommon (minor allele frequency smaller than 0.05) including rs1061170 (or Y402H) (minor C allele frequency being 0.04; compared with 0.38 in Caucasians). Extension of LD throughout the *CFH* gene was detected. Haplotypes were inferred for 13 SNPs (12 SNPs with a frequency grater than 0.05 and rs1061170) as shown in Table 1. Although three haplotypes whose pattern was similar to those in Caucasians were identified, their frequency was different between the two ethnic groups (Table 2). The ARMD-susceptible haplotype (G-C, referred as J1) which is most common in Caucasians (0.29) was much less frequent (0.033) in Japanese population. Instead, the J3 haplotype (G-T), which was reported not to confer ARMD risk in Caucasians, was most prevalent in Japanese (0.56). When 146 exudative ARMD patients were tested, in addition to 34 polymorphisms that are present in controls, 20 single-nucleotide variants (16 SNPs, 4 insertions/deletions) including 13 singletons, were identified. When these SNPs were tested for logistic regression analysis for age and gender correction, and the largest reduction was observed in rs375046 (from 0.272 to 0.0567) and the smallest P value was 0.0262 for rs2274700. Again, statistical tests failed to associate any of the 12 frequent SNPs (> 0.05) and rs1061170 or deduced haplotypes with ARMD, as summarized in Tables 1 and 2.

Discussion

We systematically identified *CFH* polymorphisms in Japanese and revealed significant differences in their allele frequencies and haplotype frequency between Japanese and Caucasians. In Caucasians, the C allele of rs1061170 is a common nonsynonymous variant and in a significant association with ARMD in multiple case–control studies. For instance, Zareparsi et al. (2005) with the largest sample size from a single center, reported the risk of carrying at least one C allele as $\chi^2 = 110.96$, $P < 1 \times 10^{-24}$. In contrast, the frequency of the C allele of rs1061170 is as low as 0.04 in Japanese. We failed to replicate Caucasian studies in Japanese ($\chi^2 = 3.19$, $P_{corr} = 0.423$) even with strictly selected cases in late stages, which corresponds to the patient subgroup in which rs1061170 showed most significant association (Hageman et al. 2005; Haines et al. 2005; Klein et al. 2005). No other ARMD-associated *CFH* SNPs, including I62V, in LD with rs1061170 or haplotypes in Caucasians were associated with ARMD in Japanese. Another Japanese research group recently reported a case–control association study for limited number of SNPs in the gene with relatively small sample size and they revealed ethnic difference in allele frequency of SNPs as we did and potential association with ARMD, which seemed nonconclusive due to limited description (Okamoto et al. 2006). Our study design was sufficient to rule out false negativity where relative risk of SNPs was as high

Table 1 *CFH* SNP associations with CNV positive ARMD in Japanese

Position[a]	dbSNP ID	Location[b]	Allele frequency[c]				X^2	P	P^{d}_{corr}
			Control		Case				
			Ref.	Var.	Ref.	Var.			
47029856	Novel	5′FL (– 258)	0.45	0.55	0.41	0.59	0.68	0.409	1.000
47051011	rs551397	Intron1 (– 36)	0.60	0.40	0.71	0.29	6.31	0.012	0.081
47051172	rs800292	Exon2 (+ 126)	0.60	0.40	0.71	0.29	6.16	0.013	0.091
47055326	rs1329423	Intron4 (– 219)	0.50	0.50	0.42	0.58	3.34	0.068	0.396
47063263	rs1061170	Exon9 (+ 45)	0.04	0.96	0.08	0.92	3.19	0.074	0.422
47091886	rs2274700	Exon10 (+ 83)	0.54	0.46	0.65	0.35	5.90	0.015	0.101
47093513	rs6664877	Intron10 (– 149)	0.94	0.06	0.97	0.03	2.98	0.085	0.470
47093514	rs6677460	Intron10 (– 148)	0.94	0.06	0.97	0.03	2.98	0.085	0.470
47104681	rs3753396	Exon13 (+ 143)	0.53	0.47	0.45	0.55	3.00	0.083	0.459
47114865	rs375046	Intron15 (– 28)	0.06	0.94	0.09	0.91	1.21	0.272	0.958
47118713	rs1065489	Exon18 (+ 26)	0.53	0.47	0.46	0.54	1.63	0.201	0.855
47119855	rs16840522	Intron18 (– 89)	0.95	0.05	0.97	0.03	1.52	0.220	0.880
47121841	Novel	Intron20 (+ 144)	0.52	0.48	0.45	0.55	2.63	0.110	0.554

[a]Number of nucleotide position in NT_004487.17

[b]Location represents number of nucleotides counted from the corresponding exon/intron boundary

[c]Ref. and Var. were defined by dbSNP. For the two novel SNPs, Ref. were nucleotides in NT_004487.17

[d]P_{corr} corrected by permutation test (no. of iterations = 10,000)

Table 2 Comparison of *CFH* haplotypes between Japanese and Caucasians

	rs800292	rs1061170	Caucasian		Japanese		χ^2	P	P^a_{corr}
	I62V	Y402H	ARMD	Controls	ARMD	Controls			
J1	G	C	0.50	0.29	0.08	0.03	3.92	0.048	0.157
J2	A	T	0.12	0.21	0.30	0.40	5.55	0.019	0.060
J3	G	T	> 0.12	> 0.19	0.62	0.57	1.91	0.167	0.453

J1, J2 and *J3* represent three haplotypes consisted of two missense SNPs, rs800292 and rs1061170. Frequency of corresponding haplotypes in Caucasians was calculated based on numbers reported by Hageman et al. (2005). Frequency of J3 in Caucasians could not be calculated precisely due to lack of data for minor haplotypes. Composition of allele of the two SNPs in haplotype J1 is the same as at-risk haplotype H1 in Caucasians, and this is also the case for J2 and H2

[a] P_{corr} corrected by permutation test (no. of iterations = 10,000)

as in Caucasians, even with the low allele frequency in Japanese. However, we could not exclude the possibility of weaker genetic contributions of exonic or exon/intron boundary SNPs or possible association of unidentified polymorphisms in intron/reguratory regions to ARMD in Japanese. Nonetheless, the genetic attributable risk in Japanese could not be substantial because the allele frequency was sufficiently small if any in the SNPs susceptible in Caucasians. The absence of *CFH* contribution to ARMD in Japanese may correlate with the ethnic differences of ARMD phenotypes. First, the prevalence of late-stage ARMD in Caucasians is much higher than in Japanese (Oshima et al. 2001). Second, the clinical features and responses to therapy reportedly differ between the two populations: (1) drusen, an inflammatory deposit containing complement factor H protein is less frequently observed in Japanese patients (Bird 2003) (our cases had soft drusen for only 6.8%) and (2) the standard photodynamic therapy for exudative ARMD is far more effective in Japanese than in Caucasians (JAT Study Group 2003). A relationship between ethnic differences in disease-susceptible genetic variants and ethnic diversity in phenotypes has also been suggested in other diseases (Horikawa et al. 2000; Tsai et al. 2001; Lesage et al. 2002; Yamazaki et al. 2002; Bottini et al. 2004; Mori et al. 2005). Recently, Gold et al. (2006) reported that the variation in factor B and complement component 2 genes is associated with ARMD in Caucasian. It would be interesting to evaluate their ethnic difference as we did in the present study and it would be helpful to dissect genetic background of ARMD and its ethnic diversity.

In summary, our data demonstrated substantial differences in the frequencies of SNPs and haplotypes in the *CFH* gene between Caucasian and Japanese populations, and no significant associations between SNPs of exonic regions and exon/intron boundaries and CNV positive ARMD in Japanese.

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A functional variant in *FcRH3*, encoding Fc Receptor Homolog 3, is associated with rheumatoid arthritis and several autoimmunities

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Abstract

Rheumatoid arthritis (RA) is a common autoimmune disease with a complex genetic etiology. Herein we identify a single-nucleotide polymorphism (SNP) in the promoter region of *FcRH3*, a member of the Fc receptor homolog family, that is associated with RA susceptibility (OR=2.15,

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COMPETING INTERESTS STATEMENT

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P=0.00000085). This polymorphism alters the binding affinity of nuclear factor-κB and regulates *FcRH3* expression. High *FcRH3* expression on B-cells and augmented autoantibody production were observed in individuals with the disease-susceptible genotype. Associations were also found between the SNP and susceptibility to autoimmune thyroid disease and systemic lupus erythematosus. *FcRH3* may thus play a pivotal role in autoimmunity.

Rheumatoid arthritis represents one of the most common autoimmune diseases, and is characterized by inflammation of synovial tissue and joint destruction. Although the disease is believed to result from a combination of genetic and environmental factors, the complete etiology of RA has not yet been clarified[1]. While specific haplotypes of human leukocyte antigen (HLA)-DRB1, usually referred to as shared-epitope (SE) sequences[2], have been repeatedly reported as conferring RA-susceptibility[3,4], other genetic components are also involved in the pathogenesis of RA[5]. This combination of HLA haplotypes and non-HLA genes accounting for disease susceptibility is also seen in other autoimmune diseases[6–8]. In autoimmune thyroid disease (AITD), for instance, studies have consistently shown that the *HLA-DR3* haplotype is associated with disease risk, in addition to a functional haplotype of a non-HLA gene, *CTLA4*, that has recently been associated with AITD susceptibility[9].

Identification of non-HLA genes associated with RA susceptibility and other autoimmunities seems difficult, due to the low relative risk of disease resulting from these non-HLA genes compared with the strong relative risk from disease-associated HLA haplotypes. In a search for non-HLA determinants of disease susceptibility, whole genome studies have been conducted for both human autoimmune diseases and experimental animal models. These studies have revealed non-random clustering of susceptibility loci for clinically distinct diseases[8,10]. This overlapping of susceptibility loci for multiple autoimmunities suggests the existence of common susceptibility genes in those regions. Intense studies of loci-clustering regions has revealed genes commonly associated with multiple autoimmune diseases, such as *CTLA4* on 2q33 (ref. [9]), *SLC22A4/A5* on 5q31 (ref.[11]) and *PTPN22* on 1p13 (ref. [12]).

Cytoband 1q21-23 is one of the regions implicated in susceptibility to multiple autoimmune diseases. The Fcγ receptor (FcγR) II/III genes are located in 1q23 and a new family of genes, Fc receptor homologs (FcRHs)[13,14] (also known as IRTAs[15,16] or SPAPs[17]) clusters nearby in 1q21 (Fig. 1a). FcRHs have high structural homology with classical FcγRs, although ligands and function remain unclear. These receptors are strong candidates for involvement in autoimmunity, as they are believed to play important roles in the pathogenesis of RA and other autoimmune diseases[18]. Region 1q23 represents a candidate locus for susceptibility to systemic lupus erythematosus (SLE), and variants in the classical FcγR II/III genes would partially account for disease susceptibility [6,19]. The FcRH gene cluster in 1q21 is also reportedly associated with SLE (Gibson, AW. *et al.* American College of Rheumatology 66th Annual Scientific Meeting, 2003). Region 1q21 represents a candidate locus for psoriasis (*PSORS4* (ref. [7,20])) and multiple sclerosis[21]. The mouse syntenic region of human 1q21, on chromosome 3, also displays susceptibility loci for multiple autoimmune disease models[8], including collagen-induced arthritis (*Mcia2* (ref. [22]), *Eae3* (ref. [23]), *Tmevd2* (ref. [24]), *Idd10* and *Idd17* (ref. [25])). Although 1q21-23 is a strong candidate region for RA susceptible genes, as above mentioned, the association of classical FcγRs with disease susceptibility remains controvertial[26,27]. The present study focused on the 1q21-23 region to identify RA-associated genes in Japanese subjects using linkage disequilibrium (LD) mapping.

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RESULTS

Case-control study by SNP-based LD-mapping in 1q21-23

To evaluate the extent of association, we analyzed linkage disequilibrium (LD) with SNPs distributed in a 16-Mb region on 1q21-23, including the FcRH gene cluster and the classical FcγRs (Fig. 1a). A total of 658 control subjects were genotyped for 742 SNPs from the JSNP database, and 491 SNPs were selected for evaluation of LD based on the following criteria: allele frequency >0.1; successful genotyping rate >0.95; and P>0.01 with Hardy-Weinberg Equilibrium (HWE) testing. The pairwise LD index Δ[28] was calculated for each pair of SNPs, identifying 110 LD blocks[11] at a threshold of Δ >0.5 (Fig. 1a).

For association testing, the Japanese set of 830 cases and 658 controls used for LD block evaluation was examined. Initially, 94 RA cases were genotyped for 491 SNPs and their allele frequencies were preliminarily compared with 658 control subjects. A total of 9 SNPs were identified as displaying allele frequencies differing by >0.1 between 658 controls and 94 cases with P<0.01. The remaining cases were genotyped for these 9 SNPs, and allele frequencies were tested for case-control association. The smallest P value was identified between an intronic SNP in the *FcRH3* gene and RA (fcrh3_6, $P=1.8 \times 10^{-5}$; association was statistically significant in both RA subgroups (94 and 736)). This SNP was located in an LD block containing 4 of the 5 FcRH genes, with the fifth in the adjacent block. We thus used these 2 LD blocks to further evaluate the origin of this association (Fig. 1b), although our results do not exclude the presence of RA and/or other autoimmunity-associated variants in other LD blocks in 1q21-23.

In addition to the 25 SNPs among 491 used SNPs for LD block evaluation in the 2 LD blocks, 16 additional SNPs were identified in exons and 5′- and 3′-flanking regions of 5 FcRH genes and 1 pseudogene (*FcRHψ4* or *LOC343265)* by searching the public database and sequencing genomic DNA from Japanese RA-patients. These additional 16 SNPs were genotyped for the identical case and control samples (830 cases, 658 controls) to increase density of variants in the targeted region. A peak of association was observed in a short segment consisting of 4 SNPs in *FcRH3* ($P<1.0 \times 10^{-4}$) (Fig. 1c, **Supplementary Table 1** online). These comprised fcrh3_3, fcrh3_4, fcrh3_5 and fcrh3_6, located at nt −169, −110, +358 (5′-untranslated region of exon 2) and +1381 (intron 3; 204 and 859bp from the 3′- and 5′-end of the flanking exons) relative to the transcription initiation site, respectively.

The smallest P value without correction was observed in recessive-trait genotype comparison of fcrh3_3 in *FcRH3* ($P=8.5 \times 10^{-7}$; odds ratio, 2.15; 95% confidence interval, 1.58–2.93) (Table 1). This P value was still significant when the most conservative Bonferroni correction was applied (comparisons for 507 SNPs; P_{corr}=0.00043). The 4 strongly associated SNPs were in LD with each other and 3 common haplotypes were inferred (Table 2; Fcrh3_3, fcrh3_5 and fcrh3_6 showed strong LD with each other, with Δ >0.99, whereas fcrh3_4 showed relatively weak LD with the other 3 SNPs (mean Δ = 0.68)).

To identify causal variants in this segment based on genotype data, a forward stepwise-regression procedure was performed with cut-off P value to proceed next step being 0.01 (ref. [29]). No SNP in FcRH genes other than *FcRH3* improved the model. None of the 4 SNPs in *FcRH3* were preferred over the others in these data (data not shown), which implicated that one of the SNPs in *FcRH3*may cause the disease, but there still remained the possibility that variants in other genes were truly associated with the disease.

To validate the case-control association test, samples (830 cases, 658 controls) were evaluated for the impact of population stratification on the case-control study. We selected 2069 SNPs,

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each of which was identified as a tagging SNP[30] in 2069 distinct LD segments that were previously identified by genotyping 74,842 SNPs distributed in autosomal chromosomes[31]. Analysis of population structure[32], and the χ^2 sum[33], were used for the evaluation of stratification. No significant evidence of population stratification was detected (**Supplementary Fig. 1** online), suggesting no or negligible stratification of our samples and supporting the validity of the case-control association results by removing this confounder from further consideration.

Regulatory effect of SNP -169 C/T on *FcRH3* expression

Since none of the 4 SNPs in *FcRH3* (fcrh3_3, fcrh3_4, fcrh3_5, and fcrh3_6) produces amino-acid substitutions, potential effects of the SNPs on transcription factor binding were assessed using TRANSFAC software. Nuclear factor-κB (NF-κB) was predicted to bind the sequence containing RA-susceptible allele fcrh3_3 (-169C) with a high score (core match 1.000, matrix match 0.957) and substitution to a non-susceptible allele T decreasing the score of NF-κB binding substantially (core match 0.760, matrix match 0.824). The other 3 SNPs were not predicted to bind to any transcriptional factor with high score and nucleotide substitution was not predicted to affect binding at any regulatory factor. We therefore focused on the 5′-flanking region of fcrh3_3 to explore the regulatory effects on expression of *FcRH3*.

Reporter gene analysis was performed using the genomic sequence of *FcRH3* from nt −523 to +203. Constructs were made corresponding to the 3 haplotypes using SNPs at nt -169 (C/T; fcrh3_3) and -110 (G/A; fcrh3_4) (Fig. 2a). These constructs were transfected into Raji cells, a Burkitt's lymphoma cell line that expresses *FcRH3* (ref. [13]) and is derived from germinal center B-cells. Luciferase activity was significantly higher in cells transfected with -169C/-110G or -169C/-110A than in cells transfected with -169T/-110G. This suggests that -169C/T is critical for regulation of *FcRH3* expression. To clarify, we cloned single or 4 tandem copies of 30-bp oligonucleotides surrounding -169T/C and control oligonucleotides into a vector with the SV40 promoter. Cells transfected with a single copy of the C allele produced significantly greater luciferase activity than in cells transfected with a single copy of the T allele. More convincingly, transfection with 4 tandem copies of the C allele enhanced luciferase activity 20-fold over those cells transfected with 4 tandem copies of the T allele (Fig. 2a).

To elucidate specific nuclear factors that bind the disease-susceptible allele, we analyzed the sequence around -169C/T. These sequences were predicted by TRANSFAC software to display binding affinity for nuclear factor (NF)-κB, which regulates a wide variety of genes in the immune system. The disease-susceptible sequence GGGAAGTCCC (underlined nucleotide represents SNP at -169) displayed higher matrix similarity to the consensus NF-κB binding motif than the non-susceptible sequence GGGAAGTCCT. Electrophoretic mobility shift assay (EMSA) was then performed to examine whether differences between the susceptible -169C allele and the non-susceptible -169T allele affected binding of nuclear proteins from Raji cells. The 30-bp labeled oligonucleotides used in the luciferase assay were used again in this study. These sequences contain the predicted NF-κB binding site. Two major bands, I and II, were observed in the presence of nuclear extracts, and intensity of band I was higher for the susceptible -169C allele than for the non-susceptible -169T allele (Fig. 2b). Competition assays with unlabelled oligonucleotides revealed that these complexes were specific for the probes. In addition, competition assays with unlabelled probes of the C allele for T and the T allele for C demonstrated that the C allele was better able to compete for binding, a result consistent with the higher binding affinity exhibited by labeled C allele probes alone. A supershift experiment was also performed with antibodies specific for NF-κB components (p50, p52, p65, RelB, cRel). Supershifts were observed in some lanes with specific antibodies for p50, p65 and cRel (Fig. 2b). Among these, only anti-p50 antibody shifted band II, suggesting the presence of a

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p50-p50 homodimer. Band I had the highest intensity and a significant allelic difference, and was supershifted by anti-p50, anti-p65 and anti-cRel antibodies. Although these findings indicate that band I comprises a mixture of heterodimers, the greater shifts caused by anti-p50 and anti-cRel antibodies suggest that the main component is a p50-cRel heterodimer.

The two *in vitro* assays indicated the potent transcriptional activity of the disease-susceptible haplotype regulated by NF-κB, suggesting expression of *FcRH3* from a chromosome with the disease-susceptible -169C allele be more than from a chromosome with non-susceptible -169T allele. To extend these findings, we quantified expression of *FcRH3* in peripheral blood B-cells from healthy donors using quantitative Taqman methods, and analyzed the effect of the number of susceptible copies on the transcript level by regression model. Regression analysis revealed a significant positive correlation between number of susceptible chromosomes and transcription level (R^2=0.49, P=0.0076) (Fig. 2c).

Allele-specific transcript quantification (ASTQ)[9,34] was also performed to confirm the effect of the SNP on transcription.. Using an Eag I restriction fragment length polymorphism (RFLP) located at position +358 in exon 2 of *FcRH3* (fcrh3_5, +358C/G), the relative contribution of each haplotype to transcript production in heterozygous individuals could be measured (Fig. 2d). The transcripts of 5 individuals with the -169C+358C/-169T+358G genotype were evaluated, and mean ratio (susceptible vs. non-susceptible haplotype) was 1.63, significantly higher than that of DNA amplicons (ratio=1.06, $P<1 \times 10^{-5}$) from the same individuals. (The quantity of template DNA from the 2 haplotypes was equal.) These results show that the expression of *FcRH3* is higher in individuals with the disease-susceptible haplotype, and suggest that higher expression of *FcRH3* is a potential cause and component of the pathological mechanism(s) leading to RA.

Expression of *FcRH3* mRNA

FcRH3 expression in multiple tissues was then quantified using TaqMan methods. Expression of *FcRH3* transcripts was high in the spleen and tonsils (Fig. 3a), which are secondary lymphoid organs. Lower expression was observed in thymus and bone marrow. Analysis of human blood fractions revealed that CD19-positive cells, which represent the B-cell population, displayed the highest level of *FcRH3* expression among peripheral blood mononuclear cells. Lower expression was observed in CD4- and CD8-positive cells (Fig. 3b). Next, the effect of B-cell stimulation on *FcRH3* expression was examined. Peripheral blood B-cells from a healthy donor were cultured for 4 h using known B-cell stimulants, and mRNA of *FcRH3* was quantified (Fig. 3c). Expression of *FcRH3* was increased using stimuli comprising anti-CD40 antibody and lipopolysaccharide (LPS).

Expression of *FcRH3* transcripts in synovial tissue was then investigated using *in situ* hybridization methods. T and B cells are known as the key players with regard to inflammation in synovial tissue, producing pro-inflammatory cytokines and autoantibodies that might be pathogenic[1]. These cells are known to show three distinct histological patterns: diffuse infiltration; clustering in aggregates; and follicles with germinal center reaction[35,36]. Aggregations of T and B cells were observed in paraffinized synovial sections from patients with RA, using immunostaining with anti-CD3 and -CD20 antibodies, respectively (Fig. 3d, e). *In situ* hybridization assay with serial sections detected *FcRH3* mRNA in aggregated lymphocytes (Fig. 3f, **g**). Although strict differentiation between B and T cells was difficult, at least some aggregated B-cells were positive, with strong expression of *FcRH3* mRNA. RA synovium from two other patients displayed similar images of lymphocyte aggregates and *FcRH3* expression (**Supplementary Fig. 2** online).

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SNP association with autoantibody and *HLA-DRB1* status

Higher *FcRH3* expression was suspected as leading to B-cell abnormalities in RA, so associations were examined in RA patients between genotype and two RA-related autoantibodies: rheumatoid factor (RF); and anti-cyclic citrullinated peptide (CCP) antibody. RF is a well-known autoantibody for the Fc region of IgG, and titers correlate with RA disease activity[37]. Anti-CCP antibody recognizes peptides containing citrulline, and is detected in RA with extremely high specificity[38,39]. RF titer in RA patients was positively correlated with the number of susceptible alleles with statistical significance (R^2=0.049, P=0.0065) (Table 3). The positive ratio of anti-CCP antibody in RA patients also differed significantly among genotypes ($P<0.05$), and correlated with numbers of susceptible alleles.

As genetic interactions between HLA and non-HLA loci have been described in susceptibility for RA and other autoimmune diseases[26,40], genotype distributions for SNP -169C/T were compared among three RA-case subgroups stratified by number of *HLA-DRB1* shared-epitope (SE)-encoding alleles. We had previously genotyped *HLA-DRB1* in our population and observed significant associations between RA-susceptibility and SE-encoding alleles[4]. Allele frequency of RA-susceptible allele, SNP -169C, in the subgroup with two copies of SE alleles was significantly higher than in the subgroup with no SE alleles (SNP -169C allele frequency: SE +/+, 0.49 (n=113); SE +/−, 0.43 (n=376); SE −/−, 0.39 (n=215)) ($P<0.05$).

Replication study of association in RA, ATID and SLE

To confirm associations between the *FcRH3* variant and RA susceptibility, a replication study was conducted (540 RA patients, 636 control subjects). Allele-frequency comparison revealed a significant association between fcrh3_3 (-169C/T) and RA susceptibility (P=0.041, allele frequency: RA patients, 0.40; control subjects, 0.36) (**Supplementary Table 2** online). No significant differences were noted between two cohorts that consisted of the replication samples. These results further confirmed the association of the fcrh3_3 -169C SNP allele with RA susceptibility in Japanese.

Since this region was known to display associations with multiple autoimmune diseases, and because several variants were known to be involved in multiple autoimmunities, associations were investigated between SNP -169C/T and two other autoimmune diseases, AITD and SLE. A total of 509 Japanese patients with AITD (Graves' disease, n=351; Hashimoto's thyroiditis, n=158 patients) and 564 Japanese patients with SLE were recruited and compared with 2037 Japanese controls. In addition, AITD, SLE and RA cases were combined as subjects with an autoimmune phenotype and tested for associations with the SNP. Individual diseases as well as combination of two AITDs and combination of AITD, SLE and RA were significantly associated with the SNP (OR=1.52 and P=0.00000084 in Japanese for a recessive model between all 4 autoimmunities considered in aggregate and controls; Table 4). As RA-specific autoantibodies were correlated with the number of susceptible alleles, anti-DNA antibody titer was higher in SLE patients with the -169C/C genotype than in subjects with other genotypes (294.1 IU/ml vs. 145.5 IU/ml; n=120; P=0.026 by Student's t-test), a conclusion not further established by regression analysis (P=0.12).

DISCUSSION

LD mapping of 1q21-23 in Japanese subjects revealed multiple LD blocks in the region, and 1 block containing *FcRH3* displayed associations with RA. This association was replicated in a second Japanese case-control set. The RA-associated allele was also associated with increased risk of other autoimmune disorders, such as AITD (Graves' disease and Hashimoto's thyroiditis) and SLE. Recent reports on autoimmune disease-associated polymorphisms show

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that some disease-susceptible variants are often limited to specific ethnic groups[12] while other polymorphisms are widely dispersed among *Homo sapiens*, but significantly associated in only specific ethnic groups[41,42]. We evaluated haplotypes consisting of 4 SNPs in *FcRH3* in African American, European American, and Asian (Korean and Japanese) subjects. Weaker LD was identified in African Americans and substantial differences in allelic frequency were noted among three major ethnic groups (**Supplementary Table 3** online).

Though the evidence presented herein for *FcRH3* being an autoimmune susceptibility gene is very powerful, additional autoimmune disease-related genes may probably exist in this region in addition to *FcRH3*. For example, 1q23 represents a strong candidate locus for SLE-susceptibility[6], particularly involving the association of the classical FcγR genes with SLE-susceptibility in the Japanese population[19], although those variants are not in LD with -169C/T in our Japanese population ($\Delta<0.05$, Fig.1a). Multiple SLE susceptibility genes are also syntenic to human 1q23 in murine models of SLE[43].

Further evaluation of polymorphism associations showed that an SNP in the promoter region of *FcRH3* alters expression of *FcRH3* via NF-κB binding. Since higher expression of *FcRH3* was observed in individuals with susceptible alleles, and augmented autoantibody production was associated with the susceptible genotype, important steps in the sequence of events leading to autoimmunity must proceed through *FcRH3*. That the susceptible allele is associated with *HLA-DRB1* in RA is consistent with *FcRH3* functioning in the context of HLA class II restriction, which is usually seen in the interaction between T-cells and antigen-presenting cells, including B-cells. Moreover, together with the dominant expression of *FcRH3* on B-cells and the critical roles of B-cells suggested by a recent clinical trial of B-cell-depleting therapy[44], the present findings might provide a genetic basis for B-cell abnormality in autoimmunity.

Although the precise function of *FcRH3* is unknown, the predicted molecular structure suggests that *FcRH3* is a membranous protein that conveys signals into cells through a cytoplasmic domain containing an immunoreceptor-tyrosine activation motif (ITAM) and an immunoreceptor-tyrosine inhibitory motif (ITIM)[14]. An *in vitro* study showing the binding of tyrosine kinases syk and ZAP70 to the ITAM region and tyrosine phosphatases SHP-1 and SHP-2 to the ITIM region[17] supports the proposed signaling function of *FcRH3*. In a previous study examining *in situ* hybridization in human tonsil, *FcRH3* was expressed in the germinal center, with particularly high expression in the light zone[16], suggesting that *FcRH3* functions predominantly in centrocytes. The present finding that CD40 stimulation, which is important in germinal center formation[45], upregulates *FcRH3* expression in B-cells could indicate that *FcRH3* is specifically expressed in germinal center centrocytes under the influence of CD40 signals. In the light zone, centrocytes undergo clonal selection and affinity maturation regulated by positive and negative signals from antigen receptors and co-receptors[46]. High expression of *FcRH3* and augmented autoantibody production in individuals with the disease-susceptible genotype is consistent with *FcRH3* influencing the fate of B-cells and augments the emergence of self-reactive cells in the germinal center.

Besides a role of *FcRH3* in lymphoid tissues, expression of *FcRH3* in synovial tissue might explain the pathological connection between *FcRH3* variants and RA. As we have shown, *FcRH3* is strongly expressed in aggregated lymphocytes. Although our synovial samples showed only T-cell/B-cell aggregates, lymphocytes in RA synovial tissue are known to form a germinal center-like structure, called an "ectopic germinal center", where T-cell dependent antibody production and affinity maturation occur[36]. Ectopic germinal center formation is also seen in tissues from patients with AITD and SLE, and *FcRH3* might be involved in pathological autoimmune reaction in these disease-specific ectopic lymphocyte aggregates.

Considering that augmented expression of *FcRH3* is associated with susceptibility to autoimmune disorders, and that *FcRH3* expression is regulated in B-cells in the secondary lymphoid organ and is detected in lymphocytes of disease-specific tissues, *FcRH3* very probably is functional molecule in immunity and potentially pathogenic in autoimmune disorders.

METHODS

Subjects

Three independent cohorts of RA patients (n=830, 217, and 323), a cohort of SLE patients (n=564), and a cohort of AITD patients (n=509; comprising Graves' disease (n=351) and Hashimoto's thyroiditis (n=158)), were enrolled for the study through several medical institutes in Japan. Four independent cohorts of unaffected control subjects (n=658, n=262, n=374 and n=752) were recruited at various sites in Japan. All subjects were Japanese. RA patients (84.2% women; mean age, 59.0 ±12.3 years; 75.0% rheumatoid factor-positive) satisfied the revised criteria of the American Rheumatism Association for RA[47]. SLE patients followed the criteria of the American College of Rheumatology for SLE[48]. Diagnosis of AITD was established based on clinical findings and results of routine examinations for circulating thyroid hormone and thyroid stimulating hormone (TSH) concentrations, serum levels of antibodies against thyroglobulin, thyroid microsomes and TSH receptors, ultrasonography, $^{[99m]}TCO_4^-$ (or $[^{123}I]$) uptake and thyroid scintigraphy.

The first control cohort was used for evaluation of LD in 1q21-23 and was compared with the first RA cohort for initial identification of RA-associated LD block and SNPs. The second and third cohorts of RA and controls were used for replication test of the first cohort result. Graves' disease, Hashimoto's thyroiditis, SLE, combination of the two AITDs and combination of RA, SLE and AITDs were tested for associations using the total pool of controls. Control subjects in three other ethnic groups, Korean (n=100), African American (n=120) and European American (n=120), were enrolled for evaluation of *FcRH3* haplotypes. Synovial tissues were sampled from individuals with RA who underwent arthroplastic surgery. All subjects provided informed consent to participate in the study, as approved by the ethical committee of the SNP Research Center, RIKEN.

SNPs and genotyping

SNPs were discovered in exons and 5'- and 3'-flanking regions of *FcRH1, FcRH2, FcRH3* and *FcRHψ4 (LOC343265)* by direct sequencing of DNA from 24 patients. Other SNPs were selected from the JSNP database and Assay-On-Demand SNP database (Applied Biosystems). SNPs were genotyped using Invader and TaqMan assays[41] as indicated by manufacturers. Probe sets for the Invader assay were designed and synthesized by Third Wave Technologies, and those for the TaqMan assay were obtained from Applied Biosystems. When assessing the results of SNP genotyping, we generally exclude successful call rates<0.95 and values of P<0.01 obtained by HWE testing in control subjects. The error rate of Invader assay was 0.0023, which was estimated by 11,092 assays in 2 replicates using 118 randomly selected SNPs (internal control data).

Luciferase assay

The promoter fragment of three haplotypes corresponding to nt −523 to +203 of *FcRH3* was cloned into the pGL3-Basic vector (Promega). Oligonucleotides were generated using the allelic sequences of nt −189 to −160 of *FcRH3* as follows: -169T (5′ GGTGAGATTACGGGAAGTCCTTGATCTGTA3′); -169C (5′ GGTGAGATTACGGGAAGTCCCTGATCTGTA3′); and Cont

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(GGAGTGTAATCGGGTTGACCAAGTACAGAT). A single copy or 4 tandem copies of these oligonucleotides were cloned into pGL3-Promoter vector (Promega). Raji cells (RCB1647; RIKEN Cell Bank) were grown in RPMI1640 medium supplemented with 10% fetal bovine serum and antibiotics. We electroporated (230 V and 975 μF) 1 $\times 10^7$ cells with 5 pmol of constructs and 1 pmol of pRL-TK vector (internal control for transfection efficiency) in a 0.4-cm gap cuvette. After 48 h, cells were collected and luciferase activity measured using the Dual-Luciferase Reporter Assay System (Promega).

EMSA

EMSA and preparation of nuclear extract from Raji cells were performed as previously described[49]. Oligonucleotides -169T and -169C were labeled with digoxigenin -11-ddUTP using the DIG gel-shift kit (Roche). We incubated 5 μg of nuclear extract with 40 fmol of DIG-labeled nucleotide for 25 min at room temperature. For competition experiments, nuclear extract was pre-incubated with unlabeled oligonucleotide (100-fold excess) before adding digoxigenin-labeled oligonucleotide. For supershift assays, 4 μg of anti-p50, p52, p65, RelB and cRel antibodies and rabbit IgG (control antibody) (Santa Cruz Biotechnology) were incubated for 15 min at room temperature after incubation of the labeled probe. Protein-DNA complexes were separated on a non-denaturing 6% polyacrylamide gel in 0.5× Tris-Borate-EDTA buffer. The gel was transferred to a nitrocellulose membrane, and signals were detected using an LAS-3000 luminoimage analyzer (Fujifilm).

RNA extraction and cDNA preparation

Peripheral blood was collected from consented healthy volunteers to obtain CD19-positive lymphocytes. Polymorphonuclear cells were separated by differential centrifugation using Lymphoprep resolving solution (AXIS-FIELD). CD19-positive lymphocytes were isolated using the MACS system with CD19 microbeads (Miltenyi Biotec), and cell purity >95% was confirmed using flow cytometry. Cells were stimulated with anti-CD40 Ab (Cymbus Biotechnology), anti-IgM Ab (Jackson Immunoresearch), Il-4 (eBioscience), APRIL (PeproTech), BAFF (PeproTech) and LPS (Sigma) for 4 h. Total RNA was isolated using RNeasy Mini Kit (Qiagen). RNAs of other normal tissues were quantified using Premium Total RNA (Clontech). Total RNA was reverse transcribed using TaqMan Gold RT-PCR reagents with random hexamers (Applied Biosystems), in accordance with the instructions of the manufacturer.

Quantification of *FcRH3* expression using real-time RT-PCR

Real-time quantitative PCR was performed using an ABI PRISM 7900 (Applied Biosystems) and Assay-on-Demand TaqMan probe and primers (Hs00364720_m1 for *FcRH3*), according to the manufacturer's instructions. A standard curve was generated from the amplification data for FcRH3 primers using a dilution series of total RNA from Raji cells as templates, and data were normalized to GUS level.

ASTQ

Allele-specific quantification was performed as previously described[34] with some modifications. Preparation of cDNA from B-cells was undertaken as described above. Both cDNA and genomic DNA were amplified by PCR for 37 cycles using primers specific for exon 2 of *FcRH3* (**Supplementary Table 4** online), and for an additional cycle using forward primer with Alexa Fluor 488 label at the 5′-end. Products were directly digested using Eag I by incubation at 37 °C for 12 h. Full digestion was monitored by the inclusion of PCR products from homozygote +358G/G. Digested products were then run on a 12.5% polyacrylamide gel, followed by quantification using an LAS-3000 analyzer.

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